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Genomic Termini of Equine Herpesvirus 1

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After cell infection with the equine herpesvirus 1 (EHV-1), the termini of the linear double-stranded DNA genome fuse to form circular forms. To investigate the mechanisms in the generation and cleavage of such replicative-form DNAs, the genomic termini, the fusion of termini from replicative-form molecules, and the junction between the short and long genome segments have been analyzed by restriction mapping, blot hybridizations, cloning, and sequencing. The data suggest that the genome ends are not redundant and that the genomic termini are fused in replicative intermediates via 3′ single-base extensions at the termini of the unique long segment (U_L) and terminal repeat (T_R). Adjacent to the EHV-1 termini are AT and γ sequence elements highly conserved among different herpesviruses. We propose that both of these sequence elements are important for the cleavage of EHV-1 replicative forms.

Equine herpesvirus 1 (EHV-1) is a significant viral pathogen of horses. Virus strains grouped as EHV-1 by biological markers and molecular biological means are associated with abortion, respiratory tract infection, and neurological disease of horses (1, 2, 6, 21, 22, 28). On the basis of molecular biological and serological properties, EHV-1 has also been found to be associated with disease conditions in bovine animals (8).

EHV-1 is an alphaherpesvirus with a linear double-stranded DNA genome of about 150 kilobase pairs (kbp) which is usually divided in different segments (see Fig. 1): a long unique segment, U_L (120 kbp); an internal repeat, I_R (10.5 kbp); a unique short segment, U_S (11 kbp); and a terminal repeat, T_R (10.5 kbp). The U_S segment is bracketed by the two inversely oriented repeats I_R and T_R. U_S is also found in either possible orientation relative to the fixed U_L segment. As a consequence, virus DNA consists of an equimolar mixture of the two isomeric forms (P and I in Fig. 1) (4, 14, 25, 34). The same genome arrangement and isomerization are also reported for bovine herpesvirus 1 (BHV-1) (11), EHV-3 (3, 29), pseudorabies virus (27), and varicella-zoster virus (9).

After infection of susceptible cells, the linear viral genomes of herpesviruses form circular molecules via ligation of the genomic ends. Later in infection the circular genome acts as a template during DNA replication, which has been postulated to occur by a rolling-circle mechanism (24). DNA replication leads to an accumulation of concatemeric viral DNA molecules which are made up of head-to-tail linked units of newly replicated virus DNA. Cleavage of these concatemers at the fused termini by an unidentified endonuclease activity provides unit-length linear virion DNAs which are concomitantly packaged during virus maturation.

All herpesviruses studied to date replicate their DNAs by the same pathways, which require fusion and subsequent cleavage of the termini of virion DNA. Those herpesvirus genomes which contain large, inversely oriented repeats also undergo genomic isomerization. Depending on the number of inversely oriented repeats, virion DNAs consist of either two (e.g., EHV-1) or four (e.g., herpes simplex virus) isomeric forms. We have focused on the relative simplicity of herpesvirus genomes which occur in only two isomeric forms. Here we report on the nucleotide sequence analysis of the genomic termini of EHV-1 virion DNA, the fusion of the termini to replicative intermediates during virion DNA replication, and a related internal junction (between U_L and I_R). The comparison of these DNA sequences indicates that the endonuclease activity which cleaves concatemeric replicative intermediates probably acts on two distinct sites, the cleavage and the recognition sites. Whereas the recognition site seems to be conserved in all fused herpesvirus termini studied to date, the cleavage site is not. The characterization of these *cis*-acting elements could help to identify the still-hypothetical viral endonuclease activity which plays a major role in herpesvirus DNA processing.

MATERIALS AND METHODS

Cells and virus strains. The EHV-1 reference strain Austria IV was isolated from the aborted fetus of a Lipizzaner horse (7) and has been used for cloning experiments. For comparison, two field isolates have been used. Army 183 is a respiratory isolate from a horse and is documented as EHV-1 (32). Strain 136/B is a bovine fetal isolate of EHV-1 (8). Viruses were propagated in equine dermal (ED) cells, as reported previously (6).

Isolation of viral DNA. DNA was prepared from supernatant virus by using sodium dodecyl sulfate and proteinase K lysis, phenol-chloroform extraction, and ethanol precipitation, as described elsewhere (7). Viral DNA from infected-cell pellets was isolated 18 h after infection (multiplicity of infection, 1). Cell pellets suspended in TE buffer (10 mM Tris hydrochloride, 0.1 mM EDTA, pH 8.3) were lysed with sodium dodecyl sulfate and proteinase K, and the DNA was isolated as previously described (6).

Origin and construction of recombinant clones. The construction of recombinant clones containing EHV-1 genomic terminal fragments has been reported previously (8). Briefly, whole-virion DNA was made blunt by being treated with the large fragment of *Escherichia coli* DNA polymerase in the presence of the four deoxyribonucleotide phosphates. 5′-Phosphorylated dodecamer *Bam*HI linkers (5′-CGCGGAT CCGCG-3′) were ligated to the blunt-ended virion DNA, and

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the ligation products were cut with *Bam*HI. The *Bam*HI left-terminal fragment T (2.25 kbp) and the *Sal*I-*Bam*HI right-terminal fragment S (1.45 kbp) (see Fig. 1) were isolated on preparative agarose gels and cloned into a pUC vector. The 6.45-kbp *Bam*HI replicative-form (Rf) DNA fragment containing the fusion of the left and right genomic termini (RfI) as well as a 3.8-kbp *Bam*HI fragment (RfII) carrying the head-to-head fused left termini (see Fig. 1) were cloned from the intracellular viral DNA. Virus-infected-cell DNA was cleaved with *Bam*HI. The sizes of the *Bam*HI fragments carrying the fusion of the genomic termini were determined by Southern blotting, and the appropriate fragments recovered from a preparative agarose gel were cloned into a pUC vector (for the RFII *Bam*HI fragment) or into λL47 (for the RfI *Bam*HI fragment). The *Bam*HI E fragment (9.8 kbp) spanning the junction between U_L and I_R (34) was cloned from the digest of virion DNA into λL47. Recombinant lambda clones were identified by plaque hybridization with appropriate radioactively labeled DNA probes. Recombinant lambda DNA was prepared from a large-scale culture of bacteriophage propagated in *E. coli* WL66 (15).

Blot hybridization. DNA restriction fragments separated on agarose gels were transferred (26) to GeneScreen nylon membranes (New England Nuclear Corp.). Nick-translated DNA probes were prepared by using [^{32}P]dCTP or [^{35}S]dCTP by the method of Rigby et al. (23).

DNA sequencing. Recombinant clone DNAs were digested with appropriate enzymes and dephosphorylated. The restriction fragments were labeled at the 5′ termini by phosphate transfer from [^{32}P]ATP (Amersham Corp.), catalyzed by T4 polynucleotide kinase (Stehlin AG), onto dephosphorylated DNA fragments. The end-labeled fragments were digested with a second restriction enzyme and were sequenced by the chemical degradation technique, as described by Maxam and Gilbert (17, 18).

RESULTS

Cloning and characterization of the genomic termini. The genomic termini of whole-virus DNA were made blunt and ligated to dodecamer *Bam*HI linkers. This method generated *Bam*HI recognition sites at both ends. The *Bam*HI T fragment (2.25 kbp; left terminus) and a 1.45-kbp subfragment (*Bam*HI-*Sal*I) of the *Bam*HI S fragment (4.2 kbp) enclosing the right terminus were cloned into the appropriate pUC8 and pUC19 sites, respectively. Physical maps were deduced from representative clones (T108 and S110) and are depicted in part in Fig. 1.

Analysis and cloning of fusion fragments in replicative-form DNA. To determine the arrangement of EHV-1 replicative viral DNA, virion DNA and infected-cell DNAs were cleaved with restriction enzymes (*Bam*HI, *Bgl*II, and *Eco*RI). Blots containing electrophoretically separated DNA restriction fragments were analyzed by hybridization with labeled plasmid DNA containing the sequence specific for the left (clone T108) and the right (clone S110) genomic termini (Fig. 1). When probed with the plasmid clone containing the left genomic terminus, one band (the *Bam*HI T fragment) was detected in *Bam*HI-cleaved virion DNA (Fig. 2A, lane 1). Two additional bands were detected with this probe in infected-cell DNA preparations (Fig. 2A, lanes 2 and 3). The fragment designated RfI (6.45 kbp) is explainable by fusion of the left (T fragment, 2.25-kbp)- and the right (S fragment, 4.2-kbp)-terminal *Bam*HI fragments. This finding was supported by probing similar Southern blots with the cloned right terminus, which detected a fragment of the same size (RfI in Fig. 3A, lanes 1 and 2). The predicted head-to-tail fusion between the two termini as it occurs in herpesvirus replicative intermediates could be confirmed by restriction enzyme mapping of the *Bam*HI RfI fragment cloned in λL47. A 2.25-kbp *Sst*I-*Hin*dIII fragment was found to encompass the fusion T_R/U_L, and this fragment was later subcloned in a pUC vector to give RfI-116 (Fig. 1). Two bands were detected in viral DNA digested with *Bam*HI when the Southern blots were probed with a plasmid carrying the right terminus (Fig. 3A). Due to nucleotide sequence redundancy (Fig. 1), this radioactive probe hybridizes to the right-terminal *Bam*HI fragment (S) as well as the *Bam*HI fragment E (9.8 kbp) carrying the junction between I_R and the right end of U_L (Fig. 3A, lane 1).

The unexpected fragment (3.8 kbp) designated RfII (Fig. 2A, lanes 2 and 3), which was detected with the left-terminal DNA fragment as a probe in infected-cell DNA only, could be characterized by molecular cloning and physical mapping as a head-to-head arrangement of two left termini. No other EHV-1 sequences are part of the *Bam*HI RfII fragment (Fig. 2B). This head-to-head arrangement of the two U_L DNAs seems to occur as frequently as the head-to-tail fusion (RfI) between U_L and T_R (Fig. 2A, lanes 2 and 3). In contrast to the size of U_L/T_R fusion fragment RfI, fragment RfII (3.8 kbp) is approximately 700 bp smaller, as shown by physical mapping of viral and molecularly cloned DNA, than the expected simple fusion of two complete left-terminal T fragments (4.5 kbp) (Fig. 1). The head-to-head arrangement in RfII has partially eliminated sequences between two inverted 87-bp repeats (see below) at the distal ends of U_L, indicating that this configuration has originated from a recombination event rather than from a fusion between two left termini of EHV-1 genomic DNA. This head-to-head arrangement was prevalent in all infected-cell DNAs of different EHV-1 reference strains as well as in EHV-1 field isolates, including an EHV-1 strain of bovine origin (Fig. 3B). Since a corresponding tail-to-tail arrangement which would involve two right termini could never be detected (data not shown), the significance of this head-to-head arrangement of the left-terminal fragments in replicative-intermediate-form DNA remains uncertain, and it is not clear whether these intermediates are aberrant products or contribute to progeny viral DNA.

Analysis of internal junction fragment U_L/I_R. The U_S segment of EHV-1 is bracketed by two large repeats (T_R and I_R) which are inversely oriented with respect to each other (Fig. 1). Since we wanted to compare the fusion of the right end of T_R and the left end of U_L (RfI clone) with the analogous situation at the internal junction of the left end of I_R and the right end of U_L (Fig. 1), we cloned this internal junction fragment as the *Bam*HI E fragment into λL47 (Fig. 1). By comparing a physical map of the *Bam*HI S fragment with that of the U_L/I_R-spanning *Bam*HI fragment, we were able to identify a *Sst*I-*Sst*I fragment (2.6 kbp) to harbor the U_L/I_R junction (clone E113; Fig. 1).

Nucleotide sequence analysis. Relevant parts of the recombinant DNA clones harboring the left (clone T108) and the right (clone S110) genomic termini, junction U_L/I_R (clone E113), and fusion U_L/T_R (clone RfI-116) were sequenced on both strands. The sequence analysis of the left and right termini and junction fragment U_L/I_R revealed the presence of multiple sets of tandemly oriented repeated sequences. We recognized 13 copies of a 17-bp (5′-AGGCCACGC CCACTGGG-3′) tandemly repeated sequence and 15 copies of a 15-bp (5′-TGGGGCTCACTGCTA-3′) tandemly repeated sequence within 1 kbp of the left end of EHV-1 DNA

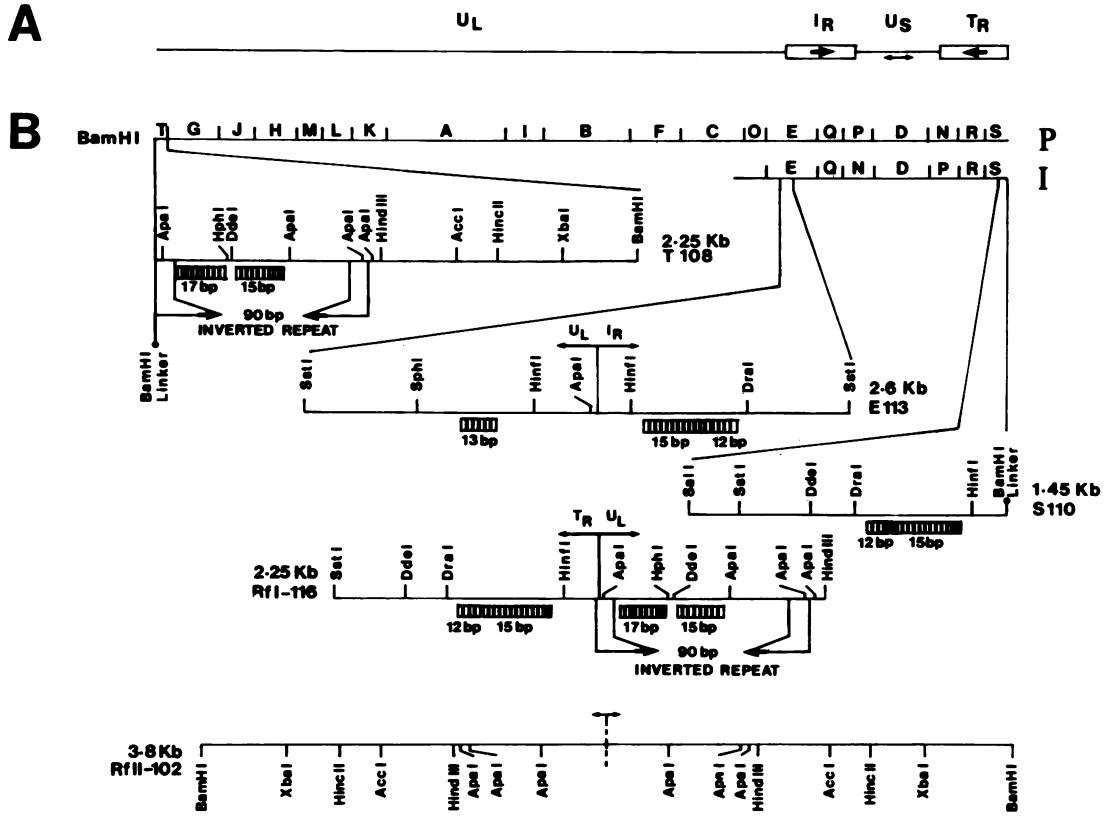

FIG. 1. (A) Genomic structure and physical map of EHV-1 DNA, showing the cleavage sites for *Bam*HI (34). The genome of EHV-1 consists of the unique long segment U_L; an internal repeat, I_R; the unique short segment U_S; and a terminal repeat, T_R. I_R and T_R are inverted relative to each other, bracketing U_S. U_S itself occurs in two different orientations (↔) relative to U_L, I_R, and T_R, giving rise to two equimolar genomic isomers (22). P and I represent the two isomeric genomic arrangements. The orientation of U_L is fixed relative to those of I_R and T_R. Letters above the depicted EHV-1 genome represent *Bam*HI cleavage products. (B) Relevant restriction enzyme sites used for generating plasmid clones and the maps of (i) the left U_L terminus (plasmid clone T108), (ii) the right T_R terminus (plasmid clone S110), (iii) the U_L/I_R junction of the right end of U_L and the left end of T_R (plasmid clone E113; only a *Sst*I-*Sst*I fragment is shown covering the junction), (iv) the RfI fusion of the left and right genomic termini (plasmid clone RfI-116), and (v) the head-to-head arranged (RfII) left genomic termini (plasmid clone RfII-102) are shown. The localization of tandemly repeated sequences in the U_L and T_R termini and the 90-bp inversely repeated sequences in the U_L terminal fragments are delineated. The points of the fusion and junction in RfI-116 and E113 are indicated (↔). The assumed axis of mirror image symmetry in RfII-102 is shown (⋮).

(Fig. 1 and 4). In the DNA sequence of the right terminus (Fig. 5), we found two sets of tandemly repeated elements. These are 22 copies of a 15-bp (5′-TTCCTGCTCCTCCCC-3′) repeat and 10 copies of a 12-bp (5′-TTGATGGGCGGG-3′) repeat. In addition, an 18-bp sequence (5′-CCCCTTCC GGTGACGTCA-3′) at positions 557 to 574 and 605 to 622 is repeated twice, close to the right genomic end (Fig. 5). Another array of tandemly arranged direct repeats (13 copies of a 13-bp repeat [5′-AGCCCCCTGCCCT-3′]) is present at the right end of U_L, close to the U_L/I_R junction (see Fig. 7). Comparison with known repeat sequences of other herpesviruses showed considerable homology of the 15-bp repeat located at the right terminus of EHV-1 to a 14-bp repeated sequence at the left terminus in BHV-1 virion DNA. This repeated sequence has counterparts in herpes simplex virus type 1 and other herpesviruses (11).

The comparison of the nucleotide sequences from the right and left termini showed no sequence redundancy (Fig. 4 and 5). By juxtapositioning the sequences of the genomic termini and comparing them with the sequences across the fusion point between U_L and T_R (Fig. 6), it became evident that a single base pair was missing at the tips of the cloned left and right genomic termini. This finding is consistent with previous reports (9, 12, 19, 30) indicating that due to the cloning procedure a single-base extension at both 3′ ends of the genomic termini was removed as a result of the 3′- to 5′-exonuclease activity of the DNA polymerase large fragment. This enzyme was used as described before in order to blunt the ends of EHV-1 virus DNA before *Bam*HI linker was attached (see Materials and Methods). This base pair is retained in the fusion fragment (Fig. 6), and therefore it was conclusive that the 3′ single-base extension is a cytosine at the left genomic terminus and a guanosine at the right genomic terminus. This was taken into account for Fig. 4 and 5. It is therefore likely that the genomic termini of EHV-1 virion DNA fuse via the complementary 3′ single-base extensions to form replicative intermediates. Consequently, replicated EHV-1 concatemeric DNA is cleaved by an endonuclease activity at the fusion U_L/T_R, giving rise to these 3′ single-base extensions at the termini in order to generate virion DNA molecules.

By comparing the nucleotide sequences at the fusion of the termini (U_L/T_R) with the junction (U_L/I_R), it became evident that these two sequences were identical except for their inverse orientations (Fig. 6 and 7). Part of this sequence identity was expected, since the EHV-1 genome carries two

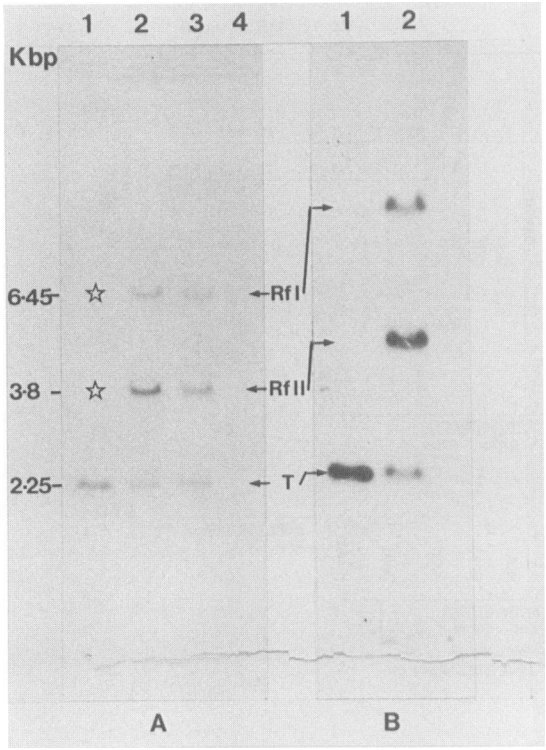

FIG. 2. Detection and analysis of replicative intermediate fragments of EHV-1 DNA by blot hybridization. (A) Autoradiographic image of a Southern blot after hybridization with the ^{35}S-labeled plasmid clone T108 DNA carrying the left genomic terminus of EHV-1. Lanes: 1, EHV-1 reference strain Austria IV (virion DNA extracted from purified virion from the cell culture supernatant); 2, whole-cell DNA of ED cells infected with EHV-1 reference strain Austria IV; 3, whole-cell DNA of ED cells infected with EHV-1 strain Army 183; 4, mock-infected-cell DNA. All DNAs were cut with *Bam*HI. Fragment T (2.25 kbp) refers to the left-terminal *Bam*HI fragment. *Bam*HI restriction fragments RfI (6.45 kbp) and RfII (3.8 kbp) detected in restricted cellular DNA but not in virion DNA (☆) refer to the fusion of left and right genomic terminal fragments (RfI, *Bam*HI T [2.25 kbp] and S [4.2 kbp] fragments) and to a head-to-head arrangement of two left genomic terminal fragments (RfII), respectively. (B) Autoradiographic image of the Southern blot after hybridization with the plasmid RfII-102 DNA carrying the cloned *Bam*HI RfII (3.8 kbp) fragment. Lanes 1 and 2 correspond to those in panel A. The detected *Bam*HI fragments T, RfI, and RfII are the same as in panel A, indicating that RfII is composed of nucleotide sequences which are present solely at the left-terminal *Bam*HI fragment T and replicative intermediates thereof.

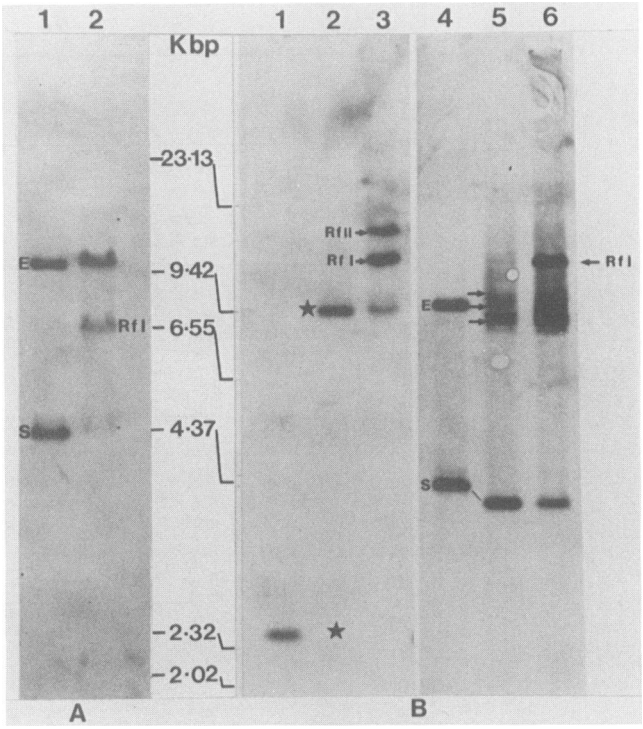

FIG. 3. (A) Detection and analysis of EHV-1 replicative-intermediate fragment (RfI) by blot hybridization with cloned DNA sequences specific for the right genomic terminus. An autoradiographic image of a Southern blot after hybridization with plasmid clone S110 DNA probe carrying the right genomic terminus of EHV-1 is shown. Lanes: 1, EHV-1 reference strain Austria IV virion DNA; 2, Austria IV-infected whole-ED-cell DNA. The DNAs were cut with *Bam*HI. Fragment S (4.2 kbp) refers to the right-terminal *Bam*HI fragment. Fragment E (9.8 kbp) is located at the U_L/I_R junction (Fig. 1). Hybridization of *Bam*HI fragments E and S with the right genomic terminus as a probe is due to identical sequences present in both I_R and T_R. The RfI fragment carrying the fusion of the left and right genomic termini is present only in infected-cell DNA. Note the absence of a RfII replicative intermediate as it was detected in Fig. 2A and B. (B) Identification and analysis of the terminal fragments and replicative-intermediate DNA in a ruminant EHV-1 isolate, 136/B. Lanes: 1 and 4, Austria IV virion DNA; 2 and 5, 136/B virion DNA; 3 and 6; total cell DNA from 136/B-infected ED cells. Lanes 1 to 3 were probed with clone T108 DNA specific for the left genomic terminus, as for Fig. 2A. Lanes 4 to 6 were probed with clone S110 DNA specific for the right genomic terminus, as in panel A. Strain 136/B is a ruminant isolate and has been characterized as EHV-1. Its *Bam*HI left-terminal fragment (lane 2) is of a larger molecular size (9.7 kbp) than the left-terminal *Bam*HI fragment in the EHV-1 reference strain (lane 1) (8). Both are indicated (★). As a consequence, replicative-intermediate DNAs RfI and RfII are of larger molecular sizes than those in Fig. 2. Note that RfII replicative-intermediate DNA is detected only with a probe specific for the left genomic terminus (compare lanes 3 and 6). The *Bam*HI junction I_R/U_L is heterogeneous in 136/B (lanes 5 and 6, arrows) compared with the *Bam*HI E fragment of the Austria IV strain (lane 4).

large inversely oriented repeats (T_R and I_R). The left end of I_R is identical to the sequence of the right end of T_R, and these ends are part of the U_L/I_R junction and the U_L/T_R fusion, respectively. However, the sequence identity extends across the fusion and junction and comprises a stretch of 32.5 bp at the left and right ends of U_L. In analogy to a very similar situation in BHV-1 (12), we have designated this sequence as a β element, and its boundaries are shown in Fig. 4 to 8 (the inverse orientation of β is called β′ in Fig. 4 and 7). Unexpectedly, the nucleotide sequence analysis of the left terminus revealed an additional β element in inverse orientation proximal to the 17- and 15-bp repeat array (at positions 969 to 1003; Fig. 4). This internal β element is part of a 90-bp duplication of the left terminus (87.5 bp) which extends 2.5 bp into the right genomic terminus (Fig. 4 and 6). Thus, the EHV-1 genome carries three β elements, and their arrangement with respect to other reiterated and nonreiterated parts can be simplified (β′ strands for an inversely oriented β element) as follows:

(32.5 bp) β-U_L-β′(35 bp)-U_L-β′(35 bp)-I_R-U_S-T_R-β(2.5 bp).

As mentioned above, the β element is based on an operational definition describing a stretch of nucleotide sequence

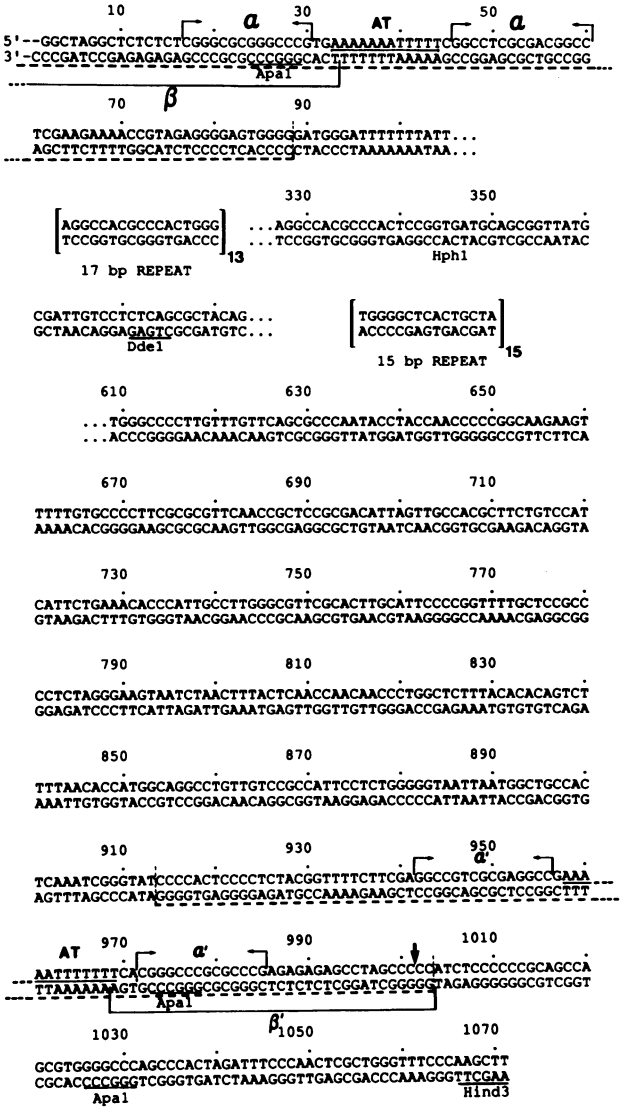

FIG. 4. Nucleotide sequence of the distal part of plasmid clone T108, encompassing the left genomic terminus. Sequences are given starting from the left genomic terminus to the most distal *Hin*dIII site. Several restriction sites are indicated for comparison with Fig. 1. The 3′ single-base extension at the natural left end of the genome is deduced from the fusion of both genomic termini. Two inversely repeated 90-bp sequence elements (----) are present at the left end of the EHV-1 genome (the left terminus contains 87.5 bp; 2.5 bp is on the right terminus) and ~1 kbp proximal in the vicinity of the *Hin*dIII site. The At stretch is shown. The designated sequence elements β and β′ (inverted orientation) are marked. The potential alternative cleavage site at the internal β element is deduced (Fig. 8) and is marked (↓). Two palindromic sequences (α and α′ [inverted orientation]) and two tandem repeat arrays are indicated.

identity which reaches beyond the sequence identity of T_R and I_R. In addition, the β element makes up the potential cleavage site for the endonuclease activity. This activity mediates the cleavage of head-to-tail fused concatemeric herpesvirus DNA molecules. The β element at the fusion of T_R/U_L encompasses such a functional cleavage site. Although all three β elements in the EHV-1 genome are identical, the endonuclease activity cleaves only within the β element spanning the T_R/U_L fusion. The two internal β

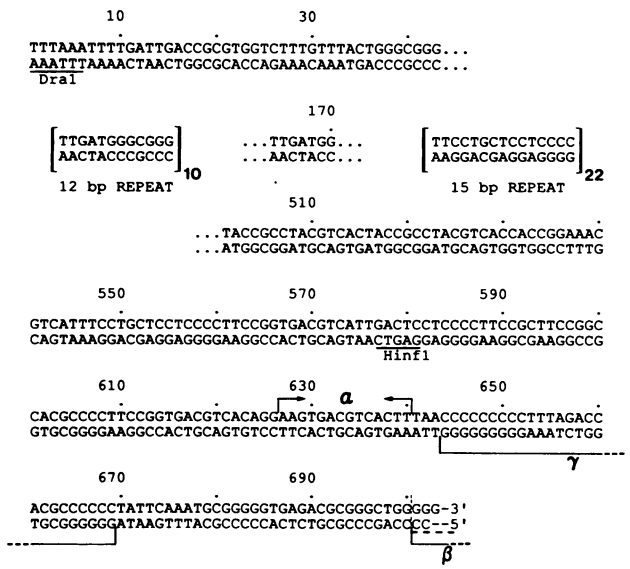

FIG. 5. Nucleotide sequence of the distal part of the right genomic terminus of EHV-1, represented by plasmid clone S110. The sequence is given starting from the most distal *Dra*I site extending to the right end (Fig. 1). The remaining 2.5 bp of the β element and the conserved γ sequence element are indicated and refer to Fig. 8. The repeat arrays and α sequence elements are indicated.

elements, present ~1 kbp proximal to the left genomic terminus in U_L and at the U_L/I_R junction, are not cleaved. Were these two β elements to be cleaved, two events should be noticeable: (i) cleavage within the β element carrying the U_L/I_R junction would yield to virion DNA molecules having

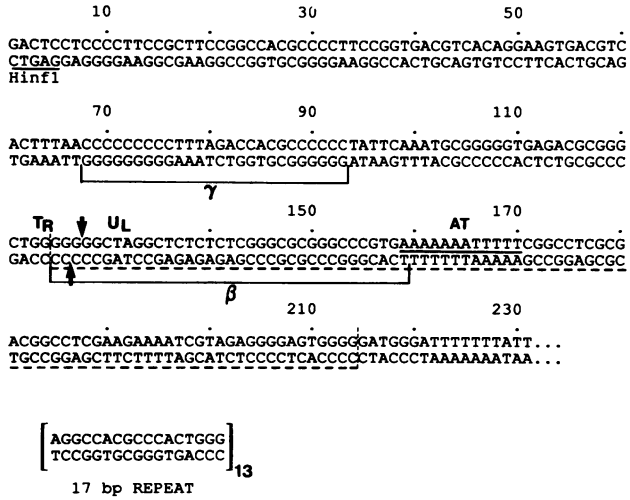

FIG. 6. Nucleotide sequence of the fusion of left and right genomic termini occurring in replicative-form DNA (RfI) of EHV-1, as represented by plasmid clone RfI-116. Sequences are given starting from the *Hin*fI site of the T_R terminus (Fig. 1) to the U_L terminus. The fusion between T_R and the left end of the U_L segment is indicated, showing the cleavage site (arrows). The cleavage site is flanked by the conserved sequences reported in the terminal nucleotide sequences of different virus genomes (10, 12), and the U_L and T_R parts are marked as in Fig. 4 and 5, respectively. The 90-bp sequence element (----) is indicated as described in the legend to Fig. 4.

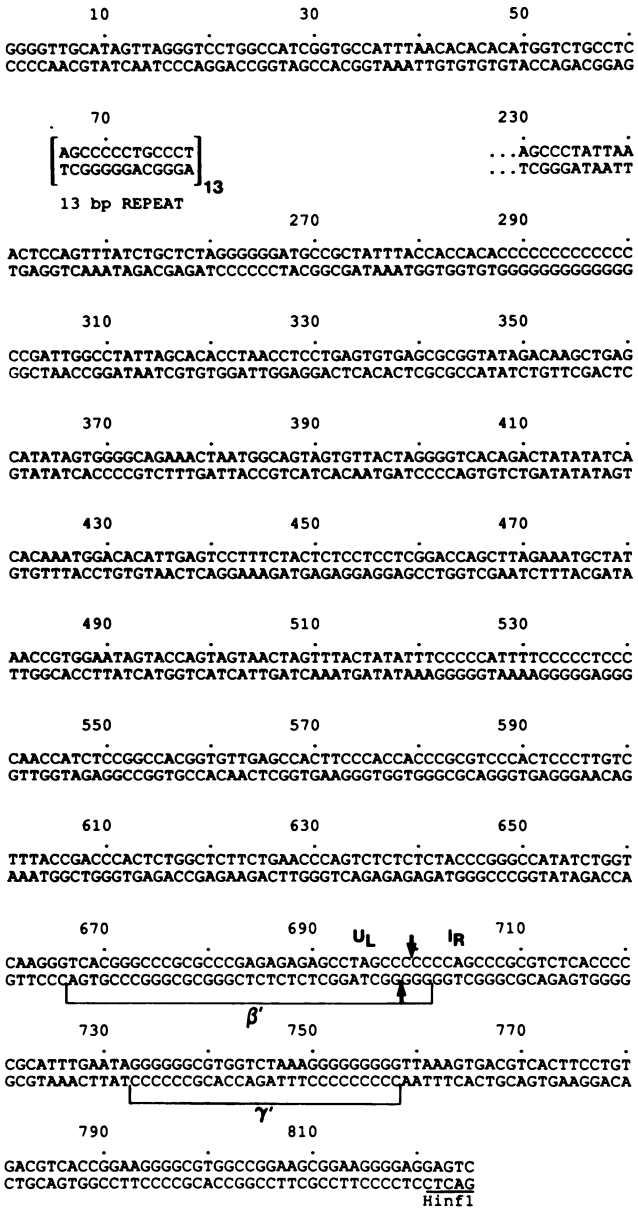

FIG. 7. Nucleotide sequence of the junction between the left end of I_R and the right end of U_L in EHV-1, as represented by plasmid clone E113. The sequence is given starting from the right end of U_L extending through the junction to the *Hin*dIII site in I_R. The junction point (arrows) is deduced by similarity with the fusion sequence shown in Fig. 6. The β′ element, which describes the nucleotide sequence identity, arising from T_R (or I_R) into the left (or right) end(s) of U_L, respectively, and the γ′ elements are indicated. The 13-bp tandem repeats are marked.

U_L inversely oriented with respect to I_R, and (ii) cleavage at the β element located ~1 kbp proximal to the left terminus would yield to virion DNA molecules with shortened (or elongated) left- (or right-) terminal restriction fragments. Neither event was detected in Southern blot hybridizations. Thus, the specificity of endonuclease activity is likely to be provided by the β element plus adjacent sequences present at the fusion of U_L/T_R but absent adjacent to the two internal β elements. Since T_R and I_R consist of reiterated sequences present at the fusion and junction of EHV-1 replicative-form DNA, the unique sequences present only at the left end of U_L are likely candidates for providing the specificity of endonuclease activity at the U_L/T_R fusion (Fig. 8). A poly(AT) stretch (A_nT_n) is located proximal to the β element at the left genomic terminus. This A_nT_n stretch is likely to be such a candidate, since such an A_nT_n stretch is missing at the right end of U_L (Fig. 8). Further support for the A_nT_n stretch being part of the recognition site stems from the observation that an A_n stretch or an A_nT_n stretch is found at the same position in almost all herpesvirus genomes (10, 12, 16).

This hypothesis seems to be invalid, considering the situation at the internal β element located ~1 kbp distal from the left terminus. Since this β element is part of an inversely oriented 90-bp duplication of the left genomic terminus (Fig. 1 and 4), the poly(AT) stretch (A_nT_n) is present right next to this internal β element. However, this β element is not cleaved during DNA processing. This finding indicates that the cleavage specificity provided by the β element plus an A_nT_n stretch alone is not sufficient but, rather, other sequences adjacent to this arrangement and unique to fusion U_L/T_R must be present. The nucleotide sequence comparison of the three β elements (Fig. 8) and the adjacent sequences revealed that the cleavage specificity within a β element might be mediated by an A_nT_n stretch close to the left genomic terminus in conjunction with sequences present at the right genomic terminus.

DISCUSSION

In this communication, we report the structure of the EHV-1 genomic termini and the fusion that occurs upon joining the termini in the replicative-concatemeric forms. Mocarski and Roizman (19) first presented evidence that the termini of herpes simplex virus type 1 each contain a single-base 3′ extension complementing each other to produce fused ends (circular or concatemeric forms) during replication. Subsequent studies have suggested similar extensions in the DNA termini of varicella-zoster virus (9), human cytomegalovirus (31), and BHV-1 (12). Recent reports on murine cytomegalovirus (16) also indicated the possibility of similar terminal structures. For pseudorabies virus, a blunt-ended L terminus and a 2-bp overhang at the S terminus have been reported (13).

EHV-1 virion DNA probably contains 3′ single-base extensions at both termini which are complementary, indicating that EHV-1 virion DNA could circularize by base pairing after infection of cells. This form of head-to-tail fusion found in replicative-form DNA of herpesviruses ensures the replication of the viral termini as well as the replication of the herpesvirus genome. DNA replication of herpesviruses is thought to occur via a rolling-circle mechanism, using the circular replicative-form DNA as a template. This mode of DNA replication yields large concatemers consisting of head-to-tail arranged virion DNA units. Mature virion DNA is generated by base-precise cleavage at the point of fusion of two ends. The cutting event seems to be a prerequisite for packaging (19). In order to identify terminal structures that are functionally essential for cleavage of herpesvirus replicative-form DNA, we focused on the structural analysis of simply arranged herpesvirus genomes such as those of BHV-1 (12) and EHV-1. The cleavage event in such type D herpesviruses (24) generates termini with unique terminal nucleotide sequences. It is conceivable that (i) the cleavage site (the β element in EHV-1) is recognized and cleaved by a virally encoded endonuclease and (ii) the nucleotide sequences at and adjacent to the cleavage site mediate enzy-

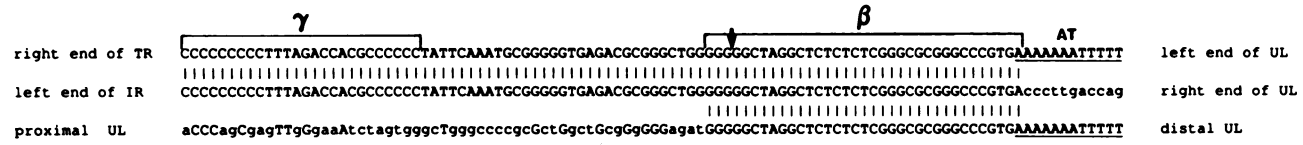

FIG. 8. Nucleotide sequence comparison of three β elements and the adjacent sequences present at the T_R/U_L fusion (line 1), the U_L/I_R junction (line 2), the left end of U_L (line 3). Identical nucleotides are capitalized and indicated by vertical lines. The boundaries of the β and γ elements are marked, and the cleavage site within the β element is indicated (↓). Cleavage is observed only at the β element of the T_R/U_L fusion (line 1) but not at the other two locations (lines 2 and 3). Note that the β elements located at the U_L/I_R junction (line 2) and within the distal U_L sequences (line 3) are not flanked by the AT element (U_L/I_R junction) or the γ element (distal U_L sequence).

matic specificity. Since the three β elements as they are present in the replicative-form DNA of EHV-1 are identical in nucleotide sequence, all three could theoretically serve as potential cleavage sites. This, however, is not the case. The facts that (i) the U_L segment is fixed in its orientation relative to I_R and (ii) only one distinct pair of EHV-1 terminal restriction fragments are produced consequently mean that only the β element at the T_R/U_L fusion is cleaved, whereas the two internal β elements are not. Hence, the cleavage site alone cannot mediate specificity of cleavage. It is therefore likely that other sequences adjacent to the cleavage site make up the recognition site for the enzymatic activity. On the basis of this assumption, a nucleotide sequence comparison of all three β elements and their adjacent sequences was done (Fig. 8). Such a comparison should help to delineate the boundaries of the recognition site. As seems obvious (see Results), an AT stretch proximal to the β element at the left terminus is a likely candidate. This possibility is further emphasized by the comparison of other terminal nucleotide sequences with EHV-1. The sequence A_7T_5 in EHV-1 is greatly conserved at the left terminus in two other D-type herpesviruses adjacent to their putative cleavage sites: pseudorabies virus, A_9T_3; BHV-1, $A_{10}T_4$ (12). Other herpesviruses show a pure poly(A) stretch with a variable size 31 to 42 bp proximal to the termini (with the exception of human cytomegalovirus) (10, 12, 16). Recently, proteins have been identified in the nuclear extract of infected cells which bind specifically to DNA sequences consisting of the AT stretch (*pac-2*) (10) and the cleavage sites (DR1) together (5). In addition, most herpesvirus termini show a palindromic structure distal to the AT or A_n element within the β element. In EHV-1, additional palindromic sequences are present in both the terminus (indicated as an α-like element in Fig. 4 and 5).

The internal β element (Fig. 4 and 8) is flanked by the same AT stretch as the β element spanning the fusion T_R/U_L. In contrast, the leftward boundary is different between those two β elements (Fig. 8). Since the β element spanning the fusion T_R/U_L is cleaved and the internal β element is not, we argue that their sequence divergency reflects the functional difference between these two β elements. Consequently, the right genomic terminus is part of the recognition site flanking the β element of T_R/U_L, along with sequences proximal to the left terminus. Similar notions underlining the implication of the presence of signals at both ends were reported previously (33, 35). Aligning the nucleotide sequences of the right termini of other herpesviruses with that of EHV-1 revealed that the stretch of conserved sequences first reported by Tamashiro et al. (30, 31) is also present in EHV-1 (designated γ in Fig. 5 and 8) (10, 12). It seems, therefore, likely that the two highly conserved sequence blocks designated γ and AT (or *pac-1* and *pac-2*) (10) contain the sequence arrangements essential for cleavage specificity within the β element. This could be tested by introduction of mutations into these sequences, which have been shown to be sufficient for cleavage in herpes simplex virus type 1 (20).

The demonstration of a replicative-form DNA consisting of a head-to-head arrangement of the two left-terminal fragments raises the possibility of recombinational events between two left termini of EHV-1 DNA. This could involve the 90 nucleotides at the end of the L component, which are found duplicated ~1 kbp proximal in the form of an inverted repeat. A simple recombination event between the left termini of two EHV-1 virion DNAs could be mediated by the two pairs of the 90-bp inverted repeats. Thus, the nucleotide sequences bracketed by these inverted repeats in the donor or recipient of the recombining molecules would be eliminated. This event would account for the loss of 824 bp (from 89 to 913 bp in Fig. 4). In fact, the head-to-head arrangement in RfII is approximately 700 bp smaller than expected (see Results). Sequence data might reveal the mechanism of this arrangement and its role in EHV-1 replication. If such a head-to-head arrangement was a functional replicative intermediate in DNA replication of EHV-1, a similar tail-to-tail arrangement of two right genomic termini would be essential to form circular or concatemeric molecules. Such an arrangement could not be shown in our investigations. Thus, an illegitimate homologous recombination event may lead to the head-to-head arrangement of the two left termini which are discarded later in the process of maturation and packaging.

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