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## COPY NUMBER OF TANDEM DIRECT REPEATS WITHIN THE INVERTED REPEATS OF MAREK'S DISEASE VIRUS DNA

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We previously reported that DNA of the oncogenic strain BC-1 of Marek's disease virus serotype 1 (MDV1) contains three units of tandem direct repeats with 132 base pair (bp) repeats within the inverted repeats of the long regions of the MDV1 genome, whereas the attenuated, nononcogenic viral DNA contains multiple units of tandem direct repeats (Maotani et al., 1986). In the present study, the difference in the copy numbers of 132 bp repeats of oncogenic and nononcogenic MDV1 DNAs in other strains of MDV1 was investigated by Southern blot hybridization. The main copy numbers in different oncogenic MDV1 strains differed: those of BC-1, JM and highly oncogenic Md5 were 3, 5 to 12 and 2, respectively. The viral DNA population with two units of repeats was small, but detectable, in cells infected with either the oncogenic BC-1 or JM strain. The MDV1 DNA in various MD cell lines contained either two units or both two and three units of repeats. The significance of the copy number of repeats in oncogenicity of MDV1 is discussed.

Marek's disease virus serotype 1 (MDV1) is the etiological agent of Marek's disease (MD), a highly contagious malignant lymphoma in chickens. The genome of MDV1 in virus particles has been shown to be a linear doublestranded DNA with a molecular weight of  $110 \times 10^6$  and buoyant density of  $1.705 \ g/cm^3$ (Lee et al., 1971; Hirai et al., 1979). The genome consists of a long unique region (U<sub>L</sub>) and a short unique region (U<sub>s</sub>) bounded by inverted repeats, like that of herpes simplex virus (Cebrian et al., 1982). Detailed analyses of MDV1 DNA with restriction endonucleases have been reported by many investigators (Hirai et al., 1979, 1981a, b: Ross et al., 1983; Fukuchi et al., 1984; Silva and Witter, 1985). However, the genes responsible for oncogenic transformation of MDV1 have not been mapped on the virus genome. We previously reported comparative studies of the structures of oncogenic and nononcogenic MDV1 DNA and showed that some restriction endonuclease fragments, such as BamHI-D and -H, of MDV1 DNA were heterogeneously expanded in size with loss of oncogenicity (Hirai et al., 1981a, 1984b). The heterogeneous DNA fragments are named BamHI-Dhet and -Hhet, respectively. This feature appears to be common to nononcogenic MDV1 isolates (Ross et al., 1983; Fukuchi et al., 1985; Silva and Witter, 1985). We showed that cloned BamHI-D and -H fragments of oncogenic MDV1 strain BC-1 both contain three units of 132 bp tandem direct repeats, whereas the nononcogenic variant DNA contains multiple units of tandem direct repeats (Maotani et al., 1986). The 132 bp direct repeats are located within the terminal repeat (TR<sub>L</sub>) and internal repeat (IR<sub>L</sub>) of the long region of the MDV1 genome and heterogeneous increase in the number of the 132 bp repeats results in the production of BamHI-Dhet and -Hhet fragments in digests of nononcogenic MDV1 DNA.

The latent MDV1 genomes in virus-nonproducer T-lymphoblastoid cell lines, established from chickens with MD, were shown to exist as closed circular DNA (Tanaka et al., 1978; Hirai et al., 1981b; Rziha and Bauer, 1982), or in an integrated form (Kaschka-Dierich et al., 1982). Recently, the latent viral DNA was found on specific chromosomes of the two MD cell lines by in situ hybridization (Hirai et al., 1986a). The BamHIcleavage patterns of MDV1 DNA in various MD cell lines indicate the presence of the whole virus genome in these cell lines (Hirai et al., 1984b). In addition, we found that BamHI digests of MDV1 DNA from MD cell lines contained BamHI-D and -H, but not BamHI-Dhet and -Hhet. Thus, the copy number of 132 bp repeats appears to be related to the oncogenicity of MDV1. In this work, we examined the copy number of the 132 bp repeats of MDV1 DNA in cells productively infected with various oncogenic and nononcogenic strains, and in various MD cell lines by Southern blot hybridization.

The MD cell lines used were as follows:

MDCC-MSB1 (Akiyama and Kato, 1974), MDCC-HP1 (Powell et al., 1974), MDCC-LS1 (Tanaka et al., 1978), MDCC-RP1 (Nazerian et al., 1977), MDCC-JP2 (Yamaguchi at al., 1979), MDCC-MSB1-41C (Yamada et al., 1983) and MDCC-BMCL1, obtained from Dr. Ohki. These cell lines were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum at 41 C. The MDV1 strains used were as follows: the oncogenic BC-1 strain at the 21th passage of

А



В



FIGURE 1. Location of oncogenic MDVI strain-specific regions within the  $TR_{\rm L}$  and  $IR_{\rm L}$  of the MDVI genomes.

(A) Structure of the MDVl genome and the location of oncogenic virus-specific fragments BamHI-D and -H. The structural arrangements of inverted repeats (open boxes) of the MDVl genomes and locations of oncogenic strain-specific fragments BamHI-D and -H fragments are based on those proposed by Fukuchi et al. (1984). (B) EcoRI cleavage map of cloned BamHI-H and MspI (or HpaII) and AccI cleavage maps of the EcoRI-1-BamHI-H of BC/LP DNA. These maps are based on those constructed by us (Hirai et al., 1984b; Maotani et al., 1986). The fragments that differ in size in BC/LP and BC/HP DNAs are indicated by solid bars. culture (BC/LP) and its nononcogenic variant at the 64th passage (BC/HP), the oncogenic JM strain at the 17th passage (JM/LP) and its nononcogenic variant at the 54th passage (IM/HP) and the nononcogenic strain C2 and highly oncogenic strain Md5 at the 5th passage. These MDV1 strains were cell-associated viruses and were propagated in primary chicken embryo fibroblasts (CEF) as described previously (Hirai et al., 1981a). Total cellular DNA was isolated from MD cell lines or virus infected CEF, digested with restriction endonucleases and subjected to Southern blot hybridization as described previously (Hirai et al., 1986b). The cloned BamHI fragments of MDV1 and their subfragments were derived from our pBR322-MDV1 (BC-1 strain) recombinant library (Hirai et al., 1984a; Maotani et al., 1986). Fig. 1A shows the locations of oncogenic strain-specific fragments, BamHI-D and -H, on the physical map of the MDV1 genome constructed by Fukuchi et al. (1984). The subfragments of BamHI-D and -H that differed in size in BC/LP and BC/HP DNAs were mapped within the TR<sub>L</sub> and IR<sub>L</sub>, respectively, and located on the restriction endonuclease maps of BamHI-H (Fig. 1B, Hirai et al., 1984b; Maotani et al., 1986). These subfragments are the EcoRI fragment 1 of BamHI-H (EcoRI-1-BamHI-H) and its subfragment, MspI-1 (or HapII-1), which contain three units of 132 bp tandem direct repeats, corresponding to AccI-fragment 3 of EcoRI-1-BamHI-H (Fig. 1B, Maotani et al., 1986). We estimated the copy number of 132 bp direct repeats in MDV1 DNA on the basis of the size, 2.1 kilo bases (kb), MspI-1 of EcoRI-1-BamHI-H, which contains three units of the repeats (Maotani et al., 1986). If MDV1 DNA contains two units of 132 bp repeats, the size of the MspI-1 fragment should be about 2.0 kb. Since MDV1 DNA was considerably methylated in MD cell lines, but not in productively infected cells (Kanamori et al., submitted), MspI was used for digestion of viral DNA instead of its isoschizomer Hpall.



FIGURE 2. Southern blot hybridization of the <sup>32</sup>Plabeled EcoRI-1-BamHI-H to blots containing BamHI-MspI digests of total DNAs extracted from productively infected cells and MD cell lines.

Samples of 0.1 and 5 µg of total DNAs from infected cells and MD cell lines, respectively, were doubly digested with BamHI and MspI. The digests were electrophoresed in 1.0% agarose gel, transferred to nitrocellulose filters, and subjected to Southern blot hybridization with 32P-labeled EcoRI-1-BamHI-H. Abbreviations for blotted DNAs are as follows. (A) Oncogenic viral DNAs. H, cloned BamHI-H of BC/LP; BL, BC/LP-infected cell DNA; JL, JM/LP-infected cell DNA; M5, Md5infected cell DNA. (B) Nononcogenic viral DNAs. BH, BC/HP-infected cell DNA; JH, JM/HP-infected cell DNA; C2, C2-infected cell DNA. (C) DNAs from MD cell lines. MS, MSBl cell DNA; LS, LSI cell DNA; HP, HPI cell DNA; RP, RPI cell DNA; BM, BMCLl cell DNA; JP, JP2 cell DNA; 41, MSBI-41C cell DNA. The sizes of DNA fragments are indicated in kb on the left sides of the pannels in this figure. The ladder of DNA fragments with sizes increasing in increments of 132 bp are indicated by arrowheads. The numbers attached to arrowheads indicate copy number of 132 bp repeats. Fragments of less than 1.5 kb are not shown.

For determination of the copy number of 132 bp repeats within the inverted repeats of MDV1 DNA, the total DNAs from virusinfected cells and MD cell lines were doubly digested with BamHI and MspI. These digests were subjected to Southern blot

hybridizations with 32P-labeled EcoRI-1-BamHI-H (Fig. 2) and the results are summarized in Table 1. The labeled probe hybridized to four MspI-BamHI fragments of MDV1 DNA of 2.1, 1.2, 0.7 and 0.5 kb (Hirai et al., 1984b). Of these fragments, the fragments corresponding to the 2.1 kb fragment varied in size in virus DNAs of cells infected with various virus strains and of MD cell lines, while the other three fragments were the same size as those of cloned BamHI-D and -H fragments, except for one of RP1 cells (data not shown). Therefore, only the variable fragments are shown in Fig. 2. The BamHI-MspI digests of BC/LP DNA contained mainly MspI-1 of 2.1 kb and included few fragments containing two units or more than four units of 132 bp repeats, while JM/LP DNA was more heterogeneous and had a larger number of 132 bp repeats, as indicated by arrowheads in Fig. 2A. The predominant population of JM/LP DNA contained 5 to 12 units of 132 bp repeats and was about 95% of the total viral DNA molecules, as estimated by a densitometry tracing. This result was expected from our previous finding that the BamHI-D and -H of JM/LP DNA were larger than those of BC/LP DNA (Hirai et al., 1981a). Since these fragments of JM/LP DNA usually appeared to expand in sizes during in vitro passages less than those of BC/LP DNA (data not shown), JM/LP DNA at further lower passages could contain a fixed lower number of repeats, probably about 5. In contrast, the DNA of the highly oncogenic MDV1 strain Md5 (Witter et al., 1980) contained only two units of 132 bp repeats (Fig. 2A). The viral DNA population with two units of 132 bp repeats was very small, but detectable (less than 0.05%) in cells infected with either BC/LP or JM/LP. Fig. 2B shows that BamHI-MspI digestion of DNAs of nononcogenic strains BC/HP, JM/HP and C2 produced a series of bands with sizes increasing in increments of about 130 bp in place of the 2.1 kb fragment of cloned BamHI-H. The number of 132 bp repeats was

estimated to vary from 3 to about 100 units from the electrophoretic mobilities of the DNA fragments containing the repeats. However, the predominant population of these nononcogenic viral DNA molecules appeared to contain about 10 to 40 units of 132 bp repeats and no fragment containing two units of repeats was detected in these nononcogenic viral DNAs. Thus, the copy number of 132 bp repeats of oncogenic MDV1 DNA increased and became heterogeneous with loss of oncogenicity during serial passage of the cells in culture.

Next, we attempted to evaluate the copy number of 132 bp repeats of the MDV1 DNA in MD cell lines (Fig. 2C). 32P-labeled EcoRI-1-BamHI-H hybridized to one or two discrete bands corresponding to the MspI subfragment 1 of EcoRI-1-BamHI-H, in addition to three other small subfragments, which are not shown in Fig. 2C. The MD cell lines were subdivided into two groups based on the copy number of 132 bp repeats: group 1 contained only two units of repeats and included the HP1, RP1 and JP2 cell lines; group 2 contained both two and three units and included the LS1, BMCL1, MSB1 and MSB1-41C cell lines (Table 1). This small number of 132 bp repeats seemed to be characteristic of the latent viral DNA in MD cell lines, as compared with viral DNA in productively infected cells, because these cell lines were passaged more than 100 times in culture since their establishment as cell lines. For example, the MSB1 cells used in the present study were passaged more than 300 times in our laboratorv, but did not contain viral DNAs with more than four units of 132 bp repeats. MSB1-41C cells, containing the same number of repeats as parental MSB1 cells (Fig. 2C), were cultured continuously at 41C in another laboratory and had acquired the ability to become attached to the culture vessels (Yamada et al., 1983). In addition, we found that MSB1 cells, which were frozen immediately after establishment of the cell lines, also contained MDV1 DNA with two and three

MD cell lir	MD cell lines or MDV1 strains		
cell line group 1	MDCC-JP2, -HP1, -RP1	2	
group 2	MDCC-MSB1, -MSB1-41C, -LS1, -BMCL1	2, 3	
oncogenic strains	Md5 BC-1 (LP) IM (LP)	2 3 (majority) 5–12 (majority)	
nononcogenic strains	BC-1 (HP), JM (HP), C2	3–100	

TABLE 1.

units of 132 bp repeats (data not shown). Therefore, the number of the repeat, was quite stable during repeated passages in cultures for long periods. These results also suggest that MSB1 cells contain viral DNAs with almost the same numbers of two and three units of repeats. In fact, the DNA of a clone obtained from MSB1 in soft agar also showed the same copy number of repeats as the parental cell (data not shown). It is noteworthy that all MD cell lines examined contained viral DNA with two units of repeats. The two units of 132 bp repeats could be the sequence essential for maintenance of oncogenic transformation of MD cell lines. BC/LP, which was rescued from MSB1 cells by cocultivation with CEF, mainly contained populations of viral DNAs with three units of 132 bp repeats, the population with only two units of repeats being very few (Fig. 2A). Therefore, we need to examine whether the oncogenicity of BC/LP is caused by small virus population with two units of 132 bp repeats in their MDV1 DNA. The virus population with three or more units of repeat in their MDV1 DNA may also contribute to the oncogenicity of BC/LP although the oncogenic potential was probably reduced with increase in the copy number of 132 bp repeats. Viruses isolated from single plaques should be used to examine this problem. The BK virus with DNA containing two units of 68 bp

tandem direct repeats was shown to be more oncogenic than that containing three units of the repeats (Watanabe and Yoshiike, 1985). The 68 bp repeats contain the sequence homologous to the enhancer sequences of SV40 and adenoviruses. Therefore, transcriptional control by repeated sequences, including the 132 bp repeats of MDV1 DNA, may play a role in oncogenic transformation. Recently, we found that three polyadenylated RNAs encoded from the region containing 132 bp repeats within the inverted repeats of MDV1 DNA of BC/LP and the 132 bp repeats were also a part of the coding regions of these RNAs (Kanamori et al., submitted). In place of these RNAs, polyadenylated RNAs of heterogeneous size were found in BC/HP infected cells. It will be interesting to investigate whether the altered RNA transcriptions in BC/HP infected cells influence the oncogenicity of MDV1. Further studies on the structure and functions of 132 bp repeats are required for understanding the oncogenicity of MDV1.

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