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Attenuation of Marek's Disease Virus by Deletion of Open Reading Frame *RLORF4* but Not *RLORF5a*

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Marek's disease (MD) in chickens is caused by the alphaherpesvirus MD virus (MDV) and is characterized by the development of lymphoblastoid tumors in multiple organs. The recent identification and cloning of *RLORF4* and the finding that four of six attenuated strains of MDV contained deletions within *RLORF4* suggested that it is involved in the attenuation process of MDV. To assess the role of *RLORF4* in MD pathogenesis, its coding sequence was deleted in the pRB-1B bacterial artificial chromosome clone. Additionally, *RLORF5a* was deleted separately to examine its importance for oncogenesis. The sizes of plaques produced by MDV reconstituted from pRB-1BΔRLORF5a (rRB-1BΔRLORF5a) were similar to those produced by the parental pRB-1B virus (rRB-1B). In contrast, virus reconstituted from pRB-1BΔRLORF4 (rRB-1BΔRLORF4) produced significantly larger plaques. Replication of the latter virus in cultured cells was higher than that of rRB-1B or rRB-1BΔRLORF5a using quantitative PCR (qPCR) assays. In vivo, both deletion mutants and rRB-1B replicated at comparable levels at 4, 7, and 10 days postinoculation (p.i.), as determined by virus isolation and qPCR assays. At 14 days p.i., the number of PFU of virus isolated from chickens infected with rRB-1BΔRLORF4 was comparable to that from chickens infected with highly attenuated RB-1B and significantly lower than that from rRB-1B-infected birds. The number of tumors and kinetics of tumor production in chickens infected with rRB-1BΔRLORF5a were similar to those of P2a chickens infected with rRB-1B. In stark contrast, none of the chickens inoculated with rRB-1BΔRLORF4 died up to 13 weeks p.i.; however, two chickens had tumors at the termination of the experiment. The data indicate that *RLORF4* is involved in attenuation of MDV, although the function of *RLORF4* is still unknown.

Marek's disease (MD) is a lymphoproliferative disease in chickens caused by a member of the *Alphaherpesvirinae* subfamily, MD virus (MDV). The pathogenesis of infection with MDV can be divided into four phases (3). Bursa-derived (B) lymphocytes are infected between 3 and 6 days postinfection (p.i.) and are the primary target cells for the first phase of lytic infection, which is followed by infection of activated $CD4^+$ thymus-derived (T) lymphocytes in the second phase. During the second phase, viral replication typically decreases and a latent infection is established between 5 and 10 days p.i. in activated $CD4^+$ T lymphocytes. The third phase is characterized by reactivation of MDV replication between 14 and 21 days p.i. After the third phase, lymphomas may develop depending on the genetic susceptibility of the chickens and the virulence of the virus strain.

The MDV genome consists of two unique regions, the unique short region (U_S) and the unique long region (U_L), flanked by inverted repeats known as the inverted repeat long (IR_L) and short (IR_S), and the terminal repeat long (TR_L) and short (TR_S). The MDV Eco Q (*Meq* or *RLORF7*) and viral interleukin-8 (IL-8) homologue (*vIL-8* or *RLORF2*) genes and the open reading frames (ORFs) *RLORF5a* (previously named ORF *L1*) and *RLORF4* have been identified within the IR_L and TR_L regions of the MDV genome (see Fig. 1). Meq is a basic leucine zipper protein with an amino acid sequence similar to the proteins encoded by the *jun* and *fos* proto-oncogenes (12), has been shown to possess transformation potential when transfected into Rat-2 cells (18), and has been found in all MDV-transformed chicken cell (MDCC) lines studied thus far (12, 16). *RLORF5a* transcripts are expressed more abundantly in MDCC lines than in infected chick kidney cell (CKC) cultures (20). Deletion of this ORF in attenuated CVI988 indicated that it is not essential for virus replication and the establishment of latency (31). *RLORF4* transcripts were recently shown to be expressed in MDCC lines and MDV-infected CKC cultures. Interestingly, in four of six cell culture attenuated MDV strains, deletions were identified within this ORF (10), although the authors erroneously referred to this ORF as *LORF4* in their report. *vIL-8* was originally identified through examination of splice variants of *Meq* (17, 23) and shares high similarity with cellular IL-8. Baculovirus-expressed MDV vIL-8 can function as a chemoattractant for peripheral blood mononuclear cells, although the classically described attraction of neutrophils was not observed (22).

The use of direct mutagenesis of the MDV genome has become a powerful tool to identify specific genes important for MDV replication and oncogenesis. Previous studies examining the functional role of vIL-8 using deletion mutants showed that deletion of all three exons of *vIL-8* decreases the ability to replicate in vivo; however, the virus was still able to become latent and cause tumors, albeit at a much lower incidence (5, 6, 22). Deletion of *Meq* in the very virulent MDV strain Md5 showed that the virus was still able to replicate in vivo but at lower levels than those of wild-type Md5, and tumors did not develop up to 8 weeks p.i. (19). Deletion of *RLORF5a* in the

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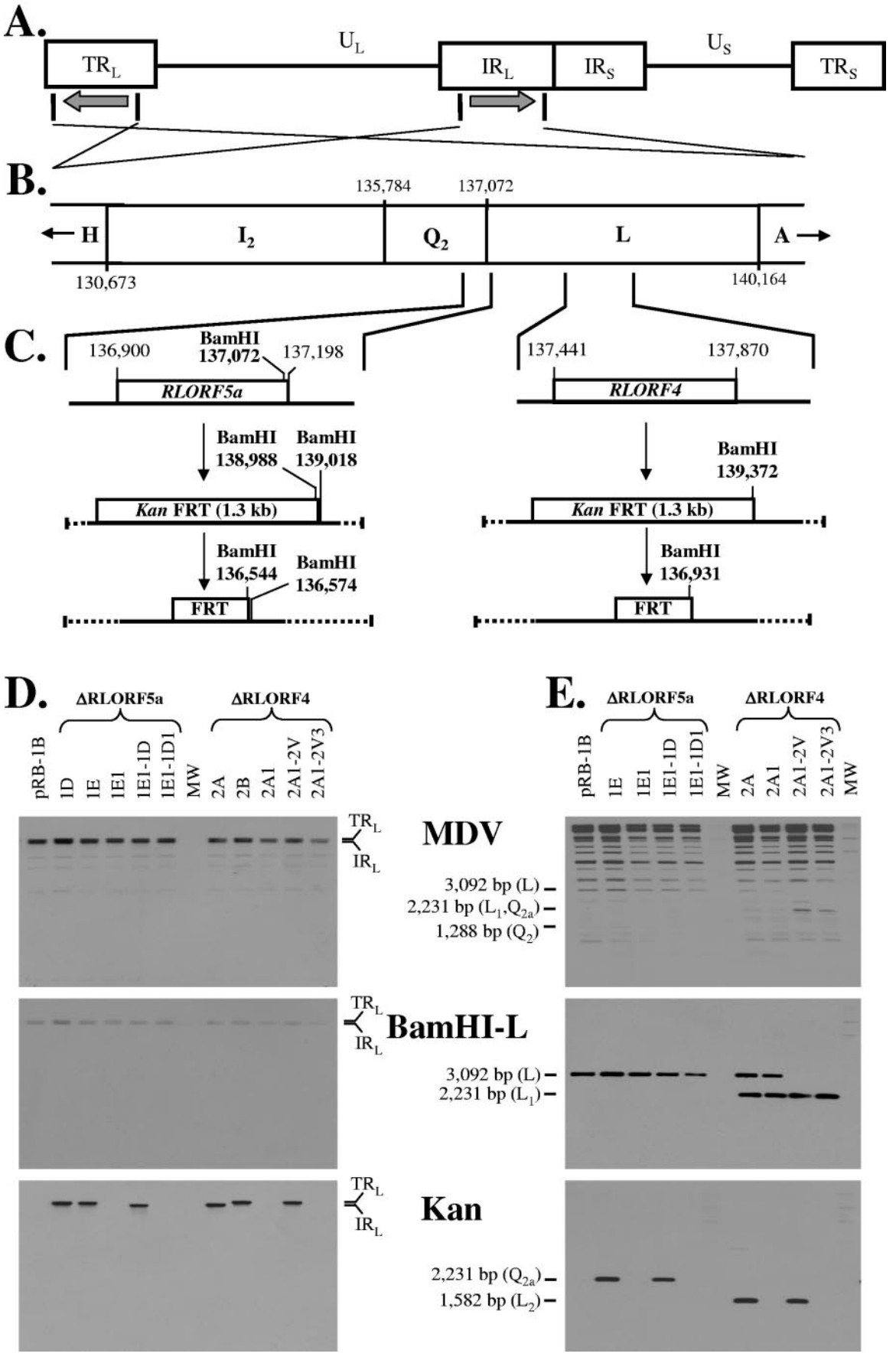

attenuated vaccine strain CVI988 indicated that it is not essential for virus replication and the establishment of latency; however, the effect on oncogenesis could not be determined using this strain of MDV (31).

Attenuated MDV shows two major characteristic changes compared to nonattenuated strains. First, attenuated strains replicate more efficiently in vitro, causing larger plaques and increased viral titer and DNA replication, than nonattenuated strains do (41). Second, attenuated strains replicate much less efficiently in vivo than nonattenuated strains do (30, 41). In this report, we examined the phenotype and oncogenic potential of two mutant viruses with deletions in ORFs encoded in the IR_L and TR_L regions of the genome, rRB-1BΔRLORF5a and rRB-1BΔRLORF4. The recombinant viruses were generated by targeted deletion from the recently established bacterial artificial chromosome (BAC) clone of RB-1B, pRB-1B (24). Chickens infected with rRB-1BΔRLORF5a replicated and produced tumors similar to those of rRB-1B, while rRB-1BΔRLORF4 caused no MD-related deaths for up to 13 weeks p.i., although two chickens had tumors at this time. These data show that RLORF4 is involved in the attenuation of MDV as hypothesized; however, its function remains unknown.

MATERIALS AND METHODS

Cell cultures and virus strains. CKC cultures were prepared from 14-day-old specific-pathogen-free (SPF) chickens by standard methods (32) and used to propagate MDV strains RB-1B/p17 (RB-1B at passage 17) (29) and rRB-1B reconstituted from pRB-1B (clone 5) (24) and the mutant viruses rRB-1BΔRLORF5a (clone 1E1-1D1), rRB-1BΔRLORF4 (clones 2A1-2V1, 2A1-2V2, and 2A1-2V3), rRB-1BΔRLORF4+/− (2A1), and rRB-1BΔRLORF4+/− (2A8). Chicken embryo cell (CEC) cultures were prepared from 10-day-old SPF embryos by standard methods (32) and used to propagate the attenuated RB-1B/p71 strain (30) and for transfections.

Construction of mutant BAC clones. Gene disruptions of *RLORF5a* and *RLORF4* in pRB-1B were performed by the method of Datsenko and Wanner (7), with recombination between short homologous DNA regions catalyzed by the phage λ Red recombinase. *Escherichia coli* EL250 cells (15) harboring the λ *red* genes and the arabinose promoter-driven *flp* recombinase gene were kindly provided by Neal G. Copeland (National Cancer Institute, Frederick, MD). Briefly, the mutagenesis strategy was to replace the targeted gene with the kanamycin resistance gene (*kan*^{r}) by homologous recombination. *kan*^{r}, flanked by *flp* recognition target (FRT) sites from pKD13 (kindly provided by Barry L. Wanner, Purdue University), was amplified by PCR using primers with ~50-base-pair extensions that were homologous to the start and end of the coding sequence of the gene to be disrupted. The sequences of the primers used for deletion of *RLORF5a* and *RLORF4* were synthesized by Integrated DNA Technologies (Coralville, IA) and were as follows (MDV-specific sequences shown in capital letters): ΔRLORF5a forward, 5′-AATATATACAGGGATGCACAGACATACTCCTATGCACCGATACACAGGCAcgtgtaggctggagctgcttc-3′; ΔRLORF5a reverse, 5′-TCTACAAAAGATCTAAATTTTACGTAATGGATCCCGTCCCGATCGTCCCCcattccggggatccgtcgac-3′; ΔRLORF4 forward, 5′-TTTTTACCTTAGTGCGTTTGTTGACGGAAATGTCCGCCATTATTTGAACcgtgtaggctggagctgcttc-3′; and ΔRLORF4 reverse, 5′-GGGCGATTTCCCTGTTATTGGGTCTCCACCAACACGTGATATTcattccggggatccgtcgac-3′. On the basis of the published sequence of MDV strain Md5 (36), nucleotides were deleted between 4,564 to 4,898 and 136,722 to 137,056 for *RLORF5a* and between 3,673 to 4,259 and 137,361 to 137,947 for *RLORF4*. The resulting PCR products were used to transform the recipient EL250 cells expressing Red recombinase using electroporation, and recombinant clones were isolated as kanamycin-resistant colonies as previously described (33). BAC DNA was isolated and examined for insertion of *kan*^{r} into the right locus using restriction digestion mapping with NheI, BamHI, and HindIII enzymes. Since there are two copies of *RLORF5a* and *RLORF4* in the MDV genome, one of each in the IR_L and TR_L regions, NheI digests were used to examine into which repeat long region the selectable marker was inserted. NheI cleaves the MDV genome such that the IR_L-specific bands (25.2 kb) and TR_L-specific bands (36.8 kb) can be readily distinguished. BamHI and HindIII restriction digests (data not shown) were used to evaluate the banding patterns of the mutated BAC clones to confirm the absence of spurious mutations elsewhere in the genome. Additionally, Southern blot analysis of each clone was performed using digoxigenin (DIG)-labeled probes for *kan*^{r}, the BamHI-L fragment (vIL-8 gene), and the whole MDV genome.

Once individual clones were examined and confirmed to lack spurious changes, *kan*^{r} was excised by induction of FLP recombination by incubation in Luria-Bertani (LB) medium containing 0.02% arabinose (Sigma, St. Louis, MO) for 12 h. Bacteria were diluted 1:1,000,000 in LB medium and plated onto LB agar containing 30 μg/ml chloramphenicol. Individual colonies were restreaked onto LB agar with chloramphenicol and LB agar with chloramphenicol and kanamycin to confirm that individual colonies were no longer kanamycin resistant. By using this technique, 100% of colonies screened were chloramphenicol resistant and kanamycin susceptible (K. W. Jarosinski, unpublished observations). Small-scale BAC DNA preparations were performed by the *E. coli* alkaline lysis method previously described (27). Individual clones were again screened as described above for the presence of *kan*^{r}, and restriction digest banding patterns were evaluated.

This protocol was repeated for deletion of *RLORF5a* and *RLORF4* in the remaining repeat to delete the second copy of each ORF. Once both copies were deleted and restriction enzyme banding patterns were proven to be correct, the regions were sequenced to confirm the absence of the ORF of interest. Unexpected sequences were not detected in this region, except for one copy of the 72-bp FRT sequence resulting from the recombination and FLP recombination process (7). Also, two clones with *RLORF4* deleted in only one repeat long region (pRB-1BΔRLORF4+/− 2A1 and 2A8) were used in one animal experiment (experiment 5). Unless otherwise noted, rRB-1BΔRLORF5a and rRB-1BΔRLORF4 were reconstituted from clones 1E1-1D1 and 2A1-2V3, respectively.

Recombinant viruses were reconstituted by transfecting CEC cultures with purified BAC DNA as previously described (33), and CEC cultures were passed onto CKC cultures at 7 days posttransfection. Each virus was then expanded in CKC cultures until stocks could be stored.

Southern blot analysis. Probes used in Southern blot analysis were prepared using either the DIG-High Prime (MDV) or PCR DIG Probe Synthesis (vIL-8 and *kan*^{r}) kits from Roche Applied Science according to the manufacturer's protocols. The complete MDV genomic probe was prepared using sonicated pRB-1B DNA that was randomly labeled with DIG (kindly provided by Sascha Trapp, Ithaca, NY). A DIG-labeled probe for the vIL-8 gene was used to identify the BamHI-L fragment and was prepared using previously published primers spanning the vIL-8 start and stop codons (10) and pRB-1B DNA as the template. The DIG-labeled *kan*^{r} probe was prepared using *kan*^{r}-specific primers (KanDIG

FIG. 1. Schematic representation of the genomic structure of MDV and the procedure for deletion of *RLORF5a* and *RLORF4*. (A) Genomic structure of MDV showing the locations of the TR_L and TR_S, U_L and U_S, and IR_L and IR_S regions. (B) Map positions of BamHI-H/D, I_2, Q_2, L, and A regions and genes located within the TR_L and IR_L regions of the genome. The TR_L is inverted for simplicity. Also included are the locations of the BamHI sites and their locations based on the published Md5 sequence (36). (C) Locations of *RLORF5a* and *RLORF4* in the BamHI-Q_2/L and -L fragments, respectively, and the subsequent insertion and excision of *kan*^{r} within these regions. The locations of each respective ORF (in roman type) and the locations of BamHI sites (in bold type) are shown. Insertion of *kan*^{r} plus FRT sites leads to an additional BamHI site that remains after excision of *kan*^{r}. (D and E) Southern blot analysis of BAC clones was used to examine insertion and deletion of *kan*^{r} at each step in the process with molecular weight (MW) markers. NheI (D) digests were used to determine which repeat *kan*^{r} was inserted into, while BamHI (E) digests were used to evaluate the banding pattern of each clone to identify any clones that may have extraneous alternations in their DNA. Probes used in each Southern blot were derived from the complete MDV genome (top panel), the BamHI-L fragment (middle panel), or *kan*^{r} (bottom panel), respectively. Specific bands are identified, and their sizes are indicated. See Results for description of bands.

forward, 5′-ACCCAGCCGGCCACAGTCG-3′; KanDIG reverse, 5′-GGGCGC CCGGTTCTTTTTG-3′) with pKD13 as the template. For hybridization, DNA was separated by 0.8% agarose gel electrophoresis, gels were stained with ethidium bromide, and DNA was transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) according to the manufacturer's instructions.

DNA extraction from tissues or cultured cells. Splenocyte preparations or CKC cultures infected with different strains of MDV were used for DNA extraction. Splenocytes were obtained by forcing spleens through a 60-μm-pore-size screen, resuspended in cold phosphate-buffered saline (PBS), and centrifuged on Ficoll-Paque Plus (Pharmacia, Piscataway, NJ). DNA was extracted from 1×10^7 splenocytes or infected CKC cultures using DNA STAT-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. Dried DNA pellets were dissolved in 100 μl DNase-free water, stored at 4°C, and used for quantitative PCR (qPCR) assays.

qPCR assays. Quantification of MDV genomic copies using qPCR was performed as previously described (9, 45). Briefly, primers and probe specific for the MDV-infected cell protein 4 (ICP4) gene were used in qPCR assays. DNA loading for each sample was normalized using primers and probe specific for the chicken inducible nitric oxide synthase (iNOS) gene.

For the generation of standard curves in qPCR assays, plasmids containing either the ICP4 or iNOS gene were used. Serial 10-fold dilutions of each respective plasmid were used for generating standard curves, starting with approximately 500 pg of plasmid. Total copy numbers were determined using the formula [(pg of input)(1 pmol/340 pg)(1/template size [in bp])(1 mol/1×10^{12} pmol)]/(6.02×10^{23} copies/mole) as previously described (10), and standard curves were generated by plotting the cycle threshold (C_T) value at each dilution with the total copies. When creating standard curves, the *y* intercept was set at 40, since a C_T value of 40 indicated no amplification of the specific target DNA. Therefore, any sample with a C_T value of 40 had a value of 0 for the target sequence. The coefficient of regression was always >0.99 for standard curves.

All qPCR assays were performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Inc.), and the results were analyzed using Sequence Detection System v.1.9.1 software as previously described (9–11). Each qPCR reaction mixture contained TaqMan Universal Master Mix (Applied Biosystems, Inc.), 100 ng DNA, 25 pmol of each gene-specific primer, and 10 pmol of gene-specific probe in 25-μl volumes. Thermal cycling conditions were as follows: 50°C for 3 min, followed by 40 cycles at 94°C for 20 s and 60°C for 1 min. Real-time fluorescence measurements were performed as previously described (10, 11). Using the standard curve generated for each gene, the number of copies for ICP4 and iNOS DNA was determined by using the C_T value for that sample. Because the MDV genome contains two copies of ICP4 and the chicken genome contains two copies of the iNOS gene, MDV genome copies were determined by dividing the number of ICP4 copies by the number of iNOS copies. This number was multiplied by 1,000,000 to create whole numbers, and the values were expressed as the number of MDV copies per 1×10^6 cells.

Virus isolation assays. In order to determine the number of MDV-infected cells in chicken spleens, 5×10^5 splenocytes from individual birds were plated onto CKC cultures in triplicate. MDV-PFU were counted 5 days p.i., and the mean number of PFU per 1×10^6 cells was determined for each treatment group and compared using Student's *t* test.

Measurement of plaque areas. After initial transfection of the mutant viruses into CKC cultures, it appeared that rRB-1BΔRLORF4 virus produced larger plaques than rRB-1B or rRB-1BΔRLORF5a do. Therefore, we examined plaque sizes more thoroughly by infecting cells seeded in six-well plates with 100 PFU per well. In the first experiment, rRB-1B/p5, rRB-1BΔRLORF5a/p5, and rRB-1BΔRLORF4/p5 (2A1-2V3) were compared, while in a second experiment, rRB-1B/p5, rRB-1BΔRLORF4/p3 (2A1-2V1), rRB-1BΔRLORF4/p3 (2A1-2V2), and rRB-1BΔRLORF4/p3 (2A1-2V3) were compared. Cells were fixed at 5 days p.i. with cold 90% acetone for 10 min and air dried. Cells were rehydrated in PBS for 10 min, PBS plus 10% fetal bovine serum was added as a blocking solution for 30 min, ~0.5 ml of anti-MDV chicken sera obtained from MDV-infected SPF chickens was added to each well at a 1:1,000 dilution, and plates were incubated at room temperature for 30 min. Cells were then washed three times with PBS for 10 min, and goat anti-chicken immunoglobulin G (heavy and light chains) labeled with Alexa Fluor 568 (Molecular Probes, Inc., Eugene, OR) was added. After 30 min, cells were washed twice with PBS, and digital images of 65 individual plaques were obtained using an epifluorescence Axiovert 25 inverted microscope and an AxioCam HRc digital camera (Zeiss, San Marcos, CA). Plaque areas were measured using the National Institutes of Health ImageJ software (rsb.info.nih.gov/ij/), and means were determined for each virus.

RNA extraction and reverse transcription-PCR (RT-PCR) assays. Total RNA was extracted from 60-mm dishes of rRB-1B-, rRB-1BΔRLORF5a-, or rRB-1BΔRLORF4-infected CKC cultures at 0, 3, and 6 days p.i. using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. The dried RNA pellets were dissolved in 100 μl RNase-free water and DNase treated with the DNA-free system from Ambion, Inc. (Austin, TX) using the manufacturer's instructions.

RT-PCR assays were performed using 1 μg total RNA using the GeneAmp Gold RNA PCR kit (Applied Biosystems, Inc., Foster City, CA) in 20-μl reaction mixtures as previously described (10). To amplify cDNA, 3 μl of the RT mixture was used with each pair of specific primers as previously described for *RLORF5a* (ORF *L1*) (44), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *RLORF4*, and *vIL-8* (10). The "touch-down" PCR procedure previously described was used to amplify specific cDNA products (43). PCRs were electrophoresed through 1.5% agarose gels, stained with ethidium bromide, and visualized using an Eagle *Eye*II still video system (Stratagene, La Jolla, CA).

In vitro replication. In vitro replication of mutant viruses was measured over time by qPCR assays. In trial 1, DNA replication was assessed in CKC cultures that were infected with 100 PFU of rRB-1B/p5, rRB-1BΔRLORF5a/p5, or rRB-1BΔRLORF4/p5 (2A1-2V3). In trial 2, rRB-1B/p5, rRB-rRB-1BΔRLORF4/p3 (2A1-2V1), rRB-1BΔRLORF4/p3 (2A1-2V2), and rRB-1BΔRLORF4/p3 (2A1-2V3) were used. For each virus, DNA was obtained from three dishes at 24-h intervals from the time of plating (0 h) to 6 days p.i. and analyzed as described above.

In vivo experiments. SPF P2a (major histocompatibility complex $B^{19}B^{19}$) chickens (38) were obtained from departmental flocks and housed in isolation units. Water and food were provided ad libitum. All experimental procedures were conducted in compliance with approved Institutional Animal Care and Use Committee (IACUC) protocols. In all experiments, chickens were inoculated intra-abdominally with 2,000 (experiment 1) or 1,000 (experiments 2 to 7) PFU of the various viruses, while control chickens were inoculated with uninfected CKC cultures. MDV does not spread horizontally between animals before 14 days p.i. (40); therefore, chickens infected for 14 days or less were housed together (experiments 2, 4, and 6). For experiments 1, 3, 5, and 7, all groups of chickens were housed in separate isolation units. Chickens were assigned to treatment groups using a randomized table.

Experiment 1. In order to determine whether rRB-1BΔRLORF4 was able to replicate in vivo, 2-day-old P2a chickens were inoculated with uninfected CKC cultures ($n = 20$), rRB-1B/p5 ($n = 20$), or rRB-1BΔRLORF4/p5 ($n = 20$). In addition to inoculated chickens, an additional 10 sentinel chickens were housed with infected chickens to serve as contact controls. At 6 days p.i., splenocytes were collected from 10 chickens from each inoculated group, and virus isolation assays were performed on each sample. At 22 days p.i., splenocytes were collected from all remaining chickens, including contact control chickens, and virus isolation assays were performed.

Experiment 2. Groups of 24 4-day-old chickens were inoculated with uninfected CKC cultures (control), RB-1B/p17, RB-1B/p71, rRB-1B/p6, rRB-1BΔRLORF5a/p6, or rRB-1BΔRLORF4/p6. At 4, 7, 10, and 14 days p.i., six chickens per group were used to evaluate thymic and bursal atrophy, as well as virus replication. Whole-bird body weights were measured prior to euthanasia. All thymus lobes and the bursa from each bird were weighed after collection, and the relative weights of the thymus and bursa to the whole body were determined. Spleens were collected, and splenocytes were used for virus isolation assays and DNA extraction.

Experiment 3. To test the oncogenic potential of rRB-1BΔRLORF5a and rRB-1BΔRLORF4, 20 4-day-old chickens per group were inoculated with the same viruses as in experiment 2, but each group was housed in a separate isolation unit. Chickens were evaluated daily for symptoms of MD and were euthanized and examined for gross MD lesions when the birds showed clinical evidence of MD. Groups with significant early incidence of MD were terminated at 6 weeks p.i. The number of birds in groups without evidence of MD was reduced at 7 weeks p.i. to provide the required space/bird according to IACUC protocols. The remaining chickens were kept until 13 weeks p.i. when the experiment was terminated. Chickens euthanized at 7 and 13 weeks p.i. were examined for gross MD lesions.

Experiment 4. To determine whether single deletions of *RLORF4* would cause the same phenotype as double deletions, 24 4-day-old chickens per group were inoculated with uninfected CKC cultures (controls), rRB-1B/p5, rRB-1BΔRLORF4+/−/p4 (2A1), rRB-1BΔRLORF4+/−/p4 (2A8), or rRB-1BΔRLORF4/p5. At 4, 7, 10, and 14 days p.i., six chickens from each group were euthanized and splenocytes were used in virus isolation assays and DNA extraction.

Experiment 5. Four-day-old chickens were inoculated with CKC cultures ($n = 10$), rRB-1B/p5 ($n = 9$), rRB-1BΔRLORF4+/−/p4 (2A1) ($n = 9$), rRB-1BΔRLORF4+/−/p4 (2A8) ($n = 10$), or rRB-1BΔRLORF4/p5 ($n = 9$) and housed in separate isolation units. Chickens were evaluated daily for symptoms

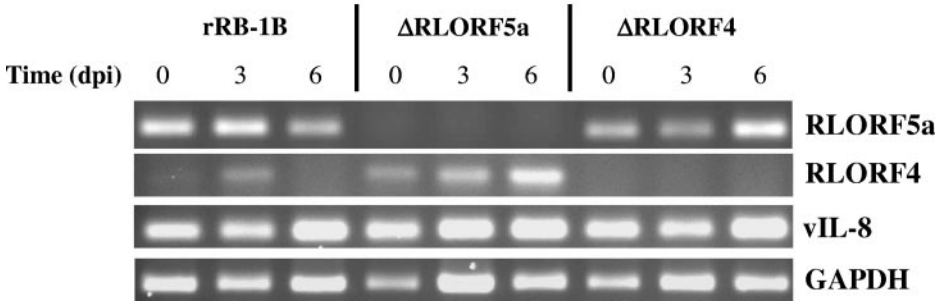

FIG. 2. Lack of *RLORF5a* and *RLORF4* mRNA expression in rRB-1BΔRLORF5a and rRB-1BΔRLORF4, respectively. RT-PCR assays for *RLORF5a*, *RLORF4*, *vIL-8*, and *GAPDH* mRNA expression were performed on total RNA collected from CKC cultures infected with rRB-1B, rRB-1BΔRLORF5a, or rRB-1BΔRLORF4 at 0, 3, and 6 days p.i. (dpi).

of MD and sacrificed when clinical symptoms became apparent as in experiment 3. The experiment was terminated at 52 days p.i., since a majority of chickens in all groups, except uninfected controls and animals inoculated with rRB-1BΔRLORF4, showed clinical MD symptoms.

Experiment 6. To determine whether the attenuated phenotype of rRB-1BΔRLORF4 clone 2A1-2V3 was clone specific, two additional clones were evaluated for in vivo replication. Chickens in each group (4 days old) were inoculated with uninfected CKC cultures (controls), rRB-1B/p5, rRB-1BΔRLORF4/p3 (2A1-2V1), rRB-1BΔRLORF4/p3 (2A1-2V2), or rRB-1BΔRLORF4/p3 (2A1-2V3). At 4, 7, 10, and 14 days p.i., four or five chickens from each group were euthanized and splenocytes were used in virus isolation assays and DNA extraction for use in qPCR assays.

Experiment 7. To examine the oncogenic potential of three individual clones of *RLORF4* deletion mutants, 10 chickens in each group of 4-day-old chickens were inoculated with the same viruses as in experiment 6. Chickens were evaluated as in experiments 3 and 5, and the experiment was terminated at 52 days p.i.

Statistical analysis. Significant differences in the means for virus isolation, plaque size measurements, and qPCR assays were determined using either Student's *t* test or Tukey-Kramer comparison of means.

RESULTS

Construction of MDV deletion mutants. *RLORF5a* is located at the junction of the BamHI-Q_2 and -L fragments, while *RLORF4* is located solely in the BamHI-L fragment. There are two copies of each ORF, with one copy in both the IR_L and TR_L regions of the MDV genome (Fig. 1A and B). Using the virulent pRB-1B BAC clone (24), *kan*^{r} was inserted into a single copy of *RLORF5a* and *RLORF4* separately by Red recombination (Fig. 1C). The positive selection marker was subsequently removed by FLP recombination, which completely removed each respective ORF and left minimal residual sequences consisting of one FRT and a BamHI site. In order to delete both copies within the IR_L and TR_L, each ORF was deleted in one repeat and then the procedure was repeated to remove the second copy in the other repeat.

Southern blot analysis of each clone during each step of the recombination/deletion process was performed following digestion of the BAC clones with NheI to distinguish each repeat long region (Fig. 1D). *kan*^{r} was first inserted into *RLORF5a* (clone 1D, TR_L; clone 1E, TR_L) and *RLORF4* (clone 2A, IR_L; clone 2B, TR_L) and then removed by FLP recombination (clone 1E1 for *RLORF5a* and clone 2A1 for *RLORF4*). The presence of bands in the respective clone that were reacting with a *kan*^{r}-specific probe is shown in the bottom panel of Fig. 1D. After the first deletion of each respective ORF, *kan*^{r} was inserted into the other repeat using the same method. Multiple clones were screened for insertion and excision, and only one clone for each deletion is shown for simplicity. *kan*^{r} was inserted into *RLORF5a* within the IR_L in the 1E1 clone (1E1-1D) and into *RLORF4* within the TR_L of the 2A1 clone (2A1-2V) and subsequently recombined out to produce the following double-deletion clones for *RLORF5a* (1E1-1D1) and *RLORF4* (2A1-2V3), which were used for further experiments. In animal experiment 5, clones 2A1 and 2A8 (not shown in Fig. 1) that were produced by the exact same methodology were used for inoculation of chickens.

Southern blot analysis after BamHI digestion of the engineered virus mutants was performed. Using a whole-genome, repeat-specific, or *kan*^{r} probe, no apparent alterations were visible in either clone, apart from the expected deletions. Insertion of *kan*^{r} and subsequent FLP-mediated removal of the *kan*^{r} resulted in an additional BamHI site. Insertion of *kan*^{r} into *RLORF5a* did not result in any discernible alterations in the restriction pattern, because the additional restriction enzyme site was inserted only 30 bp from the BamHI site, creating the Q_2 and L fragments (Fig. 1E, top panel). Following deletion of *RLORF4*, the L fragment is divided into two fragments of 2,231 bp (L_1) and 360 bp, although the latter fragment was not detected on the membrane since the probe for vIL-8 binds downstream of this fragment (Fig. 1E, top and middle panels). Insertion of *kan*^{r} into *RLORF5a* led to an increase of the size of the Q_2 fragment to 2,231 bp (shown as Q_{2a}). Using the *kan*^{r} probe, the Q_{2a} and L_2 fragments could be readily identified (Fig. 1E, bottom panel). HindIII restriction digests were also used to examine the integrity of the various mutant BAC clones, and no obvious spurious changes were seen (data not shown). The retention of the FRT sites after either ORF was replaced was further examined by nucleotide sequencing and proved to be correct (data not shown).

Figure 2 shows mRNA expression of *RLORF5a*, *RLORF4*, and *vIL-8* at 0, 3, and 6 days p.i. in infected CKC cultures. Deletion of *RLORF5a* and *RLORF4* sequences completely abolished *RLORF5a* and *RLORF4* mRNA expression, respectively, while *vIL-8* mRNA expression was not affected in vitro.

Deletion of *RLORF4* results in increased plaque sizes and replication rates in CKC cultures. First, the replication of mutant viruses was evaluated by measuring the plaque areas and the level of DNA replication in vitro. Plaque sizes were determined for rRB-1B, rRB-1BΔRLORF5a, and rRB-1BΔRLORF4 (Fig. 3). Determination of the mean plaque ar-

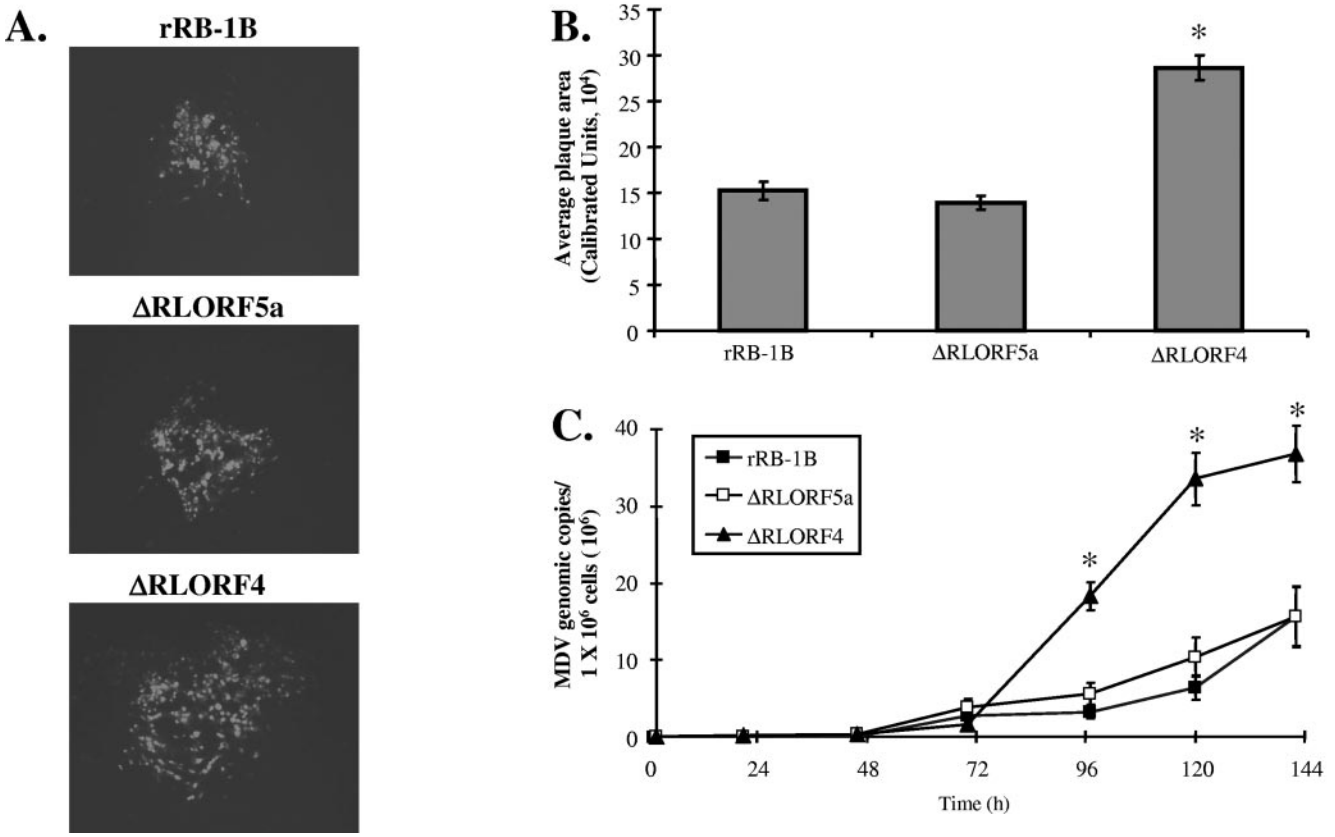

FIG. 3. In vitro replication of rRB-1B, rRB-1BΔRLORF5a, and rRB-1BΔRLORF4. (A) Panels show a representative plaque for each virus. (B) The average plaque area for each virus was determined; the standard error of the mean (error bar) for each group was also determined. Values that were significantly different ($P < 0.01$) from those of the other groups are indicated (*). (C) MDV genomic copies were measured using qPCR assays in rRB-1B-, rRB-1BΔRLORF5a-, or rRB-1BΔRLORF4-infected CKC cultures over the course of 6 days. The numbers of MDV genomic copies in CKC cultures infected with rRB-1BΔRLORF4 were significantly higher at 96, 120, and 144 h than those for both rRB-1B and rRB-1BΔRLORF5a ($P < 0.05$ [*]).

eas demonstrated that there was no significant difference in the plaque areas produced by rRB-1B and rRB-1BΔRLORF5a, while the average size of rRB-1BΔRLORF4-induced plaques was significantly larger than those of rRB-1B and pRB-1BΔRLORF5a (Fig. 3A and B).

Figure 3C shows the number of MDV genomic copies in CKC cultures infected with rRB-1B, rRB-1BΔRLORF5a, or rRB-1BΔRLORF4 using qPCR assays. At 72 h p.i. the number of genomic copies of rRB-1BΔRLORF4 was significantly higher and continued to increase at a faster rate than those of both rRB-1B and rRB-1BΔRLORF5a, consistent with the increased plaque size. From these experiments we concluded that deletion of *RLORF4* in the pRB-1B clone resulted in increased in vitro replication of the reconstituted virus, at least in CKC cultures.

In vivo replication of rRB-1BΔRLORF4 and rRB-1BΔRLORF5a. In a preliminary animal experiment, chickens infected with rRB-1B or rRB-1BΔRLORF4 were examined for virus replication at 6 and 22 days p.i. (experiment 1) using virus isolation assays. There was no significant difference between the two viruses with 0.60 ± 0.26 PFU/1 × 10^6 splenocytes isolated from chickens inoculated with rRB-1B and 0.26 ± 0.20 PFU/1 × 10^6 splenocytes demonstrated for rRB-1BΔRLORF4-infected chickens at 6 days p.i. However, at 22 days p.i., there was a significant difference between rRB-1B-infected chickens (216 ± 94 PFU/1 × 10^6 splenocytes) and rRB-rRB-1BΔRLORF4-inoculated chickens (14 ± 4 PFU/1 × 10^6 splenocytes). MDV was not isolated or detected using qPCR assays from contact chickens in both groups at 22 days p.i.

In a second animal experiment, chickens were inoculated with the following viruses including uninfected controls: RB-1B/p17, RB-1B/p71 (attenuated), rRB-1B/p6, rRB-1BΔRLORF5a/p6, or rRB-1BΔRLORF4/p6. Table 1 summarizes the relative weights of prepared thymi and bursas for each group at 4, 7, 10, and 14 days p.i. Animals in all groups, except those infected with rRB-1BΔRLORF5a, had a significant increase in the relative thymus weight compared to uninfected chickens at 4 days p.i. Apart from RB-1B/p17, which caused thymic atrophy as evidenced by decreased thymus weights compared to uninfected controls at 7, 10, and 14 days p.i., only RB-1B/p71 and rRB-1B caused thymic atrophy at 10 days p.i. compared to uninfected chickens, while rRB-1BΔRLORF5a and rRB-1BΔRLORF4 did not cause thymic atrophy at any time point. Only chickens infected with RB-1B/p17 showed bursal atrophy at 14 days p.i.

Also in experiment 2, splenocytes were collected and used

TABLE 1. Relative weights of thymi and bursas[a]

No. of days p.i.	Group	Body wt (g)[b]	Thymus wt (%)[b]	Bursa wt (%)[b]
4	Uninfected	49.9 ± 2.6	0.20 ± 0.07 A	0.29 ± 0.03
	RB-1B/p17	52.7 ± 1.5	0.38 ± 0.04 BC	0.34 ± 0.02
	RB-1B/p71	52.5 ± 1.9	0.38 ± 0.03 BC	0.33 ± 0.02
	rRB1B	50.2 ± 3.6	0.34 ± 0.03 B	0.32 ± 0.01
	rRB1BΔRLORF5A	46.9 ± 2.5	0.29 ± 0.02 A	0.31 ± 0.02
	rRB1BΔRLORF4	51.3 ± 1.3	0.45 ± 0.03 C	0.34 ± 0.02
7	Uninfected	62.7 ± 2.3	0.33 ± 0.01 B	0.30 ± 0.02
	RB-1B/p17	59.0 ± 2.1	0.14 ± 0.02 A	0.31 ± 0.03
	RB-1B/p71	58.5 ± 3.0	0.28 ± 0.02 B	0.31 ± 0.03
	rRB1B	63.6 ± 2.1	0.39 ± 0.03 B	0.35 ± 0.02
	rRB1BΔRLORF5A	64.1 ± 4.0	0.34 ± 0.01 B	0.33 ± 0.02
	rRB1BΔRLORF4	57.0 ± 2.4	0.37 ± 0.04 B	0.34 ± 0.01
10	Uninfected	78.2 ± 3.2	0.49 ± 0.06 CD	0.34 ± 0.02
	RB-1B/p17	82.9 ± 4.4	0.18 ± 0.04 A	0.24 ± 0.05
	RB-1B/p71	79.0 ± 4.2	0.35 ± 0.05 B	0.36 ± 0.02
	pRB1B	78.6 ± 4.6	0.37 ± 0.03 B	0.43 ± 0.05
	rRB1BΔRLORF5A	84.5 ± 4.5	0.43 ± 0.04 BC	0.37 ± 0.02
	rRB1BΔRLORF4	82.4 ± 7.1	0.51 ± 0.02 D	0.37 ± 0.03
14	Uninfected	121.6 ± 5.4 BC	0.53 ± 0.06 B	0.40 ± 0.03 B
	RB-1B/p17	102.6 ± 5.7 A	0.21 ± 0.05 A	0.25 ± 0.03 A
	RB-1B/p71	122.4 ± 3.3 BC	0.51 ± 0.03 B	0.39 ± 0.02 B
	rRB1B	105.1 ± 8.1 AB	0.45 ± 0.06 B	0.38 ± 0.08 B
	rRB1BΔRLORF5A	108.3 ± 6.0 AB	0.52 ± 0.02 B	0.40 ± 0.03 B
	rRB1BΔRLORF4	118.1 ± 7.6 ABC	0.54 ± 0.05 B	0.46 ± 0.04 B

[a] P2a chickens were either inoculated with CKC cultures (uninfected) or with 1,000 PFU of RB-1B/p17, attenuated RB-1B/p71, rRB-1B/p6, rRB-1BΔRLORF5A/p6, or rRB-1BΔRLORF4/p6. All thymus lobes and bursae were weighed and their weights relative to the bird body weight were calculated.

[b] Values that were significantly different from the other values in that group by a Tukey-Kramer comparison of means are indicated by the letters. Values labeled with different letters were significantly different ($P \leq 0.05$).

for virus isolation assays (Fig. 4A) and for collection of DNA to determine viral DNA copy titers (Fig. 4B). Only RB-1B/p17 and RB-1B/p71 could be isolated from infected chickens at 4 days p.i. (Fig. 4A). At 4, 7, 10, and 14 days p.i., RB-1B/p17 was isolated at significantly higher levels than all other strains. In comparisons of the two pRB-1B-derived deletion mutants to the parental rRB-1B, only rRB-1BΔRLORF4 had significantly lower viremia levels at 14 days p.i. Similar results were obtained using qPCR assays detecting the number of viral genomic copies (Fig. 4B). These data indicate that the pRB-1B-derived virus exhibited a slightly delayed onset of viremia compared to parental RB-1B and that the *RLORF4* deletion mutant appeared greatly debilitated with respect to lytic virus replication at 14 days p.i. (Fig. 4A and B).

Deletion of *RLORF4* reduces the oncogenic potential of rRB-1B. In order to examine the oncogenic potential of rRB-1BΔRLORF5a and rRB-1BΔLORF4, a third animal experiment was performed. All chickens infected with RB-1B/p17 succumbed to MD by 4 weeks p.i., while a majority of rRB-1B- and rRB-1BΔRLORF5a-infected chickens developed MD by 6 weeks (Fig. 4C). Therefore, all the remaining chickens in the rRB-1B- and rRB-1BΔRLORF5a-infected groups were euthanized at 6 weeks p.i. Because no clinical signs had occurred in animals infected with RB-1B/p71 and rRB-1BΔRLORF4, 10 animals in these two groups were evaluated for gross MD lesions at 7 weeks p.i. Postmortem examinations showed that none of the chickens infected with RB-1B/p71 or rRB-1BΔRLORF4 had developed MD lesions at that time point. From 7 to 13 weeks p.i., no chickens in the remaining groups developed MD symptoms and the experiment was terminated at 13 weeks p.i., when MD-specific lesions were found in two birds infected with rRB-1BΔRLORF4.

rRB-1B with a single deletion of *RLORF4* is phenotypically similar to rRB-1B in vivo. Since deleting both copies of *RLORF4* in pRB-1B resulted in attenuation of the virus reconstituted from the recombinant BAC, viral replication of two independent clones still containing one of the two copies of *RLORF4* was examined in a fourth animal experiment. Figure 5A and B show that viruses reconstituted from two independent clones harboring only one copy of *RLORF4*, 2A1 and 2A8, maintained virus replication rates similar to that of rRB-1B in vivo by virus isolation (Fig. 5A) and qPCR (Fig. 5B) assays. In contrast and as described in experiment 3 above, rRB-1BΔRLORF4 replicated at significantly lower levels at 14 days p.i.

In the fifth animal experiment, the oncogenic potential of the single deletion mutants was evaluated in P2a chickens over a 52-day period. Animals infected with rRB-1B or single-deletion mutant 2A1 or 2A8 started to develop MD symptoms and gross lesions by 5 weeks p.i. (Fig. 5C), similar to the rRB-1B-infected chickens in experiment 3 (Fig. 4C). Again, chickens infected with rRB-1BΔRLORF4 did not develop MD symptoms or lesions for up to 52 days p.i.

Phenotypic changes in rRB-1BΔRLORF are not clone specific. Since only one rRB-1BΔRLORF4 mutant clone was examined in experiments 1 to 5, two additional clones were

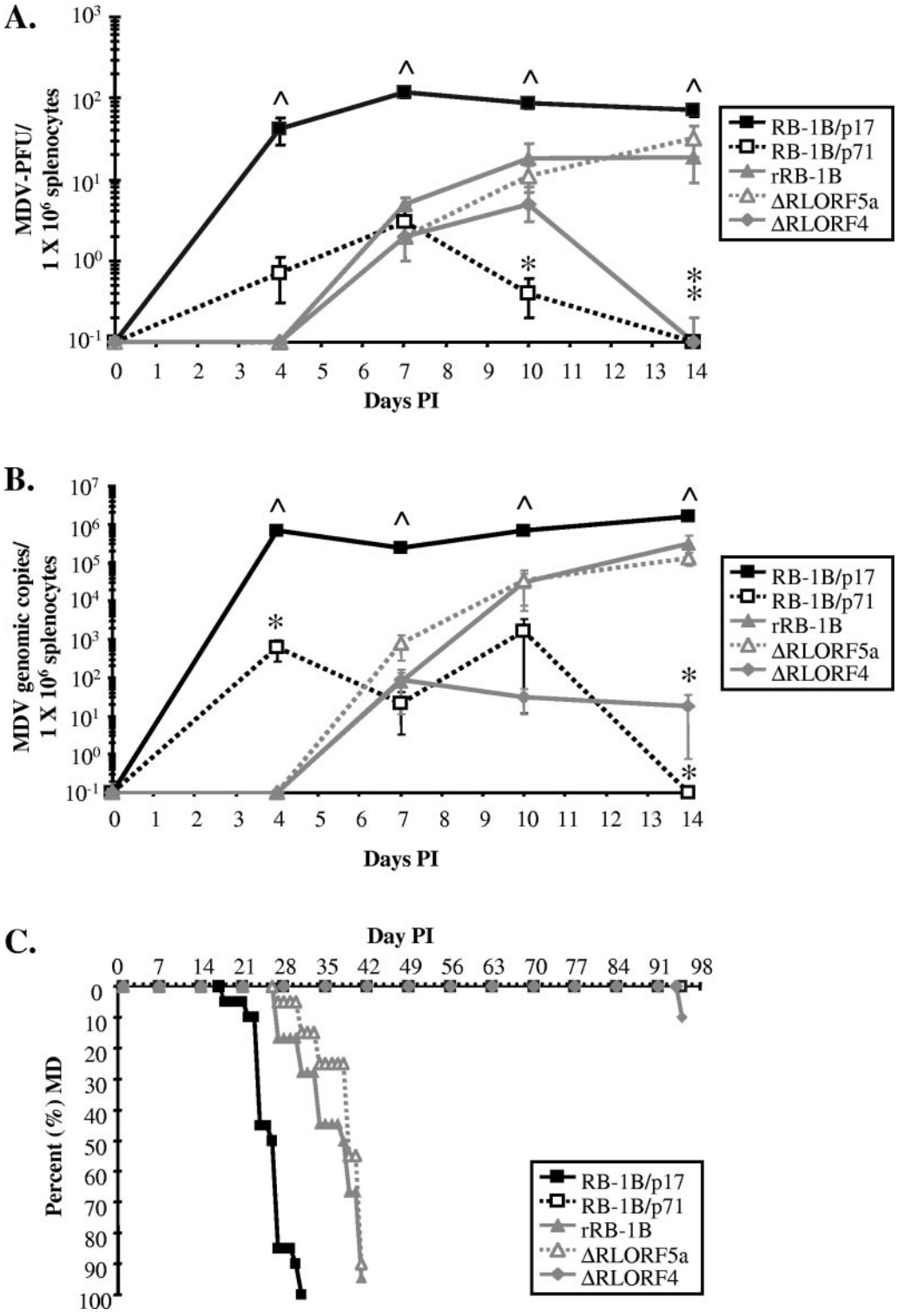

FIG. 4. In vivo replication of MDV and tumor incidence in P2a chickens inoculated with RB-1B/p17, rRB-1B, rRB-1BΔRLORF5a, rRB-1BΔRLORF4, or attenuated RB-1B/p71. (A) MDV isolation in CKC cultures inoculated in triplicate with 5×10^5 splenocytes. Plaques were counted 5 days p.i., and the results are expressed as the mean PFU/1×10^6 splenocytes ± standard error of the mean (SEM) (error bar) for each group. Values that were significantly higher (^) or lower (*) than those of all the other groups ($P < 0.05$) are indicated. (B) Mean MDV genome copy numbers/1×10^6 splenocytes ± SEM (error bar) using qPCR assays. Values that were significantly higher (^) or lower (*) than those of all the other groups ($P < 0.05$) are indicated. (C) Percent incidence of MD during a 13-week evaluation period.

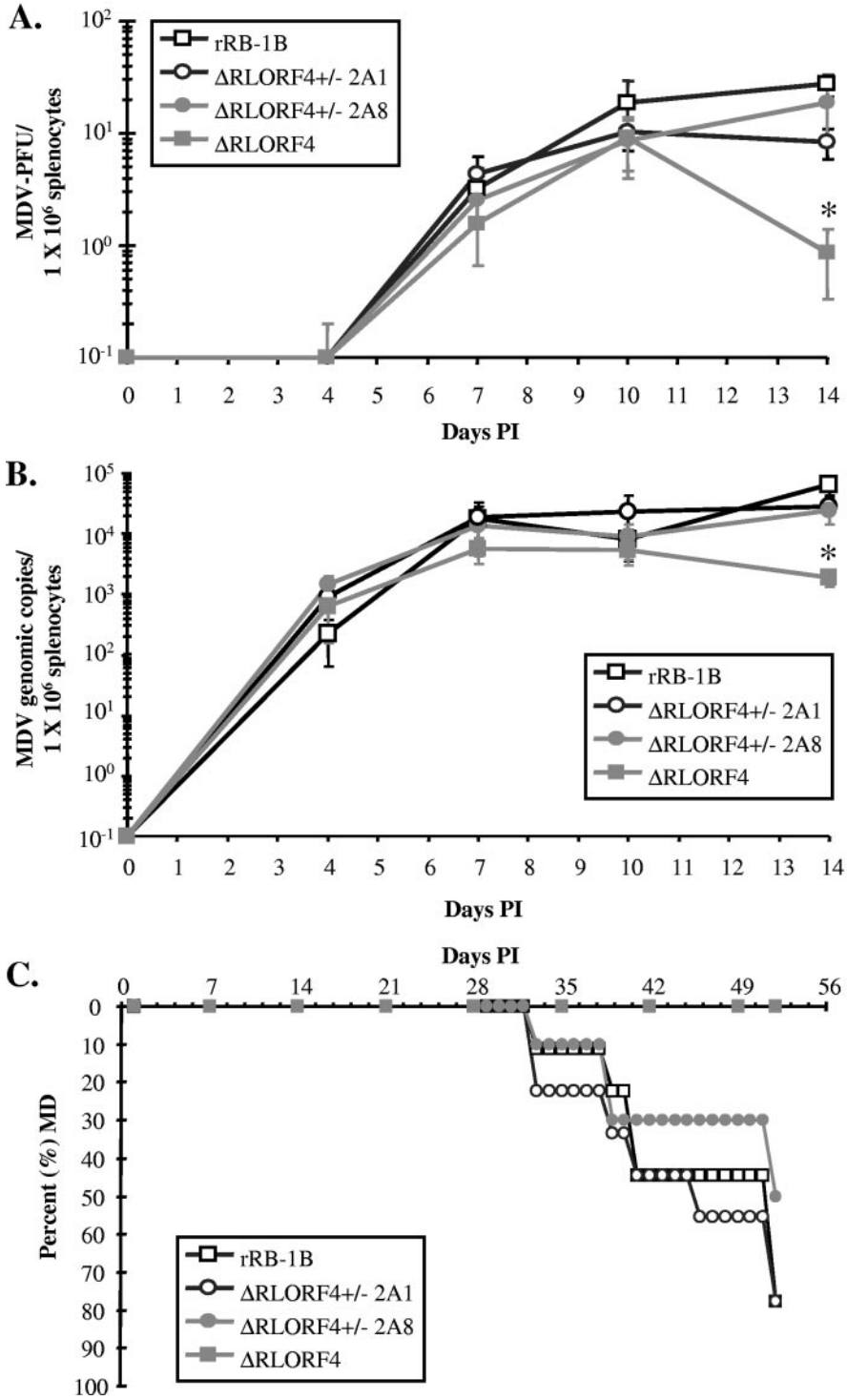

FIG. 5. In vivo replication of MDV and tumor incidence in P2a chickens inoculated with rRB-1B, rRB-1BΔRLORF4+/− (2A1), rRB-1BΔRLORF4+/− (2A8), and rRB-1BΔRLORF4. (A) MDV isolation in CKC cultures inoculated in triplicate with 5×10^5 splenocytes. Plaques were counted 5 days p.i., and the results are expressed as the mean number of PFU/1×10^6 splenocytes ± standard error of the mean (SEM) (error bar) for each group. Values that were significantly lower than those of all the other groups ($P < 0.05$) are indicated (∗). (B) MDV genome copy numbers/1×10^6 splenocytes ± SEM using qPCR assays. Values that were significantly lower than those of all the other groups ($P < 0.05$) are indicated (∗). (C) Percent incidence of MD during a 52-day evaluation period.

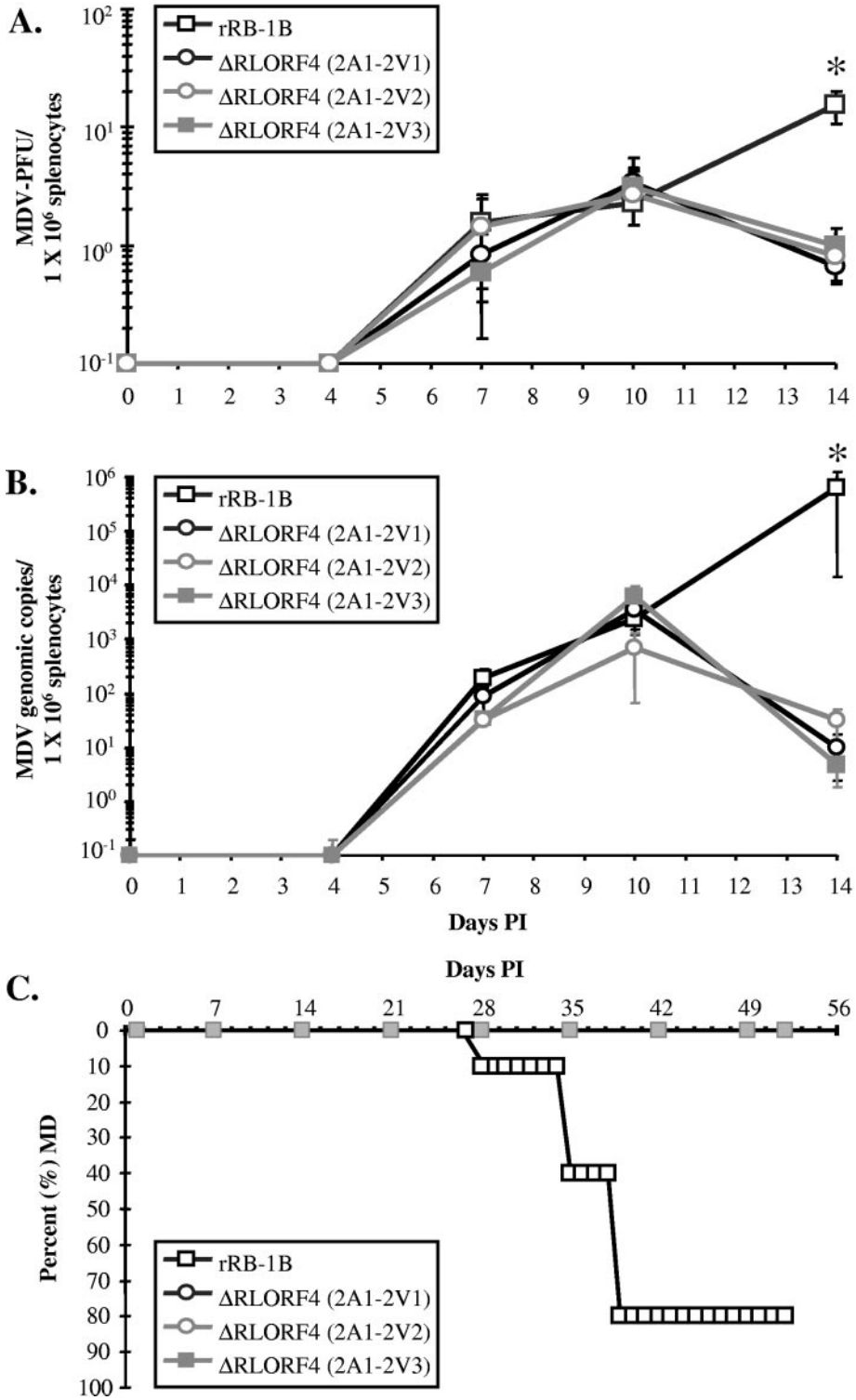

FIG. 6. In vivo replication of MDV and tumor incidence in P2a chickens inoculated with rRB-1B, or three independent clones of rRB-1BΔRLORF4 (2A1-2V1, 2A1-2V2, and 2A1-2V3). (A) MDV isolation in CKC cultures inoculated in triplicate with 5×10^5 splenocytes. Plaques were counted 5 days p.i., and the results are expressed as the mean number of PFU/1×10^6 splenocytes ± standard error of the mean (SEM) (error bar) for each group. Values that were significantly higher than those of all the other groups ($P < 0.05$) are indicated (*). (B) MDV genome copy numbers/1×10^6 splenocytes ± SEM using qPCR assays. Values that were significantly higher than those of all the other groups ($P < 0.05$) are indicated (*). (C) Percent incidence of MD during a 52-day evaluation period.

evaluated both in vitro and in vivo. In vitro analysis showed that there were no significant differences between all three rRB-1BΔRLORF4 deletion mutants (2A1-2V1, 2A1-2V2, and 2A1-2V3) using plaque measurement and qPCR assays, while each was significantly different from rRB-1B (data not shown), similar to Fig. 3. Analysis of viral replication in vivo showed that using virus isolation and qPCR assays, all three independent rRB-1BΔRLORF4 clones replicated similarly (Fig. 6A and B). Replication was significantly lower at 14 days p.i. than with parental rRB-1B. Additionally, none of the rRB-1BΔRLORF4 clones induced MD lesions for up to 52 days p.i., while rRB-1B induced MD (Fig. 6C), consistent with experiments 3 and 5.

DISCUSSION

MDV is an oncogenic alphaherpesvirus that can be attenuated by serial passage in cultured cells (41). The previous finding that four of six attenuated strains of MDV examined had deletions within *RLORF4* suggested that this ORF may be important for in vivo replication of MDV (10) because attenuated MDV does not replicate as well as nonattenuated MDV in vivo (30). However, attenuation of MDV by serial passage in CEC or CKC cultures can lead to multiple genomic changes, including expansion of the 132-bp tandem direct repeat region within the IR_L and TR_L regions (8, 25, 26), although it has been shown that this alteration does not directly cause attenuation (34). In addition, transcripts in the 1.8-kb transcript family that has been associated with the oncogenic potential of MDV (1, 2) are truncated in attenuated strains (1, 2, 14, 26, 28). Expression of glycoprotein C (gC) is greatly reduced after serial passage in vitro as well (4, 37, 39, 42). To test the hypothesis that deletion of *RLORF4* may lead to attenuation of MDV, *RLORF4* was directly deleted in the virulent pRB-1B BAC (24) clone by Red recombination.

Following attenuation of MDV, phenotypic changes in the virus include decreased potential for in vivo replication and oncogenicity and increased in vitro replication rates and plaque sizes compared to those of nonattenuated strains. When both copies of *RLORF4* were deleted in pRB-1B, the average plaque size of rRB-1BΔRLORF4 was almost double the sizes of plaques induced by the parental rRB-1B and a second mutant, rRB-1BΔRLORF5a (Fig. 3A and B). When qPCR assays were used to measure the number of MDV genomes per cell in infected CKC cultures, the number of MDV copies in rRB-1BΔRLORF4-infected cultures was significantly higher than those observed in rRB-1B- and rRB-1BΔRLORF5a-infected cultures after 72 h p.i. These data indicate that deleting *RLORF4* leads to in vitro growth of MDV phenotypically associated with attenuated viruses. These data are very similar to recent observations for the absence or presence of gC in MDV. Deletion of the gC-encoding ORF, whose expression is down-regulated by serial passage of the virus in cultured cells, results in increased plaque sizes (35). As such, the increase in plaque size after deletion of *RLORF4* is the second example of the deletion of an MDV ORF that resulted in an increase in virus-induced plaque size.

When the rRB-1BΔRLORF4 mutant virus was examined in vivo by virus isolation and qPCR assays, there was little difference between rRB-1BΔRLORF4 and the parental rRB-1B virus early during infection (Fig. 4 to 6). Generally, the numbers of infectious virus and genomic copies detected in rRB-1BΔRLORF4-infected chickens were lower than those in rRB-1B-infected chickens, although these differences were not statistically significant until 14 days p.i. and later (experiment 1 and Fig. 4 to 6). The decreased replication in vivo satisfies the second major characteristic of attenuated MDV in that deleting *RLORF4* led to decreased in vivo replication. Additionally, when the oncogenic potential of rRB-1BΔRLORF4 was examined, the level of MD incidence was reduced from 95% induction in P2a chickens infected with parental rRB-1B down to 10% in rRB-1BΔRLORF4-infected chickens 13 weeks p.i. (Fig. 4C). None of the rRB-1BΔRLORF4-infected chickens developed MD lesions for up to 8 weeks, which is generally the period over which MD incidence is studied. Examination of two independently derived single-deletion *RLORF4* mutants showed that deletion of only one copy of *RLORF4* is not sufficient to induce the phenotypic changes seen in double-deletion mutants, suggesting that the RLORF4 product is indeed important in MD pathogenesis and that one copy of the diploid ORF is sufficient for full pathogenicity. Analysis of the single-deletion mutants also indicated that it is unlikely that other significant genomic changes were introduced during the construction of mutant rRB-1BΔRLORF4, which could be responsible for attenuation. This was further substantiated by the fact that viral replication and oncogenic potential of three independent clones of rRB-1BΔRLORF4 were identical. Taken together, the in vivo and in vitro data strongly suggest that deleting both copies of *RLORF4* directly leads to attenuation of MDV.

Schat et al. (30) have speculated that attenuated strains may lose their ability to establish a primary infection in lymphocytes; however, this does not appear to be the case with rRB-1BΔRLORF4 because significant differences did not occur until 14 days p.i. This suggests that the involvement of *RLORF4* in MD pathogenesis may be primarily later during the infection, possibly during the latency/reactivation or transformation phase, since RLORF4 transcripts could be detected in both lytically infected CKC cultures and transformed MDCC lines (10). Deletion of *RLORF4* significantly reduced incidence of MD lesions, but it is unknown whether this was due to a potential involvement in transformation or an involvement in reactivation since there was less viral replication in vivo beyond 14 days p.i. in chickens infected with the *RLORF4* deletion mutant.

During the recent cloning of oncogenic RB-1B as a BAC, one clone termed pRB-1B-2 containing a 7.7-kb deletion in the IR_L and TR_L regions was obtained. Virus reconstituted from that clone was nononcogenic (24), presumably because deletion of these regions resulted in the complete loss of a number of genes or ORFs, including *Meq*, *RLORF5a*, *RLORF4*, and *vIL-8*, which strongly suggests that genes within this region are involved in oncogenesis. Deletion of *vIL-8* in oncogenic RB-1B (22) and Md5 (6) showed that vIL-8 is dispensable for the establishment of latency and transformation but is involved in the early cytolytic phase, likely by attracting B or T lymphocytes to primarily infected cells. Lupiani et al. (19) recently showed that deletion of *Meq* in the Md5 strain did not affect replication of the virus but that *Meq* was involved in transformation since no chickens produced tumors in rMd5ΔMeq-

infected chickens for up to 8 weeks p.i. Since deletion of *RLORF4* did not cause MD lesions at 7 weeks p.i. but did at 13 weeks p.i., it would be interesting to see whether other mutant viruses that have significantly reduced incidences of MD tumors up to 8 weeks continue to remain tumor-free for longer periods. These data combined indicate that *Meq*, *RLORF4*, and/or *vIL-8* are involved in attenuation of MDV, while *RLORF5a* is not.

It has previously been suggested that the region upstream of *vIL-8* contains the putative *vIL-8* promoter (10). *RLORF4* is also located in this region; therefore, deletion of *RLORF4* could also potentially affect vIL-8 mRNA expression. Although *vIL-8* mRNA production is still evident in rRB-1BΔRLORF4-infected CKC cultures using qRT-PCR assays (Fig. 2), it is unknown at this time whether the level of *vIL-8* mRNA expression is altered in vivo. We are currently studying the potential importance of levels of vIL-8 in the deletion mutants.

It is interesting that when the levels of in vivo replication and oncogenesis for RB-1B/p17 and rRB-1B were compared, the latter replicated at significantly lower levels and MD occurred with kinetics that were delayed by approximately 1 week compared to RB-1B/p17. Additionally, no contact birds were detectably infected with MDV at 22 days p.i. in experiment 1. These data suggest that the pRB-1B virus harbors some mutations compared to the original RB-1B/p17 virus used as a comparative virus in this study, although the recombinant virus generated from pRB-1B is still virulent and regularly causes between 90 and 100% MD incidence in P2a chickens (13) (Fig. 4C). There are at least two possible reasons for this discrepancy between rRB-1B and RB-1B. The first is that insertion of the mini-F plasmid pHA1 into U_S2 alters pathogenesis. Previous studies in which the U_S1, U_S2, U_S10, and the *SORFs-1*, *-2*, and *-3* were deleted by *lacZ* insertion in RB-1B showed that horizontal transmission was reduced (21). These data combined with those presented in this report suggest that U_S2 is important for MDV pathogenesis, possibly affecting the virus' ability to efficiently infect feather follicle epithelium cells. The second is that the change in pathogenesis of rRB-1B may be a clone-specific effect, since it was constructed using a clone-purified fourth passage stock of RB-1B and it was phenotypically identical to its parental strain (24).

It has previously been shown that deletion of *RLORF5a* in the CVI988 strain did not affect in vitro and in vivo replication and the establishment of latency (31); however, its involvement in oncogenesis could not be studied since CVI988 is nononcogenic. In this report, we examined the effect of deleting *RLORF5a* in the virulent pRB-1B BAC clone. Figures 3 and 4 show that deleting both copies of *RLORF5a* did not affect in vitro or in vivo replication of rRB-1B, nor did it change its oncogenic potential, conclusively showing that *RLORF5a* is dispensable for MDV replication and oncogenicity. However, deleting both copies of *RLORF4* in the virulent pRB-1B BAC clone led to changes in MDV replication, both in vitro and in vivo, two main characteristics of attenuated MDV strains. These data, in addition to the fact that a number of serially passaged MDV strains contain deletions within *RLORF4*, suggest that *RLORF4* is important in MDV pathogenesis. Future studies will concentrate on the identification of the RLORF4 gene product and its mechanism of function.

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