JOURNAL OF VIROLOGY, May 1986, p. 657–660
0022-538X/86/050657-04$02.00/0

Vol. 58, No. 2

Amplification of a Tandem Direct Repeat within Inverted Repeats of Marek's Disease Virus DNA during Serial In Vitro Passage

KUMIKO MAOTANI,[1] AKIHIRO KANAMORI,[1] KAZUYOSHI IKUTA,[1] SHIGEHARU UEDA,[1] SHIRO KATO,[1] AND KANJI HIRAI[2]*

Department of Pathology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565,[1] and Department of Molecular Biology, Tokai University School of Medicine, Bohseidai, Isehara 259-11,[2] Japan

Received 16 July 1985/Accepted 25 September 1985

DNA of the oncogenic strain BC-1 of Marek's disease virus contains three units of tandem direct repeats with 132 base pairs in the terminal repeat and internal repeat, respectively, of the long region of the Marek's disease virus genome, whereas the attenuated, nononcogenic viral DNA contains multiple units of the tandem direct repeats.

The genome of Marek's disease virus (MDV) has been shown to be a linear double-stranded DNA with a molecular weight of 110×10^6 and buoyant density of 1.705 g/cm^3 in neutral CsCl (5, 10). Cebrian et al. (1) showed by electron microscopic studies that the genome structure consists of a long unique region (U_L) and a short unique region (U_S) bounded by inverted repeats, similar to that of herpes simplex virus (13). Fukuchi et al. (2) have recently completed the physical maps of the restriction endonuclease fragments of MDV DNA, which support the electron microscopic data.

We reported previously that serial passages of oncogenic MDV in culture resulted in structural change of its DNA with loss of oncogenicity (7). The restriction endonuclease fragments *Bam*HI-D and -H in enzyme digests of oncogenic MDV DNA were lost at higher passage levels with loss of oncogenicity and were not found in digests of standard nononcogenic MDV DNA (8). These oncogenic strain-specific fragments share homology with the DNA fragments with heterogenous electric mobilities of nononcogenic MDV at higher passage levels in culture, which are larger than *Bam*HI-D and -H and are named *Bam*HI-Dhet and -Hhet, respectively (4). The presence of *Bam*HI-Dhet and -Hhet appears to be a common feature of various isolates of nononcogenic MDV (3, 14, 16). The present results show that the size variation of *Bam*HI-Dhet and -Hhet fragments is due to variation in the copy number of tandem repeats of 132 base pairs (bp). The tandem repeats were mapped within the terminal repeat (TR_L) and internal repeat (IR_L) of the long region of the MDV genome.

The BC-1 strain of MDV was propagated in primary chicken embryo fibroblasts as described previously (6). The BC-1 strain at lower passages of 16 to 23 (BC-1/LP) had the ability to form typical Marek's disease lymphomas in chickens, whereas at higher passages of 40 to 48 (BC-1/HP) it showed no oncogenicity (9). Viral DNA was extracted from Nonidet P-40-treated virions, purified by sedimentation in neutral glycerol gradients, and digested with restriction endonuclease *Bam*HI as described previously (5, 7). The *Bam*HI fragments of BC-1/LP DNA were cloned into pBR322 with *Escherichia coli* DH-1, essentially as described by Okayama and Berg (12). The *Bam*HI fragments isolated from recombinant plasmids were further digested with restriction endonucleases *Eco*RI, *Sma*I, *Hpa*II, *Taq*I, *Hha*I, *Acc*I, and *Sau*3AI (purchased from Takara Shuzo Co., Kyoto) as specified by the supplier. The sizes of the fragments obtained were determined on 1% agarose or 4% polyacrylamide gel under the conditions described by Maniatis et al. (11). The *Hin*dIII fragments of bacteriophage lambda DNA and *Hin*fI fragments of pBR322 DNA were used as molecular weight markers. For DNA sequencing analysis, DNA fragments were cloned into the M13 phage vectors mp8 and mp9 and then subjected to sequencing analysis by the dideoxy chain termination technique (15) with an M13 phage sequencing kit purchased from Takara Shuzo Co. The reaction products obtained were resolved on 8% polyacrylamide–8 M urea gels. The conditions for Southern blot hybridization were as described previously (4).

To analyze the structures of *Bam*HI-Dhet and -Hhet, we attempted to clone *Bam*HI-Dhet and -Hhet of BC-1/HP DNA into the *Bam*HI sites of pBR322. The recombinant plasmids used here were two *Bam*HI-Dhet clones (pMBDh7 and pMBDh26) and three *Bam*HI-Hhet clones (pMBHh3, pMBHh27, and pMBHh42). The sizes of *Bam*HI-Dhet and -Hhet from these recombinant plasmids were compared with those of cloned *Bam*HI-D (pMBD1) and *Bam*HI-H (pMBH8) of BC-1/LP DNA (Fig. 1A). Cleavage with *Bam*HI of these recombinant plasmids, pMBD1, pMBDh26, pMBDh7, pMBH8, pMBHh3, pMBHh42, and pMBHh27, yielded cloned MDV DNA fragments with sizes of 12.0, 14.0, 14.5, 5.3, 6.0, 7.0, and 8.0 kilobases, respectively. The cloned fragments were isolated from these recombinant plasmids and digested further with *Eco*RI. Only one subfragment in the *Eco*RI digests differed in size in *Bam*HI-D and -Dhet and in *Bam*HI-H and -Hhet. The variable sized fragments, *Eco*RI fragment 2 for *Bam*HI-D and *Eco*RI fragment 1 for *Bam*HI-H, may contain the region specific to oncogenic MDV DNA.

Figure 2A and B show the location of oncogenic strain-specific fragments, *Bam*HI-D and -H, on the physical maps of the MDV DNA genome constructed by Fukuchi et al. (2) and the restriction endonuclease maps of the cloned *Bam*HI-D and -H of BC-1/LP DNA. The physical maps of *Bam*HI-D and -H were constructed from the results obtained by single and double digestion with combinations of restriction endonucleases and by reciprocal Southern blot hybridization. The site for the U_{L1}-TR_L and U_{L2}-IR_L junctions were located close to the left end of *Eco*RI fragment 1 of *Bam*HI-D (*Eco*RI-1–*Bam*HI-D) and the right end of *Eco*RI-3–*Bam*HI-H, respectively, because *Eco*RI-1–*Bam*HI-D and *Eco*RI-3–*Bam*HI-H hybridized slightly to *Bam*HI-H and -D,

* Corresponding author.

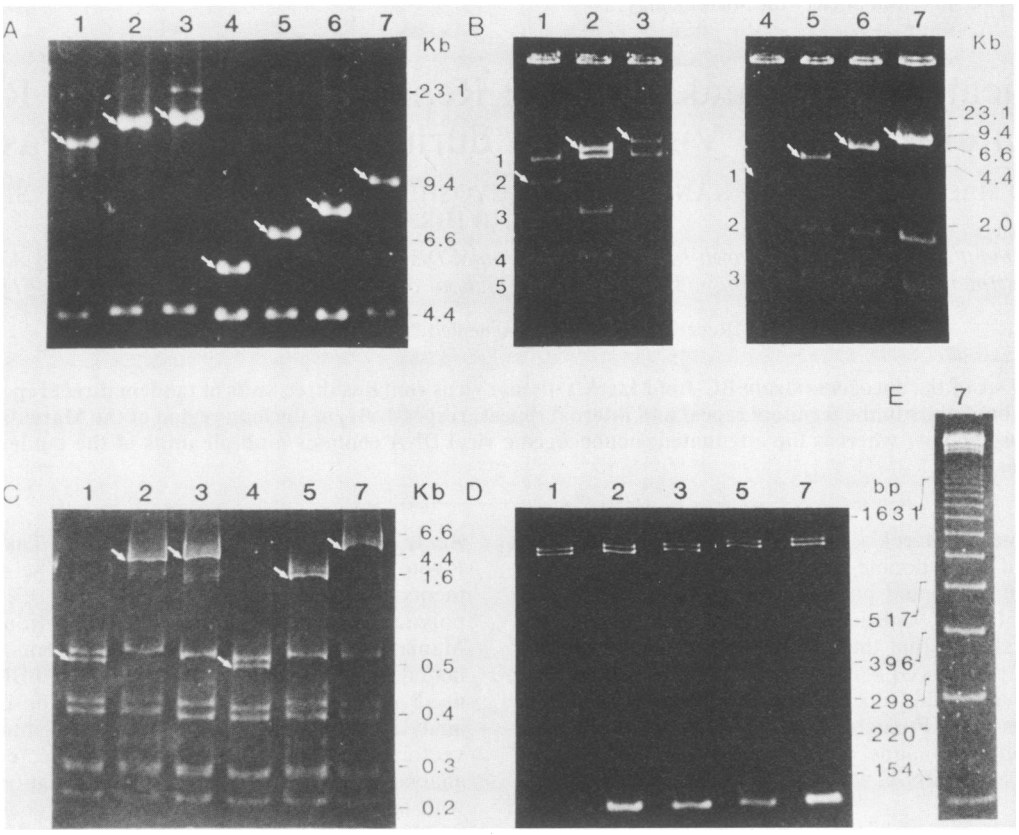

FIG. 1. Characterization of cloned *Bam*HI D, Dhet, H, and Hhet fragments from BC-1/LP and BC-1/HP DNAs. DNA fragments were cleaved with restriction endonucleases, separated by electrophoresis on 1% agarose gel, and stained with ethidium bromide. The slot numbers in A, B, C, D, and E indicate restriction endonuclease digests of cloned subfragments derived from the recombinant plasmids as follows: 1, pMBD1 (*Bam*HI-D); 2, pMBDh7 (*Bam*HI-Dhet); 3, pMBDh26 (*Bam*HI-Dhet); 4, pMBH8 (*Bam*HI-H); 5, pMBHh3 (*Bam*HI-Hhet); 6, pMBHh42 (*Bam*HI-Hhet); 7, pMBHh27 (*Bam*HI-Hhet). *Hin*dIII-digested DNA (A and B), *Hin*fI-digested pBR322 DNA (D and E), and their mixture (C) served as molecular weight markers, and values are indicated on the right of each panel. The fragments that differed in size or number in BC-1/LP and BC-1/HP DNAs are shown by arrows. (A) *Bam*HI cleavage patterns of recombinant plasmids containing *Bam*HI-D, -Dhet, -H, and -Hhet. (B) *Eco*RI cleavage patterns of cloned *Bam*HI-D, -Dhet, -H, and -Hhet. The *Eco*RI subfragment numbers of *Bam*HI-D and -H are indicated on the left of the corresponding bands (C) *Taq*I cleavage patterns of *Eco*RI-2–*Bam*HI-D, *Eco*RI-1–*Bam*HI-H and their corresponding fragments of cloned *Bam*HI-Dhet and -Hhet. (D) *Acc*I cleavage patterns of *Eco*RI-2–*Bam*HI-D, *Eco*RI-1–*Bam*HI-H, and their corresponding fragments of cloned *Bam*HI-Dhet and -Hhet. (E) Identification of the tandem repeat in *Bam*HI-Hhet. The cloned *Eco*RI-*Bam*HI subfragment including the variable region of *Bam*HI-Hhet (pMBHh27) was partially digested with *Acc*I.

respectively (data not shown). Therefore, the variable regions between BC-1/LP and BC-1/HP DNAs are located within the TR_L and IR_L.

Since *Eco*RI-2–*Bam*HI-D and *Eco*RI-1–*Bam*HI-H contain the variable region between BC-1/LP and BC-1/HP DNAs (Fig. 1B and 2B), these *Eco*RI subfragments and their corresponding fragments of cloned *Bam*HI-Dhet and -Hhet were subcloned into the *Bam*HI and *Eco*RI sites of pBR322. Then, the oncogenic strain-specific regions were further defined by comparison of the cleavage patterns with various restriction endonucleases of the *Eco*RI subfragments from recombinant plasmids. The cleavage patterns of the *Eco*RI subfragments with *Taq*I indicated that only one specific fragment in the enzyme digests differed in size in oncogenic BC-1/LP DNA and nononcogenic BC-1/HP DNA (Fig. 1C). Since *Eco*RI-2–*Bam*HI-D and *Eco*RI-1–*Bam*HI-H showed identical cleavage patterns with various restriction endonucleases including *Taq*I (Fig. 1C) and *Acc*I (Fig. 1D) (data not shown for the other enzymes), the cleavage maps with various restriction endonucleases of cloned *Eco*RI-1–*Bam*HI-H only are shown in Fig. 2C, and the oncogenic strain-specific fragments of the restriction endonucleases that showed size heterogeneity in *Bam*HI-Dhet and -Hhet subclones are indicated by solid bars on the cleavage maps. The cleavage patterns of *Eco*RI-2–*Bam*HI-D and *Eco*RI-1–*Bam*HI-H with *Acc*I were identical with those of the corresponding fragments of *Bam*HI-Dhet and -Hhet, except that a small fragment of about 130 bp was more abundant in the enzyme digests of fragments of the nononcogenic strain than in those of the oncogenic strain (Fig. 1E). Partial digestion with *Acc*I resulted in a series of bands with sizes increasing by increments of about 130 bp (Fig. 1E). These results indicate that the *Bam*HI Hhet fragment contains multiple units of about 130-bp tandem repeats in the variable regions.

For more precise determination of the structure of oncogenic strain-specific regions, the oncogenic strain-specific *Taq*I subfragment of about 540 bp in Fig. 2C was cloned into the *Acc*I site of M13 phage vector mp8 and then subjected to DNA sequencing analysis. The entire sequence of the *Taq*I fragment is 543 bp. Three identical 132-bp nucleotide sequences were tandemly repeated at nucleotides 96, 228, and 360. The 132-bp direct repeat contains 49% guanine plus cytosine residues. Each direct repeat of 132 bp

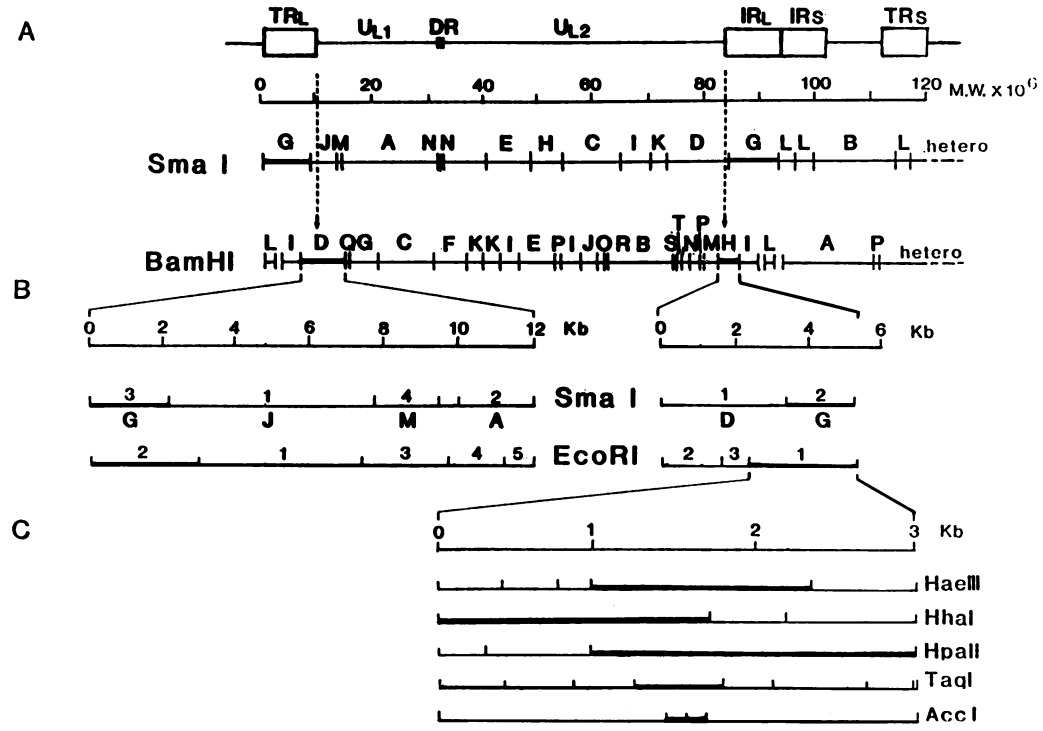

FIG. 2. Location of oncogenic MDV strain-specific regions within the TR_L and IR_L of the MDV genome. The fragments that differed in size in BC-1/LP and BC-1/HP DNA are indicated by solid bars. The structural arrangements of inverted repeats (open boxes) and the linkage maps of *Bam*HI and *Sma*I fragments are based on those proposed originally by Fukuchi et al. (3), but the fragment nomenclature of *Sma*I is revised according to our nomenclature (7). For example, *Sma*I-C and -D in this figure correspond to *Sma*I-C1 and -C2, respectively, in the nomenclature proposed by Fukuchi et al. (3). (A) Structure of the MDV genome. The vertical dotted lines indicate the positions of U_{L1}-TR_L and U_{L2}-IR_L junctions. (B) *Sma*I and *Eco*RI cleavage maps of cloned *Bam*HI-D and -H of BC-1/LP DNA. The *Sma*I fragments hybridized to each *Sma*I subfragment of *Bam*HI-D and -H were identified by Southern blot hybridization and are indicated under the lines. (C) Restriction endonuclease cleavage maps of the *Eco*RI-1–*Bam*HI-H fragment of BC-1/LP DNA.

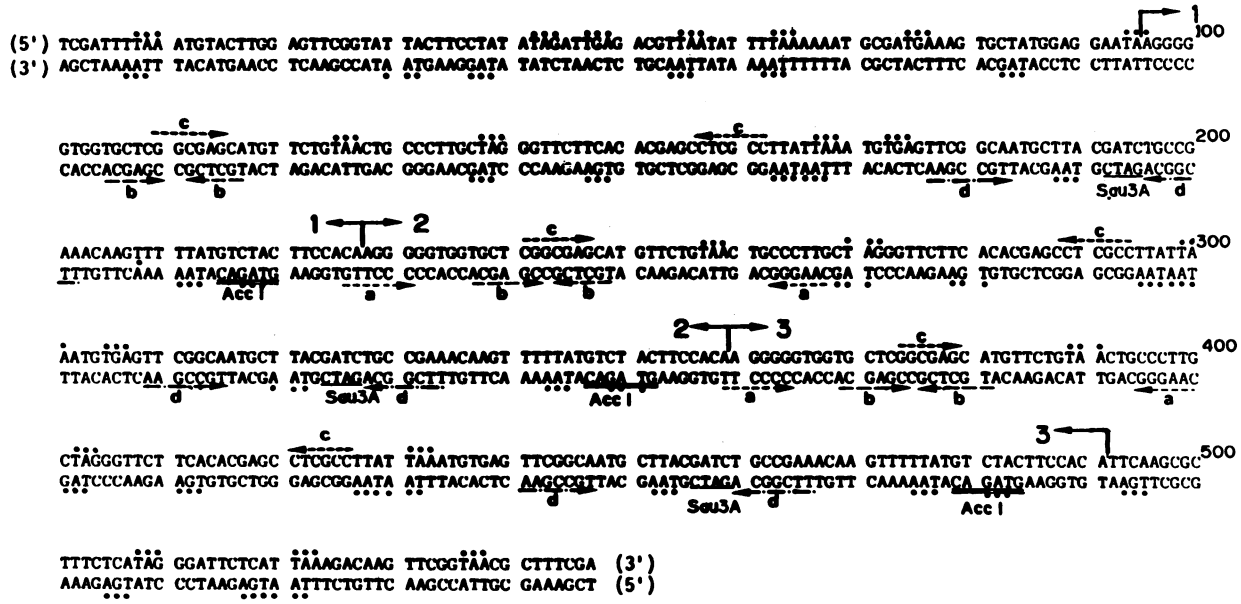

FIG. 3. Nucleotide sequence of the 543-bp *Taq*I fragment. The sequence is shown from the left end of the *Taq*I fragment in the map orientation as indicated in Fig. 2C. Three units of tandem direct repeats are presented by (<T>). The dyad symmetries within the 132-bp direct repeats are indicated by arrows with matching letters. Termination codons (TAA, TAG, and TGA) within the 132-bp repeats are shown by dots. Recognition sequences for *Sau*3AI and *Acc*I are indicated by single and double underlines, respectively.

contains four sets of inverted repeats of 6 or 7 bp separated by various numbers of nucleotides. These inverted repeats would form a secondary single-stranded structure with dyad symmetries of 6 or 7 bp and may play a role as a recognition site for a regulatory protein. Nucleotide sequencing of the 132-bp repeat also showed that there is a termination codon every six reading frames in both strands of the repeat (Fig. 3). Therefore, if mRNA were transcribed from this region, translation from either strand of the repeat would be blocked by these termination codons.

Digests with *Acc*I (Fig. 1D) and *Sau*3AI (data not shown) of the cloned *Eco*RI subfragments of *Bam*HI-Dhet and -Hhet, corresponding to *Eco*RI-2–*Bam*HI-D and *Eco*RI-1–*Bam*HI-H, respectively, yielded multiple units of the 132-bp fragment. Since the 132-bp direct repeat contains only one cleavage site each for *Acc*I and *Sau*3AI (Fig. 3), the results confirm that the *Bam*HI Dhet and Hhet fragments both contain multiple units of 132-bp direct repeats.

Several clones of recombinant M13 phage DNA containing the *Sau*3AI 132-bp fragment isolated from pMBDh26, pMBHh3, and pMBHh27 were isolated for nucleotide sequencing analysis. All *Sau*3AI 132-bp fragments from these recombinant phage DNAs showed the same nucleotide sequence as those of *Bam*HI-D and -H (data not shown). Thus, a structural difference between BC-1/LP and BC-1/HP DNAs within TR_L and IR_L was found only in the number of 132-bp tandem direct repeats. The oncogenic strain-specific regions within TR_L and IR_L of *Bam*HI-D and -H, respectively, of BC-1/LP DNA contain three units of 132-bp direct repeats. In contrast, the copy number of 132-bp direct repeats of BC-1/HP DNA was estimated as 6 to 35 units from the electrophoretic mobilities of *Bam*HI-Dhet and -Hhet of BC-1/HP, as reported by us (4).

Are the oncogenic strain-specific regions including the 132-bp tandem direct repeats responsible for oncogenic transformation? Further studies on the structure and function of the regions are required to answer this question.

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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