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Low-Level Inversion of the L Component of Pseudorabies Virus Is Not Dependent on Sequence Homology

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Pseudorabies virus has a class 2 genome in which the S component is found in two orientations relative to the L component. The L component is bracketed by sequences that are partially homologous; it is found mainly in one orientation, but a small proportion is inverted (J. M. DeMarchi, Z. Lu, G. Rall, S. Kuperschmidt, and T. Ben-Porat, J. Virol. 64:4968–4977, 1990). We have ascertained the role of the patchy homologous sequences bracketing the L component in its inversion. A viral mutant, vYa, from which the sequences at the right end of the L component were deleted was constructed. Despite the absence of homologous sequences bracketing the L component in vYa, its L component inverted to an extent similar to that of the L component in the wild-type virus. These results show the following. (i) The low-frequency inversion of the L component of PrV is not mediated by homologous sequences bracketing this component. (ii) Cleavage of concatemeric DNA at the internal junction between the S and L components is responsible for the appearance of the minority of genomes with an inverted L component in populations of pseudorabies virus. (iii) The signals present near or at the end of the S component are sufficient to allow low-frequency cleavage of concatemeric DNA; the sequences at the end of the L component are not essential for cleavage, although they enhance it considerably.

The genomes of herpesviruses consist of large double-stranded molecules that have been subdivided into three or five classes (8, 12). Class 2 (or D) genomes, such as, for example, pseudorabies virus (PrV) and varicella-zoster virus (VZV), consist of two components, a unique long (U_L) and a unique short component. The unique short component is bracketed by large inverted repeats, and the unique sequence bracketed by these inverted repeats inverts itself relative to the fixed U_L component. Class 3 (or E) genomes, of which herpes simplex virus is the prototype, consist of DNA in which both the U_L and unique short components are bracketed by large inverted repeats. Four equimolar forms of the genomes are present in populations of virions in which the two components are in different orientations relative to one another (8, 12).

This classification, although useful, is arbitrary. Thus, the genomes of VZV and bovine herpesvirus, class 2 DNA molecules, possess a U_L component that is flanked by a short inverted repeat (1, 5, 7, 9). The L component of PrV also is bracketed by what appears to be a short vestigial inverted repeat (3). In both VZV and PrV, a small proportion (approximately 5%) of the genomes have a U_L component that is found in an inverted orientation (1, 3).

Inversion of the L component can, in principle, occur either by recombination via the inverted homologies that bracket it or by cleavage of concatemeric DNA at the internal junction between the S and L components. In PrV, recognition signals necessary for cleavage of concatemeric DNA have been shown to be present both upstream and downstream of the cleavage site (14). A sequence with homology to the pac 1 element of herpes simplex virus is present at both ends of the inverted repeats bracketing the unique short component, and a sequence homologous to the pac 2 element of HSV is present at the left end of the U_L component (2, 5, 6). The low-frequency inversion of the L component of PrV could thus be due to low-frequency cleavage between the internal inverted repeat and the sequences adjacent to the internal inverted repeat that are partially homologous to those at the left end of the L component (3).

The internal junction of PrV can be a substrate for cleavage (11); mutants in which the sequence normally present at the left end of the genome have been translocated to the right end of the L component (next to the internal repeat of the S component) are cleaved at this position with high frequency. In the genomes of these mutants, the L component is completely invertible. The fact that the sequences at the right end of the U_L component are only partially homologous to those at the left end of the genome (3) could be the reason for the low-frequency cleavage at the internal junction, resulting in a low level of inversion of standard wild-type PrV genomes. Alternatively, the low-level inversion of the L component in wild-type PrV could be due to recombination via the patchy inverted homology bracketing it.

To ascertain whether the patchy homologous sequences bracketing the L component play a role in inversion, we constructed a mutant, vYa, from which 487 bp from the right terminus of the L component were deleted and replaced with 101 bp of unrelated (M13) sequences. The relevant features of the construction of vYa are as follows. A cloned 2.0-kbp *Bgl*I fragment which spans the junction between the S and L components was digested with *Sma*I, resulting in a deletion of 504 bp, ranging from positions −487 to +17 (Fig. 1). The

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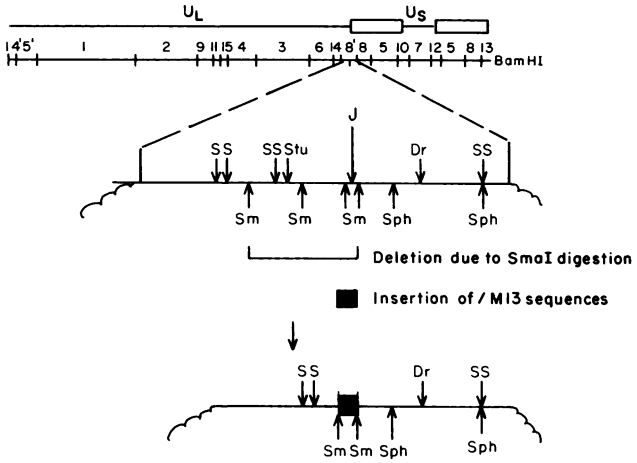

FIG. 1. Construction of plasmid pYa. The structure of the genome of PrV and the *Bam*HI restriction map are illustrated on the top two lines. The restriction map of the 2.0-kb cloned *Bgl*I fragment that spans the junction between the L and S components is shown on the third line from the top. This plasmid was digested with *Sma*I, removing 487 bp from the L and 17 bp from the S component; a 101-bp fragment of M13 DNA was inserted. The resulting plasmid, pYa, is shown on the bottom line. U_S, unique short component.

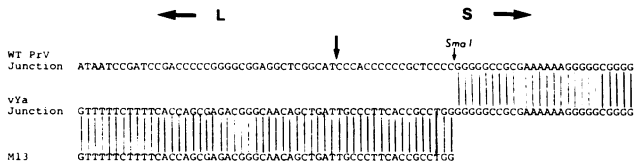

FIG. 2. Nucleotide sequence at the junction of the L and S components of vYa. The top line shows the sequence at the internal junction between the S and L components of wild-type (WT) PrV DNA. The *Sma*I site 17 bp upstream from the junction between the S and L components is indicated. In vYa, the 17 bp normally present at the ends of the inverted repeat are missing and have been replaced by M13 sequences. The M13 sequences have become part of the inverted repeat; they are present at the S terminus of the genome (11). The large downward-pointing arrow indicates the junction between the S and L components.

plasmid was religated with a *Hae*III fragment of M13 DNA (bp 5938 to 6038). The resulting plasmid, pYa, has a 17-bp deletion at the end of the internal inverted repeat and a deletion of 487 bp at the right terminus of the U_L component; it also has an insertion of 101 bp of M13 DNA.

To isolate vYa mutant virions, pYa was cotransfected with wild-type PrV DNA into RK cells by the calcium chloride precipitation method (4). Following virus-induced cell degeneration, virions were plaque assayed. Individual plaques were picked into 96-well plates in which RK cells had been grown and incubated at 37°C for 3 days. A portion of the growth medium in each well was dot blotted onto nitrocellulose filters, denatured, and probed with nick-translated M13 DNA. Once mutant plaques containing an M13 DNA insert were identified, they were plaque purified again twice. The frequency of isolation of the mutants was approximately 0.5%.

Six of the mutants (vYa), obtained from four different transfection experiments, were further characterized. They were subjected to Southern (13) analysis by using appropriate restriction enzyme fragments as probes. The results obtained indicated that all of the mutants had the expected structure; they had lost the *Stu*I site, and the sizes of the fragments generated indicated an approximately 400-bp deletion (see below and data not shown). Their internal junction fragments (the equivalent of *Bam*HI fragment 8′ of the wild-type genome [Fig. 1]) were cloned, and the region into which the modifications had been introduced was sequenced (10).

The nucleotide sequence of the junction between the S and L components of wild-type PrV and mutant vYa1 (Fig. 2) shows that the junction between the L and S components is that expected of the desired mutant; the last 17 bp of the internal inverted repeat of the S component are missing and have been replaced by the M13 sequences. Although the junction on the other side of the M13 DNA has not been sequenced, the absence of the rightmost sequences of the L component was shown by (i) the sizes of the fragments generated and (ii) the absence of *Stu*I, *Sma*I, and *Sst*II sites normally found in that region.

Since the L component of the vYa mutant is not bracketed by sequences with patchy homology (the rightmost 487 bp have been replaced by M13 DNA), this mutant was used to ascertain whether patchy homologous sequences play a role in low-level inversion of the L component of the wild-type genome. If this were the case, no inversion of the L component of the vYa genomes should be observed.

Figure 3 and Table 1 summarize the *Bam*HI, *Sal*I, and *Pst*I restriction maps and the expected sizes of the restriction fragments generated from genomes of PrV and vYa with the prototype and inverted orientations of the L component. The expected hybridization patterns to different probes of these fragments are also summarized in Table 1. The Southern blots (Fig. 4 and 5) of wild-type and vYa DNA digests show

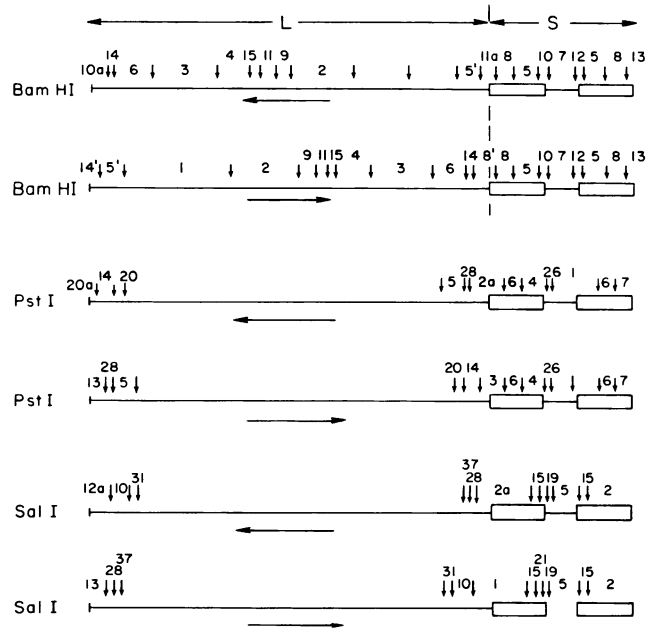

FIG. 3. Restriction maps of the PrV genome with the L component in the prototypic and inverted orientations. Open rectangles represent inverted repeats of the S component. The arrows under the L components indicate the orientations of those components. Arrows pointing to the right indicate prototype orientation, and arrows pointing to the left indicate inverted orientation.

TABLE 1. Sizes of junction and end fragments derived from the L component of the genomes of wild-type PrV(Ka) and vYa mutants

Digest and fragment	Wild type			vYa		
	Fragment designation	Size (kpb)	Fragment(s) hybridized with[a]	Fragment designation	Size (kbp)	Fragment(s) hybridized with[a]
*Bam*HI						
Prototype junction	8′	5.0	B8′, B13	8′	4.6	B8′, B13, M13
Prototype end	14′	1.4	B14′	14′	1.4	B14′
Inverted junction	11a	3.1	B8′, B13, B14′	11a	3.1	B8′, B13, B14′
Inverted end	10a	3.3	B8′	10a	2.9	B8′, M13
*Sal*I						
Prototype junction	1	10.0	B8, B13′	1	9.6	B8, B13
Prototype end	13	2.1	B13′	13	2.1	B14′
Inverted junction	2a	9.6	B8′, B13, B14′	2a	9.6	B8′, B13, B14′
Inverted end	12a	2.5	B8′	12a	2.1	B8′
*Pst*I						
Prototype junction	3	4.3	B8′, B13	3	3.9	B8′, B13
Prototype end	13	2.0	B14′	13	2.0	B14′
Inverted junction	2a	4.8	B8′, B13, B14′	2a	4.8	B8′, B13, B14′
Inverted end	20a	1.5	B8′	10a	1.1	B8′

[a] B8′, B13, and B14′ are *Bam*HI PrV DNA fragment probes; M13 is an M13 DNA probe.

that the populations of genomes obtained from both included a minority of genomes in which the L component was inverted. Thus, for example, *Bam*HI fragment 8′ should hybridize to itself as well as to *Bam*HI fragment 13 (Fig. 3). Indeed, two major bands with the appropriate sizes hybridized to the *Bam*HI fragment 8′ probe (Fig. 4). However, this probe also hybridized to two minor bands present in the *Bam*HI digests. One of the bands (fragment 11a) had the expected hybridization pattern (i.e., it hybridized to *Bam*HI fragment 13, *Bam*HI fragment 8′, and *Bam*HI fragment 14′) and the expected size (3.1 kb) of the internal junction that would be generated from mature genomes in which inversion of the L component has occurred. The other fragment, 10a, was 3.3 kb long and hybridized only to *Bam*HI fragment 8′; its hybridization pattern and size were those expected of the end fragment generated from genomes in which inversion of the L component has occurred.

The hybridization patterns of the vYa *Bam*HI digests showed that a minority of the genomes of this virion population also had an L component in the inverted orientation. The internal junction fragment (fragment 11a) generated from genomes with an inverted orientation of the L component was identical in hybridization pattern and size (3.1 kb) to that generated from wild-type genomes. However, as expected, the end fragment generated from the inverted orientation of the L component (fragment 10a) was approximately 400 bp smaller in vYa DNA than was the equivalent fragment derived from wild-type DNA; it was 2.9 kb. This was due to the deletion of approximately 500 bp from the right end of the L component and the insertion of approximately 100 bp of M13 DNA at the internal junction of the L component. Indeed, fragment 10a of vYa, as expected, hybridized to M13 as well as to *Bam*HI fragment 8′ (Fig. 4), the M13 sequences being the expected terminal sequences of the genomes with an inverted L component.

Analysis of the wild-type and vYa mutant DNAs with *Sal*I and *Pst*I (Fig. 5), as well as with *Kpn*I (data not shown), also

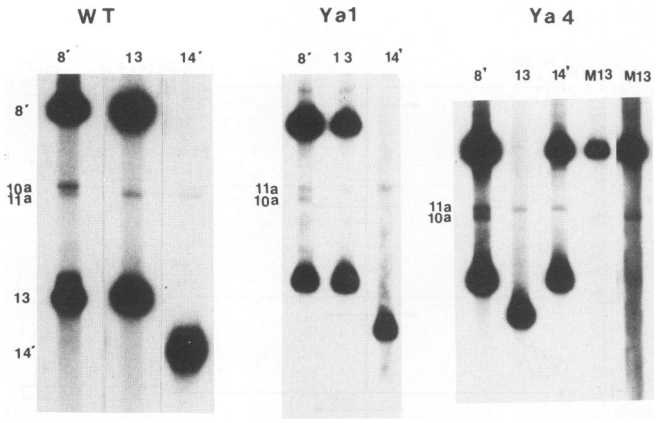

FIG. 4. Hybridization of *Bam*HI digests of wild-type (WT) and vYa DNAs. DNA was extracted from mature virions, digested with *Bam*HI, electrophoresed, and transferred to nitrocellulose filters, and strips were hybridized to the indicated nick-translated probe (PrV *Bam*HI fragments 8′, 13, and 14′ [Fig. 1 and 3 contain their maps] and M13 DNA).

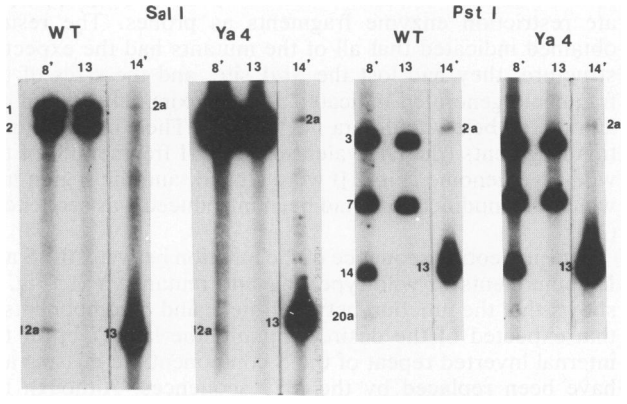

FIG. 5. Hybridization of *Sal*I and *Pst*I digests of wild-type (WT) and vYa DNAs. The experiment was performed as described in the legend to Fig. 4. The DNA was digested with *Sal*I or *Sst*I.

indicated that both populations of virions contain a minority of genomes with an inverted orientation of the L component. Faint bands with the expected sizes and hybridization patterns were observed inboth the vYa and wild-type DNA *Sal*I and *Pst*I digests (Fig. 5).

The fact that the L component in class 2 genomes inverts at low frequency has been ascribed to the presence of short (VZV) or patchy (PrV) homologies bracketing it (1, 3). As mentioned above, the low-frequency isomerization of the L component could result from its inversion via these sequence homologies or could be generated by low-frequency cleavage of concatemeric DNA at the internal junction between the L and S components. The experiments described here show that low-level inversion of the L component of the PrV genome occurs in the absence of any inverted homology between its ends; replacement of the sequences normally present at the right end of the L component (which share patchy homology with those at the left end of this component) with unrelated M13 sequences does not interfere with low-level inversion of the L component. Inversion is therefore not due to recombination between the inverted patchy homologous sequences at the ends of the L component and must result from cleavage at the internal junction between the S and L components. Davidson (1) had initially mentioned the possibility that isomerization of the L component of VZV arises by cleavage at the internal junction, and we have also favored this possibility (3).

Because the U_L component inverts at low frequency, even in the absence of the sequences that share patchy homology with the sequences at the left end of the U_L component (that are known to include *cis* signals required for cleavage [14]), low-level cleavage can occur in the absence of these sequences. Thus, even though signals with homology to pac 1 at the S terminus and signals with homology to pac 2 at the L terminus (2, 5, 6) are normally necessary for high-frequency cleavage of concatemeric PrV DNA (14), signals present only at the end of the S component are sufficient to mediate low-frequency cleavage.

Our results thus show that (i) low-frequency inversion of the L component of PrV is not mediated by homologous sequences bracketing this component, (ii) cleavage at the internal junction between the S and L components is responsible for the appearance of the minority genomes of wild-type PrV in which the L component is inverted, and (iii) signals present near or at the end of the S component are sufficient to allow low-frequency cleavage of concatemeric DNA. However, the sequences at the end of the L component, although not essential, enhance cleavage considerably.

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