Virulent Marek’s disease virus generated from infectious bacterial artificial chromosome clones with complete DNA sequence and the implication of viral genetic homogeneity in pathogenesis

Masahiro Niikura,1,2,3 Taejoong Kim,1† Robert F. Silva,1 Jerry Dodgson2 and Hans H. Cheng1

Genetic homogeneity of a test population is essential to precisely associate a viral genome sequence and its phenotype at the nucleotide level. However, homogeneity is not easy to achieve for Marek’s disease virus (MDV) due to its strictly cell-associated replication. To address this problem, two virulent infectious bacterial artificial chromosome (BAC) clones of MDV were generated from an MDV genome previously cloned as five overlapping cosmids. The Md5SN5BAC clone has the BAC vector inserted between the 3′ ends of UL3 and UL4, such that no known ORFs should be disrupted. The BAC vector is flanked by loxP sites, so that it can be deleted from the viral genome by transfecting Md5SN5BAC into a newly developed chicken cell line that constitutively expresses Cre recombinase. The Md5B40BAC clone has the BAC vector replacing a portion of US2, a location similar to that used by other groups to construct MDV-BAC clones. Although both BACs were capable of producing infectious virulent MDV when inoculated into susceptible chickens, Md5B40BAC-derived viruses showed somewhat better replication in vivo and higher virulence. Removal of the BAC vector in Md5SN5BAC-derived viruses had no influence on virulence. Interestingly, when genetically homogeneous virulent MDV generated from Md5B40BAC was mixed with avirulent virus, the overall virulence of the mixed population was noticeably compromised, which emphasizes the importance of MDV population complexity in pathogenesis.

INTRODUCTION

Marek’s disease virus (MDV, Gallid herpesvirus 2) is an alphaherpesvirus that causes Marek’s disease (MD). MD is most obviously manifested by T-cell lymphomas in chickens, its natural host, although other clinical signs such as paralysis are also associated. MD has been controlled by live vaccines since their introduction in the early 1970s (Witter, 2001). As an alphaherpesvirus, the MDV genome consists of two regions referred to as ‘long’ and ‘short’. Each of these regions has a unique sequence (unique long or UL and unique short or US) flanked by inverted repeat sequences (repeat long or RL and repeat short or RS) (Fig. 1a) (reviewed by Osterrieder et al., 2006).

One of the unique characteristics of MDV is its strict cell association, although mature virus is produced in the feather follicle epithelium of infected birds. Partly for this reason, combined with the genetic changes that are known to occur during in vitro virus replication (Spatz, 2010), it has not been easy to obtain a genetically homogeneous MDV population. For example, we demonstrated that a stock of very virulent (vv) strain Md11 actually consists of at least two genetically distinct subpopulations (Niikura et al., 2006). Genetic heterogeneity in a herpes simplex virus type-1 clinical isolate was also reported, and this genetic heterogeneity was associated with viral pathogenicity (Montgomery & Centifanto, 1989). Although it is possible to obtain MDV populations from one single virion that is artifcially extracted from infected cells (Cho, 1978),
the extraction is extremely inefficient, especially with virulent strains.

Another way to obtain a genetically homogeneous MDV population is to produce MDV from a cloned viral genome. For MDV, cloning the entire viral genome is advantageous over plaque purification because (i) the cloned homogeneous MDV genome sequence can be determined by amplifying the genome as a plasmid, which eliminates the mosaic of multiple virus genome sequences existing in a infected cell population, and (ii) generated viruses will be identical. This allows one to correlate a genomic sequence to a viral phenotype, as well as enabling methodologies to manipulate the virus genome in precise genomic sequence to a viral phenotype. To generate genetically defined MDV and, consequently, was not suitable for pathogenicity studies. To generate genetically defined virulent MDV, a set of five overlapping cosmids clones was generated from a low-passage virulent Md5 strain (Reddy et al., 2002). By co-transfecting chicken embryo fibroblasts (CEF) with these five overlapping cosmids clones, the complete MDV genome can be reconstituted through multiple homologous recombination events in the cell to generate genetically defined MDV. This reconstituted Md5 virus strain, rMd5, showed very similar characteristics to the parental Md5 virus including its virulence in susceptible chickens. Genetically defined MDV mutants based on cosmid clones were generated and used to elucidate important gene functions (Silva et al., 2004; Cui et al., 2004; Lupiani et al., 2004; Gimeno et al., 2005). A virulent MDV-derived BAC clone was generated and used for gene manipulation by Petherbridge et al. (2004). Unfortunately, the reconstituted MDV from this clone was deficient in horizontal transmission, unlike the parental virus, presumably due to mutations in three genes, UL13, gC and US2, subsequently found in this BAC clone (Jarosinski et al., 2007). Further analyses strongly suggested that the UL13 and gC gene products were involved in this defect (Jarosinski & Osterrieder, 2010).

We have been interested in MDV pathogenesis in relation to genetic variations in both the virus and host. In order to precisely assess the relationship between viral genomic sequences and virus pathogenesis, we needed a genetically homogeneous, virulent MDV clone. Toward this goal, we generated two virulent MDV-BAC clones from the Md5-derived overlapping cosmids clones and the complete nucleotide sequences of both were determined. One of the important genes were selected for the BAC insertion at a novel location on the MDV genome that does not disrupt any known MDV ORF. We also developed a cell line that can eliminate the BAC vector from the MDV BAC clones upon transfection by utilizing the Cre-loxP system. Finally, by using defined mixtures of genetically homogeneous virulent and avirulent virus preparations, we demonstrated that a few MDV-infected cells are enough to generate MD in infected birds and examined the effect of admixture with avirulent MDV on the length of survival and MD incidence caused by virulent MDV.

RESULTS

Cloning of the Md5 complete genome into BAC clones using overlapping cosmids clones

In order to generate a genetically intact virulent MDV-BAC clone, the BAC plasmid sequence was inserted into the MDV genome between UL3 and UL4 ORFs in the cosmid clone SN5 as depicted in Fig. 1. This site was selected for three reasons: (i) no clearly identified ORF was disrupted, (ii) the site was 3’ to both flanking genes, and (iii) this supposedly non-coding and non-functional region was the largest compared with other regions that satisfied the above two criteria. In parallel, we generated another construct by replacing a portion of the Us2 gene in the cosmid clone B40 with the BAC plasmid sequence, similar to the location used in previously constructed MDV-BAC clones (Fig. 1b). After insertion of the BAC sequence into the respective cosmids clones, the manipulated cosmids were transfected into CEF with the four other complementing cosmids clones. After four passages in CEF, infected cell DNA was

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extracted and MDV-BACs were recovered following transformation into *E. coli*. The recovered Md5-BAC clones were tested for their integrity by restriction enzyme digestions and Southern blots (Fig. 2 and data not shown). Two BAC clones for each construct derived from independent initial bacterial colonies were confirmed to have no noticeable deletions, insertions or rearrangements, and were denoted as Md5SN5BAC-2 and -3 and Md5B40BAC-1 and -2, respectively. Since the restriction patterns of the two clones for each construct were indistinguishable, some further experiments were conducted using only Md5SN5BAC-2 and Md5B40BAC-1.

Md5SN5BAC and Md5B40BAC clones were infectious when introduced into CEF and showed typical MDV CPE (Fig. 3a). The *in vitro* replication kinetics of MDV isolates derived from Md5SN5BAC and Md5B40BAC were closely comparable to that of rMd5, which was derived from the overlapping cosmid clones and shown to be very similar to the parental Md5 MDV in its phenotype (Fig. 3b) (Reddy *et al.*, 2002).

The complete sequence of BAC-cloned Md5

The complete sequences of Md5SN5BAC and Md5B40BAC were very similar to one another (GenBank accession nos HQ149525 and HQ149526) except for the BAC insertion sites. As expected, no deletion was made in the

Md5SN5BAC clone sequence, while a portion of US2 was replaced by the BAC vector in Md5B40BAC. Due to its highly repetitive nature, the ‘a’-like sequence was not completely sequenced. Specifically, we could not determine the exact copy number of the direct repeat sequences GGGTTA and TAGAGGGCGCCGCTGAGTTTTTCTCTA-TTTTCTGCCCCCGGCCATGGCGCGGTATG within the ‘a’-like sequences. When compared with the reported Md5 sequence (Tulman *et al.*, 2000), Md5B40BAC showed only an in-frame 18 bp deletion within *UL36* (80246–80263, numbered according to the Md5 complete genome sequence, GenBank accession no. NC_002229.3) that reduces the copy number of the direct repeat sequences from seven to six. Md5SN5BAC showed the same 18 bp deletion as Md5B40BAC. In addition, three insertions and a deletion, all of which were in homonucleotide stretches in the repeat regions, were found. The GGGG insertion in the RL regions (positions 7852 and 133780), and a G deletion and an A insertion (positions 153069 and 165375, and positions 151123 and 167335, respectively) in the RS regions were not within any known coding regions. The C insertion in the RS region (positions 15123 and 167335) is located within a potential ORF, MDV085, predicted in the original annotation, but not in the current reference sequence (GenBank accession nos AF243438 and NC_002229.3). If this ORF actually exists, the insertion will result in a frame shift, changing the 36 C-terminal amino acids of MDV085 (132 aa in total) and terminating the ORF after 127 aa.
Excision of the BAC sequence with DF-1-Cre cells

To remove the BAC vector sequences and obtain a minimally modified virulent MDV, a permissive cell line that constitutively expresses Cre recombinase was generated and designated DF-1-Cre. The expression of Cre recombinase was confirmed by Western blot using Cre-specific antibody (data not shown). The ability of DF-1-Cre cells to remove the BAC plasmid vector was examined by transfecting Md5SN5BAC and generating progeny MDV. A probe corresponding to nucleotide positions 21346–21478 that overlaps the BAC insertion site detected two EcoRI fragments (7.7 and 0.73 kb) in Md5SN5BAC and viral genome DNA generated by transfecting Md5SN5BAC into DF-1 cells (Fig. 4a). In contrast, the progeny MDV generated in DF-1-Cre cells displayed only one detectable fragment of 2.05 kb in size, which matches the expected size following the removal of the BAC sequence (Fig. 4a, b). We were unable to detect any remaining BAC sequences, indicating that the removal process was highly efficient.

The BAC insertion between UL3 and UL4 changed the transcription pattern detectable by a probe corresponding to the 3’ portion of the UL3 transcript (21055–21293). Before the removal of the BAC sequence, Md5SN5BAC-derived MDV transcribed longer, heterogeneous transcripts at the UL3 region compared with rMd5 (Fig. 4c). However, once the BAC sequence was removed, the transcripts detected by this probe were indistinguishable between Md5SN5BAC-derived MDV and rMd5 (Fig. 4d). Transcripts detectable by a probe corresponding to the 3’ of UL4 (21809–22092) did not show any difference between rMd5 and Md5SN5BAC-derived MDV (Fig. 4c).

Virulence of BAC-cloned Md5

MDV was generated from Md5SN5BAC and Md5B40BAC by transfecting either CEF or DF-1-Cre cells, and the virulence of these viruses was tested in 1-day-old chickens (Table 1). Md5B40BAC-derived viruses caused MD in all the infected birds within 8 weeks post-infection as was the case with rMd5. In the case of Md5SN5BAC-derived viruses, clone 3 MDV showed 100% MD incidence, while clone 2 MDV showed slightly less virulence at 83% MD incidence. With both Md5SN5BAC-derived viruses, the mortality within 8 weeks was less than with the Md5B40BAC-derived viruses and rMd5, although these deviations with Md5SN5BAC were not statistically significant (P > 0.05 in Fisher's exact test). In addition, there was no increase in the virulence of Md5SN5BAC-derived MDVs after the removal of the BAC sequence using DF-1-Cre cells. In all four BAC-derived MDVs, contact birds were infected by horizontal spread, although the Md5SN5BAC-derived MDVs had reduced MD incidence in contact birds.

Virus loads in peripheral blood of infected birds were evaluated at 2 weeks after infection (Fig. 5). The mean virus loads in the birds infected with three of four Md5SN5BAC-derived viruses were statistically lower than other infected bird groups though the titres fluctuated significantly. Virus loads were not altered by the removal of the BAC vector from the viral genome. The mean virus loads in the birds infected with Md5B40BAC-derived MDVs were not statistically different from that of rMd5-infected birds.

To demonstrate the effect of genetic homogeneity of infecting MDV populations on virulence, we mixed genetically homogeneous virulent (Md5B40BAC-derived MDV) and avirulent (meq-deleted rMd5) populations at...
defined ratios and used these to infect chickens (Table 2). Both parental Md5 virus at passage 16 and the genetically homogeneous virulent population caused MD in all birds at doses as low as 10 p.f.u. per bird, which was the lowest dose tested. Interestingly, when the virulent Md5B40BAC-derived virus was mixed with avirulent MDV, the MD incidence dropped significantly compared with the homogeneous inoculation with the same dose. When 10 p.f.u. of the virulent virus was inoculated alone, 100% of birds suffered from MD, while the same 10 p.f.u. caused MD only in 30% of the birds when mixed with 490 p.f.u. of avirulent virus. This effect was observed even when 100 p.f.u. of virulent virus, which is 10 times more than the necessary dose to cause MD in all infected birds, was mixed with four times as much (400 p.f.u.) avirulent virus.

All the survival curves of the birds infected with the homogeneous Md5B40BAC population were similar despite the initial infection dose, i.e. no dose dependency was observed (Fig. 6a). In contrast, birds infected with the Md5 parental population showed dose-dependence in the time-course for survival. Birds infected with a lower initial virus dose tended to survive longer, with a greater percentage surviving to the end of the experiment (Fig. 6c). Co-infection with fourfold or greater ratios of avirulent virus also increased the survival time of birds in comparison to a similar dose of virulent virus alone (Fig. 6b).

**DISCUSSION**

We have cloned the virulent MDV genome as BAC clones from existing overlapping cosmid clones and subsequently obtained the complete DNA sequences of the clones. Unlike other reported MDV-BAC clones (Schumacher et al., 2000; Petherbridge et al., 2004, 2009; Niikura et al., 2006; Baigent et al., 2006), one of our constructs, Md5SN5BAC, has the BAC plasmid inserted between UL3 and UL4 without disturbing any clearly identified ORFs. Furthermore, when transfected into DF-1-Cre cells, the BAC plasmid was effectively eliminated, leaving only 34 bp of a loxP sequence in the virus genome. After the elimination of the BAC sequence, transcripts flanking the BAC insertion site became indistinguishable from those of the parental MDV. Thus, the overall genome structure of Md5SN5BAC-derived MDV is very close to that of intact virulent MDV. In Md5B40BAC, the BAC plasmid was inserted at the US2 locus, which is similar to other reported MDV-BAC clones.

The complete sequences of our two cloned BACs, Md5SN5BAC and Md5B40BAC, were very similar to each

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**Table 1. Virulence of MDV-BAC-derived viruses**

<table>
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<th>Virus</th>
<th>Inoculated birds</th>
<th>Contact birds</th>
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<tr>
<td></td>
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<td>Death†</td>
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<td>rMd5</td>
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<td>8</td>
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<tr>
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<td>0</td>
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<tr>
<td>Md5SN5-BAC-2 (DF-1)</td>
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*Birds that died within 1 week after the infection were considered chick mortality and not included in the analyses.
†All dead birds were confirmed to have MD by necropsy.
‡Numbers of survived birds with MD lesions.
§Not statistically different from rMd5 (P>0.05 in Fisher’s exact test).
other, and the Md5B40BAC sequence was almost identical to the reported Md5 sequence. Both BAC clones were transformed into E. coli after four passages in CEF cells as MDV. The identical deletion in the repeat sequence in the UL36 ORF in both BAC clones is most probably due to a difference between the reported Md5 sequence and that of the MDV genome cloned into the overlapping cosmid clones. Other than this deletion, Md5SN5BAC had four additional differences in homonucleotide stretches compared with the reported Md5 and Md5B40 BAC sequences. In the RB-1B BAC clones, all three frame shift mutations in US2, gC and UL13 genes occurred in homonucleotide stretches and two of them contributed to the defect in horizontal spread (Jarosinski et al., 2007; Jarosinski & Osterrieder, 2010). Considering the quick change in the Md5SN5BAC clone after only four passages and the natural repair of a mutation in the A stretch within the RB-1B BAC UL13 reported by Jarosinski et al. (2007), homonucleotide stretches in the MDV genome perhaps quickly fluctuate and contribute substantially to the heterogeneity of the virus population. The genomic mutation rate of HSV-1 was calculated at ~0.003 per chromosome replication, which is similar to other micro-organisms with dsDNA genomes (Drake et al., 1998; Drake & Hwang, 2005). On the other hand, we have reported gradual genetic changes in MDV populations in CEF passages (Mao et al., 2008). In addition, cell type-specific sequential mutations during serial in vitro passages are reported for another herpesvirus, cytomegalovirus (Dargan et al., 2010). These results suggest a strong selective pressure during tissue culture passage of herpesviruses.

Table 2. Effect of mixing virulent and avirulent MDV on MD incidence

<table>
<thead>
<tr>
<th>Md5B40BAC (p.f.u.)</th>
<th>rMd5Δmeq (p.f.u.)</th>
<th>Md5 p16 (p.f.u.)</th>
<th>N*</th>
<th>Death†</th>
<th>Lesions‡</th>
<th>MD (%)</th>
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‡Numbers of survived birds with MD lesions.
§Significantly different from the birds infected with the same Md5B40BAC dose, respectively (P<0.01 in Fisher’s exact test).

Fig. 6. Effect of defined MDV mixtures on the length of survival. Chickens were infected with various doses (10–500 p.f.u.) of Md5B40BAC-derived MDV (a) or 500 p.f.u. of a mixture of Md5B40BAC-derived MDV and rMd5Δmeq at the indicated ratios (b) or parental Md5 strain stock (c). Birds were monitored for 64 days. Birds that died within 1 week after the infection were considered chick mortality and not included in the analyses. The total number of birds in each group is shown in Table 2.
MDV generated from Md5SN5BAC replicated to lower virus titres during the initial latency period and was slightly less virulent, resulting in less mortality and MD incidence, compared with the same Md5 strain derived from Md5B40BAC and the overlapping cosmid clones. Thus, Md5B40BAC-derived MDV produced MD in all the infected chickens at doses as low as 10 p.f.u., whereas Md5SN5BAC-derived MDV caused MD in 67–83 % of the infected birds even at a higher dose of 1000 p.f.u. (Table 1). The lower MD incidence byMd5SN5BAC-derived MDV held true even when the BAC sequence was removed by Cre recombinase, which indicates that the BAC insertion itself was not the cause of the phenotypic difference, unlike the outcome of a previous report (Zhao et al., 2008). There is a possibility that this apparent difference in virus load and virulence might reflect experimental fluctuations given the possibility that this apparent difference in virus load and non-virulent stocks. The most significant finding was that virulent MDV caused less MD when mixed with avirulent MDV, even when administered at identical doses. These results imply that MD incidence is affected not only by the dose of the virulent virus, but also by the composition of the infecting MDV population. MDV infection spreads in the body through viraemia in a week (Calnek, 2001). While specific humoral and cellular immune responses might be involved in this observation, the competition is apparently established at a very early stage after infection. Moreover, if the competition were solely immune-mediated, one might expect it to depend more on the amount of competitor avirulent virus rather than the ratio of avirulent to virulent inoculum. Although further analyses of virus growth kinetics in vivo are necessary to elucidate the cause, it is tempting to postulate that the avirulent virus may have interfered with the establishment of latent infection by the virulent virus for some reason in early in the infection. Latent infection is considered to be a prerequisite for the transformation of T-cells by MDV (Osterrieder et al., 2006). This possibility might partly account for the unique characteristics of MD vaccines, which are protective for avirulent virus challenge very soon after the vaccination (such as 5 days or earlier) without eliminating virulent virus from the host (Gimeno et al., 2008). One of the early effects of vaccine virus infection is that the infection of CVI988, a live vaccine for MDV, significantly reduces CD4 T-cells in the vaccinated birds for a certain period (Yamamoto et al., 1995). Also, it was reported that meg-deleted Md5 showed extensive lytic infection, but less recovery of the virus from the peripheral blood cells (Lupiani et al., 2004). In order to investigate the pathogenesis of MDV and vaccine efficacy, the possible effect of population heterogeneity in the challenge virus should be considered.

The availability of homogeneous cloned viruses allowed us to compare the effects on pathogenicity of mixed infection with characterized mixtures of virulent and non-virulent stocks. The most significant finding was that virulent MDV caused less MD when mixed with avirulent MDV, even when administered at identical doses. These results imply that MD incidence is affected not only by the dose of the virulent virus, but also by the composition of the infecting MDV population. MDV infection spreads in the body through viraemia in a week (Calnek, 2001). While specific humoral and cellular immune responses might be involved in this observation, the competition is apparently established at a very early stage after infection. Moreover, if the competition were solely immune-mediated, one might expect it to depend more on the amount of competitor avirulent virus rather than the ratio of avirulent to virulent inoculum. Although further analyses of virus growth kinetics in vivo are necessary to elucidate the cause, it is tempting to postulate that the avirulent virus may have interfered with the establishment of latent infection by the virulent virus for some reason in early in the infection. Latent infection is considered to be a prerequisite for the transformation of T-cells by MDV (Osterrieder et al., 2006). This possibility might partly account for the unique characteristics of MD vaccines, which are protective for avirulent virus challenge very soon after the vaccination (such as 5 days or earlier) without eliminating virulent virus from the host (Gimeno et al., 2008). One of the early effects of vaccine virus infection is that the infection of CVI988, a live vaccine for MDV, significantly reduces CD4 T-cells in the vaccinated birds for a certain period (Yamamoto et al., 1995). Also, it was reported that meg-deleted Md5 showed extensive lytic infection, but less recovery of the virus from the peripheral blood cells (Lupiani et al., 2004). In order to investigate the pathogenesis of MDV and vaccine efficacy, the possible effect of population heterogeneity in the challenge virus should be considered.

**METHODS**

**Virus and cells.** CEFs were prepared from line 0 chicken embryos and used as secondary cells. CEFs were maintained in a mixture of Leibovitz’s L-15 and McCoy 5A media (1:1) supplemented with FBS and antibiotics (100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹). FBS concentration was 4% for the growth medium and 1% for the maintenance medium. DF-1 cells were obtained from Dr D. Foster (University of Minnesota, USA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (Himly et al., 1998). MDV strain Md5 at passage 16 was from our in-house stock at USDA-ARS, ADOL, East Lansing, MI, USA. An MDV strain Md5 virus was generated from the overlapping cosmids clones by co-transfecting all five cosmids clones as described previously (Reddy et al., 1996, 2007; Cox et al., 1998). Therefore, it is possible that the BAC insertion or the remaining loxP sequences disrupted UL3.5 gene expression. The contribution of UL3.5 to viral replication seems to be virus-specific as it is essential for full replication in pseudorabies virus (Fuchs et al., 2007), while dispensable for varicella-zoster virus (Cox et al., 1998). We did not detect any viral replication defect in vitro with Md5SN5BAC. A probe corresponding to the 3’ end of the UL3 ORF did not detect any short transcripts potentially encoding UL3.5 (Fig. 4c).

Another possibility for the lower titre in vivo is that one or more of the spontaneous mutations observed in the Md5SN5BAC are responsible for the phenotypic difference. A major deletion in the RS region at the ICP4 promoter region in an in vitro attenuated population of MDV strain 648A was reported (Spatz, 2010). The point mutations found in the RS region of Md5SN5BAC do not overlap this deletion. However, it is possible that the mutation(s) found in this region affected the transcription of ICP4, a critical transcriptional transactivator protein, which resulted in the slightly attenuated phenotype. We also cannot formally rule out that a possible difference in ‘a’-like sequences between Md5SN5BAC and the original Md5 might affect MD incidence, though this is less likely since all the sequence components, such as DR1, DR2 and DR4 (Kishi et al., 1991), that comprise the repetitive ‘a’-like sequences exist in the BAC clones. In any case, the phenotypic difference was quantitative, not qualitative, and the reduced MD incidence caused by the Md5SN5BAC-derived virus seemed to be associated with virus replication in vivo.

The virus dose necessary to cause MD by intra-abdominal injection in all the infected birds was less than 10 p.f.u. for both Md5B40BAC-derived MDV and parental Md5. However, the survival curves for the parental Md5 showed dose-dependency, while those for Md5B40BAC-derived Md5 did not. This difference may suggest heterogeneity of the Md5 virus strain stock as in the case of Md11 (Niikura et al., 2006), considering the results with the mixed population, which also showed dose-dependency in survival times.
et al., 2002). This version of Md5 was named rMd5 and used at passage levels 3–5. A meq-deleted mutant of rMd5 was reported previously (Lupiani et al., 2004; Lee et al., 2008) and used as the avirulent population.

**Generation of DF-1 cell line constitutively expressing Cre recombinase.** A DNA fragment that encodes Cre recombinase was excised from pGIKS-Cre (ATCC) by KpnI and Smal digestions and transferred to pcDNA3.1 (Invitrogen) between the KpnI and EcoRV sites. The resultant plasmid pcDNA-Cre was transfected into DF-1 cells by Lipofectin (Invitrogen) and the cells were selected by Zeocine sites. The resultant plasmid pcDNA-Cre was transfected into DF-1 (Promega). The cloned fragment was excised by Roche). This fragment was cloned into the pGEM-T Easy vector (Novagen), HRP-conjugated anti-rabbit IgG (Zymed) and 4-chloro-1-naphthol as the substrate.

The recovered BAC clones were first evaluated for their integrity by restriction endonuclease digestions. The BAC clones were named Md5SS5BAC and Md5B40BAC corresponding to the insertion sites of the BAC plasmid. To generate genetically homogeneous MDV, the infectious BAC clones were purified with Midiprep Plasmid Purification kits (Qiagen) and transfected into CEF, DF-1 or DF-1-Cre cells. The transfection was performed either by Nucleofector with Nucleofector solution VP1002 using the program T-27 (Amaxa) for CEF or Profection calcium-phosphate transfection reagent (Promega) for DF-1 and DF-1-Cre cells according to the manufacturer’s instructions. Since the CPE by MDV on DF-1 and DF-1-Cre cells was not apparent, the transfected DF-1 and DF-1-Cre cells were blindly passaged to fresh CEF monolayers at 5 days after transfection and the subsequent MDV plaques were amplified on CEF thereafter. Due to the low concentration of FBS in the maintenance medium for CEF (1%), the replication of DF-1 cells was suppressed once passaged onto fresh CEF monolayers. The generated MDV populations were used after three or five passages in CEF.

**Generation of infectious MDV-BAC clones from the overlapping cosmid clones.** A modified SalI–SalI fragment of pBeloBAC11 was inserted into either the SN5 or B40 MDV cosmide clone (Reddy et al., 2002). Clone SN5 contains the terminal RL (TRL) and a part of the unique long region (UL) of MDV genome from strain Md5, while clone B40 contains the entire short segment and a portion of the internal RL region (IRL) of the same strain (Fig. 1a). Cosmid clone SN5 was digested by AluI at position 21384 by Reca-assisted restriction endonuclease (RARE) cleavage (Ferrin & Camerini-Otero, 1991). Briefly, a 30-mer oligonucleotide homologous to the MDV genome sequence overlapping this AluI site (5′-GAATAC-AGCTAGGAAGGTCAGTTAGTACG-3′) was incubated with the cosmide SN5 and Reca (NEB) to form a partial triplex. Then, all the remaining unprotected AluI sites in SN5 were methylated by AluI methylase (NEB). After the triplex was dissociated, the methylated SN5 was digested by AluI only at the protected site.

For the modification of the BAC plasmid, pBeloBAC11 (ResGen) was digested by SalI and the resulting 6.4 kb fragment obtained. A DNA fragment with a unique PmlI site between two loxP sequences flanked by SalI sites was generated by annealing two oligonucleotides, 5′-GTCGACATACTTGATTCGTAATATGTTGCTAAACTC-3′ and 5′-GTCGACATACTTGATTCGTAATATGCTGAGGAGTTAC-3′. The cosmid SN5 and PmlI (NEB) to form a partial triplex. Then, all the remaining unprotected AluI sites in SN5 were methylated by AluI methylase (NEB). After the triplex was dissociated, the methylated SN5 was digested by AluI only at the protected site.

For the modification of the BAC plasmid, pBeloBAC11 into cosmide clone B40, two AluI sites in the cosmide B40 at positions 157012 and 157429 were digested by RARE cleavage utilizing oligonucleotides, 5′-GGTC-TGTGCGAATAACAGCTAGTACGGTCCTC-3′ and 5′-CCCC-ACATATCATGCTAGTCTGAGGAGATTAC-3′. The digested cosmid was self-ligated, and elimination of the sequence between the two AluI sites confirmed by PCR and sequencing. This plasmid was further processed using RARE-cleavage at the newly generated AluI site by using an oligonucleotide, 5′-GGTCGTAATGCTAGTACGGTCCTAAGCT-3′. This RARE-cleaved partial B40 fragment was ligated to the modified pBeloBAC11 that had previously been digested by PmlI. After ligation with the BAC sequence, the modified cosmids SN5 and B40 were recovered using a packaging kit (Gigapack III; Stratagene). Purified SN5 and B40 cosmids with BAC insertions were co-transfected, respectively, into CEF cells with the four other complementing cosmids as described previously (Reddy et al., 2002), and the resulting MDVs were amplified by passing the transfected/infected cells onto fresh CEF monolayers four times. The infected cell DNAs were extracted with an alkali-lysis method (Niikura et al., 2006) and transformed into electrocompetent DH10B cells (Invitrogen).

**Southern and Northern blot analyses.** Southern and Northern blot analyses were performed with 32P-labelled probes (Rediprime DNA Labelling System; GE Healthcare) as described previously (Sambrook et al., 1989). Briefly, DNAs were extracted with an alkali-lysis method (Niikura et al., 2006) or by Midiprep Plasmid Purification kits (Qiagen). After restriction digestions the fragments were separated by 0.7% agarose gel electrophoresis and transferred to Hybond-N nylon membranes (GE Healthcare). For the Northern blots, RNA was extracted from infected cells with Trizol according to the manufacturer’s instruction (Invitrogen). Total RNA (10 µg per lane) was separated on 1% agarose-formalin gels and transferred to Hybond-N nylon membranes. In order to detect the entire MDV genome, the probe consisted of a 32P-labelled Md11 BAC clone (Niikura et al., 2006). For the Northern blots, PCR fragments corresponding to 3′ of UL3 and UL4 ORFs (nucleotide positions 21055–21293 and 21809–22092, respectively) were 32P-labelled and used as probes.

**Sequence of the MDV-BAC clones.** The complete sequences of both Md5SS5BAC and Md5B40BAC clones were determined by high throughput sequencing on the Roche 454 platform at the Research Technology Support Facility, Michigan State University East Lansing MI, USA. The MDV-BAC clones were purified with Midiprep Plasmid Purification kits (Qiagen) and the sequence reads of each clone totalled 2.8 Mb, which is more than 15 times coverage of the entire MDV genome. The nucleotide sequences were assembled as contigs by using the Newbler assembler (Roche). The assembled sequences were compared to the reported Md5 sequence (GenBank accession no. NC_002229.3) and the gaps filled in by sequencing PCR amplified fragments that spanned the gaps or MDV-BAC plasmids with BigDye terminator chemistry (Applied Biosystems). The complete sequence was deposited in GenBank (accession nos HQ149526 and HQ149525 for Md5SS5BAC and Md5B40BAC, respectively). Due to its highly repetitive nature, the ‘a’-like sequence region was incomplete.

**Characterization of the BAC-derived MDV.** For in vitro virus growth kinetics, 100 p.f.u. of MDV was inoculated into CEF in 6 cm tissue culture dishes and incubated for the times indicated. After the incubations, monolayers were harvested by trypsinization and kept frozen until tested. The virus titre in the sample was determined in triplicate and expressed as p.f.u. per dish.

For in vivo characterization of MDV, maternal antibody negative MD-susceptible 15I × 7I chickens (ADOL) were infected by i.a.
administration. The chickens were maintained in Horsfall-Bauer isolators and monitored daily. The virus load in peripheral blood mononuclear cells (PBMC) was determined by counting the plaques after co-culturing of 10⁴ isolated PBMC onto a CEF monolayer in duplicate for each sample. The results were expressed as the mean for individual birds. The Student’s t-test was used to determine the statistical differences between the control group and the BAC-derived MDV groups. All birds were examined by necropsy at termination (8 weeks post-inoculation). The overall mortality was statistically compared by Fisher’s exact test. For comparison of the survival curves, a log-rank test was performed using GraphPad Prism version 5.00. Animal care and management followed the ADOL animal care and usage committee policy.

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