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Stability of Marek's disease virus 132-bp repeats during serial *in vitro* passages

Brief Report

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Summary. The Marek's disease virus (MDV) genome contains 2 sets of 132-bp tandem repeat sequences. An increase in 132-bp repeat units has been associated with attenuation of oncogenicity during *in vitro* passage. By cloning entire genomes, we demonstrated that the copy number of 132-bp repeats can differ within an individual MDV genome. The stability of the 132-bp repeats during cell passage depended on the initial copy number. When both sets of repeats contained 2 copies, the copy number remained stable, while if even 1 set of repeats contained 6 copies, repeat expansion occurred relatively quickly. This expansion did not affect the *in vitro* growth curve. However, when MDV clones with low and high copy numbers were passed together, genomes with expanded repeats rapidly predominated, mimicking the behavior of naturally-occurring MDV. These results suggest that the preponderance of high-copy repeats after passage reflects intracellular copy number within individual infected cells rather than an influence on the spread of the virus.

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Marek's disease virus (MDV; family, *Herpesviridae*, genus, *Mardivirus*, species, *Gallid herpesvirus 2*) is an avian herpesvirus that causes T cell lymphomas in poultry. MDV consists of three antigenically related serotypes known as serotype 1, 2, and 3. Among these three serotypes, only serotype 1 MDV strains cause disease in chickens [1]. MDV has a genome structure co-linear with other alphaherpesviruses [8, 18]. One viral genome consists of one unique long (UL) and one unique short (US) sequence with each unique sequence flanked by inverted

repeats known as repeat long (RL) and repeat short (RS) regions, respectively. Despite the similarity in genome structure, MDV has several characteristics not associated with other alphaherpesviruses. MDV is the only alphaherpesvirus that is naturally oncogenic. Furthermore, this oncogenicity is attenuated rather quickly during *in vitro* passage, presumably due to genetic modification(s) [22]. MDV is strictly cell-associated and almost no cell-free infectious virions are detected in culture supernatants or sera of infected animals [2]. Consequently, each MDV isolate or strain is a non-clonal population of input viruses as they were originally derived from infected cells that contained multiple MDV genomes. Furthermore, since sequence polymorphisms almost invariably exist within each population, it is difficult, if not impossible, to exactly correlate variation in phenotype with a single, specific variation in genotype.

One of the best-characterized genetic changes during *in vitro* attenuation of MDV is the expansion of the 132-bp repeat region [9, 14]. Among the three serotypes, only serotype 1 MDV has 132-bp repeats. The 132-bp repeat region resides in the RL region of the genome. Consequently, one MDV genome contains 2 sets of 132-bp repeats, one in the terminal repeat long (TRL) and the other in the internal repeat long (IRL). The 132-bp repeat sequence is transcribed into multiple RNAs with several potential open reading frames [12]. However, it is not known whether the transcripts are translated, as no corresponding gene products have been detected. In virulent strains of MDV, only 2 copies of the 132-bp tandem repeats in both the TRL and IRL are detected [1]. Once MDV is passed *in vitro*, this repeat region expands quickly to copy numbers of 30 repeats or more [9]. This expansion alters the transcription pattern of this region [12]. Nevertheless, it has been impossible to investigate expansion of the repeat structure at the individual MDV genome level because of the non-clonal nature of MDV infection referred to above.

Bacterial artificial chromosomes (BACs) are F1-based plasmids that have a cloning capacity of 300 kbp or more [13] and have been used to clone entire herpesvirus genomes [10]. We recently generated BAC clones containing entire MDV genomes from a virulent strain [11], thereby allowing us to analyze properties of virulence at the individual genome level. To extend our knowledge of the role of the 132-bp repeats, we generated MDV from individual BAC-cloned MDV genomes and analyzed the expansion process *in vitro*.

Primary chicken embryo fibroblasts (CEFs) were prepared from Line 0 chick embryos maintained at our site. CEFs were maintained in a mixture of Liebovitz's L-15 and McCoy 5A media (1:1) with 1% fetal bovine serum. BAC cloning of the virulent low-passage MDV isolate Md11 and a complete sequence of one of the characterized clones (GenBank accession no. AY510475) are reported elsewhere [11]. Briefly, a BAC plasmid, pBeloBAc11, was inserted into Md11 MDV at the *US6* locus by homologous recombination in CEF. Recombinant MDV-carrying BACs (Md11-BAC) were then rescued as individual clones in *E. coli* DH10B.

To examine the copy number of 132-bp repeats in each Md11-BAC genome, clones were digested with *Taq*I, which cleaves immediately outside of the repeat region [4] and analyzed by Southern blot hybridization (Fig. 1A). The probe

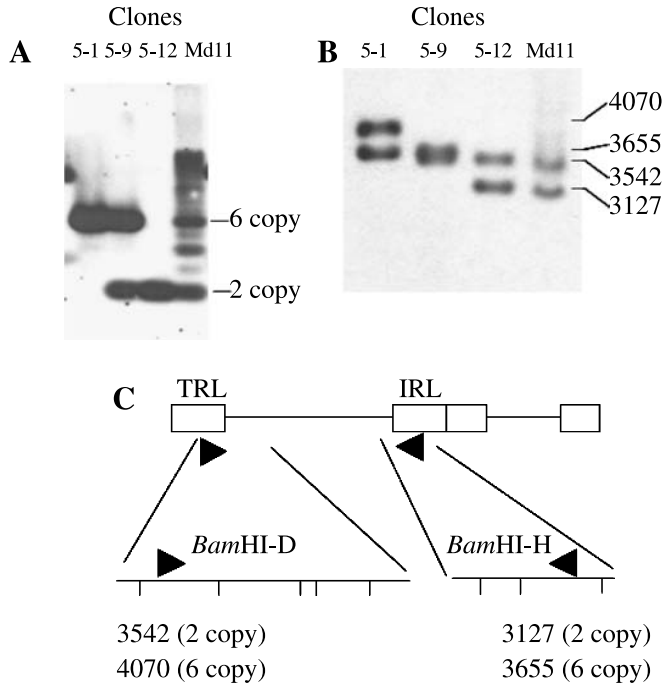

Fig. 1. MDV 132-bp repeats can be asymmetric in an individual genome. **A**. Three Md11-BAC clone DNAs (5-1, 5-9 and 5-12) were digested by *Taq*I and probed by a fragment containing 2 copies of the 132-bp repeats in a Southern blot. Positions for 2 and 6 copies of the 132-bp repeat are indicated on the right of the panel. **B**. The same Md11-BAC clones were doubly digested by *Bam*HI and *Pst*I and probed as in **A**. Expected sizes for 2 and 6 copies of 132-bp repeats in IRL and TRL are indicated on the right of the panel. **C**. Restriction map around the 132-bp repeats in the IRL and TRL. Arrowheads indicate the location of the repeats. Vertical lines within *Bam*HI-D and -H fragments (bottom) indicate *Pst*I recognition sites. Numbers below the line indicate the expected sizes of the restriction fragments containing the 132-bp repeat sequence

was generated by amplifying 2 copies of the 132-bp repeat sequence by PCR [15] and labeled using a non-radioactive system (Alkphos Direct, Amersham Biosciences, Little Chalfont, U.K.). As controls, low (passage level 15) and high passage (passage level 79) Md11 stocks were used [20, 21]. Clones 5-1 and 5-12 showed one band only, each containing an identical pair of 6- and 2-copy repeats, respectively. Clone 5-9 showed two bands corresponding to one 2-copy and one 6-copy repeat. To further analyze the location of the different repeats in clone 5-9, the clones were doubly digested by *Pst*I and *Bam*HI. As shown in Fig. 1B, the hybridization pattern indicated that 6 copies were contained in the IRL while 2 copies were present in the TRL (Fig. 1C) of clone 5-9. This demonstrates that an individual MDV genome can be asymmetric with respect to its two 132-bp repeat regions.

To analyze the expansion of 132-bp repeats during *in vitro* passage, progeny viruses were generated by transfecting these Md11-BACs into CEF by electroporation [11]. Virus was passed every 4 to 5 days by trypsin treatment of

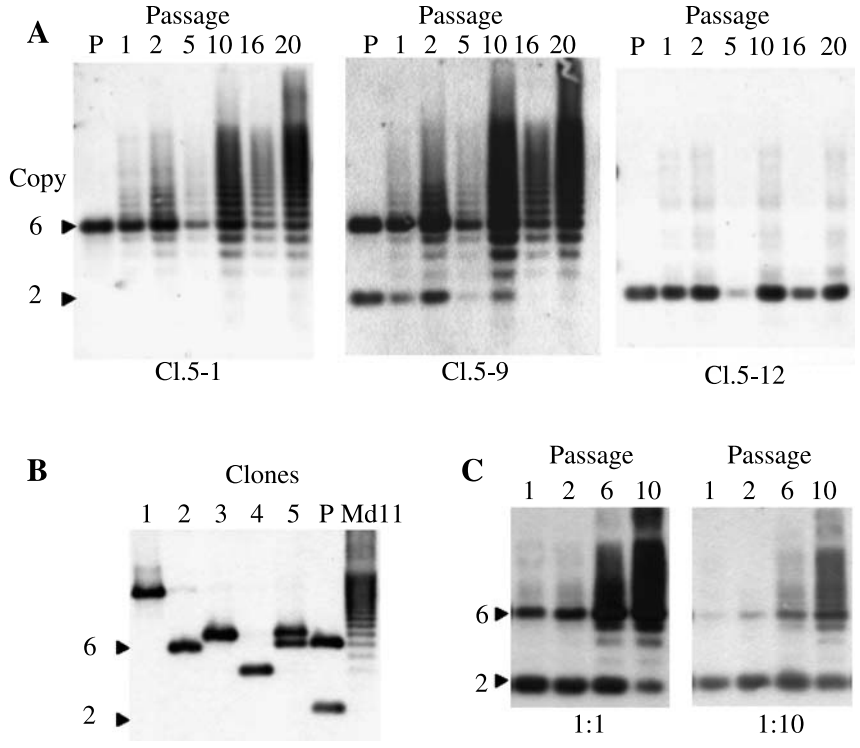

Fig. 2. Amplification of 132-bp repeats in BAC clones during *in vitro* passage. **A**. MDV was generated from three BAC clones (5-1, 5-9, and 5-12) and passed in CEF. At passage *1*, *2*, *5*, *10*, *16*, and *20*, DNA was extracted from the infected cells and analyzed as in Fig. 1A. *P*, Md11-BAC plasmid of each clone. **B**. Infected cell DNA from clone 5-9 at passage *10* was transformed into *E. coli* and Md11-BAC clones were recovered. The recovered clones (1 to 5) were analyzed as in **A**. *P*, Md11-BAC clone 5-9 plasmid. DNA from an Md11 population at passage 79 was included in the right-hand lane. **C**. Md11-BAC clones 5-1 and 5-12 were mixed at either putative 1:1 or 1:10 ratios and transfected to CEF. After the indicated numbers of passages, infected cell DNA was extracted and analyzed for copy numbers of the 132-bp repeat as in **A**. Positions for 2 and 6 copies of the 132-bp repeat are indicated on the left of each panel

infected monolayers, inoculating one-tenth of the total cells recovered onto fresh monolayers. During passage, infected CEF DNA was extracted and analyzed by Southern blot as described above. As shown in Fig. 2A, changes in repeat copy numbers in clones 5-1 and 5-9 were detected as early as the second passage. Also, the heterogeneity in copy number increased during passage. On the other hand, the 2-copy 132-bp repeats in clone 5-12 were stable for the duration of the experiment (20 passages). When 5-9 clones at passage 10 were recovered in *E. coli*, all 5 individual MDV genomes showed different copy numbers from the original 2 and 6 copies, indicating the stochastic nature of the expansion process (Fig. 2B). To analyze the relative rates of repeat expansion during *in vitro* passage, clones 5-1 (6 copies only) and 5-12 (2 copies only) were mixed at 1:1 or 1:10 ratios and electroporated into CEF (Fig. 2C). Though we aimed for a 1:1 mixture, Southern

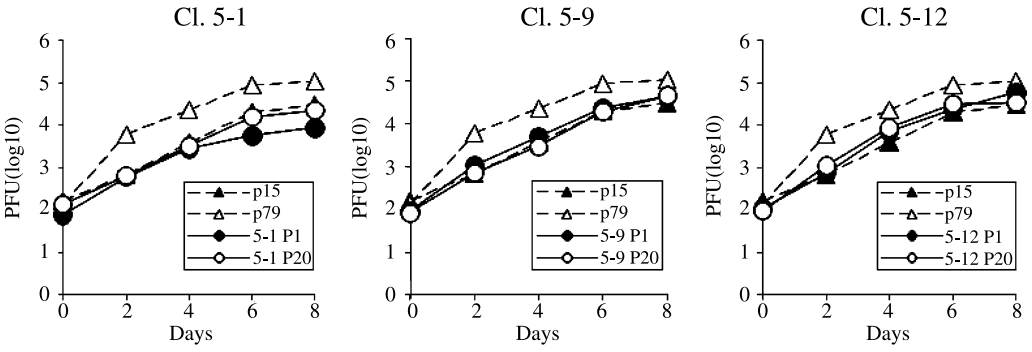

Fig. 3. Growth curves produced by each clone before and after *in vitro* passage. MDVs generated from each clone (5-1, 5-9 and 5-12) at passage 1 (filled circles) or 20 (open circles) were inoculated at 100 pfu. After 2, 4, 6 and 8 days of culture, infected CEF were harvested and assayed for MDV plaques on fresh monolayers. Individual points indicate an average of triplicates. Md11 at passage 15 (closed triangles) and passage 79 (open triangles) are shown for comparison. SDs are smaller than the individual symbols at any time points

blot results showed clone 5-12 was transfected more efficiently or, more likely, the preparation of this clone contained less contaminating non-viral DNA, so the actual input ratios were nearly 1:2 and 1:20. Nonetheless, the expanded repeat region became the dominant entity in the population following 6 to 10 *in vitro* passages, respectively.

Due to the more rapid increase of MDV genomes with expanded 132-bp repeats, we analyzed the *in vitro* growth of each clone at early and late passage levels, compared to authentic Md11 low and high passage virus stocks (Fig. 3). One hundred pfu of infected cells were inoculated into CEF, and infected cell titers were evaluated by inoculating samples harvested at the indicated time points onto fresh CEF monolayers and counting plaques 6 days later. In all 3 clones, the growth curves were similar between passage 1 and passage 20 samples and were closer to low passage Md11 than high passage Md11.

We demonstrated that 2 copies of the 132-bp repeat in the MDV genome are stable during *in vitro* passage, unlike genomes with 6 copies that quickly diversify their copy numbers (mostly by expansion). The stability of 2-copy-132-bp repeats was not a unique characteristic of clone 5-12 since we observed similar stability in an independent 2-copy-132-bp repeat BAC clone, which even had a different BAC insertion site (data not shown). Furthermore, the BAC vector is unlikely to influence repeat stability, since all of the clones used in this study carried the same BAC backbone. And though the clones used in this study carry a unique replacement of TRS with a host genome sequence [11], this is not the reason for the stability of 2 copies of the 132-bp repeats since all the clones used in this study carried the same replacement. Furthermore, independent clones with different BAC insertion sites that do not have the replacement showed similar stability. Silva et al. reported that a slight expansion of 132-bp repeats from 2 copies as early as third passages when the MDV clones were generated from the overlapping cosmid clones [16]. Although the reason for the observed expansion

in this system is not clear, we suspect the homologous recombination initially required for generating infectious virus genome in the transfected cells in this system and potential heterogeneity of the resultant clones, which could account for the slight differences among the three cosmid-derived clones and in our results.

The mechanisms behind the amplification of the repeat region in MDV are not clear. It is known that besides the 132-bp repeats, other repeat sequences are variable in MDV, though they have not been correlated with virulence or *in vitro* passage history [6, 17]. The rapid expansion of the repeat sequence may be related to the high frequency of homologous recombination associated with herpesvirus DNA replication [3]. High-frequency homologous recombination of a replicating DNA containing the replication origin of herpes simplex virus (HSV)-1, the prototype alphaherpesvirus, requires 600 + bp of homologous sequence derived from a transposon [19]. In another report, a 370-bp homologous sequence in a plasmid permitted HSV-1 replication-dependent homologous recombination [5]. If similar mechanisms function in MDV DNA replication, two 2-copies of the 132-bp repeat may be insufficient for effective recombination. Unequal recombination between two repeat regions would yield one product with only a single 132-bp sequence. Thus, it may be significant that all the RL regions generated *in vitro* (Fig. 2A) contained at least 2 copies of the 132-bp repeat.

In mixed infections, the frequency of multiple-copy 132-bp repeats is observed to increase with each cell passage compared to 2-copy 132-bp repeats. However, the repeat copy number did not influence the growth curves of individual MDV clones in infected CEF. In fact, growth curves of cells infected by Md11-BAC cloned virus either before or after 20 passages were closer to that of the low passage Md11 parent virus than the high passage Md11. One possible explanation is MDV genomes with multiple-copy repeats replicated faster relative to 2-copy repeat genomes within individual infected cells. An alternative explanation could be that intermolecular recombination occurs between the multiple-copy and 2-copy repeat genomes. Since only one set of repeats is sufficient for repeat expansion, intermolecular recombination would gradually lead to a high proportion of MDV genomes with multiple-copy repeats. This latter explanation implies that the replication rate between MDV genomes with multiple-copy and 2-copy repeats is relatively the same. The incorporation and monitoring of unique molecular tags outside the 132-bp repeat regions in the 2- and 6-copy-only MDV genomes should help to distinguish between the two explanations. In either case, the disproportional increase of MDV genomes with higher repeat numbers reflects intracellular copy number rather than an influence on the number of infected cells in the culture. Interestingly, 132-bp repeats are located in close proximity to the MDV origin of replication [4, 7], and this location may be relevant to a relationship between repeat copy number and replication, recombination, or both.

Recent results [16] indicated that the 132-bp repeat is not directly involved in MDV oncogenesis. It also seems unlikely that this repeat is directly involved in the growth of MDV in infected cells *in vitro*. The coincidental expansion of 132-bp repeat regions during *in vitro* passage along with the reduction in oncogenicity seems to result from MDV replication in cell culture without the

selective pressures exerted by growth and transmission in birds. The fact that 2-copy repeats are conserved in all known serotype 1 MDVs at the time of isolation suggests that this form of the repeat must be advantageous for MDV propagation in its natural environment, whereas it is not (relative to expanded repeats) for genome replication in cell culture. The reason behind the *in vivo* advantage of MDV genomes with only 2-copy repeats remains unknown and is worthy of further study.

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