

Further Analysis of Marek's Disease Virus Horizontal Transmission Confirms That U_L44 (gC) and U_L13 Protein Kinase Activity Are Essential, while U_S2 Is Nonessential[∇]

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Marek's disease virus (MDV) causes a devastating disease in chickens characterized by the development of lymphoblastoid tumors in multiple organs and is transmitted from the skin of infected chickens. We have previously reported that the U_S2, U_L44 (glycoprotein C [gC]), and U_L13 genes are essential for horizontal transmission of MDV in gain-of-function studies using an a priori spread-deficient virus that was based on an infectious clone from the highly virulent RB-1B virus (pRB-1B). To precisely determine the importance of each individual gene in the process of chicken-to-chicken transmission, we used the transmission-restored clone that readily transmits horizontally and mutated each individual gene in loss-of-function experiments. Two independent U_S2-negative mutants transmitted horizontally, eliminating U_S2 as being essential for the process. In contrast, the absence of gC expression or mutating the invariant lysine essential for U_L13 kinase activity abolished horizontal spread of MDV between chickens.

Marek's disease (MD) is caused by the oncogenic alphaherpesvirus *Gallid herpesvirus 2* (GaHV-2), better known as MDV. The most prominent sign of MD is the development of lymphoproliferative disease in chickens characterized by solid tumors in the viscera and other organs (3, 19). Natural infection begins through inhalation of virus, after which MDV is taken to the lymphoid organs and primary cytolytic infection in B and then T lymphocytes ensues. Following lytic infection, latency is established mainly in activated CD4⁺ T cells, which may be transformed with differing efficiencies, depending on the genotype of the infected chicken, and result in lymphoma formation. Irrespective of the transformation event, infection of feather follicle epithelial cells in the skin by migrating lymphocytes leads to the production of infectious particles that are shed into the environment, providing a continuous source of infectious virus. While the majority of the work on MDV has been focused on the transformation and reactivation of MDV during infection, little is known about horizontal transmission of virus from one chicken to another.

We recently identified genes important for horizontal transmission of MDV. We originally used a transmission-deficient virus derived from a bacterial artificial chromosome (BAC) clone of the very virulent RB-1B strain (pRB-1B-5) (35). Following sequencing of the complete BAC (40), specific genes suspected to be important for transmission were identified. We were able to restore horizontal transmission by repair of specific genes (17). We concluded that a combination of three genes, the unique short (U_S) 2, unique long (U_L) 44 or glycoprotein (g) C, and U_L13 protein kinase genes, was essential for horizontal transmission. Repair of each gene individually did

not restore spread, nor did various combinations of two genes. In this report, we further defined which genes are essential by using loss-of-function studies utilizing mutant viruses in which U_S2, U_L13, or gC was inactivated in the transmission-competent virus (17). Mutant viruses were engineered using an infectious clone and markerless Red recombination exactly as previously described (17) using primers shown in Table 1. Following confirmation of the correct modifications by restriction fragment length polymorphism (RFLP), PCR, and sequencing analyses, mutant viruses lacking the mini-F BAC sequences after *Cre*-Lox excision were reconstituted in chicken embryo cell cultures and propagated in chicken kidney cell cultures as previously described (17). Groups of P2a chickens (*n* = 10), which are highly susceptible to the development of MD (5), were experimentally infected with 1,000 PFU of the mutant viruses intra-abdominally and placed in glove box isolators with 10 age-matched, uninfected contact chickens. All experimental procedures were conducted in compliance with approved Institutional Animal Care and Use Committee (IACUC) protocols (Cornell University protocol numbers 2002-0085 and 2008-0018).

The U_S2 protein is located in the virion tegument (4) and is conserved in alphaherpesviruses. The MDV U_S2 ortholog has been shown to be nonessential for MDV replication *in vitro* and *in vivo* (33), and our earlier study (17) led to the first report suggesting U_S2 may have a functional role in MDV pathogenesis (i.e., horizontal transmission). In order to conclusively determine the role of U_S2 in this process, we generated two mutant viruses in which either the entire U_S2 open reading frame (ORF) was deleted (Δ U_S2) or the start codon of U_S2 was mutated to a stop codon (US2M1stop) in the transmission-competent virus (Fig. 1A). RFLP analysis showed that no spurious mutations were evident, apart from the expected deletion of U_S2 in the Δ U_S2 clone (Fig. 1B). The average plaque area of each virus was determined exactly as previously

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TABLE 1. Primers used for mutating Marek's disease virus genes in transmission-competent pRB-1B

Mutant ^a	Direction ^b	Sequence ^c
Δ US2	Forward	CAGTTATTAACAATAAAAAAGATTATTGGTGGAGGTGAAGTAGAATTCAGATCTGCTAGA TAGGGATAACAGGGTAATCGATT
	Reverse	GCATACATTATACGAAGTTATCTAGCAGATCTGAATTCTACTTCACCTCCACCAATAATCG CCAGTGTACAACCAATTAACC
US2M1 stop	Forward	CCCAGTTATTAACAATAAAAAAGATTATTGGTGGAGGTGAAGT <i>AA</i> GGTGTGTCCATGATA ACTATTAGGGATAACAGGGTAATCGATT
	Reverse	ATCGCATTCTACTAGAAGTGTGACTATAGTTATCATGGACACACCT <i>T</i> ACTTCACCTCCACC AATAGCCAGTGTACAACCAATTAACC
UL13 K170M	Forward	CGGAGTAGTTAAAATATTTAAGAAGACGGACATAGCCGTC <i>ATGA</i> AGTATTGGAATGTTTT AATAGGGATAACAGGGTAATCGAT
	Reverse	ATGTCATAAGTAACTCAGTTTTAAAACATTCCAATACTTT <i>CA</i> TGACGGCTATGTCCGTCTTC TGCCAGTGTACAACCAATTAACC
UL13 M170K	Forward	CGGAGTAGTTAAAATATTTAAGAAGACGGACATAGCCGTC <i>AAAA</i> AGTATTGGAATGTTTT TAATAGGGATAACAGGGTAATCGAT
	Reverse	ATGTCATAAGTAACTCAGTTTTAAAACATTCCAATACTTT <i>TTT</i> GACGGCTATGTCCGTCTTC TGCCAGTGTACAACCAATTAACC
UL13 K270A	Forward	TTCTAAACGTGCTTGTGGTTGACTCATTGGATATCG <i>C</i> ATGTGGGAATATCTTTG <i>CCA</i> GTGTACAACCAATTAACC
	Reverse	AGGACCCTCGGTGACGTTAACAAAGATATCCACAT <i>TGCG</i> GATATCCAAATGAGTCATAGG GATAACAGGGTAATCGATT
gCM1 stop	Forward	CCAAACGTAACCCTACATATCTCCCT <i>CTAG</i> CTCACGCCGCGTGTTTACGAGCTTTG <i>TA</i> GGGATAACAGGGTAATCGATT
	Reverse	AAAAAGAGTCCAGTCCACCCCAAAGCTCGTAAACACGCGGGCGTGAG <i>CTAG</i> GAGGAAGA TGCCAGTGTACAACCAATTAACC
gCstopM1	Forward	CCAAACGTAACCCTACATATCTCCCT <i>CTAG</i> CTCACGCCGCGTGTGTACGAGCTTTG <i>TA</i> GGGATAACAGGGTAATCGATT
	Reverse	AAAAAGAGTCCAGTCCACCCCAAAGCTCGTAAACACGCGGGCGTGAG <i>CTAG</i> GAGGAAGA TGCCAGTGTACAACCAATTAACC

^a Gene mutation.^b Directionality of the primer.^c Underlined sequence indicates the template binding region of the primers for PCR amplification with pEPKanS. Bold and italicized letters indicate the mutated sequences introduced into the genome.

described (18) and found to be similar for both mutant and parental viruses (Fig. 1C). Figure 1D shows MD incidence in chickens experimentally infected with each respective virus (black lines) and contact chickens (gray lines) exposed to the experimentally infected chickens. Over the course of 13 weeks, both mutant viruses and the parental virus induced MD incidences of 100%. Both Δ US2 and US2M1stop were also able to spread from chicken to chicken, with the Δ US2 virus being slightly slower in causing MD in contact chickens. By 13 weeks postinfection (p.i.), only 40% of contact chickens developed MD in this group. One chicken showed no clinical signs or gross lesions at termination, but it had MDV viremia, which was determined by previously described qPCR assays (17). From these results, we concluded that U_{S2} is nonessential for horizontal transmission, contrary to what we had originally reported (17). This is in agreement with results using another infectious MDV clone in which the U_{S2} gene was removed during BAC construction and reconstituted virus was able to horizontally spread (30).

Another gene identified to be important for horizontal transmission was the U_{L13} serine/threonine protein kinase (17). The U_{L13} gene is highly conserved, not only among the alphaherpesviruses but in all members of the *Herpesviridae*. In the case of herpes simplex virus 1 (HSV-1), U_{L13} was shown to be present in the tegument of enveloped virus and has been shown to autophosphorylate and phosphorylate a large number of viral and host proteins (6, 20, 21, 29, 31, 36). Morrison et al. (28) showed that U_{L13} protein kinase activity promotes dissociation of tegument by phosphorylation of tegument pro-

teins, while Moffat and coworkers (26) showed that the varicella zoster virus (VZV) ortholog of U_{L13} (ORF47) is required for efficient infection of T lymphocytes and skin in the SCID-hu mouse model. The MDV U_{L13} amino acid sequence contains the 11 (I to XI) conserved catalytic domains found in all protein kinases (11, 37), and the protein is catalytically active (37). The transmission-deficient MDV previously analyzed contained a frameshift mutation within the U_{L13} ORF leading to a truncated protein that encoded only the first two conserved domains (I and II) (2, 17). We hypothesized that this truncated protein lacked kinase activity and that U_{L13} kinase activity was important for transmission of MDV. The invariant lysine in the catalytic domain, positioned at amino acid 170 in the MDV U_{L13} protein, was shown to be essential for kinase activity in other U_{L13} orthologs (7, 10, 12, 20, 22, 34, 41). Therefore, we mutated lysine 170 of MDV U_{L13} domain II to a methionine (UL13K170M) and generated a revertant virus of this mutant by replacing the methionine in the original sequence (UL13M170K) (Fig. 2A). As an additional control, we mutated the lysine at position 270 in domain III (UL13K270A). RFLP analysis showed that there were no discernible differences between the parental, mutant, and revertant clones (Fig. 2B). Also, there were no significant differences in average plaque areas for each virus derived from the cloned DNA (Fig. 2C). Figure 2D shows that both mutant viruses caused 100% MD in experimentally infected chickens by 13 weeks p.i. However, when transmission from chicken to chicken was evaluated, the U_{L13} kinase mutant (UL13K170M)

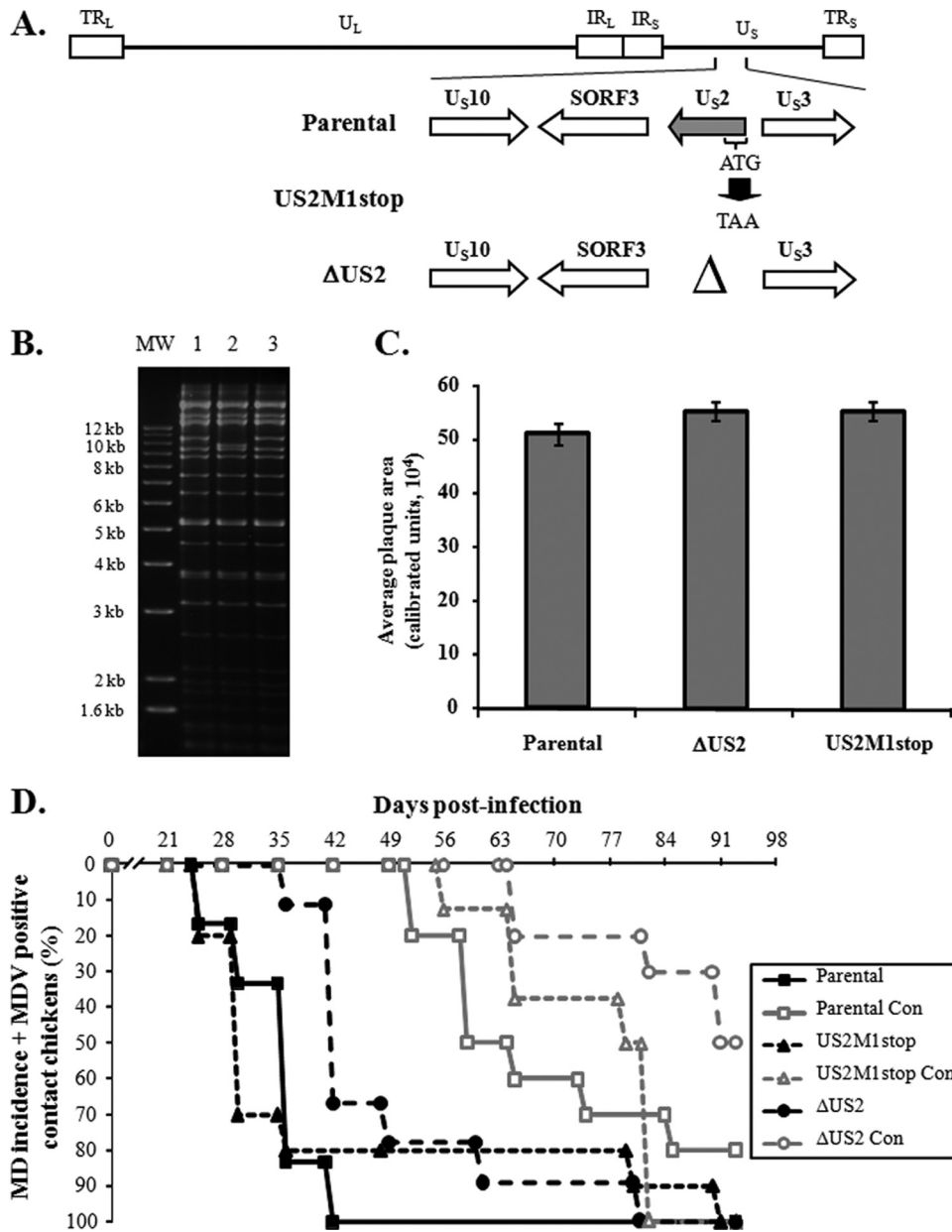


FIG. 1. Generation of U_S2 mutant MDVs and evaluation of their ability to induce MD and horizontally transmit to contact chickens. (A) Two U_S2 mutant viruses were generated, one in which the complete U_S2 ORF was deleted ($\Delta US2$) and another where the ATG start codon was mutated to a TAA stop codon ($US2M1stop$). Also shown are genes flanking the U_S2 ORF in the U_S region of the MDV genome. (B) RFLP analysis of DNA obtained from parental virus (lane 1) and $\Delta US2$ (lane 2) and $US2M1stop$ (lane 3) BAC clones using BamHI restriction patterns. Deletion of U_S2 reduces the size of the 10,354-bp fragment of the parental virus (lane 1) to 9,544 bp (lane 2). No extraneous alterations are evident in both clones. The molecular size marker (MW) used is the 1-kb Plus DNA ladder from Invitrogen, Inc. (Carlsbad, CA). (C) The average plaque area \pm standard error of the mean (SEM) for each respective virus was determined from 75 plaques exactly as previously described (18). No significant differences were seen between viruses using Student's *t* tests. (D) MD incidence of P2a chickens inoculated at 1 day of age with reconstituted BAC clones described in the text and contact (Con) chickens housed with experimentally infected chickens over the course of 13 weeks of infection. MD incidence was determined by identification of gross lesions in dead or euthanized chickens. Chickens not succumbing to MD over the course of the experiment were terminated at 92 days p.i. Blood was collected from all remaining birds and tested for MDV genomic copies using qPCR exactly as previously described (17). For determination of horizontal transmission, contact chickens positive for MDV genomic copies in the blood were included, since the presence of MDV genomes indicated spread.

did not spread to contact chickens. In contrast, both the revertant of UL13K170M (UL13M170K) and the UL13K270A viruses were able to horizontally transmit to contact chickens with efficiencies and kinetics similar to those of the parental virus. A second experiment was conducted to confirm the lack

of spread with the U_L13 kinase mutant, and, again, no transmission to contact chickens was observed (data not shown). The data strongly suggest that MDV U_L13 protein kinase activity is essential for horizontal transmission of MDV. It has been previously shown that U_L13 protein kinase activity pro-

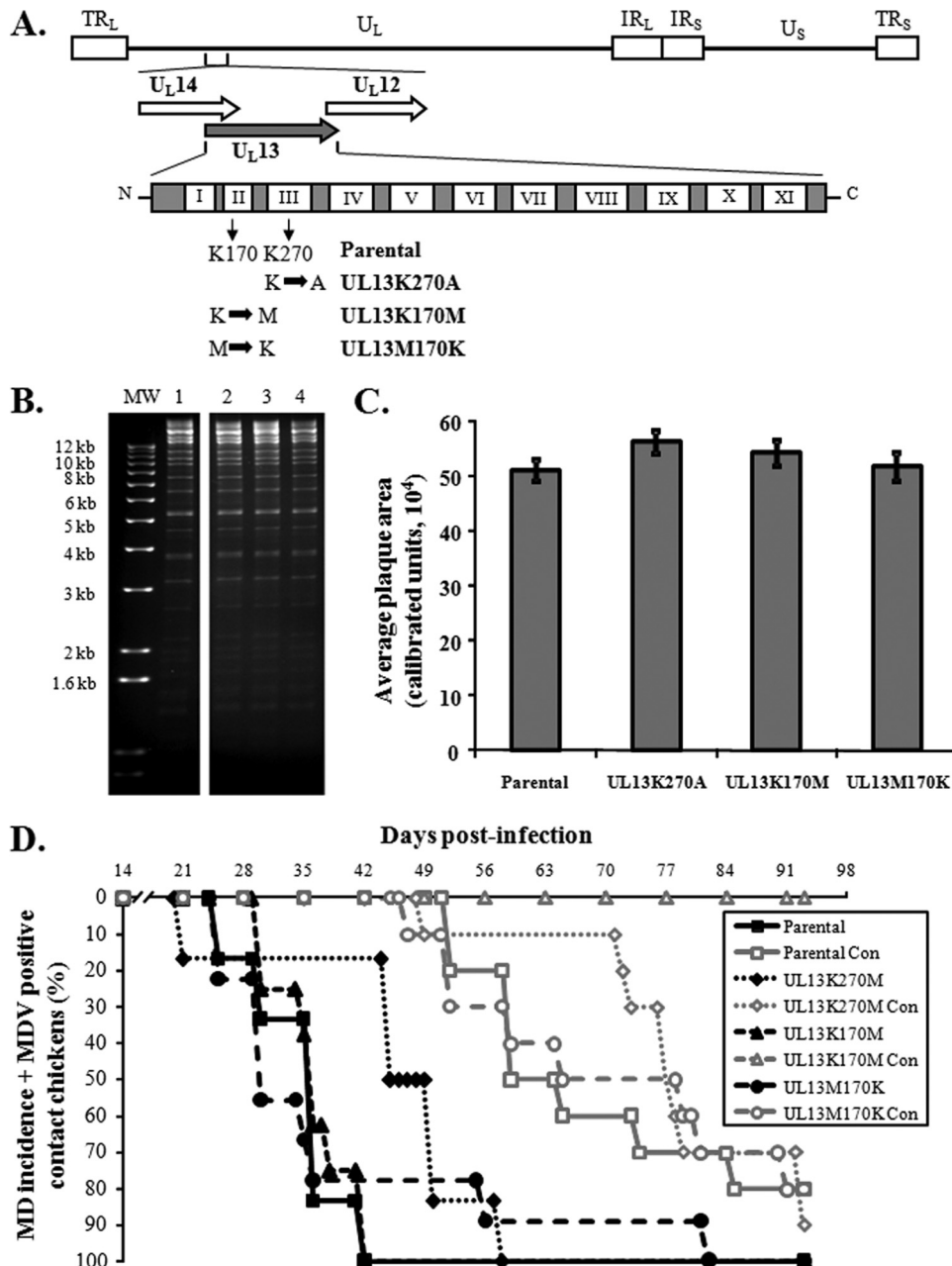


FIG. 2. Generation of UL13 mutant MDVs and evaluation of their ability to induce MD and horizontally transmit to contact chickens. (A) Schematic diagram showing the UL13 ORF flanked by overlapping UL14 and UL12 ORFs and reversed in the figure for simplicity. Also shown are the 11 (I to XI) kinase domains contained within the conserved UL13 protein. Two UL13 mutants were generated from the transmission-competent BAC clone (parental) as described in the text. (B) RFLP analysis of DNA obtained from parental virus (lane 1) and UL13K270M (lane 2), UL13K170M (lane 3), and UL13M170K (lane 4) BAC clones using BamHI restriction patterns. No extraneous alterations are evident in all clones. The molecular size marker (MW) used is the 1-kb Plus DNA ladder (Invitrogen). (C) Same as in Fig. 1C. No significant differences in plaque sizes were seen between each virus using Student's *t* tests. (D) Same as in Fig. 1D. All contact chickens housed with the UL13K170M (kinase mutant) were negative for MD lesions following necropsy and negative for MDV genomic copies in the blood using qPCR assays.

notes dissociation of tegument by phosphorylation of tegument proteins for HSV-1 (28). We hypothesize that MDV UL13 may perform a similar function during natural infection and therefore virus shed from the infected chickens that lack UL13 kinase activity are defective in cell entry. Studies are in progress to define at what point transmission from animal to animal is deficient.

Alphaherpesvirus gC orthologs have multiple functions. They play major roles in the primary attachment of cell-free virus to heparin- and chondroitin-like glycosaminoglycans on the surface of cells (25, 38), and involvement of gC in a late step of virus egress from cultured cells has been shown for the members of the *Varicellovirus* genus, pseudorabies virus (PRV-1) and equine herpesvirus 1 (EHV-1) (25, 32). Addi-

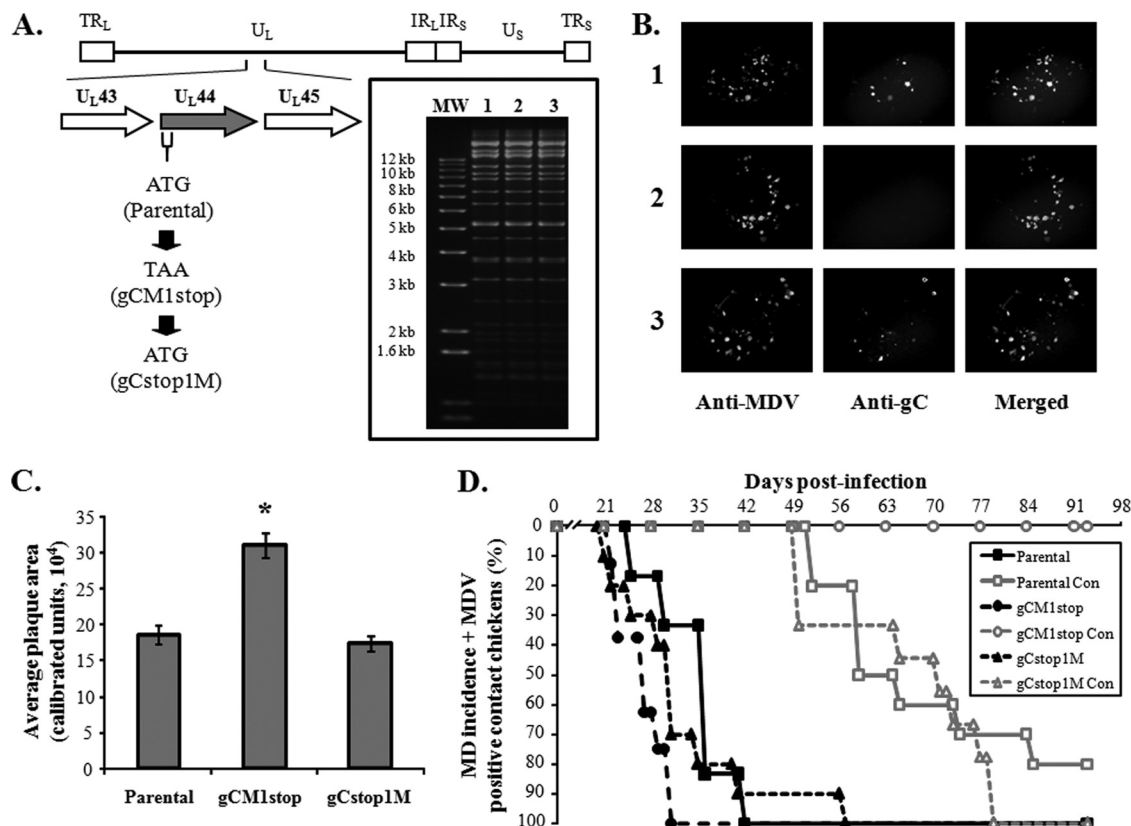


FIG. 3. Generation of U_L44 (gC) mutant MDV and evaluation of their ability to induce MD and horizontally transmit to contact chickens. (A) Schematic diagram showing the location of U_L44 (gC) in relation to U_L43 and U_L45 in the MDV genome. The start codon of gC was mutated to a stop codon (gCM1stop) in the transmission-competent BAC clone (parental). A revertant of the mutant was also produced in which the stop codon was replaced with a start codon (gCstop1M). Inset shows RFLP analysis of DNA obtained from parental virus (lane 1) and gCM1stop (lane 2) and gCstop1M (lane 3) BAC clones using BamHI restriction patterns. No extraneous alterations are evident in the clones. The molecular size marker (MW) used is the 1-kb Plus DNA ladder (Invitrogen). (B) IF assays of respective plaques for each virus using a polyclonal anti-MDV chicken antibody and an anti-gC monoclonal antibody with Alexa Fluor 568 and 488 secondary antibodies, respectively, as previously described (17, 18). Numbers are the same as in panel A. (C) Same as in Fig. 1C and Fig. 2C. The gC-null virus (gCM1stop) produced plaques approximately twice as large as parental and revertant viruses, and this was significantly different ($P < 0.001$) using Student's t tests and is indicated with an asterisk (*). (D) Same as in Fig. 1D and Fig. 2D. All contact chickens housed with the gCM1stop (gC-null) clone were negative for MD lesions following necropsy and negative for MDV genomic copies in the blood using qPCR assays.

tionally, the gC proteins of HSV-1, HSV-2, PRV-1, bovine herpes virus 1 (BHV-1), and EHV-1 are able to bind complement component C3 (1, 8, 9, 13, 14, 15). It had been suspected that gC was important for horizontal transmission of MDV, but formal proof was missing, as the constructed and tested gC mutant virus used had reduced ability to replicate in experimental chickens (27). Identification of a frameshift mutation within the U_L44 ORF in the original pRB-1B-5 BAC (35) and the subsequent repair of this mutation that restored horizontal transmission in combination with the repair of U_S2 and U_L13 strongly suggested that gC was essential for horizontal transmission (17). To definitively determine if gC is essential for animal-to-animal spread, we mutated the start codon of gC to a stop codon (gCM1stop) in the transmission-competent virus (Fig. 3A). We also generated a revertant of the gCM1stop clone in which the start codon was repaired (gCstop1M). RFLP analysis showed that there were no discernible differences between the parental, mutant, or revertant BAC clones (Fig. 3A, inset). While the gCM1stop virus plaques were negative for gC expression, both parental and revertant

(gCstop1M) viruses were reactive with the gC antibody (Fig. 3B) using an MDV gC-specific monoclonal antibody in immunofluorescence (IF) assays as previously described (18). Measurement of plaque areas of each virus showed that the gCM1stop virus lacking gC expression produced plaques approximately twice as large as the parental and revertant viruses (Fig. 3C), consistent with previous results (42). Each of the recombinant viruses induced MD in chickens infected by intra-abdominal inoculation with similar efficiencies and kinetics (Fig. 3D, black lines). However, the gCM1stop virus was unable to spread from infected to sentinel chickens, while the revertant (gCstop1M) spread efficiently (Fig. 3D, gray lines). In two follow-up experiments, the gC-null virus was repeatedly unable to spread, while its revertant transmitted like the parental virus (data not shown), clearly showing that functional gC is required for horizontal transmission of MDV. It is suspected that the functional role of MDV gC during natural infection involves binding complement. The MDV gC protein contains homologous regions (K. W. Jarosinski, unpublished observation) that have been shown to be important for com-

plement binding of other herpesvirus gC proteins (16, 39) and protecting virus from complement-mediated destruction (23, 24). Studies are in progress to address this possibility.

In conclusion, the studies presented here used loss-of-function analyses to definitely determine if expression of U_S2, gC, and U_L13 protein kinase activity individually were essential for horizontal transmission of MDV in chickens. We were able to conclusively show that U_S2 is not essential whereas both gC and U_L13 protein kinase activity, individually, are essential for horizontal transmission of MDV in chickens.

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