JOURNAL OF VIROLOGY, Dec. 1995, p. 7888–7898 Vol. 69, No. 12
0022-538X/95/$04.00+0

Retention of Oncogenicity by a Marek's Disease Virus Mutant Lacking Six Unique Short Region Genes†

MARK S. PARCELLS, AMY S. ANDERSON, AND ROBIN W. MORGAN*

Department of Animal Science and Agricultural Biochemistry, College of Agricultural Sciences, University of Delaware, Newark, Delaware 19717-1303

Received 25 July 1995/Accepted 21 September 1995

We previously reported the construction of Marek's disease virus (MDV) strains having mutations in various genes that map to the unique short (US) region of the viral genome (J. L. Cantello, A. S. Anderson, A. Francesconi, and R. W. Morgan, J. Virol. 65:1584–1588, 1991; M. S. Parcells, A. S. Anderson, and R. W. Morgan, Virus Genes 9:5–13, 1994; M. S. Parcells, A. S. Anderson, and R. W. Morgan, J. Virol. 68:8239–8253, 1994). These strains were constructed by using a high-passage-level serotype 1 MDV strain which grew well in chicken embryo fibroblasts. Despite the growth of the parent and mutant viruses in cell culture, in vivo studies were limited by poor growth of these strains in chickens. One of the mutants studied lacked 4.5 kbp of US region DNA and contained the *lacZ* gene of *Escherichia coli* inserted at the site of the deletion. The deletion removed MDV homologs to the US1, US2, and US10 genes of herpes simplex virus type 1 as well as three MDV-specific open reading frames. We now report the construction of a mutant MDV containing a similar deletion in the US region of the highly oncogenic RB1B strain. This mutant, RB1BΔ4.5*lac*, had a growth impairment in established chicken embryo fibroblasts similar to that described previously for MDVs lacking a functional US1 gene. In chickens, RB1BΔ4.5*lac* showed decreased early cytolytic infection, mortality, tumor incidence, and horizontal transmission. Several lymphoblastoid cell lines were established from RB1BΔ4.5*lac*-induced tumors, and virus reactivated from these cell lines was LacZ$^+$. These results indicate that the deleted genes are nonessential for the transformation of chicken T cells or for the establishment and maintenance of latency. On the basis of the growth impairment observed for RB1BΔ4.5*lac* in cell culture and in vivo, we conclude that deletion of these genes affects the lytic replication of MDV. This is the first MDV mutant constructed in the RB1B oncogenic strain, and the methodology described herein provides for the direct examination of MDV-encoded determinants of oncogenicity.

Marek's disease virus (MDV) is a transforming herpesvirus that rapidly induces T-cell lymphomas in susceptible chickens (7). Although it is classified as a gammaherpesvirus on the basis of its biological properties, the genomic organization and available DNA sequence data for MDV indicate that it is more closely related to the alphaherpesviruses (5, 6, 32, 34, 35, 45). The mechanism of MDV-mediated transformation of T cells remains unknown. Oncogenic MDV strains do not replicate well in cell culture and become attenuated rapidly during serial passage (10). Attenuation is accompanied by alterations in the sequence and expression of the MDV genome (3, 4, 13, 21, 41, 44), loss of horizontal transmission (10), and a decreased ability to infect lymphocytes in vivo and in vitro (39).

Previous studies of MDV transformation have focused on the identification of (i) regions of the MDV genome that undergo alteration during attenuation, (ii) viral gene products expressed in MDV-induced tumors and tumor-derived lymphoblastoid cell lines (LBCLs), (iii) viral gene products believed to be expressed exclusively by serotype 1 MDV strains, and (iv) sites of MDV integration into the host genome. These approaches have led to the identification of a number of MDV genes and regions having possible significance for transformation. The repeats (terminal and inverted, respectively) flanking the unique long (UL) region of the MDV genome (TR_L and IR_L) encode a family of RNAs that become disrupted during attenuation (3, 4). This region also contains the *meq* gene, which encodes a leucine-zipper-containing protein expressed in LBCLs (18). Genes encoding phosphoproteins pp38 and pp24 initiate in these repeats (2, 9, 20, 46). These phosphoproteins are expressed in cells lytically infected with MDV serotype 1 strains as well as in LBCLs (9). cDNAs generated from transcripts expressed from these regions in LBCLs encode small open reading frames (15, 30, 31). Antisera generated against two of these open reading frames have detected a 14-kDa protein expressed in lytically infected chicken embryo fibroblasts (CEF) as well as in an LBCL (15). Antiserum raised against another open reading frame detected a 7-kDa protein expressed in lytically infected CEF and in LBCLs (31). The repeats flanking the unique short (US) region (IR_S and TR_S) are also implicated in transformation. In particular, a cDNA expressed from IR_S and TR_S encodes an open reading frame that could specify a 94-amino-acid protein (27). Antiserum raised against this open reading frame stained RB1B-infected chicken kidney cells but not LBCLs. Finally, the examination of MDV integration sites in chromosomes of LBCLs has identified numerous sites of insertion, but none have been associated with alterations in either cellular or viral gene expression (11). Despite the contributions of this body of research to the study of MDV, the role of MDV-encoded gene products in transformation remains unknown.

We recently described the characterization of mutant attenuated MDVs containing insertions and deletions in the US region (8, 28, 29). Of these mutants, only those lacking a functional US1 gene have a discernible phenotype in cell culture, namely, a growth impairment in established CEF (29). This growth impairment correlated with decreased plaque size and numbers of PFU obtained per plaque. Although MDV is

* Corresponding author. Phone: (302) 831-2524.

† Published as Miscellaneous Paper No. 1565 of the Delaware Agricultural Experiment Station.

an attractive system for the analysis of viral gene function in the natural host, the usefulness of previously reported MDV mutants has been severely limited by their poor growth in chickens. This report describes the first mutant MDV constructed in the highly oncogenic RB1B strain of MDV. This mutant, RB1BΔ4.5*lac*, contains a deletion identical to that of GAΔ4.8*lac* (29).

MATERIALS AND METHODS

Cells and viruses. MDV was propagated in secondary CEF with growth medium consisting of M199 medium (Life Technologies, Inc. [LT], Grand Island, N.Y.) supplemented with 3% calf serum (LT), 100 IU of penicillin G per ml, and 100 μg of dihydrostreptomycin per ml (23). Passage level 7 of RB1B (RB1Bp7), a highly pathogenic strain of MDV (38), was obtained from Intervet Inc., Millsboro, Del., and passed twice in CEF for the preparation of RB1B-infected cell DNA. This RB1Bp9 DNA stock was used for mutant construction.

Plasmid constructions. Cloning was done by standard methods (37), and restriction endonuclease digestions were carried out according to the recommendations of the suppliers. Plasmid pMD190 was used to generate the RB1B deletion mutant (RB1BΔ4.5*lac*) and has been described previously (29). Briefly, a 12.2-kbp *Hin*dIII-*Bam*HI subfragment of the *Bam*HI-A fragment from the GA strain of MDV (12) was cloned into pUC18 to generate pMD163. Plasmid pMD163 was digested with *Pst*I (LT), and the 10.1-kbp vector-containing fragment was purified by preparative agarose gel electrophoresis and religated to generate pMD164. Plasmid pMD164 DNA was digested with *Pst*I and blunt ended by using the Klenow enzyme (LT). A *lacZ* cassette, derived from pCH110 (Pharmacia, Inc., Piscataway, N.J.) and modified for insertion mutagenesis (43), was blunt ended and ligated into pMD164 to generate pMD190. Plasmid pMD190 DNA was purified by using the Qiagen maxi-preparation procedure (Qiagen Corp., Chatsworth, Calif.).

RB1BΔ4.5*lac* MDV strain construction. The RB1B deletion mutant was constructed by using a modified version of a method used to construct recombinant attenuated MDV strains (8) (Fig. 1). Ten micrograms of RB1B-infected CEF DNA was cotransfected with 0.5 μg of pMD190 DNA into freshly plated CEF with calcium phosphate (23). One week posttransfection, monolayers were harvested and diluted to 20 ml with complete medium, and 200 μl was added to each well of a 96-well microtiter dish. This dish was used as a template, in that 100 μl from each well was added to duplicate 96-well microtiter dishes that had been seeded previously with 100 μl of CEF (~10^5 cells per ml). One week postplating, one microtiter dish was fixed with 95% ethanol (EtOH) and subjected to immunofluorescence analysis (IFA) with anti-β-galactosidase antiserum (5-Prime-3-Prime, Boulder, Colo.) followed by goat anti-rabbit immunoglobulin G fluorescein isothiocyanate-conjugated antiserum (Sigma Chemical Co., St. Louis, Mo.). Wells from the unfixed microtiter dish corresponding to those containing fluorescent plaques on the fixed microtiter dish, as well as numerous surrounding wells, were harvested by the addition of 50 μl of 0.5% trypsin per well; this was followed by the addition of 50 μl of calf serum (LT) per well. These wells were plated, in duplicate, onto fresh CEF. After 6 days, one of each duplicate dish was stained with Bluo-gal as previously described (8). After 10 additional rounds of picking and staining plaques, all detectable plaques were β-galactosidase positive. When this procedure was used, one recombinant was plaque purified by a total passage level of 24. This strain was designated RB1BΔ4.5*lac*. The RB1B parent virus strain was also passaged in culture an equivalent number of times so that any changes in oncogenicity and pathogenicity attributable merely to serial passage in culture could be identified. This equal-passage-level parent was designated RB1Bp24.

Southern hybridizations. For Southern blot analysis, 10 μg of DNA from MDV-infected or uninfected CEF was digested with *Eco*RI, and Southern blots were prepared by standard methods (37). The MDV-specific probes used were subfragments of the *Bam*HI-A fragment of MDV strain GA (12) (Fig. 2A). The *lacZ*-specific probe was a 2.1-kbp *Bgl*I fragment contained within the coding sequence of the *lacZ* gene (29). The probes were gel purified and biotin labelled with the Random Images (United States Biochemical Corp., Cleveland, Ohio) nonisotopic random-primed DNA labelling kit.

Northern (RNA) hybridization. Total RNA was purified by the guanidinium isothiocyanate method for RNA purification followed by cesium chloride step gradient centrifugation (1). RNA preparations were treated with RNase-free RQ1 DNase (Promega Biotec, Madison, Wis.) and quantitated by A_{260}. For Northern blot analysis, 10 μg of total RNA from each sample was separated on a 1.2% agarose-formaldehyde gel and transferred to nitrocellulose by standard methods (1). For Northern blot analysis, probes were labelled with [α-^{32}P]dCTP and the RadPrime random-primed DNA labelling kit (LT).

Growth curves. Twelve identical 60-mm-diameter tissue culture dishes of CEF were inoculated with RB1Bp24- or RB1BΔ4.5*lac*-infected CEF at approximately 200 PFU per dish. At 1, 2, 3, 5, and 7 days postinoculation, monolayers were harvested from duplicate dishes and inoculated onto fresh CEF. Titer dishes were counted after 6 days, and the mean number of plaques was determined. Titer dishes were fixed with 95% EtOH, and plaques were counted by IFA with

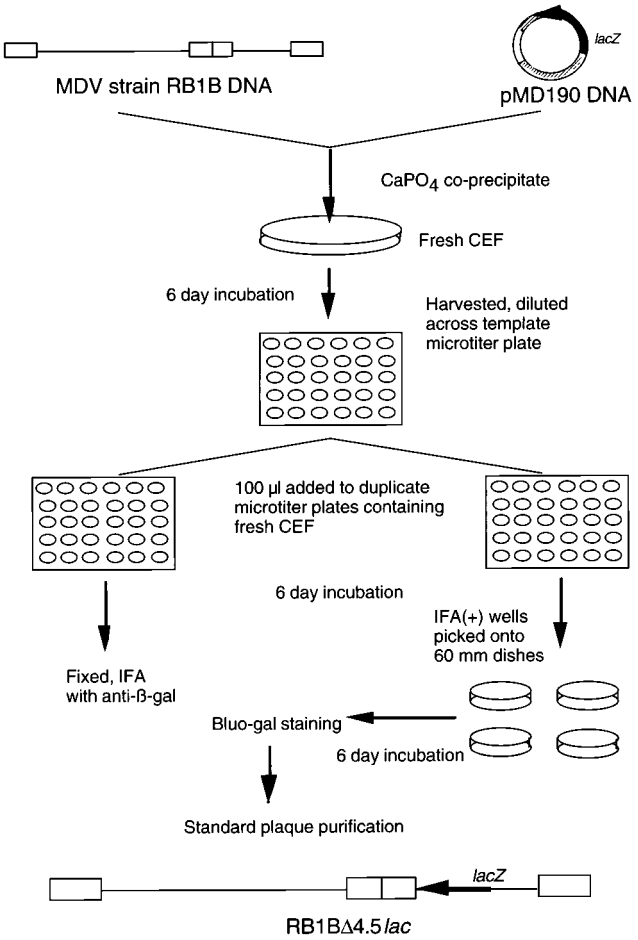


FIG. 1. Diagram of steps in the construction of RB1BΔ4.5*lac*.

polyclonal chicken antiserum to MDV; this was followed by incubation with fluorescein isothiocyanate-conjugated rabbit anti-chicken immunoglobulin G antiserum (Sigma) as previously described (29). Growth curves were done with CEF monolayers incubated at either 37 or 41°C, the latter being the body temperature of the chicken.

Virus growth in vivo. One-day-old specific-pathogen-free single-comb White Leghorn chickens were obtained from SPAFAS (Norwich, Conn.), wingbanded, randomly assigned to experimental groups, and housed in Montair Andersen gloveport isolation cabinets. The chickens were inoculated intra-abdominally with various doses of RB1Bp9-, RB1Bp24-, or RB1BΔ4.5*lac*-infected CEF or with uninfected CEF. For experiment 1, 30 chickens were inoculated per treatment group. To evaluate early cytolytic infection, virus reisolations were attempted from spleen cells and peripheral blood leukocytes (PBLs) at 4, 7, and 11 days postinoculation. At 14 days postinoculation, 10 additional one-day-old chickens were added to each isolator to assess horizontal transmission of the viruses. Virus reisolations from spleen cells and PBLs of contact-exposed chickens were attempted at 14 days postplacement. For experiment 2, 25 chickens were inoculated with uninfected CEF and 35 chickens were inoculated for the other treatment groups. Virus reisolations from the primary inoculates were attempted at 7, 14, 21, and 28 days postinoculation. At 14 days postinoculation, 10 additional one-day-old chickens were added to the control group and 15 one-day-old chickens were added to the other treatment groups. Virus reisolations from contact-exposed chickens were attempted at 7 weeks postplacement. For each experiment, inoculates and contacts were observed daily for 9 and 7 weeks, respectively, for signs of Marek's disease. Tissue samples were taken for histological analysis from representative chickens.

Virus reisolations. For virus reisolations from spleen cells, spleens were removed and pooled from three chickens per treatment group, rinsed in incomplete medium consisting of M199 (LT) supplemented with 100 IU of penicillin G per ml and 100 μg of dihydrostreptomycin per ml, and homogenized on ice with a Ten Broeck tissue grinder. Splenocytes were washed three times in incomplete medium and resuspended at concentrations of either 2×10^7 or 4×10^7 cells per ml in complete medium (incomplete medium containing 3% heat-inactivated calf

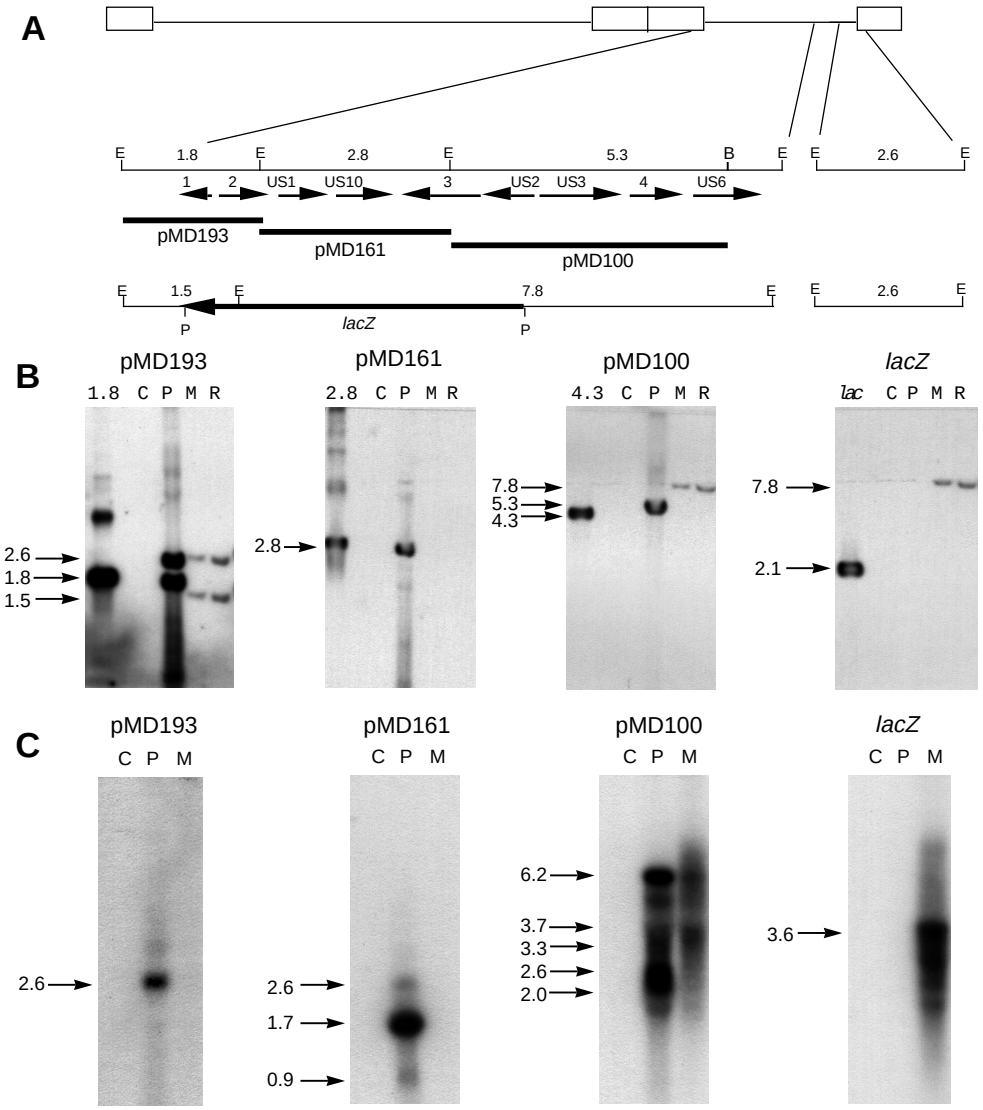


FIG. 2. Southern and Northern blot hybridization analysis of RB1BΔ4.5*lac*. (A) US region of the MDV genome, including the encoded open reading frames and relevant restriction sites. MDV-specific open reading frames are indicated by numbers 1 to 4. Abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I. Also shown are the cloned subfragments of the US region used as probes in panels B and C and the US region of RB1BΔ4.5*lac*. (B) Southern blot analysis of *Eco*RI-digested MDV-infected CEF DNA. The blots were probed with the DNA fragments shown in panel A. The *lacZ* probe used was a 2.1-kbp *Bgl*I fragment described previously (28). The first lane of each blot contained the probe used. The other lanes are as follows. Lanes C, uninfected CEF DNA; lanes P, RB1Bp24-infected CEF DNA; lanes M, RB1BΔ4.5*lac*-infected CEF DNA; lanes R, reisolated RB1BΔ4.5*lac*-infected CEF DNA. Molecular sizes of indicated bands are given in kilobase pairs. (C) Northern blot analysis of total RNA purified from uninfected and RB1B-infected CEF. Lanes C, uninfected CEF RNA; lanes P, RB1Bp24-infected CEF RNA; lanes M, RB1BΔ4.5*lac*-infected CEF RNA. Molecular sizes of indicated bands are given in kilobases.

serum; LT). For virus reisolations, 1×10^6 or 2×10^6 viable cells were plated onto fresh CEF in triplicate, and plaques were counted 6 days later by IFA.

For virus reisolations from PBLs, blood was drawn from three chickens per treatment group into one heparinized syringe, adjusted to a volume of 8 ml with incomplete medium, and placed into a conical 15-ml polypropylene tube. PBLs were purified by underlaying 3 ml of Ficoll Hypaque 1119 (Sigma) and centrifuging at $700 \times g$ for 30 min at room temperature. PBLs were removed, washed twice with 10 ml of complete medium, and adjusted to a concentration of 2×10^7 cells per ml. For virus reisolations, 1×10^6 or 2×10^6 viable PBLs were plated onto fresh CEF in triplicate, and plaques were counted 6 days later by IFA.

Cell line establishment. Tumors were removed from RB1Bp9- or RB1BΔ4.5*lac*-infected chickens, washed with incomplete media, homogenized with a Ten Broeck tissue grinder, and filtered through sterile cheesecloth. Lymphocytes were purified from the tumor homogenate with Ficoll Hypaque 1119 (Sigma). Cells were washed and resuspended in LM Hahn medium (40) and plated in 12-well dishes at concentrations ranging from 1×10^7 to 5×10^5 cells per ml. Cells were split 1:1 to 1:3 in LM Hahn medium approximately every 4 to 5 days when a drop in the medium pH was apparent until the cell lines became established. The cells were split 1:5 to 1:10 thereafter as needed. Cell lines established from RB1BΔ4.5*lac*-induced tumors have been designated MDCC-UD01, -UD02 and -UD03 (UD01, UD02, and UD03, respectively) and were derived from RB1BΔ4.5*lac*-induced ovarian, spleen, and liver tumors from separate chickens, respectively. A cell line derived from an RB1Bp9-induced kidney tumor was designated MDCC-UD14 (UD14).

Virus reactivation from MDV cell lines. Virus was recovered from lymphoblastoid cell lines by cocultivation of 1×10^5 to 1×10^6 lymphoblastoid cells with secondary CEF plated on 60-mm-diameter dishes. Seven days after cocultivation, the dishes were fixed with 95% EtOH and plaques were counted by IFA with rabbit anti-β-galactosidase polyclonal antibodies (5-Prime-3-Prime) or rabbit anti-MDV-US1 protein (graciously provided by Peter Brunovskis, Case Western Reserve University, and Leland Velicer, Michigan State University) as the primary antiserum.

RESULTS

Construction of RB1BΔ4.5*lac*. After the initial IFA screening of the EtOH-fixed microtiter dish, seven wells contained fluorescent plaques. We harvested the corresponding wells on

the unfixed sister dish and plated the contents onto duplicate 60-mm-diameter dishes of CEF. Since MDV is cell associated and trypsinization does not separate individual cells completely, we worried that the distribution of infected cells across the duplicate microtiter plates might not be equivalent. Therefore, we also harvested 14 surrounding wells and plated the contents onto duplicate 60-mm-diameter dishes of CEF. All of the remaining wells were harvested and plated onto 24-well dishes of CEF. As we suspected, some recombinant MDVs were detected in cultures derived from wells that had not corresponded to those wells containing fluorescent plaques on the fixed plate. In any event, the presence of fluorescent plaques on the fixed microtiter dish allowed us to conclude that recombinants had been generated and that they were likely to be present on the unfixed plate. One mutant, designated RB1BΔ4.5*lac*, was obtained. The deletion contained in this mutant is identical to that of GAΔ4.8*lac* (29). The published DNA sequence of this region (5, 34, 35) indicates that the deleted region is 4.5 kbp rather than the 4.8 kbp previously estimated from electrophoretic mobility.

Analysis of the RB1BΔ4.5*lac* genome. Southern blot hybridization of *Eco*RI-digested DNA from uninfected CEF or CEF infected with RB1Bp24, RB1BΔ4.5*lac*, or reisolated RB1BΔ 4.5*lac* indicated that insertion of the *lacZ* cassette at the site of the 4.5-kbp deletion occurred as expected and that the recombinant virus stock was free of detectable parent virus (Fig. 2). In addition, hybridization patterns were essentially identical to those previously reported for the GAΔ4.8*lac* mutant (29). A 1.8-kbp DNA probe (pMD193) detected two bands in MDV-infected cell DNA of 2.6 and 1.8 kbp in the case of RB1Bp24 and 2.6 and 1.5 kbp in the case of the mutant virus DNAs (Fig. 2B). The 2.6-kbp band was shown previously to encode the US-TR_S junction fragment which bears some sequence identity to the 1.8-kbp IR_S-US junction fragment (29). The 1.5-kbp fragment results from the deletion of a portion of this region in the mutant genome and the insertion of an *Eco*RI site which is present in the *lacZ* cassette. The adjacent 2.8-kbp probe (pMD161) detected a 2.8-kbp fragment in the parent virus DNA which was deleted from the mutant (Fig. 2B). The downstream 4.3-kbp *Eco*RI-*Bam*HI probe (pMD100) detected a 5.3-kbp fragment in the parent and a 7.8-kbp fragment in the mutant virus DNAs (Fig. 2B). This 7.8-kbp band results from the deletion of two *Eco*RI sites in the mutant and the insertion of one *Eco*RI site present in the *lacZ* cassette. A *lacZ*-specific probe detected the 7.8-kbp band specific for the mutant virus DNAs (Fig. 2B).

Mutagenesis-associated changes in MDV gene expression. To examine changes in MDV gene expression associated with mutagenesis, Northern blot hybridization with total RNA purified from uninfected and RB1Bp24- and RB1BΔ4.5*lac*-infected CEF was done (Fig. 2C). The probes used were identical to those used for Southern hybridization (Fig. 2A), and the Northern hybridization pattern was like that previously reported for the GAΔ4.8*lac* mutant (29). Probe pMD193 detected a 2.6-kb transcript in RB1Bp24-infected but not RB1BΔ4.5*lac*-infected CEF RNA (Fig. 2C). The adjacent probe, pMD161, detected the 2.6- as well as the 1.7- and 0.9-kb transcripts in RB1Bp24- but not RB1BΔ4.5*lac*-infected CEF RNA (Fig. 2C). The pMD100 probe detected several transcripts present in both RB1Bp24- and RB1BΔ4.5*lac*-infected CEF RNA (Fig. 2C), indicating that the region downstream of the deletion was actively transcribed. The *lacZ*-specific probe detected an abundant 3.6-kb transcript in RB1BΔ4.5*lac*- but not RB1Bp24-infected CEF RNA. These data indicated that the deletion dramatically affected MDV transcription in the region. In addition, the pattern of immunofluorescence staining of RB1Bp24 and RB1BΔ4.5*lac* with a rabbit anti-US1 polyclonal antiserum (provided by Peter Brunovskis, Case Western Reserve University, and Leland Velicer, Michigan State University) was identical to that previously reported for GAatt85 and GAΔ4.8*lac*, respectively (data not shown) (29). These results further demonstrated that the recombinant virus stock was free of detectable parent virus.

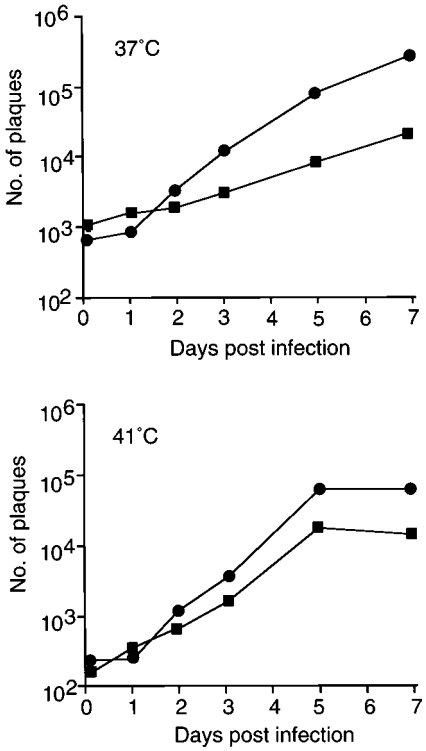


FIG. 3. RB1BΔ4.5*lac* growth curves. Growth curves were done at 37 or 41°C. Each time point is the mean of three dilutions plated in duplicate. ●, RB1Bp24; ■, RB1BΔ4.5*lac*.

RB1BΔ4.5*lac* growth in cell culture. RB1BΔ4.5*lac* showed a growth impairment in cell culture similar to that of attenuated GA strain recombinants, US1*lac* and GAΔ4.8*lac*, both of which lack a functional US1 gene (Fig. 3) (29). These viruses replicated with kinetics similar to those of their parent viruses for the first few days postinfection but showed a decrease in virus yield 3 to 5 days postinfection. This decrease resulted in an approximately 5- to 10-fold reduction in virus yield from the infection. Plaques formed by RB1BΔ4.5*lac* were notably smaller than those formed by RB1Bp24, and generally the PFU-per-plaque yield for RB1BΔ4.5*lac* was 20 to 25 compared with 40 to 50 for RB1Bp24.

RB1BΔ4.5*lac* growth in chickens. (i) Virus reisolations. To characterize RB1BΔ4.5*lac* in vivo, we inoculated chickens with uninfected CEF or with various doses of RB1Bp9-, RB1Bp24-, or RB1BΔ4.5*lac*-infected CEF and attempted virus reisolations from spleen cells and PBLs at various times postinoculation. In experiment 1, the level of infection established by RB1BΔ4.5*lac* was much lower than that established by RB1Bp9 or RB1Bp24 at both doses used and in both spleen cells and PBLs (Fig. 4). This observation suggested that deletion of this region of the genome affects the level of early cytolytic infection established by MDV.

To extend these findings, we repeated the experiment with two modifications. First, we increased the virus doses, and

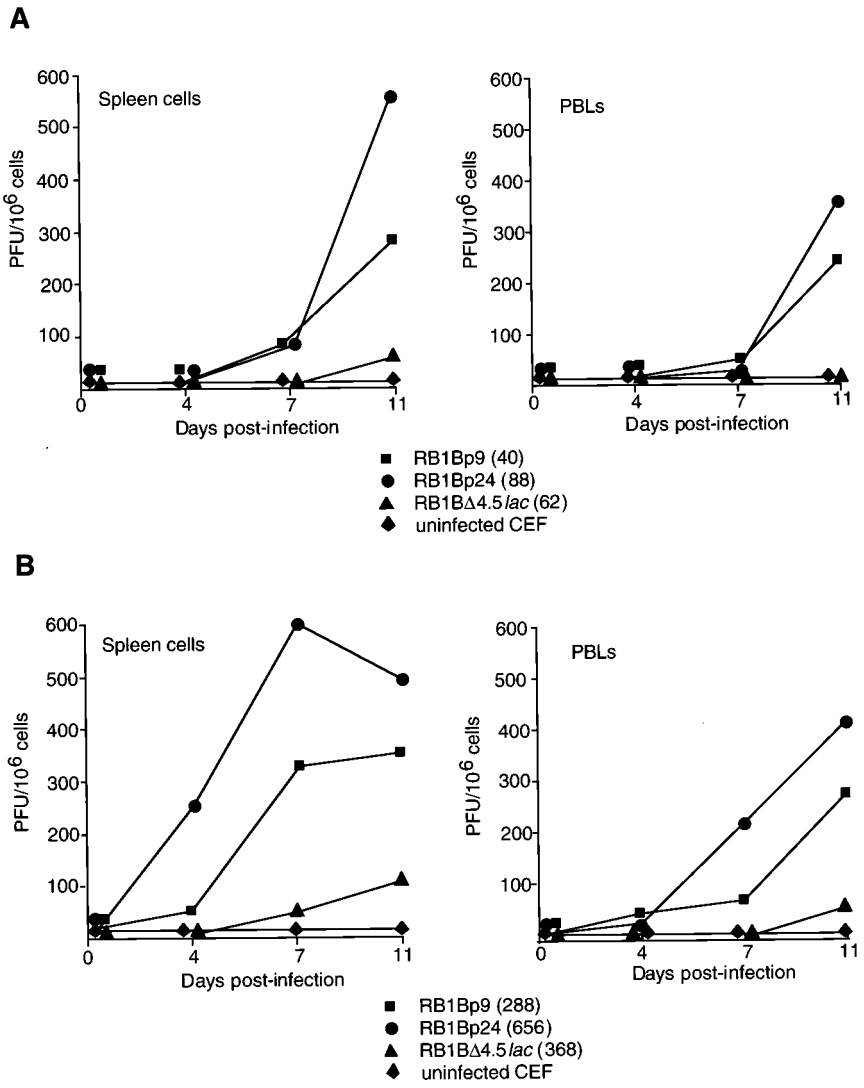


FIG. 4. RB1BΔ4.5*lac* early cytolytic infection of chickens, experiment 1. Shown are virus reisolation results for chickens inoculated with different doses of uninfected or RB1Bp9-, RB1Bp24-, or RB1BΔ4.5*lac*-infected CEF. Virus doses in PFU per chicken are given in parentheses. Each point is the mean number of plaques obtained per 10^6 cells plated. Plaques were counted by IFA.

second, we attempted virus reisolations over a 4-week interval postinoculation. By expanding the number and timing of virus reisolations, we hoped to better evaluate the progression of the viruses through the chicken. In experiment 2, RB1BΔ4.5*lac* again showed a decrease in virus accumulation in both spleen cells and PBLs compared with RB1Bp9 and RB1Bp24 (Fig. 5). At high virus input (approximately 2,500 PFU per chicken) 1 week postinfection, RB1BΔ4.5*lac* was reisolated from both spleen cells and PBLs at a frequency comparable to that of its parent virus (Fig. 5B); however, by two weeks postinfection, the level of reisolated RB1BΔ4.5*lac* had not increased significantly and the level of reisolated RB1Bp9 and RB1Bp24 had increased nearly fivefold (Fig. 5B). Despite the lower levels of virus reisolation for the RB1BΔ4.5*lac*-infected chickens, a notable progression of infection was seen in both spleen cells and PBLs (Fig. 5). Virus accumulated in the lymphoid organs and peripheral blood 1 to 2 weeks postinfection and then generally decreased 2 to 3 weeks postinfection; this was due perhaps to the influence of host factors or the death of infected lymphocytes. At 4 weeks postinfection, the levels of all three viruses increased at the high dose (Fig. 5B) but only the parent viruses showed this marked increase at the low dose (Fig. 5A). This increase probably stemmed from the proliferation of latently infected and transformed T cells, since some of the chickens taken for virus reisolations at 4 weeks postinoculation had grossly visible tumors. Also, by this time there was a notable onset of mortality in the chickens infected with the parent viruses (Fig. 6).

(ii) Mortality. In both in vivo experiments and at all virus doses tested, both RB1Bp9 and RB1Bp24 caused 80 to 90% mortality in inoculated chickens by 9 weeks postinfection (Fig. 6). RB1BΔ4.5*lac*-induced mortality, however, showed a dose dependence. In both experiments, RB1BΔ4.5*lac*-induced mortality was less than that of either RB1Bp9 or RB1Bp24, and only at a dose greater than 2,500 PFU per chicken did the mortality of RB1BΔ4.5*lac*-infected chickens reach 80% (Fig. 6D). Moreover, the onset of mortality for RB1BΔ4.5*lac*-infected chickens was delayed by at least 1 week.

Although MDV-induced mortality for RB1Bp9- and RB1Bp24-infected chickens was consistently high, the level of

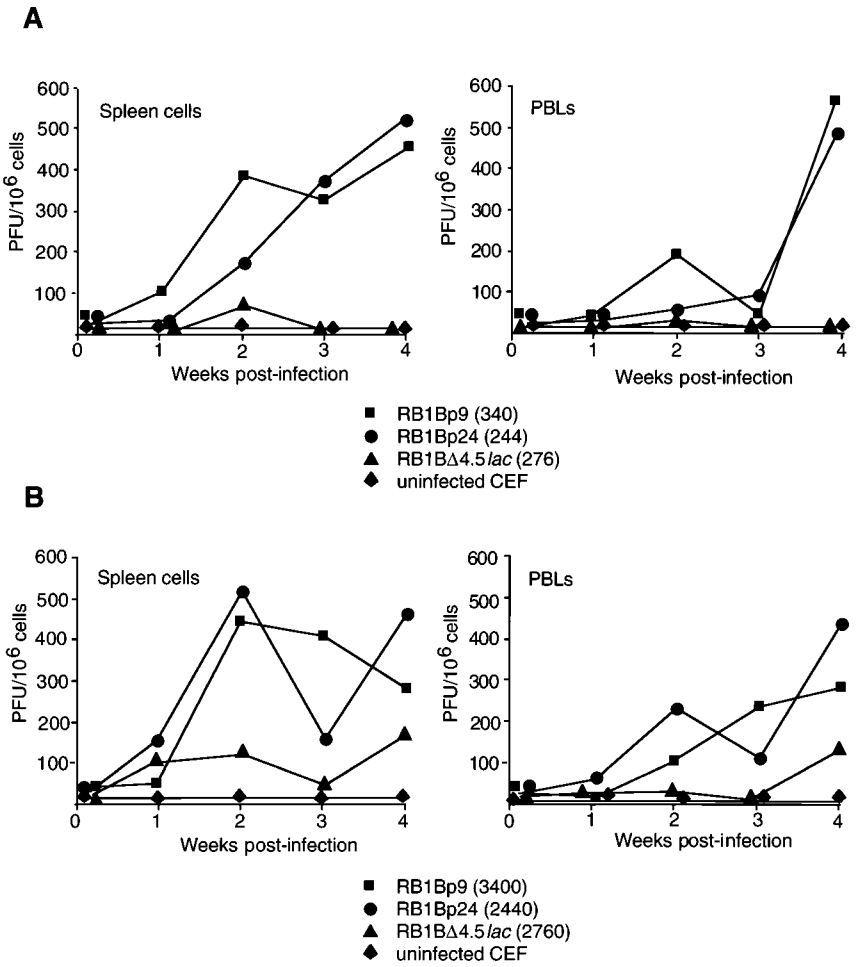


FIG. 5. RB1BΔ4.5*lac* infection of chickens, experiment 2. Shown are virus reisolation results for chickens inoculated with different doses of uninfected or RB1Bp9-, RB1Bp24-, or RB1BΔ4.5*lac*-infected CEF. Virus doses in PFU per chicken are given in parentheses. Each point is the mean number of plaques obtained per 10^6 cells plated. Plaques were counted by IFA.

mortality among contact-infected chickens from these groups was lower (Fig. 7). At low virus doses (<100 PFU per chicken), no mortality was observed for any of the virus-exposed groups (Fig. 7A) despite high mortality among the RB1Bp9 and RB1Bp24 inoculates (Fig. 6A). This may have been due to the shortened period of observation for the contact-exposed chickens (7 weeks), since many of these chickens had tumors (Fig. 8A). For RB1Bp9-exposed contacts placed with chickens inoculated with greater than 200 PFU per chicken, the mortality reflected that of the inoculates, reaching approximately 50% by 7 weeks postexposure (Fig. 6B to D and 7B to D). RB1Bp24 showed a consistently lower level of mortality in the contact-exposed chickens (Fig. 7B to D) compared with RB1Bp9, suggesting that RB1Bp24 had sustained some attenuation during passage in cell culture. In these experiments, RB1BΔ4.5*lac* induced no mortality in contact-exposed chickens (Fig. 7). RB1BΔ4.5*lac* could not be recovered from contact-exposed chickens despite several virus reisolation attempts (data not shown).

(iii) Tumor incidence. The incidence of MDV tumors is characteristically high among chickens infected with RB1B (38), which has made it a challenge strain of choice for the evaluation of MDV vaccine efficacy. Like the observed incidence of mortality (Fig. 6), the incidence of gross lymphomas was high (85 to 100%) among chickens inoculated with RB1Bp9 and RB1Bp24 (Fig. 8). The tumor incidence among chickens placed in contact with RB1Bp9-infected birds was also high (80 to 100%), whereas the tumor incidence among chickens in contact with RB1Bp24 was generally lower and more variable (30 to 85%) (Fig. 8). The tumor incidence in RB1BΔ4.5*lac* inoculates was generally lower than that in RB1Bp9 and RB1Bp24 inoculates. In addition, the tumor incidence in RB1BΔ4.5*lac* inoculates was proportional to the dose of virus inoculated. In these experiments, no gross tumors were seen in chickens placed in contact with RB1BΔ4.5*lac*-inoculated chickens. No tumors were observed in chickens exposed to uninfected CEF (data not shown).

Reactivation of MDV from RB1BΔ4.5*lac*- and RB1Bp9-derived lymphoblastoid cell lines. Cell lines were established from RB1BΔ4.5*lac*-induced (UD01, UD02, and UD03) and RB1Bp9-induced (UD14) tumors, and virus could be spontaneously recovered from these cell lines by cocultivation with CEF monolayers (Fig. 9). MDVs reactivated from UD01, UD02, and UD03 expressed β-galactosidase but not the MDV US1 protein (Fig. 9 and data not shown). MDV reactivated from UD14, however, did not express β-galactosidase but had high levels of the MDV US1 protein (Fig. 9). Southern blot analysis indicated that DNAs purified from CEF infected with

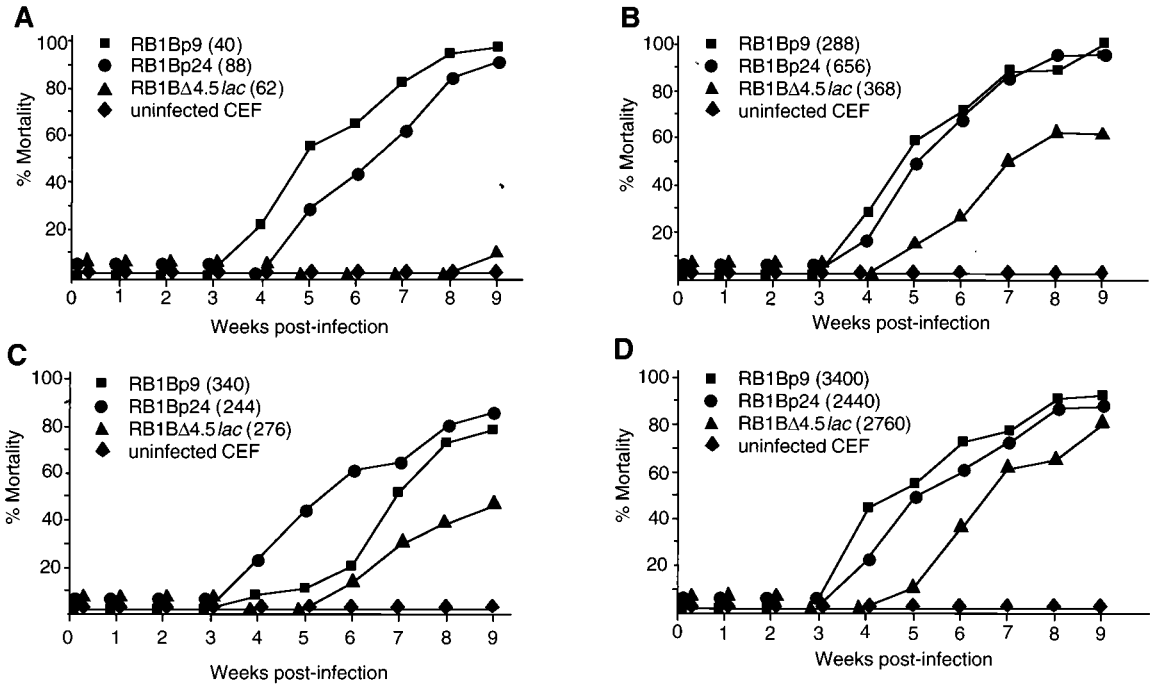


FIG. 6. MDV-induced mortality from in vivo experiments with MDV-inoculated chickens. Shown are the onset and incidence of mortality of the inoculates from experiments 1 (A and B) and 2 (C and D). Virus doses in PFU per chicken are given (in parentheses) for each set of curves. In experiment 1, the total number of inoculates was 30 chickens. In experiment 2, the number of chickens inoculated with uninfected CEF was 25 and the number of chickens inoculated with virus-infected CEF was 35.

virus reactivated from UD01, the initial RB1BΔ4.5*lac* stock, or RB1BΔ4.5*lac* reisolated from chickens were indistinguishable (data not shown).

DISCUSSION

Reports of MDV mutants are accumulating. MDV mutants that have *lacZ* insertions into homologs of the US2 (8), US1 (14, 25, 29), US10 (14, 25, 29), US3 (36), and US6 (gD) (28) genes within the US region of herpes simplex virus type 1 have been described. In addition, an MDV mutant having a large deletion that removes US1, US2, US10 and the neighboring MDV-specific open reading frames has been reported (29). A number of mutant viruses that have sustained retroviral insertions resulting in the stable integration of long terminal repeats into the MDV genome have been reported (16, 17, 19). These insertions have been clustered at the US-TR_S and US-IR_S junctions, at the UL-TR_L and UL-IR_L junctions, and within the US6 (gD) coding region. Herpesvirus of turkeys insertion mutants, including mutants with disruptions in the thymidine kinase gene (33) or, the US10 homolog gene (24, 26, 43) and a mutant with the *gpt* gene inserted in the L-S junction region (22), have been reported. In addition, serotype 2 MDV mutants with pUC18 inserted into IR_L (42) or with the *gpt* gene inserted into IR_L (22) have been reported. These previously reported mutants, with the exception of some of the retrovirus insertion mutants, have been constructed by using attenuated or nononcogenic MDV strains or herpesvirus of turkeys as the parent strain. Most of the studies have led to conclusions that the particular genes disrupted are not required for replication of the virus in cell culture. In general, the usefulness of these mutants has been limited with regard to studying virus-encoded determinants of oncogenicity, since the parent viruses used cannot cause tumors.

We now report a deletion mutant, RB1BΔ4.5*lac*, constructed in the genetic background of the highly oncogenic strain RB1B. RB1BΔ4.5*lac* contains a deletion identical to that of GAΔ4.8*lac*, spanning genes US1, US10, and US2 as well as MDV-specific open reading frames 1, 2 and 3. Southern blot analysis of DNA purified from CEF infected with RB1BΔ4.5*lac*, RB1BΔ4.5*lac* reisolated from chickens, and RB1BΔ4.5*lac* reactivated from a tumor-derived cell line (UD01) all indicated that the deletion mutant was structurally stable and that the virus stock was free of the parent virus. We attribute our ability to construct RB1BΔ4.5*lac* to three factors. First, we were able to purify RB1Bp9 DNA that had a relatively high transfection efficiency, namely, 200 to 250 plaques per 10 μg of DNA. Second, we found that infection of CEF with oncogenic MDVs can be readily detected by IFA despite the presence of few or no visible cytopathic effects. Third, we reasoned that since plaques induced by the transfected RB1B are small and since homologous recombination between the plasmid and the viral genome is uncommon, additional passage in culture prior to screening might amplify the number of recombinants.

Deletion of 4.5 kbp from the US region of the RB1B genome was accompanied by a loss of transcripts expressed from this region. The transcripts identified in RB1Bp24- and RB1BΔ4.5*lac*-infected CEF appeared identical in size and location to those recently reported for GAatt85 and GAΔ4.8*lac*, respectively (29). These findings, coupled with the similarity of restriction enzyme recognition sites and the high level of sequence identity for the US regions of strains RB1B and GA (5, 32, 34, 35), suggested that much of the US region of these strains is essentially identical.

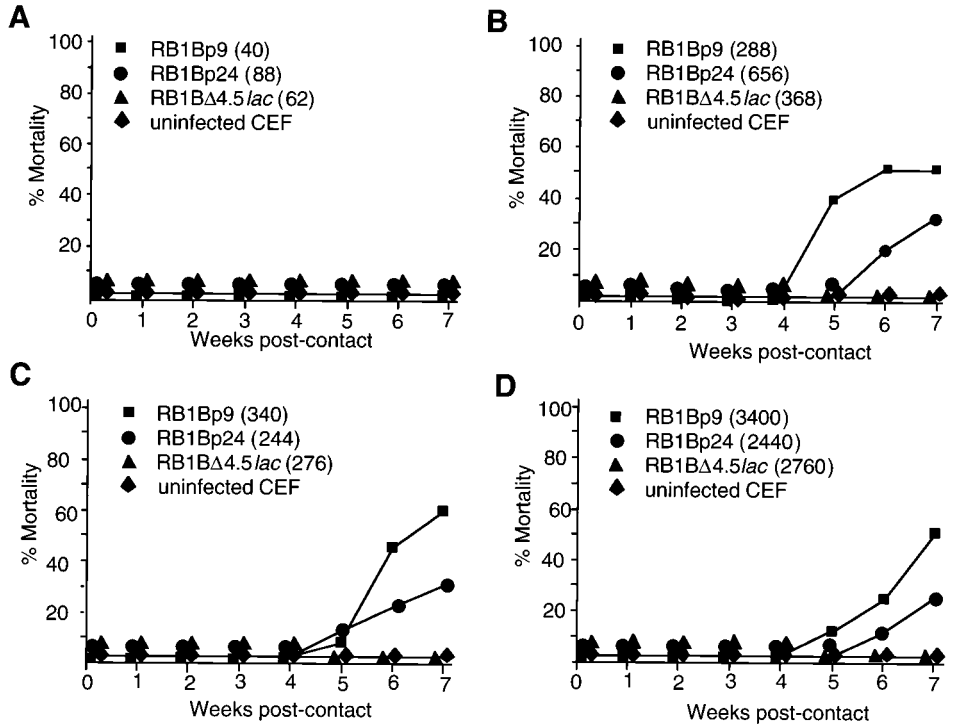
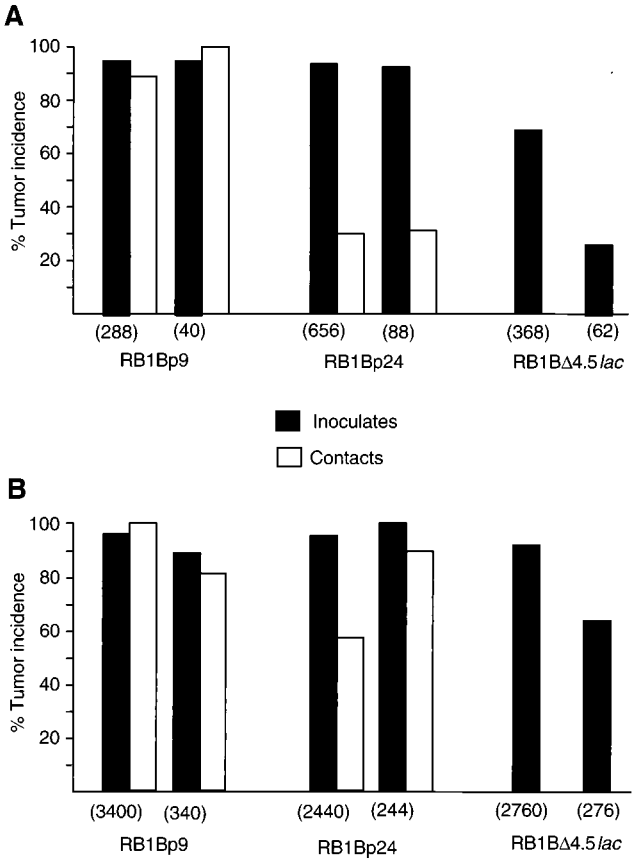


FIG. 7. MDV-induced mortality from in vivo experiments with contact-infected chickens. Shown are the onset and incidence of mortality of the contact chickens from experiments 1 (A and B) and 2 (C and D). In each panel, the doses (in PFU per chicken) of the inoculates occupying the cages in which the contacts were placed are given in parentheses.



RB1BΔ4.5*lac* showed a consistent growth impairment in chickens at the doses examined (62 to 2,760 PFU per chicken). Over a 4-week period following infection, RB1BΔ4.5*lac* never accumulated to the same levels of infection established by RB1Bp9 and RB1Bp24. Likewise, mortality associated with RB1BΔ4.5*lac* infection was consistently lower than that resulting from infection with RB1Bp9 or RB1Bp24. These results are consistent with the known phenotypes of independently isolated MDV mutants, namely, US1*lac* and GAΔ4.8*lac*, that lack a functional US1 gene (29). These mutants showed a growth impairment in nondividing CEF, decreased plaque forming efficiency on established versus freshly plated CEF monolayers, small plaque size, and low PFU/plaque ratios.

Despite the apparent growth impairment in chickens, RB1BΔ4.5*lac*-induced tumors in inoculated chickens. Virus reisolated from these tumor-bearing animals was $LacZ^+$, and virus reactivated from lymphoblastoid cell lines established from RB1BΔ4.5*lac*-induced tumors was $LacZ^+$. These results argue strongly that deletion of US1, US2, US10 and the neighboring MDV-specific open reading frames from the US region of the RB1B genome does not affect the inherent oncogenicity of MDV.

Although virus reactivated from the lymphoblastoid cell lines established from RB1BΔ4.5*lac*-induced tumors was $LacZ^+$, 98 to 99% of the lymphoblastoid cells themselves were

FIG. 8. Incidence of gross tumors in MDV-inoculated and contact-infected chickens. Shown is the incidence of macroscopic tumors in experimentally-infected chickens in experiments 1 (A) and 2 (B). The virus dose for each pair of groups (in PFU per chicken) is given in parentheses. Black bars indicate inoculates, and white bars indicate chickens placed in contact with inoculates. Chickens were observed daily for signs of Marek's disease. Chickens remaining at 9 weeks postinoculation were euthanized and necropsied. No lesions were found in chickens inoculated with uninfected CEF.

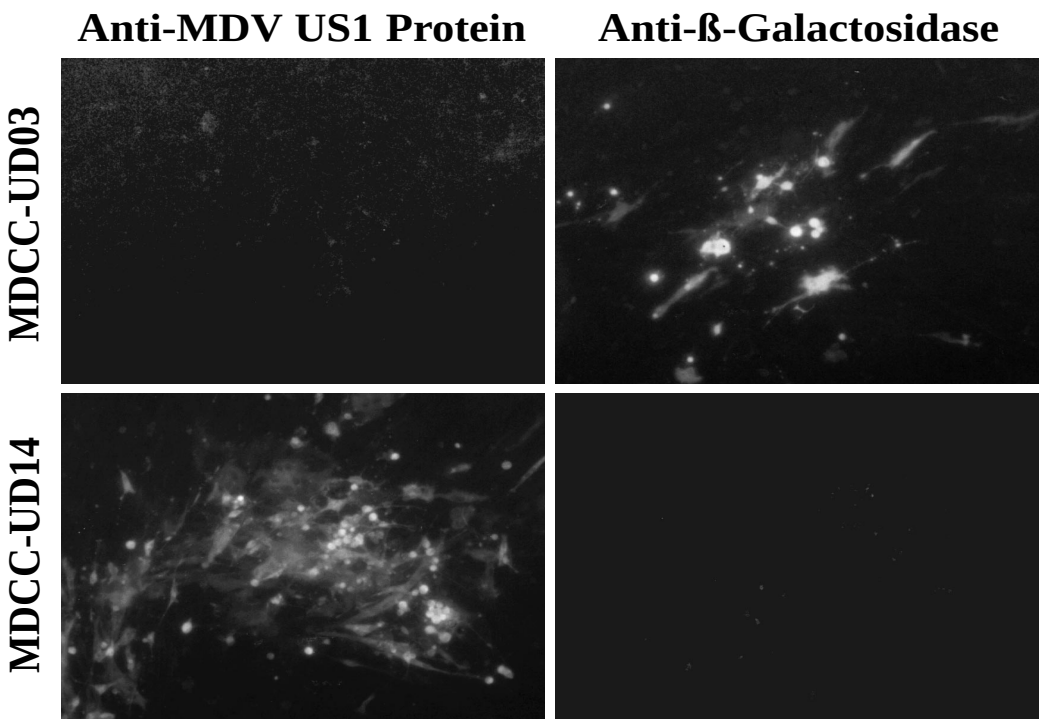


FIG. 9. Immunofluorescence analysis of MDVs reactivated from lymphoblastoid cell lines. CEF monolayers were cocultivated with 10^6 MDCC-UD03 (RB1BΔ4.5*lac*-derived) or MDCC-UD14 (RB1Bp9-derived) cells. The infected monolayers were fixed with EtOH 7 days postcocultivation and stained. The antibodies used were rabbit polyclonal antiserum to the MDV US1-encoded protein (provided by Peter Brunovskis, Case Western Reserve University, and Leland Velicer, Michigan State University) (left panels) and rabbit polyclonal antibodies to β-galactosidase (5-Prime-3-Prime) (right panels).

$LacZ^-$. Treatment of UD01 cells with 5-iododeoxyuridine resulted in a marked increase in the percentage of cells expressing both β-galactosidase and MDV antigens (data not shown). RB1BΔ4.5*lac*-induced lymphomas were largely $LacZ^-$ when stained with Bluo-gal (LT) but contained occasional foci that stained blue (data not shown). These results suggest that in these transformed cells, the *lacZ* gene is down-regulated, as are many MDV genes. During lytic infection, *lacZ* expression appears to be constitutive. The down-regulation of *lacZ* expression in RB1BΔ4.5*lac*-induced tumors and lymphoblastoid cell lines derived from them is particularly interesting, since the *lacZ* gene is expressed from the simian virus 40 early promoter in these strains, a promoter that is constitutive in most cell types. Further examination of the regulation of the *lacZ* gene in RB1BΔ4.5*lac*-derived cell lines may provide insight into changes in gene regulation that accompany latency and transformation.

Horizontal transmission of RB1BΔ4.5*lac* was not observed in these experiments. Several possibilities may account for the loss of horizontal spread of RB1BΔ4.5*lac*. First, RB1BΔ4.5*lac* may be unable to reactivate normally from infected lymphocytes and establish secondary cytolytic infection at peripheral sites. Second, it may be incapable of efficiently infecting feather follicle epithelium. Third, it may be unable to infect a cell type essential for entry of the virus into contact-exposed chickens. Fourth, the general level of infection established by RB1BΔ4.5*lac* may be too low to drive horizontal transmission of the virus.

The in vivo studies with RB1BΔ4.5*lac* and RB1Bp24 indicate that RB1B retains oncogenicity and much of its virulence up to passage level 24. The impairment in horizontal transmission observed for RB1Bp24 and the apparent loss of horizontal transmission for RB1BΔ4.5*lac* suggest that cell culture passage reduced horizontal transmissibility earlier and/or more severely than it affected oncogenicity. Previous studies of the attenuation of MDV strains have not resulted in the dissociation of tumorigenicity and horizontal transmission. We have examined the 132-bp repeat regions of the RB1Bp9, RB1Bp24, and RB1BΔ4.5*lac* genomes. This repeated region is amplified during attenuation and has been correlated with a loss of oncogenicity (13, 21, 41). RB1Bp9, RB1Bp24, and RB1BΔ4.5*lac*, as well as these strains reisolated from chickens and RB1Bp9 and RB1BΔ4.5*lac* reactivated from lymphoblastoid cell lines, had predominantly two copies of the 132-bp repeat as determined by PCR analysis (18a). However, an independently obtained derivative of RB1B at passage level 21 (RB1Bp21) showed an increased number of 132-bp repeats (18b). The factors that cause amplification of the 132-bp repeat region are unknown. Our results indicate that amplification can occur relatively quickly during cell culture passage but does not necessarily do so. Nevertheless, the possibility of amplification has important implications for mutant analysis, and careful attention must be paid to unplanned events that can occur during construction and passage of strains. As long as mutants retain oncogenicity in animals, results will be interpretable. However, if mutants lose oncogenicity in animals, the generation of rescuants and/or of multiple independently isolated mutants with different defects but similar phenotypes will be critical.

The construction of RB1BΔ4.5*lac* has important implications for the functional analysis of MDV genes, in that it allows gene function to be assessed in a context relevant to the pathology induced by MDV. The development of methods for generating rescuants of these RB1B-based mutants remains a high priority. Nevertheless, the use of MDV RB1B-based mutants coupled with the excellent in vivo system for analyzing mutant phenotypes in the natural host promises to provide

insight into herpesvirus latency and oncology in general and into the particulars of how herpesviruses interact with cells of lymphoid lineage.

ACKNOWLEDGMENTS

We thank Peter Brunovskis and Leland Velicer for sharing antisera and for helpful conversations and Marie Meyer for technical assistance with the in vivo experiments.

This work was supported by the Delaware Research Partnership; Intervet International, Boxmeer, The Netherlands; and American Cancer Society grant VM-44.

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