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Specificity of Cleavage in Replicative-Form DNA of Bovine Herpesvirus 1

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The linear double-stranded DNA genome of herpesvirus as it is present in infectious virions needs to be circularized after infection of host cells and before DNA replication. Replicative-form genomes have to be cleaved into linear unit-length molecules during virion maturation and are most probably the substrate for inversion of the short segment relative to the long segment of the bovine herpesvirus 1 (BHV-1) genome. Those regions of the BHV-1 genome which are functionally involved in these processes have been analyzed at the molecular level by cloning and sequencing the genomic termini, the fusion of both termini from replicative-form molecules, and the junction between the short and the long genome segment. On the basis of the simple genome arrangement of BHV-1, it was inferable that the cleavage of replicative-form genomes by a hypothetical BHV-1 terminase activity may be specified by a sequence at the left end of U_L (A_n element), which is located proximal to a reiterated β element that makes up the cleavage site itself. The relationship of those elements in BHV-1 and the comparison to similar regions of other herpesviruses indicate consensus sequence elements which are functionally important for cleavage and isomerization of viral DNA during maturation of virions.

Bovine herpesvirus 1 (BHV-1) causes infections of economic importance in cattle. Defined clinical symptoms lead to the differentiation of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis virus. Attempts to define biological markers of organ tropism and virulence correlating with the two clinical BHV-1 subtypes (infectious bovine rhinotracheitis and infectious pustular vulvovaginitis) gave divergent results. By restriction enzyme analysis, one can differentiate at least two groups of BHV-1 isolates. Whether these groups do or do not correspond to the clinical subdivision into BHV-1 subtypes remains unclear (11, 12, 26). Immunological investigation of viral proteins and polypeptide patterns do not elucidate the relatedness of clinical subtypes of BHV-1 and serological groups (25, 26).

The life cycles of herpesviruses include virus-specified recombinational events. All herpesviruses studied to date replicate DNA during the productive phase of their life cycles by pathways which require the specific joining and eventual severing of the ends of the viral DNA found in virions. Many of the herpesviruses also recombine DNA at internal sites such that their virions contain one DNA molecule, which is one of either two or four possible isomeric forms. These recombinational events have been studied most with herpes simplex virus type 1 (HSV-1). Its DNA occurs in four isomeric forms and is therefore more complex than that of BHV-1, which has only two isomers. We have capitalized on the relative simplicity of BHV-1 to identify signals within its DNA that specify cleavage of replicating species to yield virion DNA and that specify recombination at internal sites.

BHV-1 is a member of the α subfamily of the bovine herpesvirus (19). The 135-kilobase (kb)-spanning genome of BHV-1 is composed of a unique long segment U_L (100 kb), an internal repeat I_R (11 kb), a unique short segment U_S (13 kb), and a terminal repeat T_R (Fig. 1A). The repeats are inversely oriented with respect to each other. The U_S segment situated in between I_R and T_R appears in two alternative directions with respect to U_L. Thus, DNA isolated from virions exhibits two isomeric forms in roughly equimolar amounts. The two isomers are designated prototype (P) and inversion type (IS) of U_S (11, 13, 23). Nothing is known about possible functions of this flip-flop mechanism. But it is noteworthy that, in contrast to U_S, the orientation of U_L is fixed. The biological implications of this inversion are not known.

The BHV-1 genome structure represents a D-class herpesvirus (33) and is also exemplified in pseudorabies (PsR) virus (3), varicella-zoster virus (VZV) (6), and equine herpesvirus 1, 3, and 4 (5, 16, 39).

Here we report the results of an investigation on regions of the genome which are functionally important for viral DNA replication. Initially after cell infection, the termini of the linear genome are supposed to become ligated to form a circular molecule. Such circularization to replicative-form genomes allows the complete replication of otherwise linear molecules, e.g., by the rolling-circle model leading to the accumulation of endless concatemers in the cell (18). By cleavage of these concatemers, complete, linear virion DNA units are provided for packaging during virus maturation.

The less-complicated situation with regard to isomerization in the BHV-1 genome compared with that in HSV-1 provides a better chance to define functionally important sites for the recognition and formation of genomic termini, as well as for the recombination event which leads to the inversion of U_S. The simplicity of the BHV-1 genome compared with the HSV-1 genome allowed us to define the recognition and cleavage sites of an endonuclease performing cleavage of concatemers into virion DNA and probably inversion of U_S. The functional DNA sites of the enzyme(s) called terminase-recombinase are conserved in herpesviruses with different genome organizations. An improved knowledge about the mechanism to generate replicative and mature forms of herpesvirus genomes could possibly provide tools for therapy of herpesvirus infections.

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MATERIALS AND METHODS

Cells and virus strain. BHV-1 B1 (Schönböken) and the cell-culture-adapted derivative B4 were a generous gift from O. C. Straub, Federal Research Center for Virus Diseases of Animals, Tübingen, Federal Republic of Germany. Virus was propagated in Georgia bovine kidney cells as reported earlier (13).

Preparation of viral DNA and restriction endonuclease cleavage. DNA was obtained from lysed nucleocapsids, which were purified from cell culture supernatant as previously described (13). Viral DNA from infected cells was isolated 10 h after infection (multiplicity of infection, 0.1). Cells were scraped off and lysed with sodium dodecyl sulfate and proteinase K. The DNA was further purified by equilibrium density centrifugation in CsCl-ethidium bromide gradients. Restriction endonuclease cleavage and analysis were done as previously described (13).

Derivation and construction of recombinant plasmids. Cloning of terminal DNA fragments of BHV-1 was performed after modifying the genomic termini. Whole-virion DNA was treated with a large fragment of DNA polymerase I in the presence of the four deoxynucleoside triphosphates to generate blunt ends. ^{32}P-phosphorylated dodecamer *Eco*RI linkers (for the left-hand terminus) or decamer *Hin*dIII linkers (for the right-hand terminus) (New England BioLabs, Inc.) were ligated with T4 DNA ligase to the virion DNA. Linker molecules and virion DNA were cleaved with a combination of two restriction enzymes (*Hin*dIII and *Eco*RI). Cut linkers and cleaved virion DNA fragments were separated on analytical agarose gels. The gels were dried and exposed to X-ray films. Autoradiographs allowed the identification of the terminal fragments in the *Eco*RI-*Hin*dIII double digests of virion DNA. Genomic terminal fragments were isolated from preparative gels and ligated to an *Eco*RI-*Hin*dIII-cut pUC8 vector. This procedure ensured cloning of the terminal fragments in defined orientations. Competent JM83 cells (24) were transformed (14), and recombinant clones were identified by colony hybridization, restriction enzyme mapping, and Southern blot hybridization. The series of plasmid clones bearing the left terminus was called M (13), and the series bearing the right terminus was called E.

Clones of the BHV-1 fragment carrying the internal junction between U_L and I_R were obtained after isolation of the *Eco*RI-*Hin*dIII fragment spanning the region from the right end of U_L to I_R (Fig. 1). The plasmid clones were designated LS. Cloning of the BHV-1 fragment carrying the fusion of left and right genomic termini appearing in replicative-form molecules was achieved after cleavage of total DNA of virus-infected cells with *Pst*I and *Xho*I, separation of the resulting fragments on a preparative agarose gel, elution of the fusion fragment, and ligation to the appropriately cleaved (*Pst*I and *Sal*I) pUC8 vector. Recombinant plasmid clones designated RF harboring the fusion of the left and right genomic termini were identified by colony hybridization.

DNA sequencing. DNA was sequenced by the method of Maxam and Gilbert (21, 22) after end labeling the cloned fragments with polynucleotide kinase (Stehelin AG) and [γ-^{32}P]ATP (Amersham Corp.). Left-terminal DNA fragments were sequenced by the chain terminator method according to Sanger et al. (34) as described earlier (13). Reaction products were separated in 20, 8, and 6% polyacrylamide-urea gels which were bound to glass plates, dried after electrophoresis, and autoradiographed with XAR-5 film (Eastman Kodak Co.).

Blot hybridization. Restriction fragments were transferred (35) after electrophoresis on agarose gels to GeneScreen membranes (New England Nuclear Corp.). In vitro labeling of recombinant plasmid DNA by nick translation (31) in the presence of [α-^{32}P]dCTP or [α-^{35}S]dCTP and hybridization to the immobilized DNA fragments were done as described previously (13).

RESULTS

Cloning and characterization of the genomic termini. We modified the terminal fragments of BHV-1 DNA such that the left terminus was tagged with synthetic DNA containing an *Eco*RI recognition site and the right terminus was tagged with a synthetic DNA containing a *Hin*dIII recognition site. Cleavage of the modified virion DNAs both internally and within the synthetic DNAs yielded terminal fragments with characteristic orientations (Fig. 1). It was possible to ligate such fragments in defined orientations into the appropriately cleaved vector, pUC8, with a reasonable efficiency. As described earlier (13), recombinant M-series plasmid clones bearing the left-end *Hin*dIII fragment of the genome included a set of clones containing fragments ranging in size from 2.4 to 2.8 kb. In contrast, the recombinant E-series plasmid clones containing the right-end *Eco*RI fragment of T_R had inserts of only one size, 8.2 kb. Physical maps of the genomic termini were deduced and are presented in part in Fig. 1.

Cloning and characterization of the internal junction fragment of U_L-I_R. T_R and I_R are two large (11-kb) inverted repeats bracketing U_S (Fig. 1). To locate precisely the junction between U_L and I_R, the *Eco*RI-*Hin*dIII LS fragment (9.9 kb) carrying the junction was cloned in pUC8. We generated a physical map of the LS fragment and compared it to the physical map of the right genomic terminus. This comparison identified an *Ava*I-*Nar*I fragment (210 base pairs [bp]) that contained both the left end of I_R, which is identical to the right end of T_R, and the right end of U_L. Thus, this *Ava*I-*Nar*I fragment harbors the internal junction between U_L and I_R.

Identification, cloning, and characterization of the fusion fragment. The free termini as present in virion DNA of herpesviruses disappear after infection of cells. This apparent loss has been described for D-type herpesviruses, such as PsR virus (2, 3) and VZV (7), as well as for herpesviruses with different genomic arrangements, such as HSV-1 (30). Left and right genomic termini became fused in head-to-tail arrangements, which are considered to be replicative intermediates of the viral genome. BHV-1 virion DNA and total DNA of BHV-1-infected cells were cleaved with restriction enzymes (e.g., *Hin*dIII). The cleavage products were separated on analytical agarose gels, transferred to nylon membranes, and probed by hybridization with DNA sequences specific for the left genomic end of BHV-1. Virion DNA exhibited only one signal on the autoradiograph, representing the left-terminal DNA fragment (Fig. 2). In contrast, cellular DNA preparations showed two signals after *Hin*dIII digestion, one signal corresponding to the left-terminal fragment and the second, weaker signal representing two DNA fragments of approximately 15 to 17 kb (the two fragments are not separated in Fig. 2). The size of these newly identified fragments is explainable by fusion of the left-terminal fragment (2.4 to 2.8 kb) to the right-terminal fragment (12.5 or 14.5 kb), as would be expected in replicative-form molecules. Similar analysis of DNA cleaved by other restriction enzymes gave cleavage patterns which were consistent with the presence of a fusion fragment in intra-

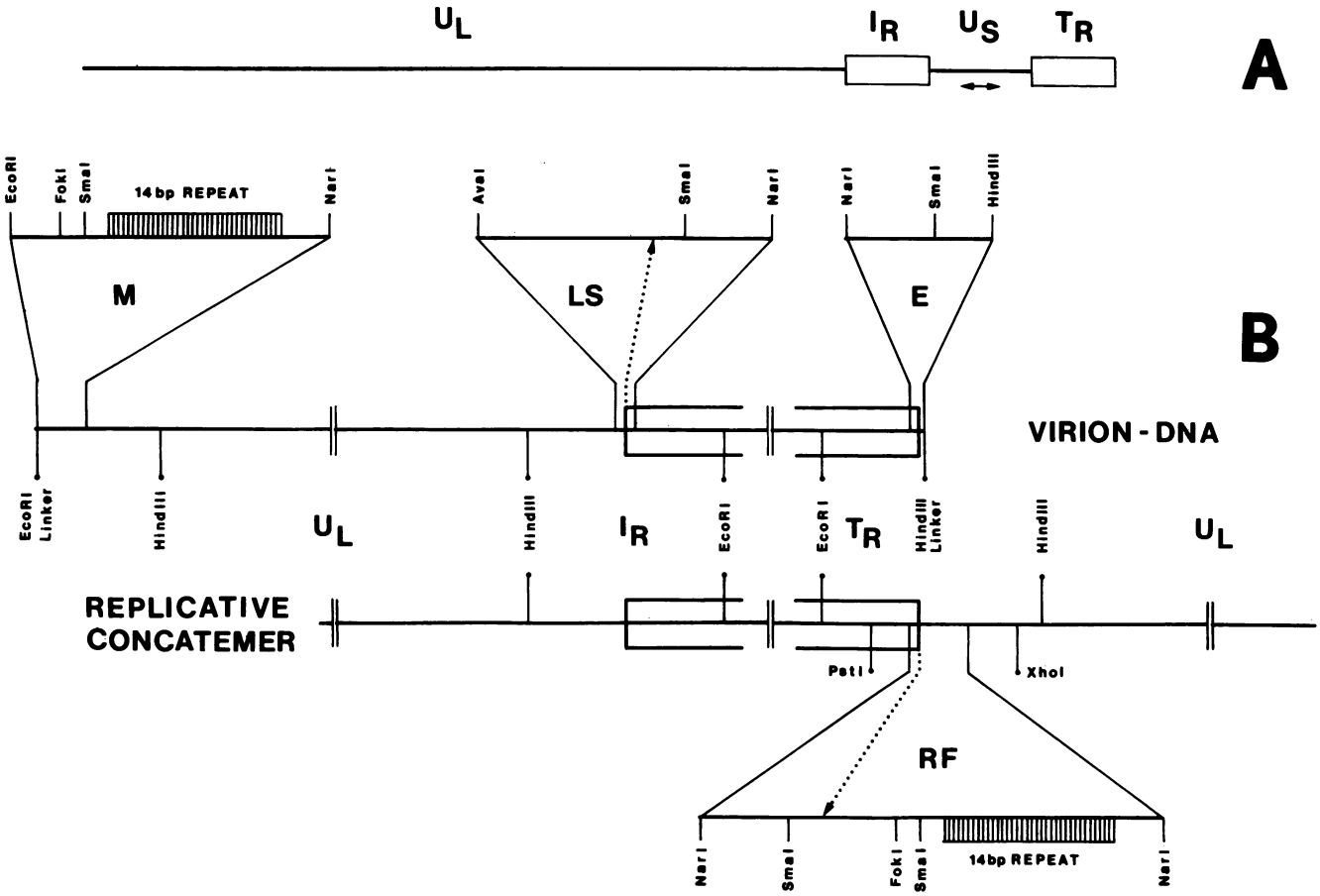

FIG. 1. Genome arrangement of BHV-1 and cloning strategy for the left-hand (M-series plasmids) and right-hand (E-series plasmids) genomic termini, the internal junction U_L-I_R (LS-series plasmids), and the fusion of the right and left genomic termini (RF-series plasmids). (A) The genome arrangement of BHV-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 (indicated by a double-headed arrow). The orientation of U_L is fixed relative to I_R and T_R. (B) Relevant restriction enzyme sites used for generating clones of the two termini and the U_L-I_R junction as they occur in the virion DNA molecule (M, LS, and E series, top line), as well as of the fusion fragment of the left and right genomic termini in the replicative-form molecule (RF series, bottom line), are sketched. The localization of the 14-bp repeat array at the left genomic terminus is shown. The detailed restriction enzyme maps of the different regions are based on the nucleotide sequences (Fig. 4 to 6).

cellular viral DNA. Furthermore, these Southern blots did not reveal any additional signals even when overexposed (data not shown). The observations demonstrate that U_L, in contrast to U_S, does not invert relative to I_R in virion DNA. Inversion of U_L with respect to I_R would create a new *Hin*dIII left-hand DNA fragment differing in size from the cloned left-terminal fragment. In addition, were U_L to invert, the inversion would create a new discernible junction with I_R. In contrast to other D-type herpesviruses (6), this putative second junction with I_R was never observed for BHV-1.

Nucleotide sequence determination and sequence analysis. Both strands of the inserts in the recombinant DNA clones harboring the left (M clones) and right (E clones) genomic termini, the junction fragment U_L-I_R (LS clone), and the fusion fragment (RF clones) (Fig. 1) were sequenced. The nucleotide sequence derived from six plasmid clones of the M series is shown in Fig. 3. As reported earlier, the terminal *Nar*I fragment (Fig. 1) includes a 14-bp tandem repeat array, giving rise to size heterogeneity of this fragment due to variations in the copy number (8 to 38) of the 14-bp repeats (13). The 235-bp-long DNA sequence distal to the repeat array was identical in five of six clones sequenced. The sixth clone, M297, showed an additional base pair at the position at which the linker had been ligated to the left genomic terminus and a few differences within the following 25-bp stretch compared with the five other M-series plasmid clones. The reason for these sequence differences is not known.

Relevant DNA sequences of the right terminus are displayed in Fig. 4. A total of six individually cloned right termini were sequenced. Comparison of the nucleotide sequence from the right and left termini showed that the termini do not exhibit sequence redundancy. Aligning the sequences of the cloned termini and the sequence spanning the fusion of U_L and T_R (Fig. 5; a total of six clones were sequenced) revealed that a single base pair at the fusion point was missing for all cloned and sequenced fragments of the genomic termini, except for the already mentioned plasmid clone M297. This finding indicates that because of the cloning procedure (see Materials and Methods), a single-base extension at the 3′ end of the genomic termini was lost, presumably as a result of the 3′-to-5′ exonuclease activity of DNA polymerase used previously to blunt the ends of the

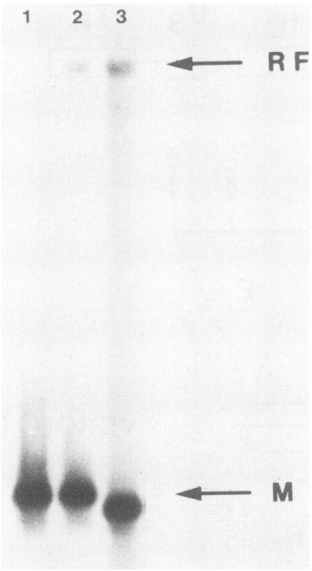

FIG. 2. Detection of the fusion of left and right genomic termini of BHV-1 by Southern blotting. Virion DNA (extracted from purified capsids [lane 1]) and whole-cell DNA of GBK cells infected with different plaque-purified BHV-1 stocks (lanes 2 and 3) were digested with *Hin*dIII, separated on agarose gels, blotted (35) to nylon membranes, and hybridized to plasmid clone M23. The autoradiograph shows signals which refer to the left-terminal *Hin*dIII fragment (M). Their size heterogeneity is due to a different copy number of the 14-bp repeats in plaque-purified virus stocks (13). In restricted cellular DNA, additional fragments can be detected which correspond to the *Hin*dIII fusion fragment of the left and right genomic termini (RF).

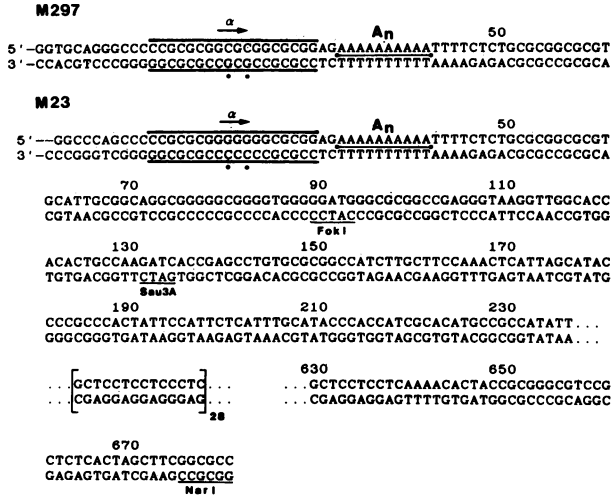

FIG. 3. Nucleotide sequence of plasmid clones M23 and M297 covering the left-hand genomic terminus. Sequences are given starting from the left genomic terminus to the most distal *Nar*I site. Several restriction sites are indicated for comparison with Fig. 1. The 3′ single-base extension at the natural left end of the genome is deduced from the fusion of both genomic termini. The sequences of the functionally important α elements are boxed, positions of variable nucleotides are marked by dots underneath the sequence, the arrow gives the relative orientation of the α elements, the A_n stretch is marked, and the 14-bp repeat array is shown in brackets.

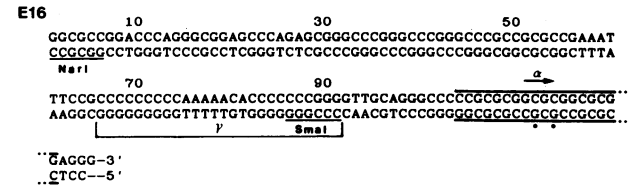

FIG. 4. Nucleotide sequence of the natural right genomic termini of BHV-1 represented by plasmid clone E16. The sequence is given starting from the most distal *Nar*I site extending to the right end. The α element and its relative orientation is indicated, the localization of the γ element is shown, and restriction enzyme sites given in Fig. 1 are indicated.

virion DNA molecules. If the single-base extension had been at the 5′ end, then our cloning procedures would have maintained this nucleotide (see Materials and Methods). A similar artifact has been reported in the cloning of HSV-1 (29), VZV (6), and human cytomegalovirus (HCMV) (40) termini. Since the missing base pair is retained in fusion fragments, we conclude that for the right terminus the 3′ single-base extension is a guanosine, whereas for the left terminus the 3′ base extension is a cytosine, which was taken into account in Fig. 3 and 4. As a result of the sequence of the fusion fragment, the genomic termini fused via the complementary 3′ base extensions.

By comparing the nucleotide sequence at the fusion of the termini with the sequence at the junction U_L-I_R, it became evident that the fusion and junction sequences were identical, considering their inverse orientations (Fig. 5 and 6). Additionally, a 18-bp sequence element, consisting of C and G only, is reiterated fourfold in the genome, always located close to the fusion and junction. This 18-bp reiterations, referred to hereafter as α elements, display remarkable features. (i) The α sequence 5′-CCGCGCGGCGCG GCGCGG-3′ differs from a palindromic structure only at positions 7 (G) and 12 (G). These two differences define an orientation for the α elements. (ii) Single α elements are located close to the left terminus and to the right terminus (Fig. 3 through 6). Both of these α elements have the same orientation. (iii) The α element located at the left terminus has a variant sequence, as determined from sequencing different plasmid clones. Transversions appear at position 9 (C to G) and at position 11 (C to G), resulting in an increased divergence from a palindromic structure. However, these transversions are located in the middle of the palindrome and

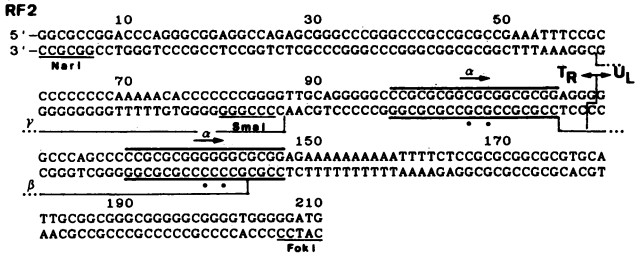

FIG. 5. Nucleotide sequence of the fusion of left and right genomic termini occurring in replicative-form DNA represented by plasmid clone RF2. The fusion between T_R and the left end of the U_L segment is indicated, showing the cleavage by a terminase activity. The cleavage site is part of the β element, encompassed by two α elements which are arranged in the same relative orientation. The consensus sequence γ of the right-hand termini of different herpesvirus genomes is underlined. Restriction sites refer to Fig. 1.

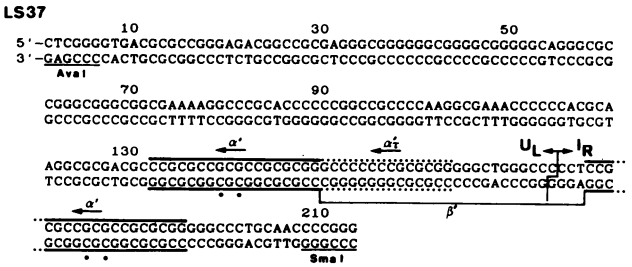

FIG. 6. Nucleotide sequence of the junction between the right end of U_L and I_R. The derivation of this DNA sequence is shown in Fig. 1 with corresponding restriction enzyme sites. The junction point is deduced by similarity with the fusion sequence shown in Fig. 5. The junction is located within a β element, which is inverted relative to the fusion fragment (β'). The β element is surrounded by two complete (α') and one partially duplicated (α'_T) α elements. Note that in contrast to the fusion fragment, an A_n stretch is not present in this sequence (Fig. 5).

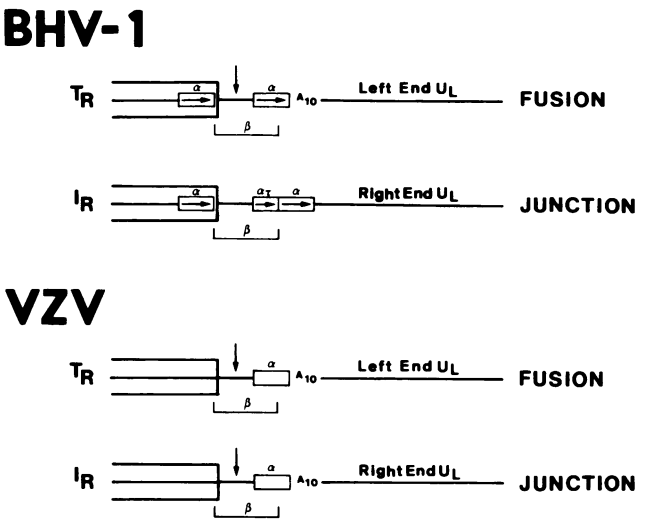

FIG. 8. Schematic comparison of the fusion and junction of BHV-1 contrast to VZV. The segments (U_L, T_R, and I_R) make up similar nucleotide sequence elements (α elements and A_n stretch) in both virus genomes. The cleavage of those genomes in their replicative form is indicated by vertical arrows. In contrast to VZV, cleavage occurs in the BHV-1 genome only in the fusion fragment of T_R and U_L. In the VZV genome, putative cleavage sites are encompassed by identical sequence elements (I_R, T_R, an α-similar element [boxed], and the A_n stretch [A_{10}]). In contrast, the putative cleavage site in the fusion of replicative-form DNA in BHV-1 is composed of a unique arrangement of sequence elements compared with the internal junction I_R-U_L.

could form a loop in a possible hairpin structure. (iv) Since the α element at the right terminus is part of T_R and is oriented rightwards by definition (Fig. 4), the analogous α element in I_R should be inversely oriented. This interpretation was confirmed by sequencing the junction (Fig. 6). (v) At the junction between U_L and I_R, the right end of U_L contains a complete α element, which is directly preceded by a truncated (14-bp) α element (α_t). U_L is therefore bracketed by two inversely oriented α elements. This arrangement is also found in the region I_R-U_S-T_R. These findings are depicted in Fig. 7.

Alignment of the nucleotide sequence of the T_R-U_L fusion fragment and the related I_R-U_L junction fragment in an inverse orientation (Fig. 5, 6, and 8) leads to two conclusions. (i) The nucleotide sequences at the fusion point of T_R and U_L and at the junction of U_L and I_R are identical. The sequence identity between the parts of T_R and I_R was expected. However, the homology extends across the fusion and junction and includes a stretch of 28 bp at both ends of U_L. We have designated this 28-bp region, which covers 14 bp of the α-element sequence, a β element (Fig. 7). (ii) Cleavage of replicative-form DNA generating free genomic termini of virion DNA occurs within the β element and leads to 3' single-base extensions (Fig. 5). Thus, the sketch (Fig. 7) of the BHV-1 genome can be completed and described by the combination of reiterated and nonreiterated elements (β' stands for inversely oriented β element):

(24.5 bp) β-U_L-β' (28 bp) I_R-U_S-T_Rβ (3.5 bp)

The β elements make up the cleavage site for the enzyme(s) performing the cleavage event in the concatemeric molecules to generate mature, virion DNA. We propose to call this enzymatic activity BHV-1 terminase. The concatemeric arrangement of BHV-1 genome units in a head-to-tail manner results in the appearance of two β elements in one virion genome unit, which can be depicted as:

-β-U_L-β'I_R-U_S-T_Rβ-U_L-β'I_R-U_S-T_Rβ-U_L-

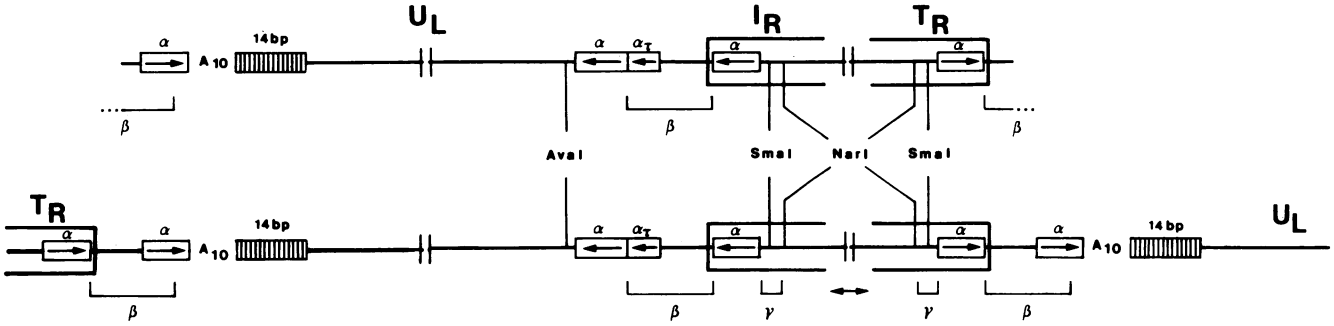

FIG. 7. Skeleton sketch of BHV-1 virion DNA molecule (top line) and of a part of the replicative-form DNA occurring in concatemers (bottom line). The sketch is not to scale, and the pairs of vertical lines interrupting each molecule indicate where large areas of the DNA are not depicted for convenience of presentation. See Fig. 1 for a less-detailed sketch. The virion DNA molecule is built of U_L, I_R, and T_R harboring morphologically important arrays (14-bp repeats and A_n stretch [A_{10}]) and elements (α, β, and γ), as shown in Results. In the replicative-form DNA, the virion DNA units are linked to each other, forming a complete β element at the fusions. A sequence-identical β element occurs in the junction U_L-I_R. The virion DNA molecule shows two parts of a single cleaved β element at the termini. The localization of restriction enzyme sites are also given in Fig. 1, 4, and 6.

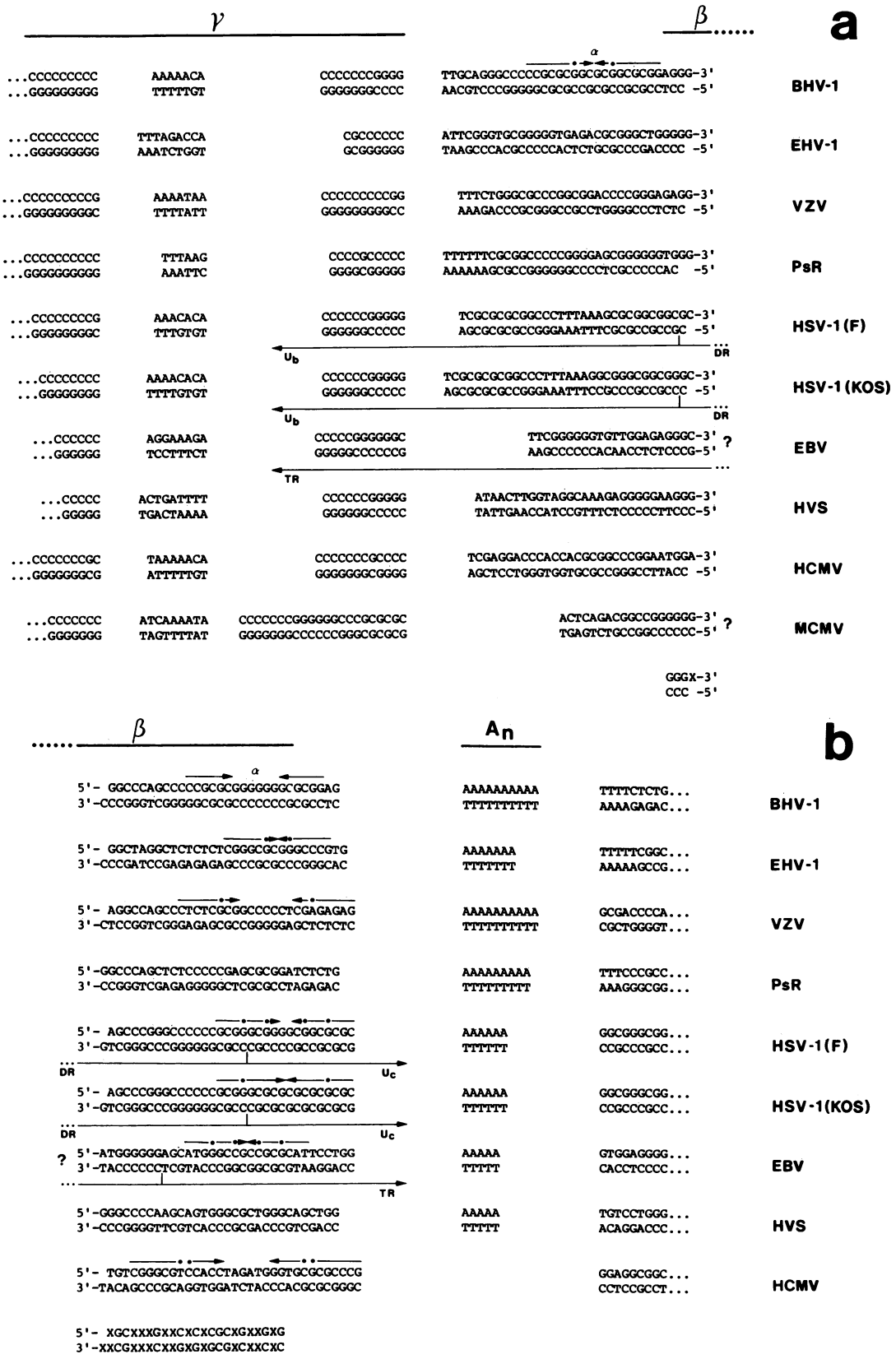

FIG. 9. Nucleotide sequence comparison. The following are compared: the right (a) and left (b) natural genomic termini of BHV-1 (this study), equine herpesvirus 1 (EHV-1) (Chowdhury and Hammerschmidt, in preparation), PsR (15), VZV (6), HSV-1 F (29), HSV-1 KOS (43), Epstein-Barr virus (EBV) (20), herpesvirus saimiri (HVS) (1), HCMV (41), and mouse cytomegalovirus (MCMV) (41). Different sequence elements that have been described are depicted (α, β, γ, and A_n), and the natural ends of the herpesvirus genomes are indicated by giving

During BHV-1 DNA maturation, the terminase cleaves only the β element at the fusion T_R-U_L but not the β element at the junction I_R-U_L. Cleavage at that second β element would lead to virion DNA molecules carrying U_L inversely oriented with respect to I_R. Such inversions were not detected in BHV-1 virions. However, both β elements do show an identical potential cleavage site for the terminase. This finding indicates that the specificity of the BHV-1 terminase reaction is mediated not only by the β element but also by sequences outside of the β element and unique to the fusion T_R-U_L. Since T_R and I_R consist of sequences which are reiterated, the specific recognition site for BHV-1 terminase is located within unique sequences at the left end of U_L.

A poly(A) stretch (A_n) is located proximal to the left-end α element (Fig. 3 and 8). In contrast, such an A_n stretch is missing at the right end of U_L, and instead there is a truncated (α_t) plus a complete α element. Consequently, it seems likely that A_n is at least part of the recognition site for the BHV-1 terminase which provides the specificity required to recognize the fusion of T_R-U_L.

DISCUSSION

To gain further information about the involvement of the genomic termini of herpesviruses in DNA replication and virus maturation and to identify structures that are functionally essential for these processes, we chose to study the relatively simple arranged herpesvirus genome of BHV-1. Apparently two major characteristics contribute to the genome structure. (i) First, precise cleavage of concatemers leads to virion DNA units generating unique sequences at both termini. The cleavage has to be performed at a distinct site by a endonuclease activity, a terminase. One has to postulate that recognition of the cleavage site is based on a nucleotide sequence. The two β elements in a concatemeric BHV-1 genome unit could theoretically serve as a putative cleavage site to generate virion DNA units. The fact that a U_L segment of the BHV-1 genome is fixed in its orientation with respect to I_R consequently means that only the potential cleavage site in the β element at the left end of U_L is cleaved by the putative terminase, whereas the β element at the right end of U_L is not. Thus, the recognition site can not coincide with the cleavage site of the terminase. (ii) Second, inversion of U_S generates two isomeric molecules of BHV-1. Virus isolated from a single plaque in cell culture gave rise to progeny consisting of an equal proportion of both isomers (data not shown). Provided that one virion has the capacity to form a plaque, then one has to conclude that isomerization is an early event in a single cycle of virus multiplication or a rather frequent one. Genome isomerization requires a recombination event involving cleavage and rejoining of DNA molecules. The similarities between the U_L-I_R junction and the T_R-U_L fusion in BHV-1 are consistent with the notion that both inversion of U_S and cleavage of concatemers are events that are probably executed by a similar or identical enzyme(s) with different cofactors.

To define the recognition site of the terminase, we compared the terminal nucleotide sequences of other herpesvirus genomes with BHV-1. As we have already emphasized (13), BHV-1 exhibits the sequence 5′-GAGAAAAAAAAAA-3′ from position 29 to 41 (Fig. 3), a sequence which is also present in the VZV genome within the terminal repeat of its U_L segment 29 nucleotides away from the genomic terminus (6). This is the only extended sequence homology shared at the U_L termini between BHV-1 and VZV. We propose that this conserved A_n stretch is part of the recognition site of the BHV-1 terminase (see Results). To gain further information about the conservation of this A_n sequence element, we compared available terminal sequences of different herpesvirus genomes, including the termini of equine herpesvirus 1, a class D-herpesvirus genome (S. I. Chowdhury and W. Hammerschmidt, manuscript in preparation) (Fig. 9).

It is remarkable that except for HCMV, all herpesviruses bear a more or less extended A_n element 31 to 42 bp proximal to their termini, if sequences are aligned accordingly. In addition, most herpesvirus termini possess a palindromic structure located distal to the A_n stretch, similar to the α element of BHV-1. There is no obvious sequence identity in the palindromic sequences of comparable α elements in other herpesviruses despite the high GC content. In no case has a perfect palindrome been found; the stem of the possible hairpin structures consists of 6 bp disrupted by one or more mismatches. An α element can be detected even in HCMV lacking the A_n stretch, whereas herpesvirus saimiri and PsR virus which possess an A_n sequence element exhibit a less convincing α element. Aligning the nucleotide sequence of the right terminus of BHV-1 and other available terminal sequences (Fig. 9a) confirmed that the conserved sequence first reported by Tamashiro et al. (40, 41) also occurs in BHV-1. In accordance with our nomenclature designating α and β elements, we propose to call this conserved area from position 67 to 93 (Fig. 4 and 9a) in the BHV-1 genome the γ element. It is obvious that in contrast to the α element present at the right-hand terminus in BHV-1, there is no equivalent α element at the right terminus of other known herpesviruses. Similar sequence comparisons were published recently (9, 10, 41).

In HSV-1, the A_n stretch is part of the U_c sequence (Fig. 9b) and the γ element is part of the U_b sequence (Fig. 9a). U_c and U_b are unique nucleotide stretches in the "a" sequence of HSV separated by a repeat array consisting of tandemly reiterated strain-dependent sequences (8, 27). This array of tandemly reiterated sequences is structurally similar to the 14-bp repeats of BHV-1 (13). Distal to the U_b and U_c stretches, two direct repeat sequences (DR) (Fig. 9a and b) are arranged in the same orientation. The location of reiterated "a" sequences of HSV-1 can be depicted as

$$a_nb\text{-}U_L\text{-}b'a'_mc'\text{-}U_S\text{-}ca$$

(n and m may vary from 1 to 10). The U_L and U_S components invert relative to each other, generating four equimolar isomeric forms of the HSV-1 genome (for a review, see

the strand orientations (5′ or 3′). A question mark points to unknown end structures. For Epstein-Barr virus, the terminal sequence within T_R is inferred from sequence homology. It is noteworthy that this sequence alignment and the depicted sequence elements do not allow to postulate any distinct length-measuring mechanism performing an accurate cleavage of the β elements. To address this question, the bottom line shows a β-element consensus deduced from this sequence alignment mainly making up sequences of the left genomic ends of different herpesvirus genomes. This consensus sequence could contribute to the base precise cleavage event within the β element of these herpesvirus genomes. The following scores show an extended structural similarity to the proposed β-element consensus sequences (number of nucleotides out of 15): BHV-1, 14; EHV-1, 13; VZV, 11; PsR, 12; HSV-1 (F), 14; HSV-1 (KOS), 15; Epstein-Barr virus, 11; herpesvirus saimiri, 10; and HCMV, 10. Arrows indicate palindromic sequence elements; dots point to mismatches within the palindrome. U_b, U_c, DR, and TR refer to defined sequence elements in HSV-1 (29, 43) and Epstein-Barr virus (20).

reference 32). Cleavage during DNA maturation occurs in DR, which is part of element "a" (Fig. 9a and b). As a result of the variable orientations of U_L and U_S components, cleavage of the concatemers can occur at each "a" sequence in any DR. This is in contrast to the less-complicated genome organization of BHV-1, in which cleavage for virus maturation occurs only in a certain β element. It is of interest that the cleavage of HSV-1 is not mediated by DR itself but by other internal elements of "a" (42). These sequence-unspecific cleavages require a complete U_c sequence, whereas U_b can be deleted. Additional data support this observation. In amplicon constructs (36, 37, 43), intact or deleted "a" sequences of HSV-1 can be tested for cleavage before packaging into defective interfering particles. Deiss et al. (9) have shown with such amplicon constructs that deletions in U_c spanning the A_n stretch inactivated the cleavage function, whereas U_b could be omitted without influencing the cleavage and packaging efficiency. These findings with HSV-1 support our hypothesis that the cleavage site bearing β element does not mediate cleavage specificity, whereas the A_n stretch does.

In BHV-1, it is likely that at the U_L-I_R junction an A_n stretch as part of the recognition site of the terminase has been replaced by an α element during evolution (Fig. 6). This replacement eliminates not only the A_n stretch in the U_L-I_R junction but also a potential secondary hairpin structure of the single α element. Because of the partial duplication $\alpha+\alpha_t$, the loop is either larger or dislocated with respect to the putative cleavage site. The functional importance of this region is underscored by a comparison of the nucleotide sequences of the junction U_L-I_R of BHV-1 and VZV (Fig. 8). Although both genomes display in general the same arrangement of U_L, I_R, U_S, and T_R, the U_L segment of VZV is bracketed by two inverted repeats T_{RL} and I_{RL} spanning 88.5 bp at the left and right ends of U_L. Thus, the A_n stretch, as well as the α element of VZV, is duplicated in inverse orientation at the junction U_L-I_R. Consequently, two identical putative recognition sites and two cleavage sites occur, which should give rise to inversion of U_L relative to I_R, provided that both cleavage sites are equivalent. VZV shows this predicted inversion of U_L although the two isomers do not represent equimolar proportions (6).

It is noteworthy that two reports emphasize the universal function of the terminase at the cleavage-recognition sites of herpesviruses. With amplicon vectors containing a HSV-1 origin of replication and "a" sequences of two herpesviruses on the same molecule, Spaete and Mocarski (38) showed that the HCMV "a" sequence is cleaved if HSV-1 helper virus is present. Although HSV-1 and HCMV share no detectable sequence homology (17), the "a" sequences of HCMV were recognized by the terminase of HSV-1. By applying the same approach, similar findings were reported with the "a" sequence of HSV-1 and a related herpes simplex virus type 2 strain as a helper to enable cleavage-packaging of tandemly arranged multimers of HSV-1 sequences in defective particles (37).

Site-specific inversion in the HSV-1 genome requires the "a" sequence (27, 28) and involves both cleavage and joining of the inverting sequences (4). In HSV-1, unique sequences bracketed by two "a" sequences invert, contributing to the generation of four equimolar genomic arrangements in herpes simplex virus. In contrast, the BHV-1 genome contains only two genomic isomers. It is obvious that the inverting short segment of BHV-1 in virion DNA molecules is bracketed by two inversely oriented α elements and the adjacent sequences of the γ elements (Fig. 7). In contrast, the noninvertable U_L segment is bracketed by two α elements only. This observation leads to the assumption that the two γ elements may contribute signal(s) required for the inversion of U_S of BHV-1.

It is not yet clear what role the "a" sequences perform in the inversion of U_L and U_S of HSV-1. In vivo recombinants of HSV-1 carrying an additional complete or deleted "a" sequence in U_L give rise to different results. Whereas Varmuza and Smiley (42) reported low levels of inversion in these mutants, Mocarski and Roizman (27, 29) have described similar mutants which are active in inversion. Additionally, deletions of the internal DRs of "a" reduce inversion significantly.

Our hypothesis that the A_n sequences of herpesvirus genomes are essential for the maturation of replicative-form DNA and the formation of the genomic isomers can be tested by the introduction of mutations into α and β elements and into the A_n stretch of BHV-1. The use of BHV-1, which has a less complicated genome structure than HSV-1, is likely to aid in the detailed understanding of these recombinational events common to many herpesviruses.

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