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Acquisition of an Additional Internal Cleavage Site Differentially Affects the Ability of Pseudorabies Virus To Multiply in Different Host Cells

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The translocation of the 325 leftmost bp of the genome of pseudorabies virus (PrV) to the internal junction between the L and S components confers upon the virus a growth advantage relative to wild-type PrV in chicken embryo fibroblasts (CEFs) and chickens and a growth disadvantage in rabbit kidney (RK) cells and mice. To clarify the molecular basis for the species-specific growth characteristics of the translocation mutants, we have compared several parameters of the virus growth cycle in CEFs and RK cells infected with wild-type PrV and with translocation mutants. The salient findings are as follows. (i) The synthesis of early-late and late proteins is not as effective in CEFs as it is in RK cells, and these proteins, in particular, the major capsid proteins, accumulate less abundantly in CEFs than in RK cells. (ii) Cleavage of concatemeric DNA to genome-size molecules is also not as effective in CEFs as it is in RK cells. (iii) The internal junction present in translocation mutants is a functional cleavage site. (iv) In RK cells, translocation mutants are hypercleaved and a significant proportion of the total viral DNA is cleaved into subgenomic fragments. (v) In CEFs infected with translocation mutants, subgenomic fragments also accumulate but most of the viral DNA remains in concatemeric form. A model which postulates that the cell-specific growth advantage or disadvantage of the translocation mutants is related to the presence of a second cleavage site within their genomes and is affected by the efficiency of cleavage of concatemeric DNA in particular infected cell types is presented. The significance of these findings as they relate to the evolution of herpesviruses with class 2- and class 3-like genomes is discussed.

Previously, we have shown (11) that the duplication and translocation of the 325 leftmost bp of the genome of pseudorabies virus (PrV) to the internal junction between the L and S components is able to confer upon the virus a growth advantage relative to wild-type PrV in chicken embryo fibroblasts (CEFs) and chickens. These mutants, however, have a growth disadvantage relative to wild-type PrV in rabbit kidney (RK) cells and mice. Here we address the basis for the altered growth characteristics of these mutants.

To attempt to understand the molecular basis for the species-specific growth characteristics of the translocation mutants, we have compared several growth parameters of wild-type PrV and of v160, a prototype translocation mutant, in CEFs and RK cells. The results obtained have led us to speculate that the cell-specific growth advantage or disadvantage of the translocation mutants is related, at least in part, to the efficiency of cleavage of concatemeric viral DNA in these cells. The presence on the translocation mutant viral genomes of a second, alternative cleavage site provides a growth advantage or disadvantage in a given cell type, depending on the efficiency of cleavage of viral concatemeric DNA in these cells.

MATERIALS AND METHODS

With the exception of the following, the procedures as well as the cells and virus mutants used are the same as those described previously (11).

Medium and solution. EDSsAA is Eagle's medium without amino acids and plus arginine plus 5% dialyzed calf serum. SSC-Sark is 0.15 M NaCl–0.015 M sodium citrate (pH 7.2) plus 2% sodium lauryl sarkosinate.

Analysis of intracellular viral DNA. Cells were infected with the appropriate mutant and incubated at 37°C. At the indicated times the cells were harvested and DNA was extracted, digested with the appropriate restriction enzyme, and analyzed by the Southern technique (12).

Sucrose gradient analysis of DNA. RK cells or CEFs were infected (multiplicity of infection, 5 PFU per cell) and incubated in Eagle's medium. At various times postinfection, the medium was lifted and the cells were scraped in SSC-Sark, heated at 60°C for 10 min, and treated with pronase (1 mg/ml). An aliquot was layered onto a 5 to 20% neutral sucrose gradient, and the gradient was centrifuged at 12,000 rpm for 19 h in an SW27 rotor (Beckman) as described previously (1). Fractions were collected, dialyzed, digested with *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and probed.

Polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis procedures have been described previously (4).

RESULTS

Translocation mutants have a growth advantage in CEFs and a growth disadvantage in RK cells relative to wild-type virus (11). To understand the basis for the species-specific growth characteristics of the mutants, we have attempted to ascertain what differences exist between the growth characteristics of wild-type virus in CEFs and in RK cells.

PrV grows to a higher titer in RK cells than in CEFs.

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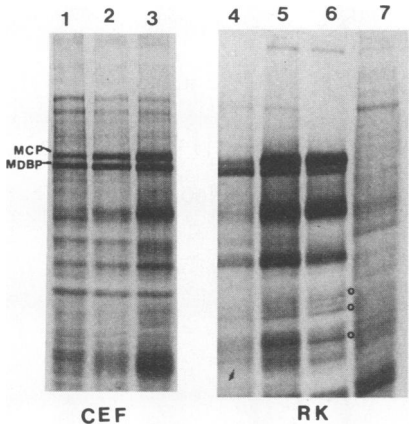

FIG. 1. Viral protein synthesis in RK cells and CEFs. Cell monolayers (10^6 cells) were infected (5 PFU per cell) with wild-type PrV(Ka) and incubated in Eagle's medium at 37°C. At various times thereafter the medium was removed, replaced with EDSsAA containing [^{3}H]leucine (50 μCi/ml). The cultures were further incubated for 60 min, when the cells were harvested and their proteins were electrophoresed. Lanes 1 to 3: CEFs; lanes 4 to 6, RK cells; lane 7, uninfected cells. Lanes 1 and 4, cells labeled between 2 and 3 h postinfection; lanes 2 and 5, cells labeled between 4 and 5 h postinfection; lanes 3 and 6, cells labeled between 6 and 7 h postinfection. The gels were exposed for 72 h. MCP (an early-late protein) and MDBP (an early protein) are indicated. Other minor late proteins are indicated with open circles.

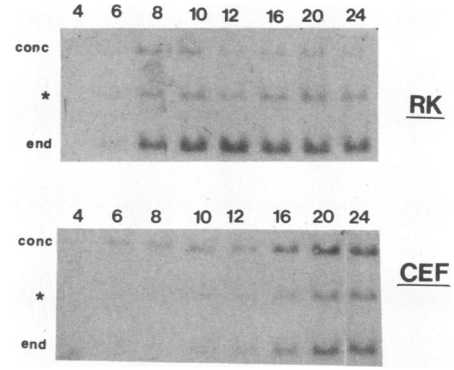

FIG. 2. Synthesis of viral DNA and cleavage of concatemeric viral DNA in infected RK cells and CEFs. Cell monolayers (10^6 cells) were infected (5 PFU per cell) with wild-type PrV(Ka). At the indicated times (in hours) after infection, the cells were harvested and total DNA was extracted. The DNA was digested with *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and probed with nick-translated *Bam*HI fragment 14′. End, *Bam*HI fragment 14′ originating from the left end of the genome (see Fig. 3 for map); conc, the concatemeric junction fragment consisting of the two end fragments (*Bam*HI fragments 13 and 14′) joined together. This band is present in the infected cells but not mature virus. (The starred bands are found in intracellular DNA and hybridize to *Bam*HI fragment 14′ only. They represent defective DNA and are not pertinent to the data discussed here.) The filter with RK cell DNA was exposed for 24 h. The filter with CEF DNA was exposed for 48 h.

While in RK cells the titer of infectious virus at the end of the growth cycle is between 200 and 1,000 PFU per cell (depending upon the experiment), the titer obtained from CEFs is consistently lower and is only between 20 and 100 PFU per cell (8). To attempt to identify the factors that may contribute to the better growth of PrV in RK cells than in CEFs, two parameters of the infectious cycle were monitored: (i) viral protein synthesis—to ascertain whether the accumulation of early or late gene products occurs similarly in both cell types—and (ii) viral DNA synthesis and the efficiency of viral DNA concatemer resolution.

Synthesis of viral proteins in RK cells and CEFs. To ascertain whether viral protein synthesis differs in CEFs and RK cells, cell monolayers were infected with wild-type PrV and at various times after infection were incubated for 1 h in medium containing [^{3}H]leucine. The cells were harvested, and the proteins were electrophoresed on acrylamide gels (Fig. 1). Inspection of the results of this and other experiments showed that while early proteins were synthesized similarly, early-late and late protein synthesis was not as efficient in CEFs as in RK cells. This observation is best illustrated by focusing on two abundantly synthesized viral proteins: the 136,000-molecular-weight (136K) major DNA-binding protein (MDBP)—an early protein—and the 142K major capsid protein (MCP)—an early-late protein (2).

At 2 h postinfection the pattern and the amounts of the viral proteins synthesized in CEFs and RK cells were similar (Fig. 1, compare lanes 1 and 4); in both, MDBP was more abundant than MCP. In RK cells, by 4 and 6 h postinfection (lanes 5 and 6), the MCP was much more abundant than the MDBP. The MCP is an early-late protein and is expressed at early stages of infection, but its expression is considerably increased during the late phase. The abundant expression of the MCP thus is an indication of the transition from early- to late-phase protein synthesis. This transition has occurred rapidly and efficiently in RK cells. In infected CEFs, the transition from early to late protein synthesis was considerably less efficient. The relative amounts of MDBP to MCP synthesized remained approximately the same throughout the experiment; at no time after infection (up to 24 h; data not shown) was the MCP synthesized as efficiently in CEFs as it was in RK cells. Furthermore, the MDBP continued to be expressed abundantly in CEFs even at late stages of infection. A comparison of the relative synthesis of minor, less prominent, early and late viral proteins in CEFs and RK cells is somewhat difficult because of the large number of viral proteins involved and because of the background of continued synthesis of cellular proteins. Some late proteins are indicated in Fig. 1. Differences in their synthesis in RK cells and CEFs could be observed. Furthermore, an analysis of the samples on gels with different concentrations of acrylamide confirmed that late proteins were not synthesized as abundantly in CEFs as in RK cells (data not shown). No differences in the patterns of protein synthesis between the wild type and translocation mutants were observed in either RK cells or CEFs.

Kinetics of accumulation of viral DNA and cleavage of concatemeric DNA in RK cells and CEFs. Monolayers of primary RK cells and CEFs were infected with wild-type PrV. At various times after infection, the cells were harvested and DNA was purified, digested with *Bam*HI, and hybridized with nick-translated *Bam*HI fragment 14′ (Fig. 2). *Bam*HI fragment 14′ is derived from the left genome terminus (see Fig. 3). Hybridization of *Bam*HI digests of mature PrV DNA with nick-translated *Bam*HI fragment 14′ generates a signal corresponding to a single band, i.e., *Bam*HI fragment 14′. Digestion of intracellular viral DNA with *Bam*HI generates an additional band that hybridizes to *Bam*HI fragment 14′. This band consists of the two end fragments of mature DNA juxtaposed and covalently linked and is generated from concatemeric DNA. The band derived

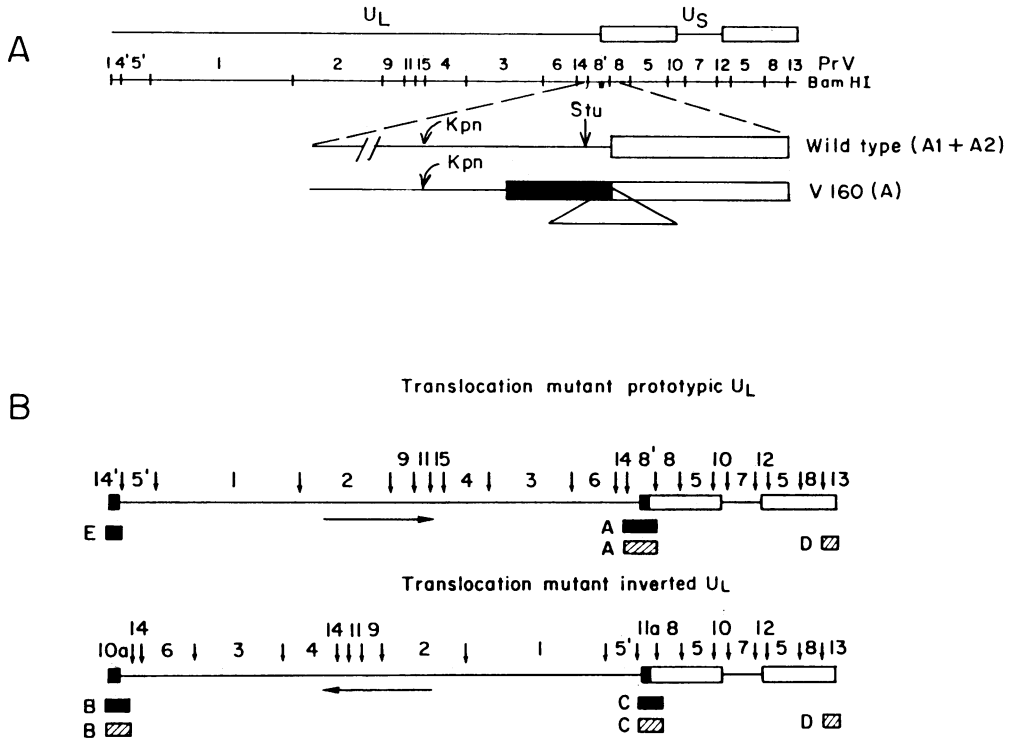

FIG. 3. *Bam*HI restriction maps of the wild-type and v160 genomes. (A) The top two lines show the structure and the *Bam*HI restriction map of the wild-type PrV genome. Open rectangles indicate sequences of the inverted repeat bracketing the S component. The third line shows an enlargement of the internal junction fragment of wild-type DNA; the *Stu*I site (Stu) is indicated. The fourth line shows the internal junction fragment of v160 DNA. The deletion in this DNA which removes the *Stu*I site is indicated. The translocated sequences derived from the left end of the unique long sequence (U_L) are indicated by black rectangles. Kpn, *Kpn*I. (B) The black rectangles under the restriction maps indicate the restriction fragments generated from v160 that hybridized to the sequence derived from the left end of the viral genome, *Bam*HI fragment 14', or the leftmost 600 bp of the genome. The striped rectangles denote the fragments that will hybridize to the sequences of the internal junction fragment of the wild-type genome, *Bam*HI fragment 8'.

from concatemeric DNA represents a much larger proportion of the total DNA in CEFs than in RK cells (Fig. 2). Even at the end of the growth cycle (24 h postinfection), a large part of the viral DNA in CEFs remains in concatemeric form. Similar results were also obtained when the gels were probed with *Bam*HI fragment 13, i.e., with the sequences derived from the S terminus of the genome (Fig. 3); the concatemeric junction fragment remained prominent in CEFs even at late stages of infection (data not shown).

Cleavage of concatemeric DNA was quantitated by scanning appropriately exposed films, and the relative amounts of DNA in the concatemeric and free terminal bands that hybridized to the sequences either at the left terminus (*Bam*HI fragment 14') or the right terminus (*Bam*HI fragment 13) were quantitated. In RK cells, as early as 6 h postinfection, 67% of the total DNA in the infected RK cells (concatemeric plus free terminus) was present as free termini; by 10 h postinfection, more than 90% was in the form of free termini. In CEFs, cleavage of concatemeric DNA into mature genomes was considerably delayed. Even at 24 h postinfection, only approximately 50% of the total viral DNA was in the form of cleaved mature genomes. Thus, cleavage of concatemeric DNA occurred at a faster rate and proceeded much more efficiently in RK cells than in CEFs. Furthermore, the amount of viral DNA that accumulated in RK cells was approximately two to three times higher than in CEFs; although the filters in Fig. 2 were probed with the same PrV probe, the filters with DNA obtained from infected CEFs were exposed twice as long as those with DNA from infected RK cells.

The modified growth characteristics of the translocation mutants are not mediated by *trans* functions. The experiments described above show that PrV grows poorly in CEFs. We have shown previously that the translocation next to the inverted repeat of a segment of DNA derived from the left end of the L component confers upon the mutant a growth advantage over wild-type virus in CEFs and a growth disadvantage in RK cells (11). The experiments described below were designed to elucidate the basis for the cell-specific growth characteristics of the translocation mutants.

We first ascertained whether the expression of a *trans* function may be at the root of the modified growth characteristics of the translocation mutants. The nucleotide sequence of the translocated fragment of DNA as well as that spanning the junctions between the translocated DNA and the adjacent regions was examined. While in some strains an open reading frame crosses the junction between the S and L components of concatemeric DNA, in others this is not the case (3). Furthermore, in none of the strains have we been able to detect a transcript that spans this junction (data not shown). The lack of detectable transcripts spanning the junction does not, however, preclude the possibility that the presence of the junction sequences somehow affects the transcription of adjacent (or even remote) viral genes, an

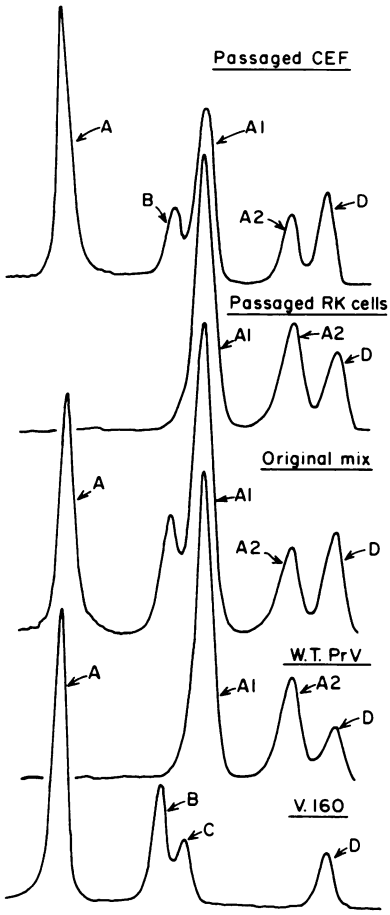

FIG. 4. Passage at high multiplicity of a mixture of wild-type PrV and v160 in CEFs and RK cells. RK cells or CEFs were infected with a mixture of wild-type (W.T.) PrV and v160 at a multiplicity of 6 PFU per cell each. After cell degeneration, the progeny virions were used to inoculate fresh cultures at a multiplicity of 4 to 6 PFU per cell. The process was repeated eight times. The initial virus population (prior to passage) as well as the virus population after the passaging regimen was purified, and viral DNA was extracted, digested with *Bam*HI and *Stu*I, electrophoresed, transferred to nitrocellulose filters, and hybridized to a probe consisting of nick-translated *Bam*HI fragment 8′. Appropriately exposed films were scanned.

effect that could be responsible for the altered growth characteristics of the translocation mutants. To test this possibility and to ascertain whether *cis* functions may be responsible for the growth characteristics of the translocation mutants, a mixture of wild-type PrV and v160 was passaged at high multiplicity several times in CEFs and in RK cells. The enrichment or loss of the junction fragment diagnostic of v160 or of the wild type was ascertained by analyzing the DNA in the virus population prior to and after virus passage (Fig. 4).

The internal junctions of wild-type PrV and v160 DNA can be differentiated from each other because wild-type PrV DNA has a *Stu*I site in *Bam*HI junction fragment 8′ while the *Stu*I site has been deleted from v160 (11) (Fig. 3). Thus, digestion of wild-type DNA with *Bam*HI and *Stu*I yields two fragments derived from *Bam*HI fragment 8′ (A1 and A2), while similarly digested v160 yields a single fragment (A). A comparison of the DNA in the initial virus mix with DNA obtained from virions produced by RK cells shows a marked decrease in the amount of the v160 junction fragment (peak A) relative to that of wild-type junction fragments (peaks A1 and A2), indicating that DNA with the junction fragment characteristics of v160 has a growth disadvantage in RK cells (Fig. 4). This decrease is marked even after a single cycle of growth of the virus mixture in RK cells (data not shown). On the other hand, the junction fragment characteristic of v160 was enriched for after passage in CEFs. The enrichment of peak A over peak A1 observed after passage in CEFs was slow but became significant after several passages at high multiplicity (Fig. 4). These results show that even at a high multiplicity of infection, when wild-type and v160 viruses can, in principle, provide helper functions to each other, the junction fragment of v160 was lost from RK cells and was enriched for in CEFs. Thus, it appears that it is the acquisition of a *cis* function that must lend the viruses their altered growth characteristics.

Identification of the *cis* functions responsible for the modified growth characteristics of translocation mutants. The terminal 325 bp, the translocation of which is sufficient to provide the virus with a growth advantage in CEFs and a growth disadvantage in RK cells (11), are known to include two *cis* functions: (i) signals that may function as an origin of replication (5, 13) and (ii) sequences that are required for efficient cleavage of concatemeric DNA into unit-length genomes. The unique ends of the mature PrV genomes become covalently linked in concatemeric DNA, and signals derived from both ends are required for cleavage (13). Because in all naturally occurring class 3-like PrV mutants that have been analyzed, the translocation directly abuts the internal repeat (9), the nucleotide sequence at the newly formed junction duplicates the sequence that is normally found at the concatemeric junction; i.e., an additional cleavage signal has been created at the internal junction between the S and L components.

In the next set of experiments, we determined whether *cis* functions involved either in DNA replication or in cleavage of concatemeric DNA could be responsible for the altered growth characteristics of the mutants.

(i) The genomes of translocation mutants do not have a replicative advantage over wild-type virus genomes. To ascertain whether the translocation confers upon PrV an advantage at the level of DNA replication, a mixture of an approximately equal number of wild-type and mutant v160 virions was made. Part of this virus mixture was used to infect either RK cells or CEF monolayers. After adsorption, the monolayers were washed to remove unadsorbed input virus and incubated for various times, and then the cells were harvested and the DNA was extracted. DNA was also purified from the original virus mixture used to infect the cultures. The DNA was digested with *Bam*HI and *Stu*I and analyzed by Southern blotting with *Bam*HI fragment 8′ as a probe. The results of a representative experiment are shown in Fig. 5.

A comparison of the ratio of the DNA bands obtained from DNA in the original virus mixture with the DNA obtained from cells at various times after infection reveals that the additional origin in v160 provided no detectable replicative advantage. This is best ascertained by comparing the relative intensities of representative internal bands derived from wild-type PrV (Fig. 5, closed circles) and v160 (open circles) DNA. (End fragments will be underrepresented in intracellular DNA because at least some of the DNA is in concatemeric form.) Densitometric scans of the Southern blots revealed no increase in the intensity of the

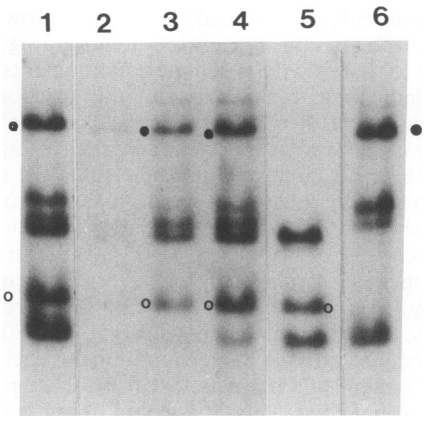

FIG. 5. The DNA of translocation mutants does not have a replicative advantage. CEFs were infected with a mixture of wild-type PrV and v160 virions (approximately 3 PFU per cell each). At various times after infection, the cells were harvested and DNA was extracted, digested with *Bam*HI and *Stu*I, electrophoresed, and transferred to nitrocellulose filters. The filters were hybridized to nick-translated *Bam*HI fragment 8′. (*Stu*I cleaves once within the *Bam*HI fragment 8′ of the wild-type PrV genome; this cleavage site has been deleted from v160 DNA.) Lane 1, original mixture of virions used to infect the cells; lane 2, DNA obtained from cells at 4 h postinfection; lane 3, DNA obtained from cells at 5 h postinfection; lane 4, DNA obtained from cells at 6 h postinfection; lane 5, wild-type PrV DNA; lane 6, DNA from v160. The ratio of wild-type DNA to v160 DNA can be estimated from the ratios of internal DNA bands generated from each virus (the end fragments are absent from intracellular concatemeric DNA). Closed circles, representative internal band characteristic of v160; open circles, representative internal band characteristic of wild-type PrV; see Fig. 3 for maps.

bands derived from v160 DNA relative to the intensity of those derived from wild-type DNA. The experiment illustrated in Fig. 5 was performed using CEFs; identical results were also obtained with RK cells. Thus, the additional putative origin of replication present at the internal junction of the genome of mutant v160 does not measurably affect DNA synthesis and therefore does not appear to be the reason for the altered host cell-specific growth characteristics of the mutant.

(ii) The internal junction between the S and L components of v160 can function as a cleavage site. The experiments described in this section were designed to ascertain whether the additional putative cleavage site created by the juxtaposition in the genome of mature virions of the translocated end fragment with the internal inverted repeat is functional. This experiment was based on the premise that if the second cleavage site at the internal junction is functional, cleavage at this position as well as at the normal concatemeric junction should generate some fragments corresponding to a free L component (90 kbp) and a free S component (45 kbp) as well as genomic-length (135-kbp) DNA.

To ascertain whether such subgenomic fragments could be detected in v160-infected cells but not in wild-type-infected cells, intracellular viral DNA was harvested at various times postinfection and centrifuged on a neutral sucrose gradient. The positions in the gradient to which the 135-kbp (genome-size), 90-kbp (L-component), and 45-kbp (S-component) molecules sediment were ascertained in parallel gradients by using appropriate size markers. (Mutant vTN155 [11] contains a unique *Eco*RI site at the junction between the S and L components; *Eco*RI-digested and undigested vTN155 DNA served as size markers for the 135-, 90-, and 45-kbp molecules.) To ensure the identity of the DNA present in positions in the gradient occupied by the 45- and 90-kbp molecules, the DNA in all of the fractions recovered from the gradients was digested with *Bam*HI and analyzed by Southern blotting, using a mixture of probes consisting of *Bam*HI fragments 4 and 7 (see Fig. 6 for a map). *Bam*HI fragment 4 originates from the middle of the unique long sequence and should hybridize to the intact genome (135 kbp) as well as to the L subgenomic component (90 kbp); *Bam*HI fragment 7 originates from the unique short sequence and should hybridize to the intact genome (135 kbp) as well as to the S subgenomic component (45 kbp).

Figure 6 shows the results of a representative experiment in which intracellular DNA from RK cells infected with v160 or wild-type PrV was analyzed. Under the experimental conditions used, mature virus DNA sediments around fractions 30 to 33, the 45-kbp fragment sediments in fractions 22 to 24, and the 90-kbp fragment sediments in fractions 27 to 29. The *Bam*HI fragment 7 probe, which originates from the S component, hybridized more strongly than did the *Bam*HI fragment 4 probe to fractions 22 to 24 of the gradient containing v160 DNA. The *Bam*HI fragment 4 probe, which originates from the L component, hybridized more strongly than the *Bam*HI fragment 7 probe to fractions 27 to 29 of that gradient (Fig. 6A). Both probes hybridized equally to genome-size molecules (fractions 30 to 33). Some hybridization of both probes was observed throughout the gradient; this is probably the result of nonspecific breakage of DNA. The differential hybridization of the probes to DNA present in the gradient in regions to which the 45- and 90-kbp DNA molecules migrate indicates the presence of subgenomic fragments, corresponding to a free S component and a free L component, in cells infected with mutant v160. This was corroborated by hybridization of the appropriate fractions with other probes originating from the S and L components of the genome (data not shown). In the gradient in which DNA obtained from wild-type-infected cells was run, all fractions hybridized with approximately the same intensity to both probes (Fig. 6B), indicating that, as expected, during wild-type PrV infection subgenomic fragments corresponding to the S and L components were not formed in sufficient amounts to be detected.

The DNA obtained from purified (extracellular) v160 virions was similarly analyzed. Subgenomic 45- and 90-kbp fragments could not be detected. Thus, subgenomic fragments of the v160 genome, although generated in the infected cells, are not packaged into virions.

The amounts of concatemeric DNA (i.e., DNA sedimenting to the bottom of the gradient) of 135-kbp (genome-size), 90-kbp (L-component), and 45-kbp (S-component) DNA present in v160-infected RK cells and CEFs was quantitated after scanning of the Southern blots. These data provide a rough estimate of the relative amounts of different-size molecules present in the cells at different times after infection (Table 1). The proportion of total viral DNA that was cleaved (i.e., was not in concatemeric form) was, as expected, considerably lower in CEFs than in RK cells. The ratio of free S and L components to intact genomes present in CEFs and RK cells infected with v160 was approximately the same. Thus, in both CEFs and RK cells, some of the viral DNA accumulating in cells infected with a translocation mutant is cleaved at both the normal concatemeric junction and at the internal junction between the S and L components, thereby generating free S and L subgenomic components.

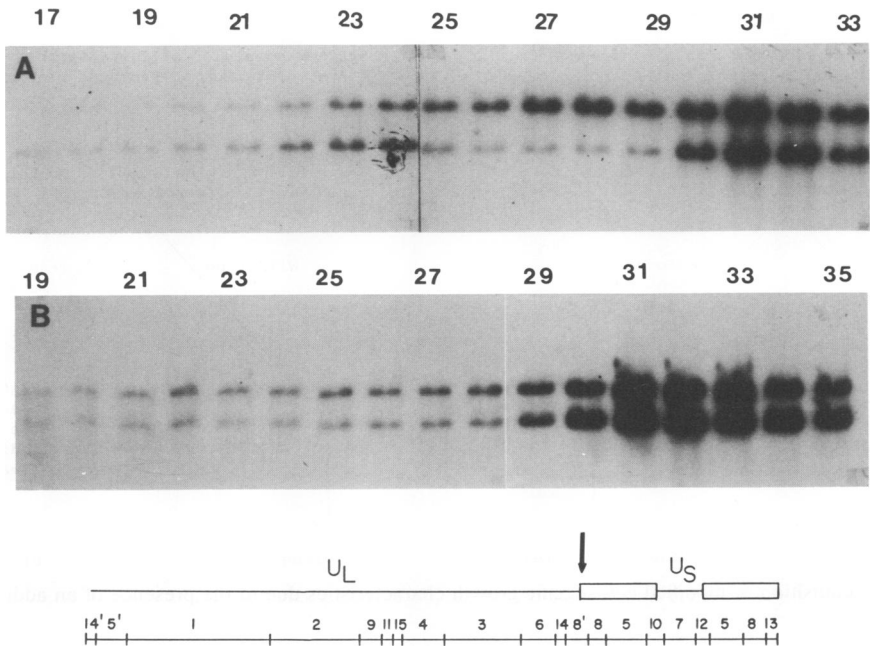

FIG. 6. Sucrose gradient analysis of DNA obtained from cells infected with v160 or wild-type virus. RK cells were infected (5 PFU per cell) with either v160 (A) or wild-type virus (B). At 16 h postinfection, the cells were harvested, lysed with sodium dodecyl sulfate, heated at 60°C for 15 min, pronase treated, and sedimented on a 5 to 20% neutral sucrose gradient. Fractions (0.5 ml) were collected, dialyzed, digested with *Bam*HI, electrophoresed, and hybridized to a probe consisting of a mixture of *Bam*HI fragments 4 and 7. (Bottom) *Bam*HI restriction map of PrV DNA. The vertical arrow indicates the position of the putative internal cleavage site in v160. U_L and U_S, unique long and short sequences, respectively.

To further corroborate these findings, the temporal appearance of free ends in RK cells and in CEFs infected with mutant v160 was determined. Figure 7 shows the pattern of hybridization of DNA obtained from the cells at various times after infection to a probe that consists entirely of sequences that have been translocated next to the internal inverted repeat (the leftmost 600 bp of the genome). In mature v160 DNA (lane V), four bands (A, B, C, and E) hybridized approximately equally to the probe. Two of these bands (A and E) originate from virions with a prototype orientation of the unique long sequence, and two bands (B and C) originate from virions with an inverted orientation of the unique long sequence (Fig. 3). At 8 h postinfection in RK cells, the internal junction fragments (bands A and C) and terminal fragments (bands B and E) were represented approximately equimolarly, indicating that junction and end fragments were present in approximately equal amounts in the cells at that time after infection. (The starred bands

TABLE 1. Estimate of the amounts of different-size molecules of viral DNA present in infected cells[a]

Cell type and time (h)	% of total DNA			
	135 kbp (unit size)	90 kbp (L component)	45 kbp (S component)	Concatemeric
RK				
6	50	14	11	25
12	73	14	8	5
18	75	11	9	5
CEF				
6	13	4	3	80
18	45	5	4	46

[a] RK cells or CEFs were infected with v160 (5 PFU per cell). At the indicated times, the cells were lysed with sodium dodecyl sulfate, heated to 60°C, and digested with pronase and aliquots were layered on neutral sucrose gradients, as detailed in Materials and Methods and in the legend to Fig. 6. Films exposed for different lengths of time were scanned, and the relative amounts of DNA in the different fractions of the gradient were quantitated. The DNA sedimenting to the bottom of the gradient was concatemeric DNA, as indicated by the considerable underrepresentation of end fragments.

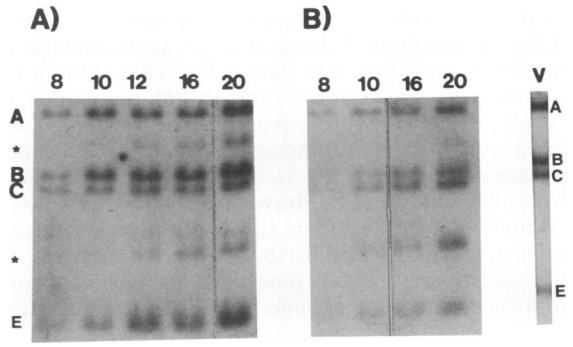

FIG. 7. Cleavage of v160 DNA in RK cells and CEFs. RK cells (A) and CEFs (B) were infected (5 PFU per cell) with v160 virions. At the indicated times, the cells were harvested and the DNA was extracted, digested with *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and hybridized to a nick-translated probe consisting of the 600 leftmost bp of the wild-type viral genome. Lane V, v160 virion DNA. Bands A and C are junction fragments. Bands B and D are terminal fragments (Fig. 3). The times (in hours) after infection at which the cells were harvested are indicated at the top of the gels.

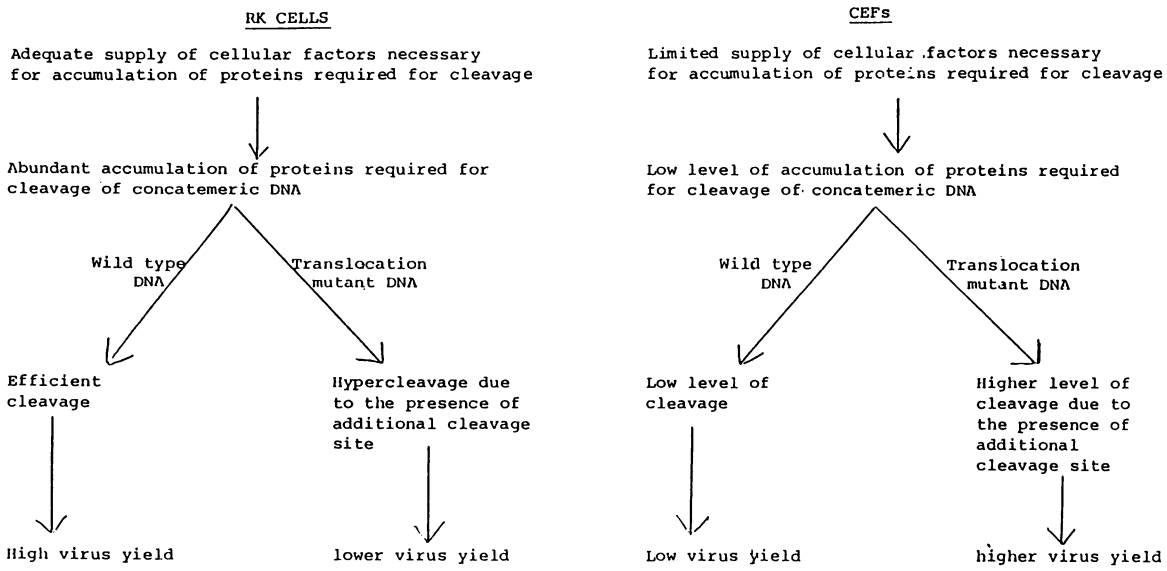

FIG. 8. Model for acquisition of modified cell-specific growth characteristics due to the presence of an additional cleavage site.

correspond to a defective product. They consist of head-to-head junctions of the ends of the L component [10]; their presence is not relevant here). By 10 h postinfection, as well as at later times, the relative amount of the end fragments exceeded that of the junction fragments. Since in mature-genome-size DNA the four bands are present in equimolar amounts but in infected RK cells the end fragments became more abundant than the junction fragments, some of the concatemeric DNA must have been cleaved at both the standard and the alternative cleavage sites, generating free S and L components. The fact that both end fragments generated from either orientation of the L component accumulated similarly indicates that both cleavage sites, i.e., the normal concatemeric junction and the alternative junction created by the translocation, were used with equal frequency.

In CEFs, the relative intensity of hybridization of the internal junction fragments (bands A and C) in all cases exceeded that of the terminal fragments (bands B and E), indicating that v160 concatemeric DNA was cleaved poorly in CEFs. As mentioned above (Fig. 2), concatemeric resolution of wild-type PrV also occurs less efficiently in CEFs than in RK cells. Two other translocation mutants—v173/3 and v160/2.5X (11)—were similarly analyzed. The results obtained were identical to those shown in Fig. 7 for mutant v160 (data not shown).

Two conclusions can be drawn from the results given in Fig. 6 and 7 and in Table 1: (i) cleavage of concatemeric DNA is poor in CEFs, and (ii) the alternative cleavage site in the translocation mutants is functional and is used, resulting in the formation of subgenomic free S and L components.

DISCUSSION

The experiments presented in this paper were designed to identify the molecular basis for the growth advantage in CEFs and disadvantage in RK cells of translocation mutants relative to wild-type virus. The results show the following. (i) Early-late and late protein synthesis (as exemplified by the accumulation of the major capsid protein) is inefficient in CEFs compared with that in RK cells. (ii) Resolution of concatemeric viral DNA is also poor in CEFs. (iii) The cell-specific growth characteristics of the translocation mutants are determined by *cis* functions present at the modified internal junction fragment of the translocation mutants. (iv) The growth advantage or disadvantage of the translocation mutants is not related to the duplication of the origin of replication. (v) The additional internal cleavage-encapsidation site present in the translocation mutants is functional.

A model depicting how the presence of an additional functional cleavage site in the translocation mutants may provide them with a growth advantage in CEFs and a growth disadvantage in RK cells is illustrated in Fig. 8. A paucity of cellular factors required for the accumulation of the appropriate viral gene products in CEFs (or the presence of inhibitory factors) results in an impaired synthesis of the early-late and late gene products that are responsible for concatemeric cleavage (7), as exemplified by the major capsid protein. Their reduced accumulation in the infected cells may limit cleavage of concatemeric DNA and its encapsidation. Alternatively, cellular factors may directly affect concatemeric cleavage rather than doing so indirectly by affecting the expression of viral products that mediate cleavage. Whatever the case may be, under conditions in which factors required for cleavage-encapsidation are limiting the probability of a cleavage event occurring may be increased with an increasing number of cleavage sites; concatemeric DNA containing two functional cleavage sites per unit-size molecule would be more likely to generate unit-length molecules and generate infectious virus than would virions containing only one such site. However, under conditions in which the cellular factors required for late viral gene expression (or directly required for cleavage) are not limiting and cleavage occurs efficiently, such as in RK cells, the presence of two cleavage sites per genome-size molecule would confer a growth disadvantage. In this case, the concatemeric DNA would be cleaved frequently at both sites, resulting in subgenomic fragments and a lower yield of infectious virus. Thus, the model predicts that the more efficient the cleavage of concatemeric DNA in a particular cell type is, the greater is the growth disadvantage of translocation mutants relative to wild-type virus. On the

other hand, the less efficient the cleavage of concatemeric DNA in a given cell type is, the greater is the growth advantage of translocation mutants over wild-type virus in these cells.

Interestingly, a mutant, vLD68, which is similar in structure to translocation mutant v173/3 but from which 68 bp spanning the internal junction (64 bp from the L component and 4 bp from the S component) have been deleted does not exhibit the host cell-specific growth characteristics of translocation mutants. This mutant behaves like wild-type virus in terms of its cell-specific growth characteristics (11); it also is not cleaved at the internal alternate cleavage site (6). Thus, the cell-specific growth characteristics of the translocation mutants and the presence of a functional cleavage site at the alternative junction created by the translocation appear to be linked.

It is to be noted that cleavage at the internal alternative site or at the standard concatemeric junction of translocation concatemeric DNA generates genomes in which the L component is in different orientations relative to the S component. Thus, the inversion of the L component in the translocation mutants could result from cleavage at the internal alternative cleavage site. Evidence that this is indeed the case has been obtained (6). This is not to say that inversion of the S component of PrV occurs by a similar mechanism; alternative cleavage cannot account for the inversion of that component in the wild-type PrV genome.

The findings described in this paper may have some bearing on the evolution of herpesviruses with class 2 genomes (in which only the S component is bracketed by inverted repeats and inverts, generating two isomeric forms) to class 3 genomes (in which both the S and L components are bracketed by inverted repeats and invert, generating four isomeric forms). It has been postulated previously (9) that class 3-like PrV molecules arise by a rather infrequent double-crossover recombinational event between two inversely oriented concatemeric class 2 molecules. In cells in which the cellular factors affecting the efficiency of cleavage are abundant (such as RK cells), the resulting mutants would be eliminated from the virus population because of their growth disadvantage in these cells. On the other hand, these mutants would be selected for in cells in which cleavage of concatemeric DNA is a limiting event, such as, for example, in CEFs; they would have a growth advantage in these cells. That gene expression of different herpesviruses occurs to a different extent in different cell lines has been widely documented. It is therefore plausible that the emergence of viral populations with type 2 and type 3 genomes is an adaptation to the prevailing conditions in a given host or target tissue and that it may be related, in some cases at least, to the number of cleavage-encapsidation sites per genome.

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