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Short Repeats Cause Heterogeneity at Genomic Terminus of Bovine Herpesvirus 1

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Analysis of the genomes of different bovine herpesvirus 1 strains revealed a U_L terminal *Hin*dIII fragment differing in size (from 2.4 to 2.8 kilobases). This fragment polymorphism occurred in the DNA of a wild-type isolate, in highly passaged, apathogenic tissue culture derivatives, and in plaque-purified substrains. This heterogeneity was due to variations in the copy number of a 14-base-pair tandem repeat comprising the base sequence 5′-GCTCCTCCTCCCTC-3′, which also exists, with some differences, in other short reiteration sequences of herpes simplex virus type 1, Epstein-Barr virus, and related human cellular DNA. Furthermore, the tandem repeat array was located in close proximity to the left end of the viral genome and may functionally be involved in viral replication.

Bovine herpesvirus 1 (BHV-1) includes infectious bovine rhinotracheitis and infectious pustular vulvovaginitis (IPV) viruses, which cause defined clinical symptoms. Virulent strains isolated from different clinical entities and apathogenic BHV-1 vaccine strains are immunologically similar and have almost identical polypeptide patterns, with minor strain-specific variations (7, 15). 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 (11 kb), which is inverted with respect to I_R (6, 12; T. M. Beck, Ph.D. thesis, University of Zurich, Zurich, Switzerland, 1984). This genome structure exemplifies a class-D herpesvirus (18) and is also found in pseudorabies virus (1), equine herpesviruses 1 and 3 (10, 22), and varizella-zoster virus (2). A consequence of such a genome structure is the ability to invert U_S relative to U_L, leading to two isomeric structures of the DNA molecule. Physical maps of the genomes of BHV-1 strains K22, Cooper, and B-Schönböken have been established for a few restriction endonucleases (6, 12; Beck, Ph.D. thesis). Specific differences in the restriction endonuclease (*Hpa*I and *Hin*dIII) cleavage patterns of infectious bovine rhinotracheitis and IPV virus strains have been reported (5, 7, 11). Analysis of the restriction endonuclease cleavage patterns of the DNAs from more than 100 BHV-1 strains allowed three different groups to be distinguished, but these did not correlate with the epidemiological features (15).

Heterogeneity in the sizes of restriction fragments located at the left-hand genomic terminus on the U_L segment of strain B4 was observed earlier in our laboratory. Strain B4 is an apathogenic descendant of an IPV virus isolate. Thus, the question arose as to whether deletions of sequences might have occurred, causing the loss of pathogenicity of this strain. Further interest was caused by the fact that the target area of the genome variability is in close proximity to the left-hand genomic terminus. This area plays an important role in the circularization or concatemerization of BHV-1 genomes during DNA replication and in cutting into complete linear virus genomes during virus maturation.

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MATERIALS AND METHODS

Cells and virus strains. The BHV-1 strains used in this study are listed in Table 1. Strains B1 (Schönböken) and B4 were a generous gift from O. C. Straub, Federal Research Center for Virus Diseases of Animals, Tübingen, Federal Republic of Germany. Strain B1 is a vaginal isolate from a cow affected with IPV. This strain was propagated less than 12 times in cell cultures and is able to induce the typical clinical symptoms of IPV. In contrast, strain B4 is a highly passaged tissue culture derivative which is avirulent. Bayferon (Bayer AG) is a commercially available strain B4 applied as an interferon inducer in veterinary medicine. Virus strains either were used without further purification or were plaque purified (Table 1). Viruses were propagated in Georgia bovine kidney (GBK) cells grown in Dulbecco modified Eagle minimal essential medium (GIBCO Laboratories) supplemented with 5% fetal calf serum. Viruses passaged once after low-multiplicity infection (10^{-3}) were kept at −80°C.

Preparation of viral DNA and restriction endonuclease cleavage. Nucleocapsids were obtained from the supernatant of an infected cell culture by centrifugation through a 30% sucrose cushion. They were lysed with sodium dodecyl sulfate and proteinase K (Serva) in combination. The DNA preparation was further purified by equilibrium density centrifugation in CsCl-ethidium bromide gradients. Restriction endonuclease cleavage and analysis were done as described previously (4). For comparative studies, DNA was prepared from reference strains, including bovine herpesviruses 2, 3, and 4, pseudorabies virus, equine herpesvirus 1, and herpes simplex virus type 1 (HSV-1), from the collection at the Institut für Virologie der Freien Universität Berlin.

Construction of recombinant plasmids. Whole virion DNA was treated with DNA polymerase I-large fragment in the presence of the four deoxynucleoside triphosphates to ensure blunt ends. Dodecamer *Eco*RI linkers (New England BioLabs) were phosphorylated with T4 polynucleotide kinase (Boehringer GmbH) in the presence of [^{32}P]ATP and

TABLE 1. Characteristics of the original strain (B1) and substrains (B4) used in this study

Strain	No. of cell culture passages	Plaque purification of virus stocks	U_L terminal DNA clones in pUC8[a]
436B1	<12	No	M44 and M94
440B4	>450	No	Not done
438B4[b]	>450	No	Not done
442B4	>450	Yes[c]	M10, M22, and M23
446B4	>450	Yes[c]	M297

[a] Derived plasmid clones containing terminal fragments from the U_L segment of the genome.
[b] Commercially available as an interferon inducer in cattle (Bayferon).
[c] Plaque-purified virus stocks were independently derived from strain 440B4.

ligated with T4 DNA ligase to the virion DNA. Ligated linkers and virion DNA were cleaved with a combination of at least two restriction endonucleases (*Eco*RI and *Hin*dIII). The digested DNA was analyzed on an agarose gel. After electrophoresis, the gel was dried, and the fragments representing the genomic termini were identified by autoradiography. The major part of the digested DNA was applied to a preparative agarose gel, and the genomic end fragments were isolated from the gel. Because the fragments had *Eco*RI-cut ends, which were supplied through the linker ligated directly to the genomic termini, and *Hin*dIII-cut ends, which were generated at the internal site distal from the first *Eco*RI site, ligation under forced cloning conditions to an *Eco*RI-*Hin*dIII-cut pUC8 vector (13) was possible. Competent JM83 cells (14) were transformed with the ligation mixture. Recombinant clones were identified by restriction site mapping and hybridization experiments.

DNA sequencing. DNA was sequenced by the chain terminator method described by Sanger et al. (19). Appropriate subclones were constructed in M13mp8 or M13mp11 replicative-form (RF) DNA (13). Recombinant phages released from transfected JM103 cells (14) were purified by CsCl gradient centrifugation, and their single-stranded DNAs were extracted. After being annealed to a 26-mer (Bethesda Research Laboratories) or 15-mer (New England BioLabs) sequencing primer, these DNAs served as templates for the sequencing reactions in an appropriate mixture of deoxynucleoside and dideoxynucleoside triphosphates and [^{35}S]dCTP. Polymerization was carried out with DNA polymerase I-large fragment (New England Nuclear Corp.). Synthesis products were separated in 0.2-mm 8% polyacrylamide–urea gels, which were bound to glass plates, dried after electrophoresis, and autoradiographed with XAR-5 film (Eastman Kodak Co.).

Hybridization experiments. After electrophoresis on an agarose gel, restriction fragments were transferred (21) to Gene Screen membranes (New England Nuclear). In vitro labeling of recombinant DNA by nick translation (17) with [^{35}S]dCTP and hybridization to the immobilized DNA fragments on Gene Screen membranes were done as described previously (4).

RESULTS

Comparison and localization of the *Hin*dIII K fragment of different B strains. As we observed previously, a twofold plaque-purified BHV-1 strain (446B4; Table 1) had a discrete genomic *Hin*dIII K fragment which was about 400 bp smaller than the corresponding fragment in the original strain (436B1). This finding had first been interpreted by us as a DNA deletion which might be involved in the loss of pathogenicity of the cell culture-adapted, attenuated vaccine strain. However, it became obvious that in the non-plaque-purified laboratory strain (440B4) as well as in the commercially available vaccine strain (438B4), the *Hin*dIII K fragment showed up as a smear in agarose gels. The sizes of the fragments ranged from 2.4 to 2.8 kb (Fig. 1). This heterogeneity indicated a mixture of different DNA genomes containing different *Hin*dIII K fragments. Therefore, different plaque-purified derivatives of strain 440B4 were isolated, and the DNAs were subjected to *Hin*dIII digestion. The resulting DNA cleavage patterns revealed discrete *Hin*dIII K fragments, with characteristic mobilities, in the predicted range from 2.4 to 2.8 kb (Fig. 1). To identify the region of size variation precisely, we constructed a physical map of the *Hin*dIII cleavage sites. Terminal *Hin*dIII fragments were labeled with ^{32}P-phosphorylated *Eco*RI linkers, which were ligated to the blunt ends of the BHV-1 genome as described in Materials and Methods. Subsequent cleavage with different restriction endonucleases and autoradiography of the agarose gel used for separating the cleavage products revealed the terminal arrangement of the *Hin*dIII K fragment (Fig. 2). An independently established *Hin*dIII restriction map (Beck, Ph.D. thesis) is in complete agreement with our data.

Cloning and characterization of terminal *Hin*dIII K fragments. We modified the *Hin*dIII K fragments in such a way that an artificial *Eco*RI site, linked to the genomic terminus, formed one end and the genome-internal *Hin*dIII site represented the other end (Materials and Methods and Fig. 2). This made it possible to ligate such fragments with a defined

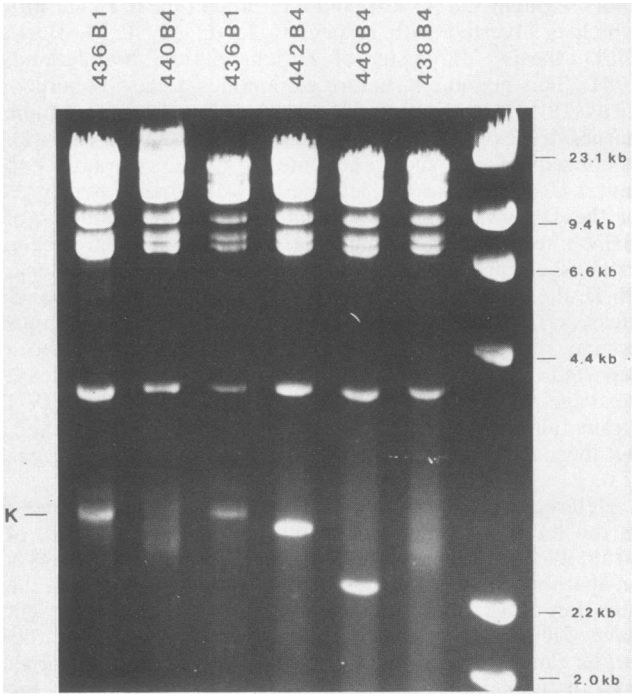

FIG. 1. Comparison of *Hin*dIII K fragments of the B1 strain and different B4 strains. The code numbers of the strains are listed in Table 1. Discrete *Hin*dIII K fragments of strains 436B1, 442B4, and 446B4 ranged from 2.4 to 2.8 kb. In contrast, strains 440B4 and 438B4 showed a DNA smear within this range. The right-hand lane shows λ DNA digested with *Hin*dIII as molecular weight marker.

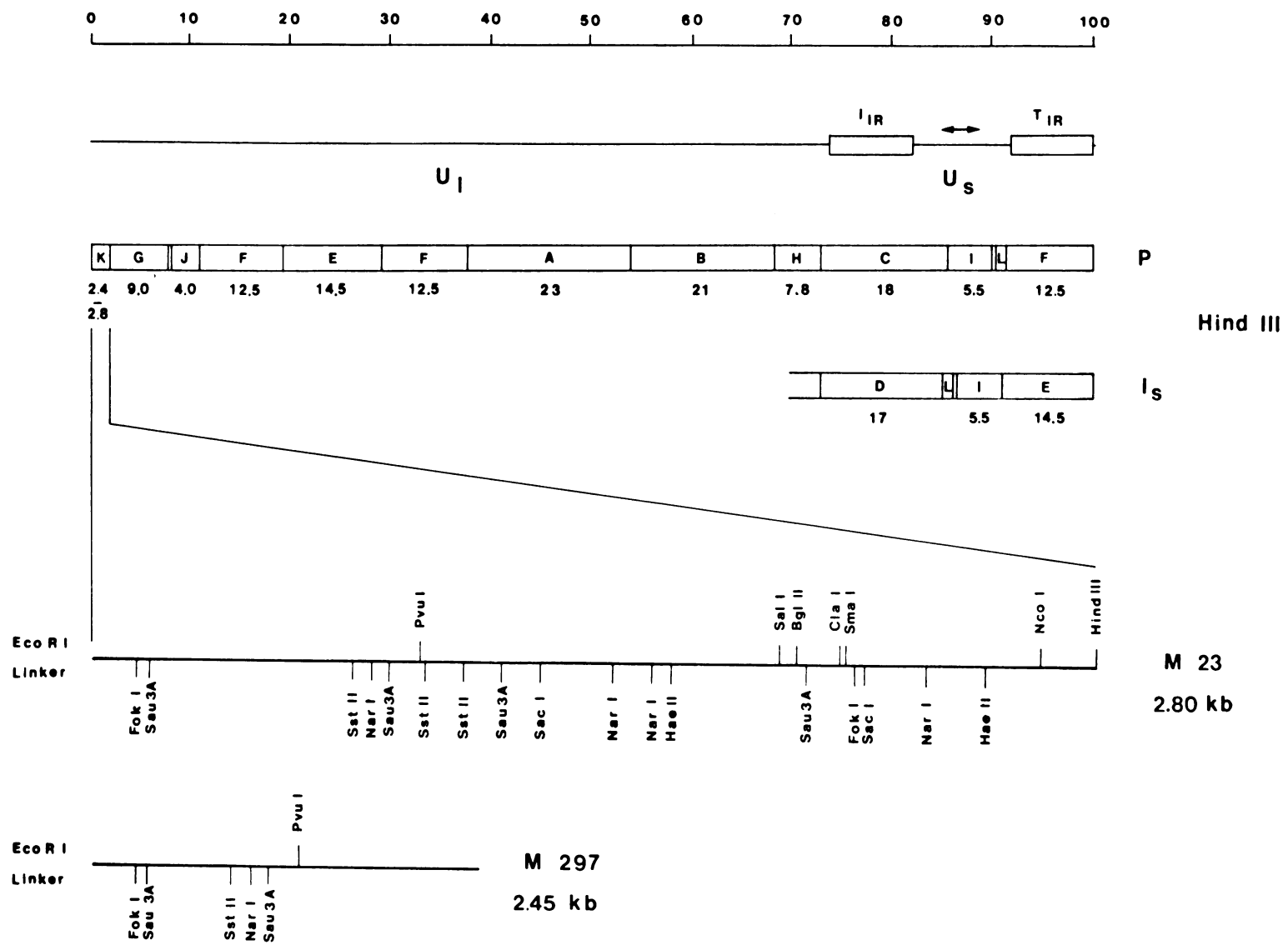

FIG. 2. Genome structure (line 1) and physical map of BHV-1 B strains showing the cleavage sites for *Hin*dIII. Fragments are lettered according to size as given in kilobases below lines 2 and 3. They sum up to a total size of 135 kb. P and I_S represent the two isomeric genome arrangements. A detailed restriction site map of the *Hin*dIII K fragment (from plasmid clone M23) is shown in line 4. For comparison, the heterogeneous region between the left-hand *Sau*3AI and SstII sites is also shown for plasmid clone M297 (line 5). Abbreviations: U_l, unique long segment; U_s, unique short segment; I_{IR}, internal repeat; T_{IR}, terminal repeat. I_{IR} and T_{IR} are inverted with respect to each other.

orientation into the appropriately cleaved vector pUC8. Recombinant plasmid clones were derived from the original strain (436B1) and from two plaque-purified substrains (442B4 and 446B4; Table 1). The cloned *Hin*dIII K fragments were analyzed by digestion with various restriction endonucleases and agarose gel electrophoresis. The data showed that the heterogeneous region is located within a certain *Sau*3AI-*Sst*II subfragment (Fig. 2) which varies in size between 250 bp (M297) and 650 bp (M44).

Analysis of the *Hin*dIII K fragment from plaque-purified isolate 442B4. Substrain 442B4 was generated by two plaque purification steps, starting with a plaque from BHV-1 strain 440B4 (Table 1). Plaque-purified stock virus was amplified once in GBK cells. From this virion DNA the *Hin*dIII K fragment was cloned. Ten recombinant clones bearing the desired insert were isolated and characterized by restriction site mapping. Three different size classes were found (Fig. 3). A representative of each was chosen for sequence analysis (plasmid clones M10, M22, and M23; Table 1).

Sequencing of the heterogeneous fragments. To characterize the region of variability precisely, we subcloned the *Eco*RI-*Nar*I subfragments spanning the variable region of the recombinant plasmids (Table 1) in M13 and sequenced the DNA by the chain terminator method (19). In general, we found the sequences to be identical, with the exception of clone M297 (see below). However, different copy numbers were observed for the sequence 5′-GCTCCTCCTCCCTC-3′, which was reiterated in tandem several times. Plasmid clones M10, M22, M23, M44, and M94 contained 24, 23, 28, 38, and 36 copies of this C-rich repeat, respectively, whereas plasmid clone M297 possessed only 8 copies. Furthermore, at the proximal end of the array of tandem repeats, plasmid clones M10, M22, and M23 were slightly divergent in a single repeat unit. All B4-derived plasmid clones had a transition from T to C in one repeat unit at nucleotide position 594 (Fig. 4), except for plasmid clone M297. The transition always lay at the same position with respect to the proximal end of the repeat array. An incomplete unit with the sequence 5′-GCTCCTCCTC-3′ finished the reiteration of minirepeats at the right end of the array (Fig. 4). The 200-bp-long nucleotide sequence distal from the repeats was in perfect agreement in five of the six clones. Only plasmid clone M297 had differences of a few base pairs within the first 25 bp, as counted from the genomic terminus of BHV-1, thus accounting for an additional *Apa*I site (Fig. 4). The restriction patterns of all six cloned fragments were identical for the regions not sequenced, and there was no indication of further sequence diversity.

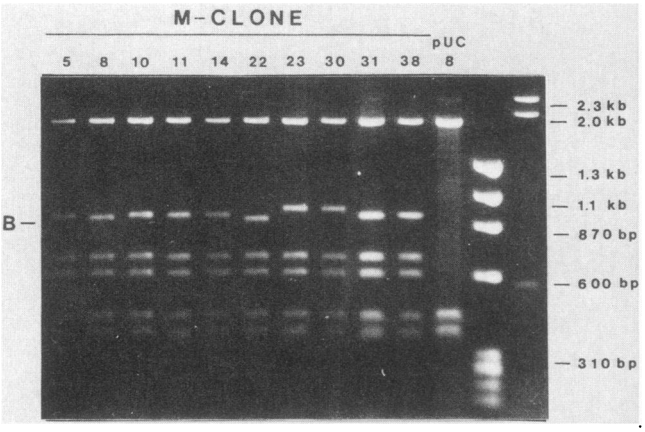

FIG. 3. *Hae*III digests of different clones derived from the *Hin*dIII K fragment of a twofold plaque-purified strain (442B4) as described in Results. Variants of the *Hae*III B fragment are grouped in three size classes; a representative of each (clones M10, M22, and M23) was sequenced. The different sizes reflect a divergency in the array of the 14-bp tandem repeat: M10, M22, and M23 contain 24, 23, and 28 copies, respectively.

Hybridization experiments. ^{35}S-labeled recombinant plasmid clones carrying the *Hin*dIII K fragment of BHV-1 were hybridized to Southern blots of restriction fragments obtained from virion DNAs of BHV-1, bovine herpesviruses 2, 3, and 4, pseudorabies virus, equine herpesvirus 1, and HSV-1. There was no hybridization signal other than that from the *Hin*dIII K fragment of BHV-1 itself.

DISCUSSION

The results showed that the size heterogeneity in the U_L terminal *Hin*dIII fragment was due to differences in the copy number of a 14-bp tandemly arranged repeat unit (5′-GCTCCTCCTCCCTC-3′). The reiteration frequency varied from 8 to 38 copies in the analyzed BHV-1 strains, resulting in a size heterogeneity of about 400 bp.

We never observed a change in frequency when the recombinant plasmid clones were propagated in *Escherichia coli* host cells. However, in a viral DNA preparation obtained after infection of GBK cells with a twofold plaque-purified virus stock, heterogeneous reiteration frequencies were observed (Fig. 3). It seems likely that the BHV-1 particle which led to the plaque used for the purification of strain 442B4 contained 24 repeat units, like 7 of the 10 isolated plasmid clones did (e.g., M10; Fig. 3). This means that a detectable loss and gain of repeat units had occurred after at least two rounds of multiplication, exemplified by plasmid clones M22 and M23. The rapid change in the number of reiterations demonstrated for strain B4 led, in this respect, to a mixture of viruses. This finding was also supported by plasmid clones M44 and M94, which had different numbers of reiterations and were derived from the virulent strain B1. Thus, it seems obvious that the previously observed size heterogeneity of restriction fragments from the left-hand genomic terminus of BHV-1 is not the explanation for the loss of pathogenicity of the attenuated strain B4.

The appearance of a repeat unit with a T-to-C transition in three out of four sequenced clones derived from strain B4 indicates that the proximal end of the repeat array is, in general, not the region where changes occur. The fact that the isolate with only 8 repeats (M297) lost the repeat unit with the T-to-C transition (but not the incomplete repeat) suggests that the inner part of the repeat array is subject to change, leaving at most two repeats at the proximal end and at least six repeats at the distal end in the case of isolate M297, although we do not have an explanation for further sequence changes in this isolate (Fig. 4).

Analogous to the 14-bp repeats of BHV-1 described here, variations in the copy number of multiple short reiteration sequences have been recognized as a reason for the size heterogeneity of restriction fragments from different HSV-1 strains, which are called reiteration I and III (3), DR 2 and DR 4 (16), and a 15-bp repeat (23). In addition to their relatively high number of deoxycytidyl residues, their sequences show additional homology in one strand of the DNA duplex (Table 2). Moreover, there is a very high homology to the sequence of internal repeat 3 (IR3) of Epstein-Barr virus, strain B95-8, which contains only three triplets: GGG, GGA,

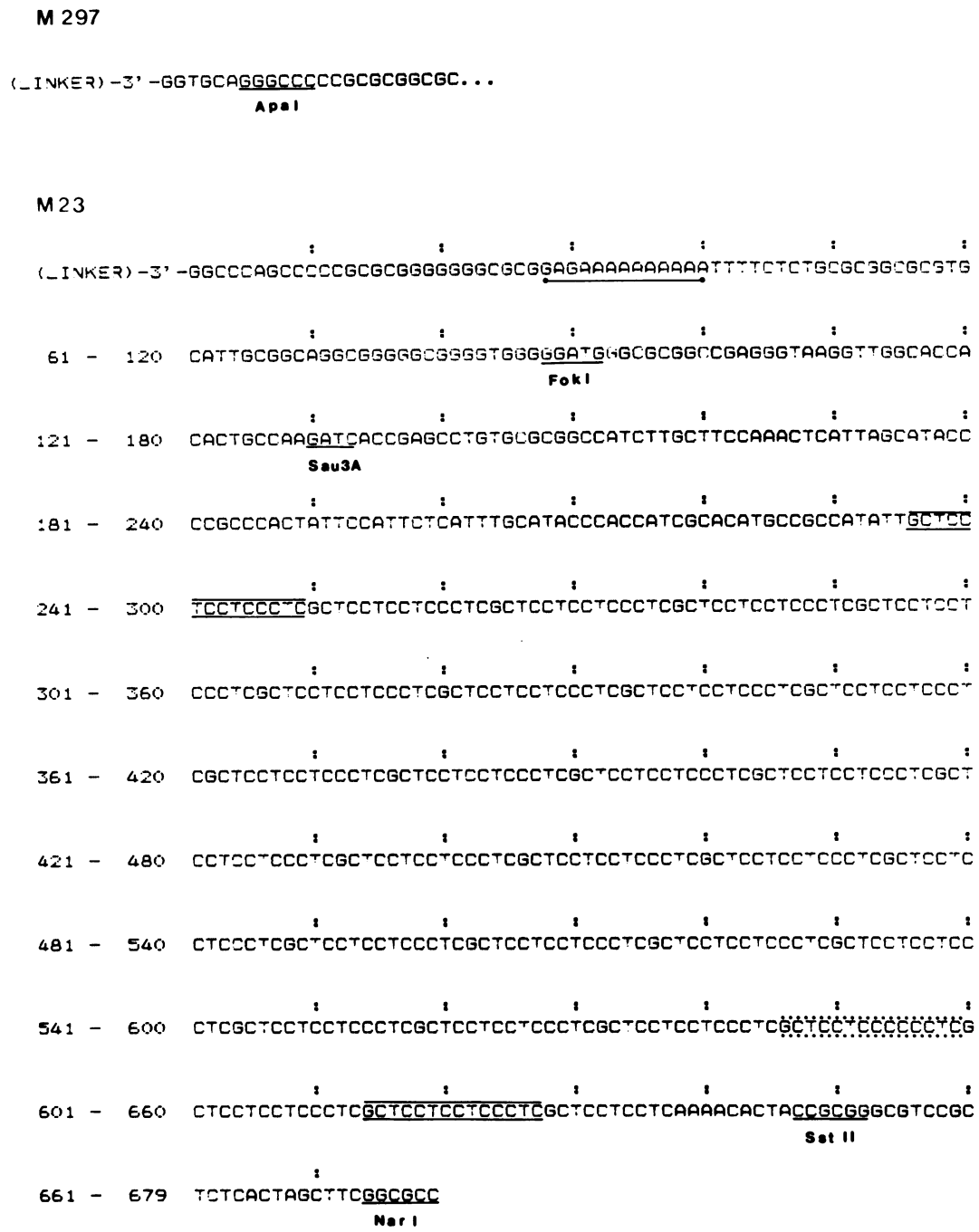

FIG. 4. Nucleotide sequence of the left end of the BHV-1 genome as represented by plasmid clones M23 and M297. Numbering starts at the left end of the genome and extends to the first *Nar*I restriction site. The recognition sites for *Fok*I, *Sau*3AI, *Sst*II, *Nar*I, and *Apa*I are underlined. ——, Consensus sequence of BHV-1 and varicella-zoster virus; ═, 14-bp repeat unit; ⋯⋯, 14-bp repeat unit with a single T-to-C transition.

and GCA (9). This 708-bp spanning IR3 region can be constructed entirely by repeating the following four complementary sequence elements: GCTCCT, GCCCCTCCT, GCCCCTCCTCC, and GCCCCTCCTCCT (for comparison, the complementary strand, not the published [9] coding DNA strand, of IR3 is given). Only the underlined nucleotides of the 6- to 12-bp-long IR3 sequence elements differ from the nucleotides of the 14-bp repeats of BHV-1. With the IR3 region of Epstein-Barr virus strongly cross-hybridizing, tandem arrays of short repeats in mouse and human cellular DNAs have been described on the nucleotide sequence level (8). IR3-related repetitive sequences have also been found in other organisms, including bovines, by Southern blot hybridization (8). Hence, we assume that the 14-bp repeat region of BHV-1 would hybridize to bovine cellular DNA, too, under appropriate conditions.

TABLE 2. Comparison of known repetitive short sequences of other herpesviruses with BHV-1 14-bp repeats

Sequence	Location in genome	Virus strain	Base sequence 5′ to 3′ in analogous arrangement[a]	Source or reference
14-bp tandem repeat, major sequence	U_L	BHV-1 B1 and B4	GCTCCTCCTCCCTC →	This study
14-bp tandem repeat, minor sequence	U_L	BHV-1 B4	GCTCCTCCCCCCTC →	This study
Epstein-Barr virus nuclear antigen triplet repeat[b]	IR3	Epstein-Barr virus B95-8	GCTCCT GCTCCT → GCCCCTCCTCCT GCTCCTCCTCCC →	9
Reiteration I	"a" Sequence	HSV-1 17 (United States)	CCGCTCCTCCCC ···CCGCTCCTCCTC··· →	3
Reiteration III	"c" Sequence	HSV-1 17 (United States)	CCTCCCCTCGCCCCCTC ···C-TCCC-TCGCTCC-TC··· →	3
Reiteration II[c]	"c" Sequence	HSV-1 17 (United States)	CCCTCCCCAGCCCCAG ···TCCTCCCTCGCTCC--··· → CCCTCCCCCGGCCCCAG ···TCCTCCCTCGCTCC--··· →	3
DR 2	"a" Sequence	HSV-1 F	CGCTCCTCCCCC ···CGCTCCTCCTCC··· →	16
DR 4[d]	"a" Sequence	HSV-1 F	CGCTCCTCCCCGCTCCCGCGG- GCTCCTCCTCCCTC → -CCCCGCCCCCAACGCC	16
DR 6	"c" Sequence	HSV-1 F	GCCCCAGCCCTCCCCA GCTCC--TCCTCCCTC →	16
15-bp tandem repeat	U_S	HSV-1 Patton	CCACTCC-CCACCCAC GCTCCTCCTCCCTC →	23

[a] Common nucleotides shared by sequences are connected by vertical dashes. Deletions are marked within the nucleotide sequences by horizontal dashes. Arrows indicate position G of the 14-bp tandem repeat of BHV-1. Dots (···) indicate that the BHV-1 14-bp repeat sequence is permutated for comparison. DR, Direct repeat.

[b] For comparison with the BHV-1 14-bp repeat, the cDNA strand of IR3 (9) is given.

[c] Reiteration II of HSV-1 17 (United States) contains two different repeated sequences; both are shown.

[d] DR 4 of HSV-1 F (United States) is 37 bp long and therefore occupies two lines in the column.

How BHV-1 genomes change the frequency of their 14-bp repeats and the function of these are not yet known. However, the recombination events discussed by Smith (20) or a slippage-repair model discussed by Umene et al. (23) could, in principle, explain this heterogeneity. Furthermore, we propose that the 14-bp repeats might functionally be involved in joining or cutting at the genomic termini of BHV-1 during replication because (i) preliminary data indicate that such joining occurs approximately 235 bp distal from the 14-bp repeat array, (ii) similar sequences are directly involved in cutting and joining of HSV-1 genomes (Table 2), and (iii) another DNA region of HSV-1, including a similar 15-bp repeat array, seems to be involved in a nonhomologous interaction between specific sites (23). Moreover, it has been shown for bovine herpesvirus 4 that the actual genomic termini are part of polyrepetitive sequences of several hundred nucleotides (4).

Besides the repeat array, additional remarkable features exist in the nucleotide sequence of the BHV-1 genomic terminus. (i) Within the repeat array, no translational stop codons can be formed. This may mean that the 14-bp repeats lie in an intergenic region, as was shown to be the case for

the 15-bp repeats analyzed by Umene et al. (23). (ii) The sequence displayed in Fig. 4 has a total G+C content of 67%, such that the sequence GAGAAAAAAAAAA (from position 28 to position 40) forms an exception. Exactly the same sequence is present in the varizella-zoster virus genome 28 to 29 nucleotides away from the genomic terminus in the terminal repeat (and internal repeat) of the L segment (2). Because this is the only extended sequence homology at L-segment termini between BHV-1 and varicella-zoster virus, we suggest that this homology may indicate an important functional site conserved in both herpesviruses. (iii) Within the first 30 nucleotides of the sequence shown in Fig. 4 a variety of possibilities exist for constructing hairpin-loop structures. In addition, the first 15 nucleotides show an imperfect but extended complementarity to the sequence from position 72 to position 95. Whether these complementary sequences represent important structures in vivo is unknown.

To our knowledge this is the first report of a tandemly reiterated sequence in the U_L segment of a class-D herpesvirus genome. Investigations concerning the functional significance of the described structure are in progress.

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