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Inversion and Circularization of the Varicella-Zoster Virus Genome

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The genome of varicella-zoster virus (VZV) is a linear, double-stranded molecule of DNA composed of a long (L) region covalently linked to a short (S) region. The S region is capable of inverting relative to a fixed orientation of the L region, giving rise to two equimolar populations. We have investigated other forms of the VZV genome which are present in infected cells and packaged into nucleocapsids. That a small proportion of nucleocapsid DNA molecules also possess inverted L regions has been verified by the identification of submolar restriction fragments corresponding to novel joints and novel ends generated by such an inversion. The presence of circular molecules has been investigated by agarose gel electrophoresis. Bands corresponding to circular forms were present in small amounts in both VZV-infected cell DNA and nucleocapsid DNA. Southern blot analysis verified that these bands contained VZV sequences. We therefore conclude that the VZV genome may occasionally contain an inverted L region or exist in a circular configuration.

Varicella-zoster virus (VZV), one of five human herpesviruses, is the causative agent of chicken pox (varicella) and shingles (zoster). The VZV genome is a linear, double-stranded DNA molecule with a molecular weight of ~80 × 10^6 (120 kilobase pairs [kbp]) and an overall guanine-plus-cytosine content of 47%. VZV DNA has been subjected to extensive restriction endonuclease mapping, and sequences spanning the entire genome have been molecularly cloned in several laboratories (5, 7, 8, 12, 18, 29). These studies have shown that VZV DNA is similar in arrangement to the DNAs of pseudorabies virus and equine herpesvirus types 1 and 3 (1, 21, 24, 27, 30). The genome is divided into two parts, long (L [~100 kbp] and short (S [≤20 kbp]). The L region is composed almost entirely of quasi-unique sequences (U_L) bounded by a very small inverted repeat (88.5 base pairs) (4). The S region is composed of a set of quasi-unique sequences (U_S, 5.2 kbp) flanked by an inverted terminal repeat (IR_S/TR_S, 7.3 kbp) which is much higher in guanine-plus-cytosine content than the U_S and U_L sequences (3, 23a). The DNA is believed to be present in two predominant, equimolar isomeric forms which result from the inversion of the S region with respect to an invariant configuration of the L region.

Recently, however, reports from several laboratories have documented the existence of novel fragments in restriction endonuclease digests of VZV DNA isolated from purified virions and nucleocapsids (4, 9, 23a). These fragments are present in small quantities and correspond in size to fragments (novel joints and novel ends) which would result from the inversion of the L region. As an example of this, we will consider the VZV *Hin*dIII fragments involved in such an inversion (see Fig. 1 and 2). In the normal configuration, *Hin*dIII-J would be the left-hand terminal L fragment, *Hin*dIII-N would be the right-hand terminal S fragment, and *Hin*dIII-H would span the junction of L and S. If L were inverted with respect to S, two new fragments would be observed. One of these would be a truncated *Hin*dIII H′ fragment, i.e., *Hin*dIII-H minus the fraction of this fragment that extends into IR_S. The second fragment would be a novel joint fragment made up of *Hin*dIII-J plus the fraction of *Hin*dIII-H that extends from the junction of L and S into the IR_S (in this case, corresponding to *Hin*dIII-N). The right-hand terminal fragment of S would still be *Hin*dIII-N. Ecker et al. (9) reported the presence of minor populations of *Hin*dIII fragments which correspond in size to the above described novel joint and end fragments but did not identify them. A recent report (23a) described the cloning and characterization of a novel joint fragment composed of sequences from *Bam*HI-R and *Bam*HI-Y (see Fig. 1 and 2). Davison (4) reported cloning and sequencing *Sal*I fragments corresponding to novel ends and joints; it was concluded that these novel fragments resulted from the inversion of L. Thus, there is a growing body of evidence that a small population of packaged VZV DNA molecules contain inverted L regions and that the presence of minor populations of DNA fragments in restriction endonuclease digests of the viral DNA can be explained by such an inversion.

The presence of novel joint fragments, however, could also be due to circularization of the VZV genome, resulting in the fusion of L and S termini. Straus et al. (28) found evidence of circular and partially relaxed supercoiled molecules upon observation of VZV DNA in an electron microscope, and Ecker et al. (9) have also recently described circular molecules in VZV DNA preparations.

In the case of the *Hin*dIII digest, the joint fragment resulting from circularization would be made up of *Hin*dIII-J linked to *Hin*dIII-N and thus would be indistinguishable from the joint fragment generated by the inversion of L. However, since circularization produces only novel joint fragments (and no novel end fragments), a population of VZV DNA molecules containing both circular genomes and genomes with L inverted would contain novel joint fragments in excess of novel ends. In addition, other restriction endonucleases such as *Pst*I and *Pvu*II (see Results and Fig. 1) should theoretically be able to differentiate between circularization and inversion directly, since the two DNA structures (circles and inversions) would yield novel joint fragments of different sizes. In this paper, we exploit a variety of techniques to investigate further the occurrence

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and properties of circular VZV genomes and VZV genomes with inverted L segments.

MATERIALS AND METHODS

Cells and viruses. VZV strains Ellen and Oka were obtained from the American Type Culture Collection. Strain Scott was obtained from G. Fisher, Department of Pediatrics, Uniformed Services University of the Health Sciences. All viruses were grown in human foreskin fibroblasts as previously described (23a, 28).

DNAs. VZV DNA was purified from nucleocapsids as described previously (28). Restriction endonuclease cleavage of DNA was carried out with Bethesda Research Laboratories enzymes under the conditions recommended by the manufacturer. VZV DNA fragments generated by digestion with *Eco*RI and *Bam*HI were cloned into lambda gtWesB or pBR325 as described previously (23a, 29). The bacterial plasmid pCG86 was a kind gift of R. Holmes, Department of Microbiology, Uniformed Services University of the Health Sciences. Cultures of pCG86 in *Escherichia coli* HB101 were grown to stationary phase, washed in 50 mM Tris hydrochloride–10 mM EDTA (pH 7.5) and either frozen at −20°C in equal portions or subjected to a "cleared lysate" procedure, followed by purification on cesium chloride-ethidium bromide gradients.

Electrophoresis of DNAs and whole cells. Gel electrophoresis of restriction endonuclease-cleaved DNA was carried out in 0.7% agarose gels run at 65 mA for 18 h. Electrophoresis of intact viral and plasmid DNAs and infected or uninfected cells was carried out by the procedure of Eckhardt (10) as modified by Gardella et al. (11). Briefly, a solution of 0.75% agarose in TBE (0.1 M Tris borate [pH 8.3]–0.01 M EDTA) was poured and cooled at 4°C to form vertical gels. Cells or DNA were suspended in a buffer containing TBE, 15% Ficoll, RNase (25 μg/ml, pancreatic, Sigma Chemical Co.,), and 0.01% bromophenol blue. Samples (75 μl each) were loaded into the agarose gel and gently overlaid with 100 μl of a buffer containing TBE, 5% Ficoll, pronase (1 mg/ml), 1% sodium dodecyl sulfate, and 0.01% xylene cyanol. Samples were subjected to electrophoresis at 0.8 V/cm for 3 h and 7.5 V/cm for 10 to 18 h. Electrophoresis was carried out at 4°C. The DNA, stained with ethidium bromide, was illuminated with UV light and photographed. The gel was then treated for hybridization, or the DNA was eluted.

Transfer of DNA to nitrocellulose. Transfer of DNA to nitrocellulose sheets (BA85, Schleicher & Schuell, Inc., Keene, N.H.) was accomplished by the method of Southern (26). Blots were prehybridized and then probed with cloned DNA fragments that had been labeled to high specific activity (10^6 to 10^8 counts per μg) by nick translation with [α-^{32}P]dCTP (ICN Pharmaceuticals, Inc., Irvine, Calif.). Fragments were hybridized in a buffer containing 30% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.02% Ficoll, 20 mM sodium phosphate (pH 6.5), and 10% dextran sulfate at 50°C for 18 to 24 h. Blots were washed five times in 2× SSC at 50°C and exposed to Kodak XAR-5 film at −70°C with a DuPont Cronex Hi-Plus intensifying screen.

Elution of DNA from agarose. Elution of DNA from 0.7% low-melting-point agarose gels was performed by excision of agarose containing the DNA and extraction with the detergent hexadecyltrimethyl ammonium bromide, as described by Langridge et al. (17). Eluted DNA was then digested with restriction enzymes, labeled with [^{32}P]dCTP by nick translation, or both (23).

RESULTS

Identification of novel VZV DNA fragments. To investigate further the suggestions that VZV L region is able to undergo inversion and that VZV DNA may circularize (4, 23a, 28), purified DNA obtained from VZV nucleocapsids was digested with *Bam*HI and *Hin*dIII and probed with cloned fragments of VZV DNA. Figure 1 shows physical maps of the relevant fragments generated by these restriction enzymes. *Hin*dIII and *Bam*HI are particularly suitable enzymes in the above context, since these enzymes cleave inside both IR_S and TR_S of the S region. This would eliminate any confusion generated by the inversion of the S region, since either orientation of the U_S region would generate an identical terminal fragment (Fig. 2). Figure 3a shows an autoradiogram of the *Bam*HI-digested DNA after it was probed with *Eco*RI-E (Fig. 1). This probe hybridizes to VZV BamHI DNA fragments B, J, K, Q and R, with the 2 M J fragment being derived from both TR_S and IR_S (Fig. 2). The faint "band" above fragment J is not reproducible. In addition to these fragments, two other regions of hybridization are seen, one migrating ahead of *Bam*HI-B and a second migrating between *Bam*HI-K and *Bam*HI-Q. The latter migrates at a position identical to *Bam*HI-N and corresponds in molecular weight to the size predicted for a novel joint fragment (R + Y). The larger fragment is of the size predicted for a truncated form of *Bam*HI-B as generated by the inversion of L (B′). In addition to these, a fragment corresponding to *Bam*HI-Y which also hybridizes to the probe can be resolved on higher-percentage agarose gels (data not shown). This is due to an 88.5-base-pair sequence present at both ends of U_L (4).

Figure 3b shows the autoradiogram of a second experiment in which VZV DNA was digested with *Hin*dIII and probed either with a mixture of *Kpn*I fragments I, L, and O or with *Bam*HI fragment Z (Fig. 2). *Hin*dIII fragments C, G, H, M, and N all hybridize to the mixed fragment probe, as expected. In addition, two novel submolar fragments hybridize. One migrates between *Hin*dIII fragments G and H and corresponds in size to J + N, a novel joint fragment; the other corresponds to a novel end fragment, H′ (Fig. 2).

The identity of the novel joint fragment was further confirmed by the finding that *Bam*HI-Z (which is the left-hand subterminal *Bam*HI fragment [P. R. Kinchington, unpublished data]) hybridized to *Hin*dIII-J and to the novel *Hin*dIII joint fragment J + N (Fig. 2). The existence of such novel joint and novel end fragments indicates that inversion or circularization events or both occur in a small fraction of VZV DNA.

To determine more clearly the relative proportions of circular molecules and molecules with inverted L regions present in VZV DNA preparations, studies were initiated to identify unique restriction endonuclease fragments that would indicate circularization or inversion. To this end, two restriction enzymes, *Pst*I and *Pvu*II, were used to analyze a single preparation of VZV DNA. These enzymes were chosen since their largest restriction fragments, the A fragments, start at the right-hand terminus of the genome and continue through both repeat segments, extending into U_L (Fig. 2, 4b, and 4d).

After *Pvu*II digestion of VZV DNA, one would expect to find *Pvu*II A fragments of 26.6 kbp for the normal configuration, 21.75 kbp for an inversion of U_L, and 27.6 kbp for a circularization event (Fig. 4b). That is, inversion would lead

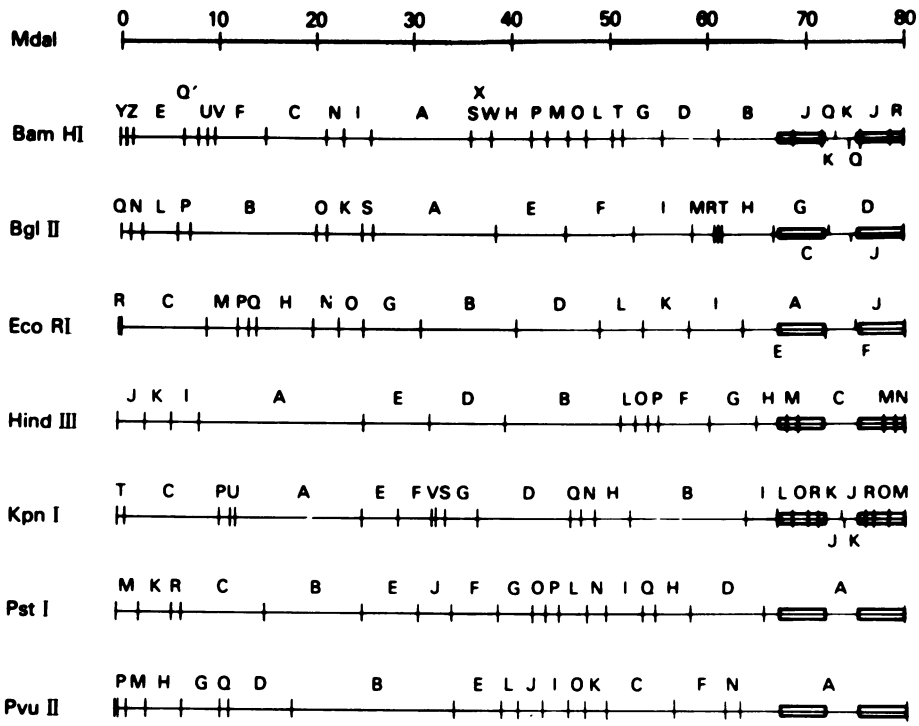

FIG. 1. Restriction enzyme maps for VZV DNA. The data shown are derived from our own studies and from those of the authors in references 5, 7, 8 and 18.

to a decrease in size of 4.85 kbp, and circularization would lead to an increase in size of 1 kbp. Fragments of 26.6 and 27.6 kbp could not be resolved. However, a submolar fragment of 21.75 kbp was observed, and this fragment hybridized to ^{32}P-labeled *Eco*RI-A on a Southern blot (Fig. 4a). *Eco*RI-A is homologous to most of the *Pvu*II A fragment (Fig. 2). One would also expect to find the 6.65-kbp part of *Pvu*II-A which, due to the inversion event, has been relocated to the left-hand terminus. A fragment of this size which hybridized to ^{32}P-labeled *Eco*RI-A was observed between *Pvu*II fragments G and H (Fig. 4a). This autoradiograph has been overexposed to show the 6.65-kbp species; all other species that hybridize to the probe are partial digest fragments.

In the *Pst*I restriction pattern, one would expect *Pst*I A fragments of 23.3 kbp for the normal VZV DNA configuration, 24.2 kbp for the inversion of U_L, and 27.6 kbp for a circularization event (Fig. 4d). Thus, inversion would lead to an increase of 0.9 kbp, which would be unresolvable from normal *Pst*I-A, whereas circularization would lead to a resolvable increase of 4.25 kbp.

Since the larger band was not observed after ethidium staining (Fig. 4c), it would seem that there was less circular VZV DNA than inverted L-form DNA in this preparation. In an attempt to detect small amounts of the 27.6-kbp fragment, the *Pst*I-cut VZV DNA was transferred to nitrocellulose and probed with ^{32}P-labeled *Bam*HI-E, which is homologous to *Pst*I fragments C and K and part of fragment M (Fig. 2). If inversion or circularization occurred, one would expect hybridization to occur to an enlarged *Pst*I A fragment (see Fig. 4d). In fact, homology was detected with a fragment of slightly larger than normal *Pst*I-A (Fig. 4c). The observed band was rather diffuse, and it was difficult to discriminate absolutely between the results of inversion, circularization, or a combination of the two, although the position of the fragment does suggest circularization. After cutting out the bands and counting them in scintillation fluid, it was estimated that the enlarged *Pst*I-A band is approximately 5 to 6% as prevalent as the molar bands.

Agarose gel electrophoresis of intact VZV DNAs and VZV-infected cells. Since it was difficult to determine absolutely the presence of unique restriction enzyme fragments that could be generated only by circularization of VZV DNA, the detection of circular molecules was also attempted by a different method. Eckhardt (10) developed an agarose gel electrophoresis technique for the resolution of circular plasmid DNAs in procaryotic cells. In this technique, DNA is very gently released from the bacteria and directly electrophoresed into agarose gels, in which circular plasmid DNA migrates at a much slower rate than does the bulk DNA. This technique has since been used to demonstrate covalently closed circular molecules in eucaryotic cells infected with herpesviruses (11, 19), and we have employed it to study the DNAs present in VZV-infected cells.

Uninfected or VZV-infected human foreskin fibroblasts showing 80 to 100% cytopathic effect were harvested, washed in phosphate-buffered saline, and subjected to agarose gel electrophoresis as described in Materials and Methods. Figure 5 (lanes 1 to 4) shows an ethidium-stained gel of such an experiment. Lanes 1 and 3 indicate uninfected cells, and lanes 2 and 4 indicate VZV-infected cells (lanes 4 and 3 have five times more material than do lanes 2 and 1). A large amount of ethidium-stained material remains at the origin of the gel, presumably reflecting DNA-protein networks which are trapped at the origin. The DNA migrates in two positions; the faster is that expected for linear DNA, and the slower form corresponds to circular DNA. It should be noted that we routinely detected DNA only at the position expected for linear DNA in uninfected cells. The circular form migrates at approximately the same position as does

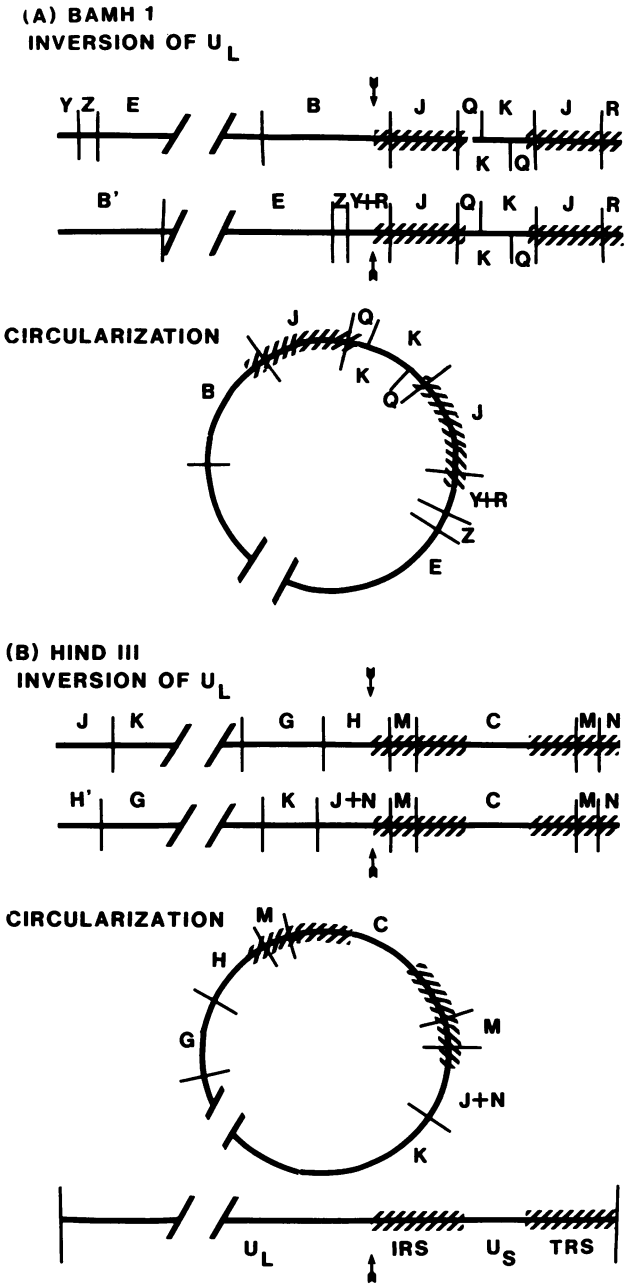

FIG. 2. Diagramatic representation of the VZV genome showing novel restriction enzyme fragments formed by inversion and circularization after the DNA has been digested with *Bam*HI (A) and *Hin*dIII (B). In each case, only the fragments mapping to U_S, IR_S, and TR_S and at either end of U_L are shown. In each figure, the upper linear representation shows the normal conformation of the DNA, the lower linear representation shows the conformation in which U_L is inverted, and the circular representation shows the conformation in which the two termini of the normal conformation are joined. Arrows indicate the junction of the L and the S regions of VZV DNA. Lines that extend above and below the representation of the genome indicate restriction sites in either conformation of the S region (see text). A representation of the VZV genome is shown at the bottom.

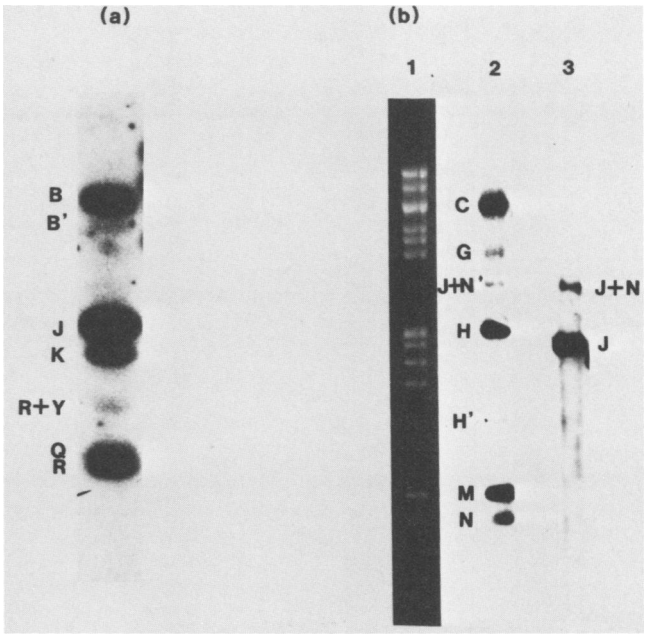

FIG. 3. Agarose gels and Southern blots showing novel restriction fragments. (a) *Bam*HI-digested VZV DNA separated on a 0.8% agarose gel, Southern blotted, and probed with ^{32}P-labeled *Eco*RI fragment E. The submolar novel fragments (B and Y + R [see Fig. 2]) are clearly present. (b) *Hin*dIII digest of VZV DNA separated on an agarose gel and stained with ethidium bromide. Lane 2, Gel blotted and probed with a mixture of ^{32}P-labeled *Kpn*I fragments I, L, and O eluted from agarose gels. The fragments that are expected to hybridize to the probe are labeled, and the novel fragments are also indicated (H', J + N [see Fig. 2]). Lane 3, a second *Hin*dIII digest of VZV DNA separated, Southern blotted, and probed with cloned ^{32}P-labeled *Bam*HI-Z.

pCG86 circular DNA (data not shown), suggesting that circular molecules of approximately the same size (i.e., 120 kbp) are present in VZV-infected cells. We noted that the resolution of the linear and circular forms depends on the quantity of cells loaded into the gel, in agreement with the observations of Gardella et al. (11) and Eckhardt (10).

To verify that the circular DNA bands in the infected cell lanes were composed of VZV sequences, the agarose gel described above was Southern blotted and probed with VZV DNA *Eco*RI fragments A to E cloned in pBR325 (Fig. 2). Figure 5 (lanes 5 to 8) shows that the circular form of VZV DNA seen in these gels (lanes 2 and 4) hybridizes to VZV DNA (lanes 6 and 8). In other experiments in which uninfected cell DNA was run and entered the gels, no hybridization was seen between uninfected cell DNAs and a VZV DNA probe. These results indicate that VZV DNA can exist in a circular form in infected cells.

Similar experiments were performed to determine whether circular VZV DNA is present in nucleocapsids. DNA was carefully isolated from purified VZV nucleocapsids, suspended in a buffer containing 5% Ficoll, 0.01% bromophenol blue, and 0.01% xylene cyanol, and electrophoresed on a vertical agarose gel. pCG86 DNA isolated from cesium chloride-ethidium bromide gradients was electrophoresed as a size marker for circular DNA. Figure 6 shows the resulting ethidium-stained gel. The vast majority of VZV DNA migrates at the position expected for linear DNA. However, a small fraction of the DNA migrates at the position corresponding to circular DNA, as shown by coelectrophoresis

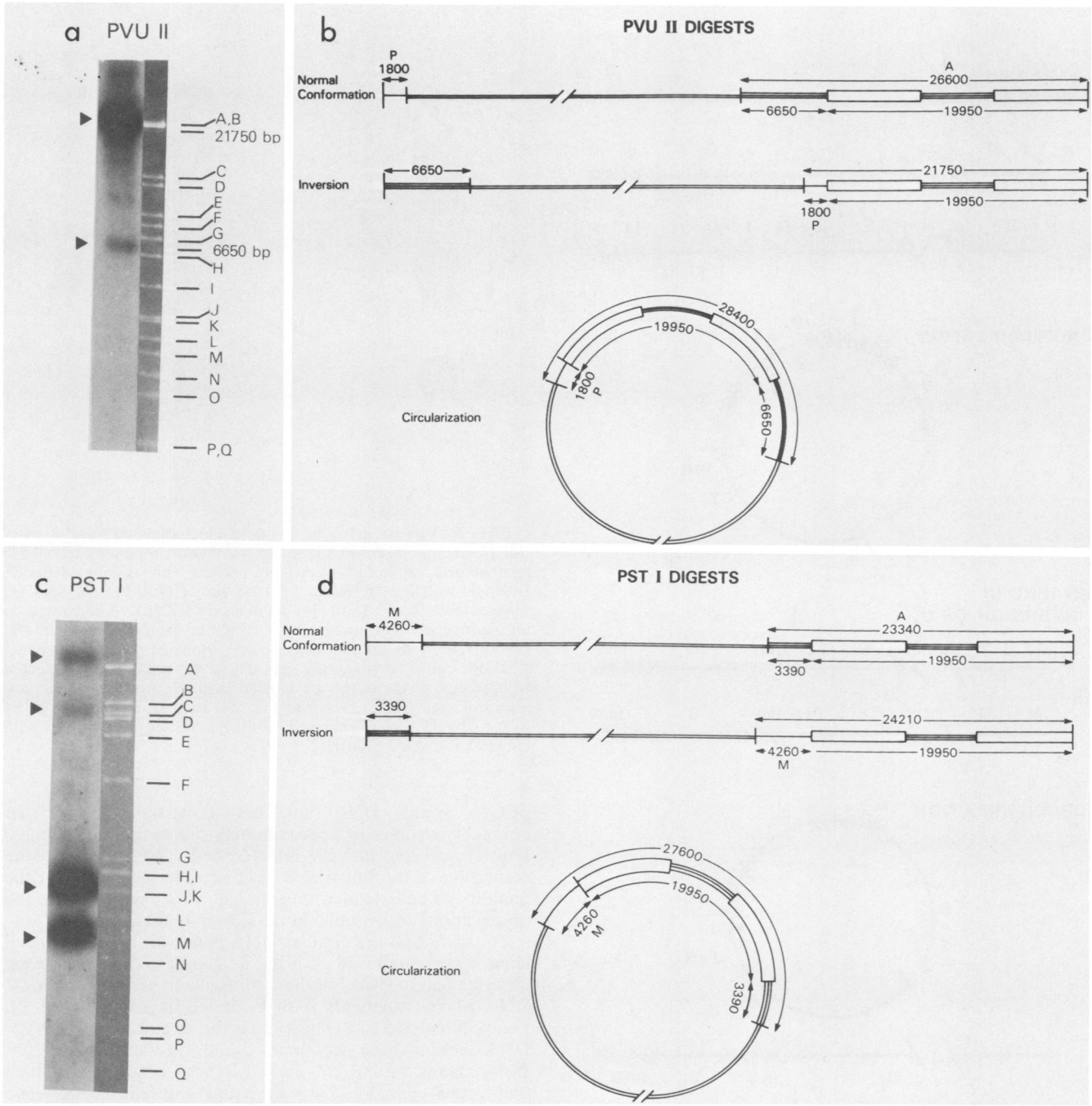

FIG. 4. Agarose gels and Southern blots showing restriction fragments of VZV DNA after digestion with *Pvu*II (a) and *Pst*I (c). A photograph of the ethidium-stained gel is shown to the right in panels a and c. The separated fragments were Southern blotted and probed with ^{32}P-labeled *Eco*RI-A (a) and ^{32}P-labeled *Bam*HI-E (c). Arrows indicate fragments that hybridize to the probes. Also shown is a representation of the restriction enzyme fragments formed by inversion and circularization after VZV DNA has been digested with *Pvu*II (b) and *Pst*I (d). In each case, only the fragments pertinent to the differences in conformation are shown (see text). In each figure, the upper linear representation shows the normal conformation of VZV DNA, the lower linear representation shows the conformation in which U_L has been inverted, and the circular representation shows the conformation in which the two termini of the normal conformation are joined. Sizes of the expected fragments are given in bases to the nearest 50-base unit.

with pCG86 (Fig. 6). We have estimated from this and other experiments that these circles form only 0.1 to 5% of the total DNA from nucleocapsids and have also detected DNA corresponding to circular DNA when intact VZV nucleocapsids were subjected to Eckhardt (10)-type gel electrophoresis (data not shown). These results indicate that VZV DNA isolated from nucleocapsids also contains a small population that is in the form of covalently closed circles.

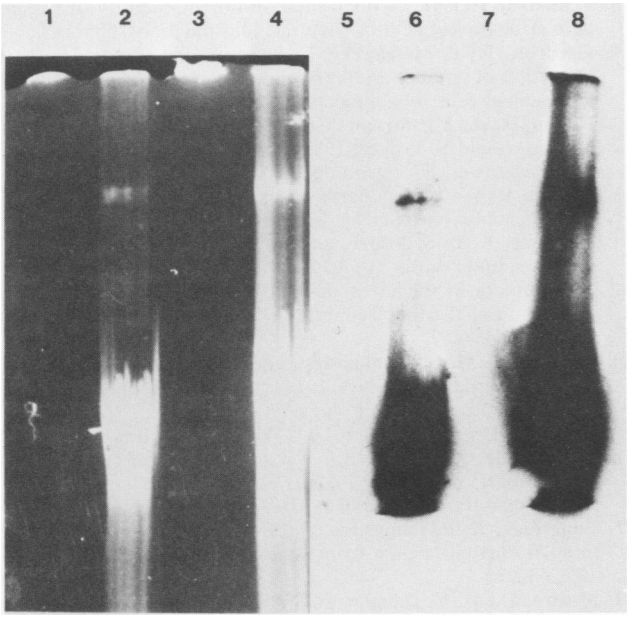

FIG. 5. Agarose gel electrophoresis and Southern blots of mock-infected cell DNA (lanes 1, 3, 5, and 7) and DNA from cells infected with VZV strain Oka (lanes 2, 4, 6, and 8). Infected or uninfected cells were lysed and separated on 0.7% agarose gels as described in Materials and Methods. The ethidium bromide-stained gels (lanes 1 to 4) were Southern blotted and probed with a mixture of ^{32}P-labeled cloned VZV DNA fragments as described in the text (lanes 5 to 8).

DISCUSSION

In this paper, we have presented data which support and extend previous observations (4, 23a, 28) that a small proportion of VZV DNA molecules possess inverted L segments or exist as covalently closed circles.

One of the observations suggesting that these forms of VZV DNA exist involved the identification of a finite population of novel restriction enzyme fragments in VZV DNA, representing both an unusual terminus and an unusual joint region. Such fragments can be generated by (i) inversion of the L region with respect to the S region, (ii) circularization of the genome, and (iii) formation of concatemeric molecules generated by head-to-tail fusion of full-length DNA. Such concatameric molecules have been shown to be present in pseudorabies virus-(1) and herpes simplex virus (HSV)-infected cells (14). However, we and others (4, 9) have shown that novel joint fragments are present in DNA from mature virions and nucleocapsids, and it seems unlikely that concatamers could be packaged into nucleocapsids due to size constraints.

The identification of both novel joint and novel end fragments in restriction endonuclease digest of VZV DNA supports the work of Davison (4), who has cloned and sequenced both novel joint and novel end fragments of VZV DNA. He has proposed that, after the formation of concatemeric VZV DNA, the DNA is packaged with package-cleavage recognition sequences at the novel U_L-U_S joint, which is predominantly recognized, and also at the normal U_L-U_S joint which is recognized less frequently. From this, it is clear that these novel restriction fragments should be found in much less than molar quantities. We find that approximately 5% of the submolar fragments corresponding to the novel joint and novel end are present in packaged VZV DNA. Thus, our data agree with the Davison hypothesis. However, the factors that determine the low frequency of recognition of one of the two proposed packaging sites are not yet clear.

Three lines of evidence suggest that VZV DNA is also able to form circular structures. Initially, we reported the presence of circular DNA molecules in preparations of VZV nucleocapsid DNA, based on electron microscopic studies (28). Such circular molecules formed only a small fraction of the total DNA, and the size of the circles seemed to be quite variable. The presence of circular molecules is now further supported by the finding of novel joint fragments in VZV DNA preparations that may be generated by circularization of the genome. The third and strongest line of evidence for the existence of circular VZV DNA forms stems from the finding of VZV DNA molecules which comigrate in agarose gels with a circular bacterial plasmid of equivalent size. Such circular molecules have been identified in both VZV-infected cells and in purified VZV nucleocapsid DNA. However, it should be emphasized that the amount of DNA in a circular configuration varies considerably from preparation to preparation of VZV DNA.

Ben-Porat and Veach (2) have proposed that the termini of pseudorabies virus circularize soon after infection. In addition, Davison and Wilkie (6) concluded that the genomic termini of HSV may ligate. Davison (4) and Mocarski and Roizman (20) have proposed that in VZV and HSV-1, respectively, a single complementary nucleotide overlap exists at each end of the DNA and thus could facilitate ligation. Rolling circle replication (a likely mechanism for herpesvirus DNA replication) requires the formation of circular DNA, and it is quite likely that circular VZV DNA is present in infected cells as a replicative intermediate (15). Circular DNA forms are also found as episomes in tumor cells after infection with lymphotropic herpesviruses (16, 25).

The presence of circular molecules in the nucleocapsids of VZV is in contrast to most findings with other herpesviruses,

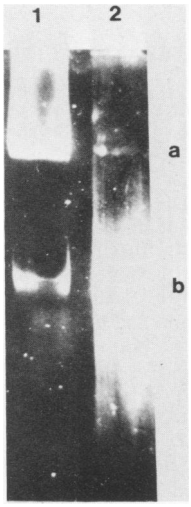

FIG. 6. Agarose gel electrophoresis of VZV and pCG86 DNAs. VZV strain Ellen nucleocapsids were purified, and DNA was extracted from them as described in Materials and Methods and electrophoresed (lane 2). pCG86 DNA was purified from *E. coli* HB101 on cesium chloride-ethidium bromide gradients and electrophoresed (lane 1). Gels were stained with ethidium bromide and photographed. Circular forms (a) and linear forms (b) are shown.

but the agarose gel analysis of VZV nucleocapsid DNA supports the initial observations by Straus et al. (28) that some circular molecules are packaged in the VZV system. It would be of some interest to determine the infectivity of VZV DNA circles, but given the awkward biological system, it would be a difficult task.

We have not as yet determined whether closed circular VZV DNA forms have covalently linked termini or whether the circle is maintained by a tightly bound protein. Electron microscopic analysis of nucleocapsid DNA suggests that some circular DNA is associated with dense proteinaceous material (W. T. Ruyechan, unpublished data). Wu et al. (31) and Hyman (13) have suggested that tightly but noncovalently bound proteins are present at the termini of HSV-1 DNA. It is possible, therefore, that circular molecules are held together by proteins that resist the disruption and denaturation steps used before electrophoresis. Indeed, if the structure of the joined termini were somewhat unstable, this might account for the wide variation seen in the quantity of circular structures observed in agarose gels and by electron microscopy.

Evidence has recently been reported which indicates that HSV virions may contain circular molecules. Poffenberger and Roizman (22) have suggested that the cleaved termini of packaged HSV-1 DNA may be rejoined, either by a linking protein or by covalent religation, to form a circle. This was based on evidence for head-to-tail terminal linkages in a novel mutant which normally does not invert either the L or S segments of HSV DNA. It seems reasonable to suggest that some VZV DNA may be packaged and circularized in a similar fashion. Further studies on the nature of the terminal linkage of VZV DNA circles and on the mechanism of circularization should shed light on the importance of these novel structures. This work is currently under way.

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