

A full UL13 open reading frame in Marek's disease virus (MDV) is dispensable for tumor formation and feather follicle tropism and cannot restore horizontal virus transmission of rRB-1B in vivo

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Abstract – Marek's disease virus (MDV) is an oncogenic alphaherpesvirus that is highly contagious in poultry. Recombinant RB-1B (rRB-1B) reconstituted from an infectious genome cloned as a bacterial artificial chromosome (BAC) is unable to spread horizontally, quite in contrast to parental RB-1B. This finding suggests the presence of one or several mutations in cloned relative to parental viral DNA. Sequence analyses of the pRB-1B bacmid identified a one-nucleotide insertion in the UL13 orthologous gene that causes a frame-shift mutation and thereby results in a theoretical truncated UL13 protein (176 aa vs. 513 aa in parental RB-1B). UL13 genes are conserved among alphaherpesviruses and encode protein kinases. Using two-step "en passant" mutagenesis, we restored the UL13 ORF in pRB-1B. After transfection of UL13-positive pRB-1B DNA (pRB-1B*UL13), the resulting, repaired virus did not exhibit a difference in cell-to cell spread (measured by plaque sizes) and in UL13 transcripts in culture compared to parental rRB-1B virus. Although 89% of the chickens inoculated with rRB-1B*UL13 virus developed tumors in visceral organs, none of the contact birds did. MDV antigens were clearly expressed in the feather tips of rRB-1B infected chickens, suggesting that the UL13 gene mutation did not alter virus tropism of the feather follicle. The results indicate that the correction in UL13 gene alone is not sufficient to restore in vivo spreading capabilities of the rRB-1B virus, and that other region(s) of pRB-1B might be involved in the loss-of-function phenotype. This finding also shows for the first time that a full UL13 ORF is dispensable for MDV tumor formation and feather follicle tropism.

Marek's disease virus / UL13 / pathogenesis / horizontal spread / chicken

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1. INTRODUCTION

Marek's disease virus (MDV) is a highly contagious avian herpesvirus. MDV is the etiological agent of Marek's disease in chickens, a multi-faceted disease, most widely recognized by the induction of a malignant T-cell lymphoma. The virus spreads very efficiently from infected to uninfected chickens by direct or indirect contact. It is believed that free infectious virus is shed from the feather follicle epithelium [1, 4]. In all other cell types – in cell culture, or in vivo – MDV is strongly cell-associated and does not release detectable quantities of free infectious virions. The molecular mechanism involved in the release of free virus from feather follicle cells remains unknown and to date no viral genes have been implicated in this biological property.

The MDV genome is approximately 185 kbp in size and encodes at least 103 proteins [32]. Several MDV genomes have been cloned as bacterial artificial chromosomes, including an avirulent strain (584Ap80C) (yielding the bacmid BAC20) and the highly virulent strain RB-1B (yielding the bacmid pRB-1B) [21, 26]. The viruses derived from these bacmids are able to replicate in cell culture. Recombinant virus from pRB-1B is highly oncogenic in chickens, but is unable to spread from infected to naïve chickens [10, 11]. Horizontal spread, thus far, is the only property lacking in rRB-1B to make it a full model to study MDV biology. Despite this shortcoming, pRB-1B and its derivatives are great tools to determine the molecular determinants involved in MDV horizontal dissemination in birds by individual, consequential or concomitant repair of genes that are found to be different from authentic, wild-type MDV [17, 32].

The UL13 gene is conserved in all members of the *Herpesviridae*. The MDV UL13 gene consists of a 1539-bp ORF encoding a

513 amino acids (aa) protein with an apparent molecular weight of 60 kDa [24]. MDV UL13 presents a high homology with the orthologs of HSV-1 and VZV which encode the unique-long protein kinases [24]. Analysis of the MDV UL13 aa sequence reveals that UL13 MDV is also a protein kinase [24].

The HSV-1 UL13 protein (pUL13) is a tegument protein. It can phosphorylate itself and at least five other viral proteins: VP22, ICP22, ICP0, the unique-short protein kinase pUS3, and gE [5, 6, 12, 19, 20, 22]. pUL13 can also phosphorylate cellular factors such as the eukaryotic elongation factor δ EF-1 [13]. The biological functions of pUL13 are still unclear. Studies on mutant viruses indicate that UL13 is dispensable for replication in cell culture [5]. The suppression of the UL13 kinase activity is associated to the formation of smaller plaques [30]. The examination of these HSV-1 UL13 mutants in mice has never been described. The VZV ORF47 protein (pUL13 ortholog) also autophosphorylates and phosphorylates the major immediate-early transactivator IE62 protein, IE63 protein, gE, as well as the ORF9 and ORF32 products [16, 23, 29]. ORF47, while not necessary in cultured cells, is required for skin and T-cell infection in SCID-hu mice [9, 18].

In this study, we report on the identification of a single mutation in the UL13 gene in the pRB-1B bacmid. We took advantage of this mutation to investigate the role of the UL13 gene in pathogenesis in vivo. We also investigate whether repairing this gene could restore the horizontal in vivo dissemination of pRB-1B-derived virus.

2. MATERIALS AND METHODS

2.1. Cells and bacmids

Chicken embryonic skin cells (CESC) were prepared and cultivated as previously described [7] from 12-day chicken embryos (LD1 Brown Leghorn chicken

strain) [25]. The pRB-1B bacmid, herein referred to as 'pRB-1B', consists of the genome of the highly virulent RB-1B strain cloned into a bacterial artificial chromosome (BAC). This bacmid was a kind gift from V. Nair (IAH Compton, UK) [21].

2.2. Antibodies to MDV antigens

The mouse monoclonal antibodies (Mabs) F19 (IgG1) and H18 (IgG1) against VP5, the major capsid protein, were used¹ [8]. K11 Mab (IgG1) against MDV-1 gB was obtained by immunizing a Balb/c mouse with baculovirus-expressed gB, and following a procedure described earlier [7]. The K11 Mab was found to react with both MDV and the serologically related herpesvirus of turkeys (HVT). MAb E21 (IgG1) directed against ICP4 was obtained using the same protocol.

2.3. Sequencing of nucleotide regions in pRB-1B

The entire UL13 and UL48 ORF of pRB-1B bacmid were sequenced as well as the regions corresponding to the cytoplasmic domain (CD) of gB, gD, gE and gM encoded by UL27, US6, US8 and UL10 respectively (MWG-biotech, Ebersberg, Germany). These sequences were compared to the ones of the Md5 complete genome (GenBank accession number AF243438) with MegAlign software version 3.08 from DNASTAR Inc. The nucleotide positions of the regions sequenced are the following ones: UL13 (36339 to 37880), UL48 (109813 to 111096), CD gB (61055 to 61405), CD gD (161002 to 161076), CD gE (163643 to 163882) and CD gM (34251 to 34490).

2.4. Generation of the pRB-1B*UL13 bacmid by "en passant" mutagenesis

The pRB-1B*UL13 bacmid, corresponding to the pRB-1B bacmid with a

deletion of a single T nucleotide in the UL13 gene, was generated by using "en passant" mutagenesis, exactly as previously described by Tischer et al. [31]. The UL13 gene from pRB-1B*UL13 was sequenced in its entirety on both strands (MWG-biotech) using UL12-13, UL14-13, and UL13rseq primers (Tab. I).

2.5. Generation of the pRB-1B*UL13-derived virus

pRB-1B*UL13 (3 µg) was transfected into 50% confluent CESC grown in 60-mm dishes by using the calcium phosphate precipitation method [14]. Six days later, cell monolayers showing viral plaques were harvested and amplified on fresh CESC.

2.6. DNA analyses

Virus DNA was purified from approximately $0.5-1 \times 10^7$ infected CESC grown in a 100-mm dish as described previously [27] and was amplified by PCR using UL12-13 and UL14-13 primers located in UL13 flanking sequences. The PCR product was purified with micropure EZ column (Millipore, Bedford, MA, USA), concentrated on Microcon YM30 (Millipore) and directly sequenced on both strands (MWG-biotech) using UL12-13, UL14-13, and UL13rseq primers (Tab. I).

2.7. Transcription examination of the UL13 region by RT-PCR

Infected CESC ($0.5-1 \times 10^7$) grown in a 100-mm dish were trypsinized and washed in Phosphate Buffered Saline solution (PBS). The pellet of cells was immediately resuspended in RNable solution (Eurobio, Les Ulis, France). Total RNA extraction was performed according to the manufacturer's instructions. RNA preparations were treated with RNase-free RQ1 DNase (Promega, Madison, WI, USA) and quantified by measuring the optical density at 260 nm. One microgram of

¹ Kut E., Rasschaert D., unpublished data.

Table I. Primers for mutagenesis and sequencing.

Primers	Sequence (5'-3')	Md5 sequence position	Description
UL14-13f	gctattctcgaaaaggaggacattc	37969-37993	Specific sequence of UL14 gene in 3'
UL12-13r	ttgccgaaggtataattgg	36164-36183	Specific sequence of UL12 gene in 5'
UL13rseq	cctactccatttaaggcc	36814-36831	Specific inner sequence of UL13 gene
UL13r574-556	gtaatgcacattcaccggc	37307-37325	Specific inner sequence of UL13 gene
5fUL13Wtpepk	gtagttaaaatattaagaagacggacatagccgtc <u>aaaaa</u> gattgggaatgttttaaAGGATGAC GACGATAAGTAGGG	37350-37409	Complementary sequences of UL13 gene are in lower case, mutation site is underlined, and complementary sequences to the I-SceI site in 5fUL13Wtpepk and to the Kanamycin gene are in upper case.
3rUL13Wtpepk	atgtcataagtaactcagttttaaacattccaatact <u>ttttg</u> acgctatgtccgCAACCAATTAA CCAATTCTGATTAG	37331-37387	

Table II. Primers used for RT-PCR analysis of MDV mRNA.

Target gene	5' primer name Sequence (5'-3')	3' primer name Sequence (5'-3')	Size of PCR product in bp
UL13	UL13f943-962 Agttctgataaaaatccagtc	UL13rseq cctactccatttaaggcc	125
UL13	UL13seq Tgctgggagtgccagctac	UL13rseq	668
UL13	5'fUL13BglII CCAGATCTatggataactgaatcaaaaaac	3'rUL13EcoRI CCGAATTCtagttccataacaacaatc	1558 (entire ORF)
UL49	MDV31f CCCTCGAGatgggggattctgaaagcgcg	MDV31r CCCTCGAGttattcgtactactgctacg	766 (entire ORF)
US2	5fUS2Xho CCTCGAGatgggtgtgtccatgataact	3rUS2HdIII CCAAGCTTctaatgactaccggtctacatt	812 (entire ORF)

total RNA was reverse transcribed using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase according to the manufacturer's recommendations (Promega). Five percent of the synthesized cDNA was then subjected to PCR amplification with 1 μ M of each primer (total volume of reaction mixture, 25 μ L). The primers used for the RT-PCR are described in Table II. Twenty micro-

liters of each RT-PCR mixture was electrophoresed on a 0.8% or 1.5% agarose gel and visualized by ethidium bromide staining.

2.8. Measurement of virus plaque size

Cells (1.5×10^6) were infected with 200 PFU of recombinant RB1B (rRB-1B)

or rRB-1B*UL13 viruses. Four days post-infection, the cells were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and stained with a cocktail of three of the Mabs described above (F19 anti-VP5, K11 anti-gB, and E21 anti-ICP4; each diluted at 1:1000) followed by a goat anti-mouse Alexa 488 (Invitrogen, Carlsbad, CA, USA). Plaques were observed with a 4× Achroplan lens (NA 0.1; Zeiss, Göttingen, Germany) mounted on an Axiovert 200 M inverted epi-fluorescence microscope (Zeiss). Measurements were performed on images captured from 100 plaques through a CCD AxioCam MRm camera (Zeiss) using the Axiovision software (Zeiss). Statistical analysis (ANOVA) was performed with the SAS software version 8.

2.9. In vivo experiments

Sixty-five one-week-old specific-pathogen-free B13/B13 white leghorn chicks were randomly allocated to 4 different groups: nine birds were inoculated with rRB-1B and housed with ten naïve contact birds (group 1); nine birds were inoculated with rRB-1B*UL13 and housed with ten contact birds (group 2); nine birds were injected with parental, wild-type RB-1B virus and housed with ten contact birds (group 3); eight negative control birds (group 4). Chicks were injected intramuscularly in the pectoral region with 0.2 mL of a cell suspension containing 1 000 plaque-forming units (pfu) of each virus stock. For pRB-1B-derived viruses, virus stocks consisted of infected CESC grown in culture; for the RB-1B virus strain, the inoculum consisted of a suspension of peripheral blood leukocytes from previously infected chickens. The injected chicks were raised with the naïve chicks (contacts). Birds were observed twice a day for clinical disease. Birds presenting severe clinical signs were killed. All surviving birds were killed on day 162.

All birds were examined for gross pathological lesions. Statistical analyses were performed using the Pearson's chi-square test and the Mann-Whitney U Test.

2.10. Detection of MDV in feather tips

Feather tips from actively growing feathers were collected from the pectoral zone of birds injected with pRB-1B-derived virus. The biopsies were 5–10 mm long and 2–4 mm wide. For indirect fluorescence analysis (IFA), feather samples were embedded in Shandon Cryomatrix (Thermo electron corporation, Waltham, MA, USA), immediately frozen in dry ice, and stored at -80°C until use. Sections (8- μm thick and perpendicular to the axis of the feather) were performed with a cryostat at -20°C (Leica Microsystems, Wetzlar, Germany), collected on Superfrost Plus glass slides, dried, fixed, and permeabilized in ethanol/acetone (1:1) at -20°C . The feather tip sections were stained with the anti-ICP4 E21 Mab, followed by a goat anti-mouse antibody conjugated to Alexa Fluor 594 (Invitrogen), and with the anti-VP5 H18 MAb directly coupled to Oregon Green with the FluoroReporter kit (Invitrogen). Sections were inspected with the Axiovert 200 M inverted epi-fluorescence microscope (Zeiss) described above with the Apotome system. For immunohistochemistry, feather samples were immediately placed into histocassettes and fixed in 3% paraformaldehyde for 48 h. Samples were processed for dehydration and paraffin embedding in a TP1020 automat (Leica Microsystems) during 20 h. Embedded samples were kept at -20°C until sectioning on an AS325 Shandon microtome (Thermo electron corporation). Sections (6- μm thick) were collected on Superfrost slides, dried at 37°C for 2 days, and stained with the anti-VP5 F19 MAb after removal of the paraffin and rehydration. Endogenous peroxidase activity was blocked by incubating the sec-

tions in methanol containing 1% H₂O₂. To avoid non-specific binding of antibodies, the sections were blocked by incubation with normal goat serum in a 1:5 dilution in phosphate-buffered saline (PBS) for 20 min. The secondary goat anti-mouse antibody coupled to horseradish peroxidase was diluted at 1:1000 in ChemMate antibody diluant (Dako) and revealed by the peroxidase DAB system (Dako). Sections were inspected with a B×41 right microscope (Olympus, Rungis, France) and pictures were taken with a DP50 color digital camera (Olympus).

3. RESULTS

3.1. Identification of a single mutation in the UL13 gene in the pRB-1B BAC clone

In order to determine why this virus does not spread from infected to naïve chickens, we determined the nucleotide sequence of selected genes or region of genes that might be involved in the spreading. The complete UL48 ORF and the sequences encoding the cytoplasmic domains of gB, gD, gE and gM were identical to the ones of the Md5 sequence. A single point mutation, an additional T nucleotide at position 37368 of the Md5 sequence, was found in the UL13 homologous open reading frame. The pRB-1B UL13 nucleotide sequence was submitted to GenBank and assigned the accession number EF110558. This additional nucleotide in UL13 ORF is responsible for a frame-shift and the introduction of a premature stop codon. Consequently, the predicted UL13 ORF is shortened from 1541 to 528 bp (Fig. 1A) and the theoretical protein product from 513 to 176 amino acids (Fig. 1B). The shortened pRB-1B pUL13 is devoid of the serine/threonine protein kinase active sites (involved in phosphate transfer) predicted at amino acid positions 264-276 in pUL13 according to ScanProsite². We

speculated that this important modification of the unique-long serine/threonine protein kinase may be associated with a loss-of-function of the protein or a complete absence of protein expression.

3.2. Construction and analysis of a UL13-repaired pRB-1B virus

To investigate the function of UL13 for horizontal spread of MDV *in vivo*, a repaired pRB-1B bacmid with a restored UL13 ORF (termed pRB-1B*UL13) was constructed. For this, the additional T in the coding sequence of the pRB-1B genome was removed using two-step "en passant" mutagenesis. In a first step, a linear PCR fragment encoding kan^R, an I-SceI site and 58 bp of the UL13 gene covering the region to repair was obtained using 5FUL13Wtpepk and 3RUL13Wtpepk primers (Tab. I). The PCR fragment was electroporated into EL250 containing the pRB-1B bacmid target to allow homologous recombination. Individual kanamycin and chloramphenicol double-resistant colonies harboring BAC clones were selected. One of these colonies was chosen to perform the second mutagenesis step. Electrocompetent cells were prepared from this colony and transformed with the pBAD-I-SceI plasmid containing the *I-sceI* gene under the control of an arabinose inducible promoter. This second recombination step conducted to the elimination of the Kan^R marker. The transformants were selected on LB agar plates containing ampicillin, chloramphenicol and 1% of arabinose.

One of the positive colonies (Cm^R Kan^S) from this final mutagenesis was selected for further analysis. First, the entire UL13 ORF from the bacmid isolated from *E. coli* was verified by sequencing. Then, the BAC DNA was transfected into primary CESC, amplified for the production of a master stock of cell-associated virus, and frozen in liquid nitrogen. The

² www.expasy.org/prosite.

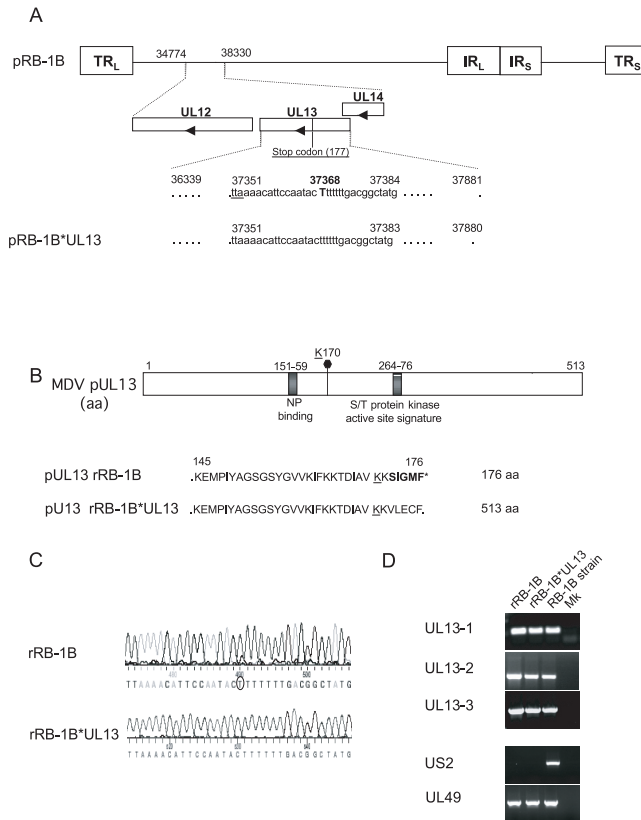


Figure 1. Generation and characterization of pRB-1B*UL13, a pRB-1B derived BAC harboring a full-length UL13 ORF. **A.** Schematic representation of the MDV genome in the UL13 region and part of the sequence for pRB-1B UL13 and for the repaired pRB-1B*UL13. The three underlined nucleotides correspond to the premature stop codon in UL13 ORF of pRB-1B due to the presence of an extra T. In pRB-1B*UL13, the additional T (position 37,668 of UL13 in Md5 sequence) has been removed. **B.** Schematic illustration of the UL13 protein with its functional domains and the theoretical amino acid sequences of pUL13 for rRB-1B and rRB-1B*UL13 between aa145 and aa176. The functional domains were determined using ScanProsite. **C.** Sequence around nucleotide 514 in UL13 gene from rRB-1B and rRB-1B*UL13. Viral DNA of each virus was obtained from infected CESC and approximately 1800 bp was amplified by PCR with UL12-13 and UL13-14 primers (Tab. I) and was sequenced. The sequence of each virus was identical, with the exception of the desired T deletion in rRB-1B*UL13. **D.** UL13 mRNA expression. Total RNA were purified from CESC infected with rRB-1B, rRB-1B*UL13 or parental RB-1B viruses, 5 days post-infection. Mock represents non infected CESC. RT-PCR were performed for UL13 with three couples of primers, UL13f943-962 and UL13rseq (couple UL13-1), UL13seq and UL13rseq (couple UL13-2) and 5'fUL13BgIII and 3'rUL13EcoRI (couple UL13-3) (Tab. I). The expression of US2 or UL49 encoding the major tegument protein VP22 was also done as controls. The pRB-1B viruses derived from bacmids are lacking US2 since the mini-F cassette was introduced in the US2 locus for the bacmid construction. This gene allows distinguishing the pRB-1B derived viruses from the RB-1B strain. We showed that the region UL13 is transcribed for the two pRB-1B derived viruses as well as for the RB-1B strain.

UL13 gene from the rRB-1B*UL13 viral genomic DNA was analyzed by PCR using UL12-13 and UL14-13 primers (Tab. I). As expected, this PCR fragment was similar in size to the PCR fragment obtained from pRB-1B viral DNA (not shown). The sequence of the 1 830 bp PCR product was verified and found to be identical to the Md5 and the repaired BAC sequences (Fig. 1C). We concluded that the mutation was stably introduced into the genome of the derived virus. The expression of UL13 mRNA was evaluated by RT-PCR with three couples of primers from total RNA extracted from infected CESC. We showed that UL13 mRNA are expressed for the two pRB-1B derived viruses as well as the RB-1B strain (Fig. 1D). No protein analysis of the pUL13 by Western blot or immunofluorescence could be performed since all our efforts to develop antibodies against pUL13 remained unsuccessful.

3.3. Cell-to-cell spread of the pRB-1B*UL13-derived virus in cell culture

To study the cell-to-cell spread of both viruses in vitro, plaque size measurements were performed on CESC. The cells were coseded with 200 pfu of each virus, fixed four days post-infection, and analyzed by fluorescence microscopy. Mean plaque areas measured for both viruses were 1.908 ± 0.054 and $1.820 \pm 0.059 \times 10^5 \mu\text{m}^2$ for rRB-1B and rRB-1B*UL13, respectively, showing no significant difference in cell-to-cell spreading ($p > 0.05$; ANOVA after square root transformation).

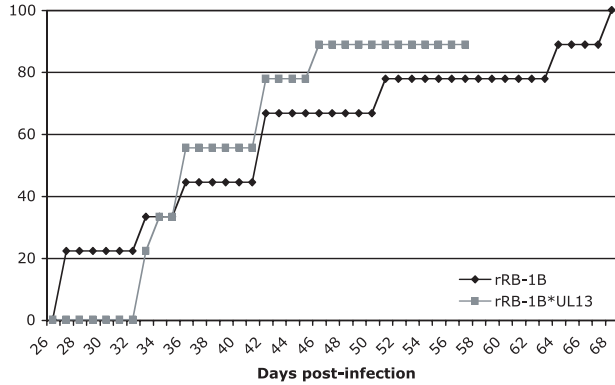
3.4. Pathogenesis and horizontal dissemination of rRB-1B*UL13 compared to rRB-1B and RB-1B strain

Three groups of nine 1-week-old B13/B13 chickens were infected with

10^3 PFU of one of the three viruses: rRB-1B, rRB-1B*UL13, or the authentic, parental RB-1B strain. One group of eight negative control chickens was also included. Each infected group was raised with ten 1-week-old naive chickens (contacts). The four groups of chickens were monitored for clinical signs and gross pathological lesions. All the birds injected with lymphocytes infected with the highly virulent RB-1B died within 16 days. rRB-1B and rRB-1B*UL13 viruses induced visceral tumors in 100% and 89% of the birds injected, respectively, with a mean time to disease onset of 42 and 36 days (Fig. 2A). The difference in the percentage of birds developing tumors due to the absence or presence of the UL13 gene was not statistically significant (Pearson's chi-square test). The difference in mean time to the onset of the disease was also not statistically significant (Mann-Witney U test). The tumors from both groups of chickens were predominantly observed in kidneys, liver, and in the muscle. These results showed that rRB-1B and rRB-1B*UL13 virus do not differ in terms of pathogenesis in inoculated birds. Moreover, we could conclusively demonstrate that a full UL13 ORF is not essential for MDV pathogenesis and does not influence disease progression in the pRB-1B background.

All (100%) chickens in the rRB-1B and in the rRB-1B*UL13 contact groups survived during the 162 days of the experiment, whereas 100% of the RB-1B strain contacts developed tumors and died within the first 55 days, with a mean survival time of 48 days (Fig. 2B). In the rRB-1B and rRB-1B*UL13 contact groups, none of the surviving chickens developed clinical disease or showed evidence of gross lesions. Therefore, we concluded that the restoration of the UL13 ORF was not sufficient to rescue the horizontal spread of MDV in the pRB-1B background.

A Cumulative incidence of MDV in birds infected with the pRB-1B-derived viruses.



B Percentage of survival in the contact birds

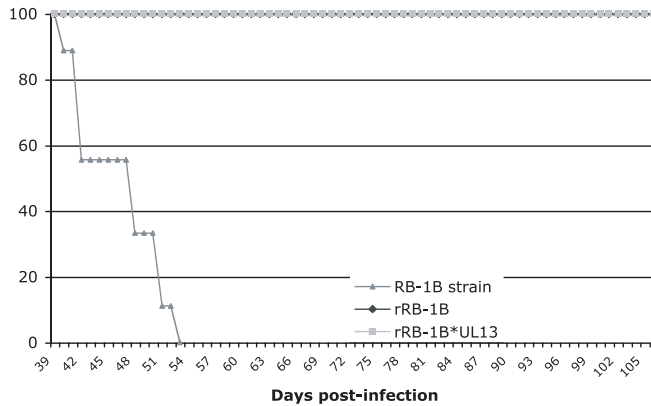


Figure 2. In vivo characterization of the pRB-1B*UL13 derived virus. **A.** Cumulative incidence of MD in birds infected with rRB-1B*UL13 and rRB-1B viruses. Virus stocks (10^3 pfu) were inoculated by intra-muscular route into groups ($n = 9$) of 1-week-old specific pathogen-free B13/B13 chickens. The cumulative incidence of the disease in the first 69-day period, time to death, is expressed as a percentage. **B.** Percentage of survival of naïve birds in contact with birds injected with rRB-1B*UL13, rRB-1B, or parental RB-1B viruses. Each injected group was raised with a naïve group ($n = 10$) of one-week old specific-pathogen-free B13/B13 chickens.

3.5. MDV antigens in feather tips of chickens infected with pRB-1B-derived virus compared to the RB-1B strain

In order to determine whether the lack of dissemination of the rRB-1B-derived

virus is due to an absence of feather tropism, the feathers of birds injected with rRB-1B or the RB-1B strain were analyzed by immunofluorescence and immunochemistry. In birds injected with pRB-1B-derived virus as well as in birds injected with the RB-1B strain (Fig. 3A),

MDV lytic replication was detected in feather follicle epithelial cells by staining with a monoclonal antibody to VP5, the major capsid protein. Double immunofluorescence staining also clearly showed the colocalisation of VP5 and ICP4, a protein with immediate-early kinetics, to the epithelial layer (Fig. 3B). The VP5 antigen was found by immunohistochemistry in the nuclei of cells which appeared to be of epithelial lineage, although no markers for epithelial or lymphoid cells could be used in this experiment. Both viruses appeared to infect the same cells in the same tissue region and the infection was consistently restricted to the uppermost zone of the skin epithelium in contact with the feather quill. These results show that the pRB-1B-derived virus is not impaired in feather tropism and that the UL13 gene is not dispensable for skin tropism *in vivo*.

4. DISCUSSION

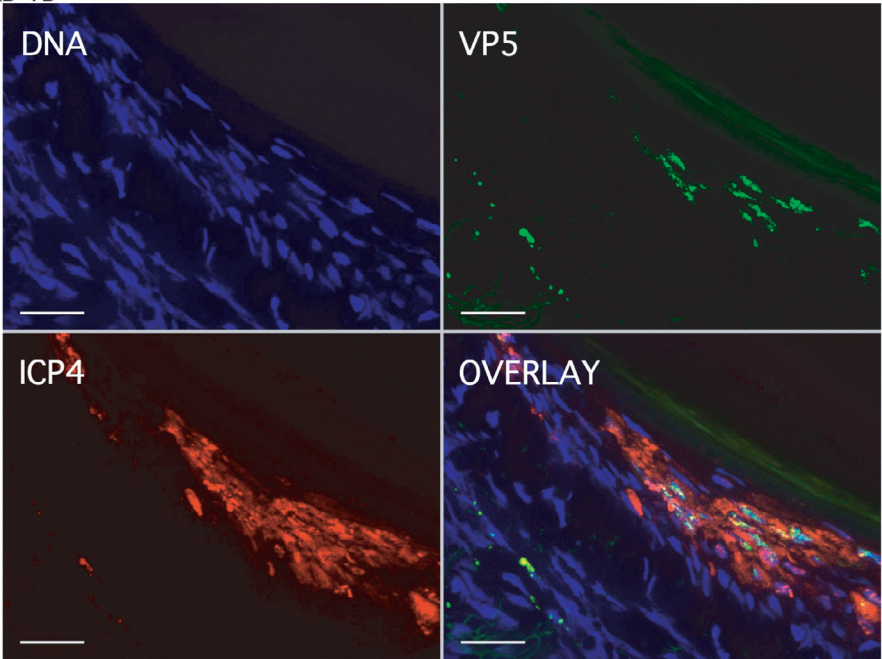
The identification of a mutation in the UL13 gene of pRB-1B and the construction of a repaired BAC clone, named pRB-1B*UL13, indicated that the MDV UL13 homologous gene is not essential for some of the MDV properties *in vitro* and *in vivo*. Experiments with two recombinant MDV reconstituted from infectious DNA demonstrated that a full-length UL13 ORF is dispensable for *in vitro* infectivity. This was in agreement with the results obtained for other alphaherpesviruses. Indeed, the UL13 ortholog was found to be non essential for the replication of PRV, HSV-1, and VZV in cultured cells [5, 9, 15]. In this study, we also report that no difference can be observed between rRB-1B and rRB-1B*UL13 with respect to plaque size. This was in accordance with the results described for VZV where no apparent differences in plaque morphology were observed between a mutant lacking ORF47 protein expression and the parental P-Oka virus in

a human melanoma cell line [9]. Curiously for PRV and HSV-1, UL13 mutants exhibit slight plaque size reductions [15, 30]. A possible explanation is that pUL13 function involved in cell-to-cell spread *in vitro* is compensated by a viral or a cellular protein expressed in the cell types used to grow VZV and MDV.

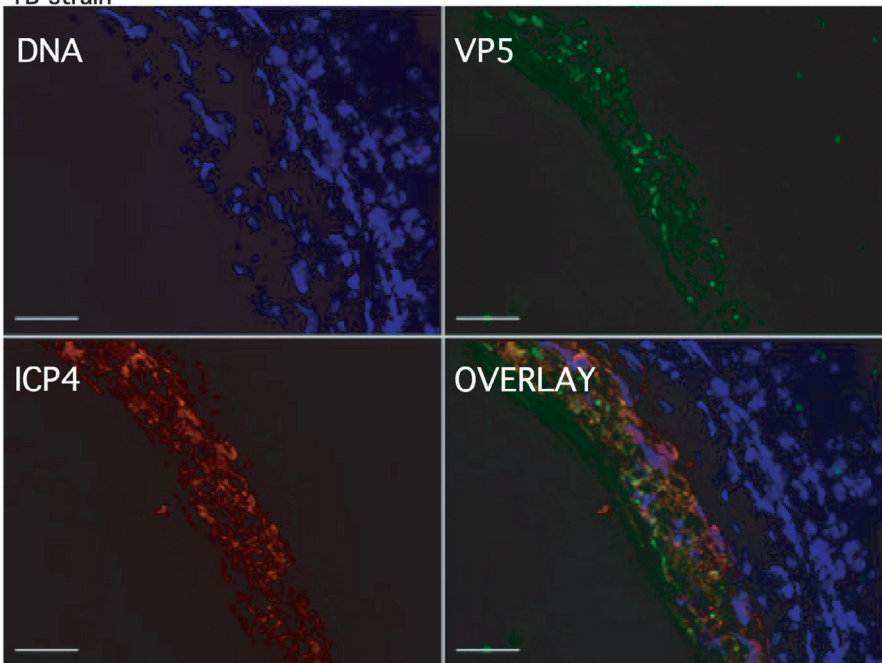
The results reported here also show that a full-length UL13 ORF is not essential for oncogenicity of MDV. This was the first time such an observation was made on an oncogenic herpesvirus. In terms of cellular tropism, this result also shows that the rRB-1B devoid of a full UL13 ORF is capable of spreading to T cells *in vivo*. This result was surprising since the VZV ORF47 is required for efficient replication in T lymphocytes *in vitro* and for growth in human fetal lymphocytes implanted in mice with severe combined immunodeficiency (SCID) [3, 28]. This obvious discrepancy suggests that there is a difference between VZV and MDV with respect to the viral determinants involved in the T-cell tropism of the two viruses in their target species. Alternatively, the blockade in the cytolytic process into T-cells (due to the

Figure 3. Detection of MDV antigens in the feather tips of chickens after infection with the rRB-1B-derived viruses and the RB-1B strain. **A.** Immunofluorescence of VP5 and ICP4 antigens. Each image corresponds to a projection of eleven 0.275- μm thick slices VP5 (light grey/green), localized in the nucleus which is stained with Hoechst 33342 (dark grey/blue). ICP4 (grey/red) is localized in the nuclei and cytoplasm of infected cells. Bars represent 20 μm . **B.** Immunohistochemistry of VP5 antigen (MCP). MCP antigen was detected in the nuclei of the cells forming the epithelial layer in contact with the feather shaft. The feather shaft is not visible on the feather tip of the rRB-1B-infected chicken. Bars represent 20 μm . (A color version of this figure is available at www.edpsciences.org/vetres.)

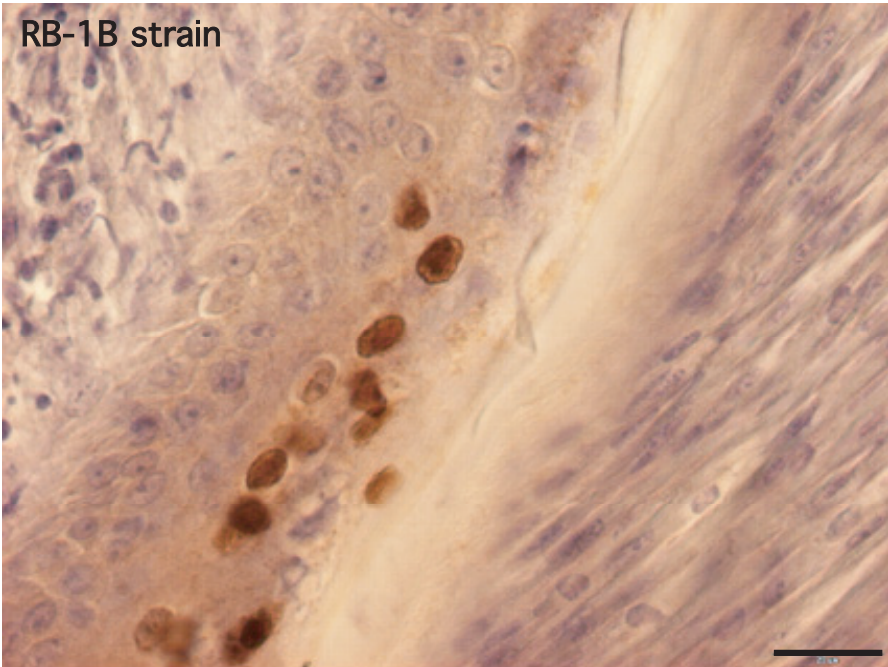
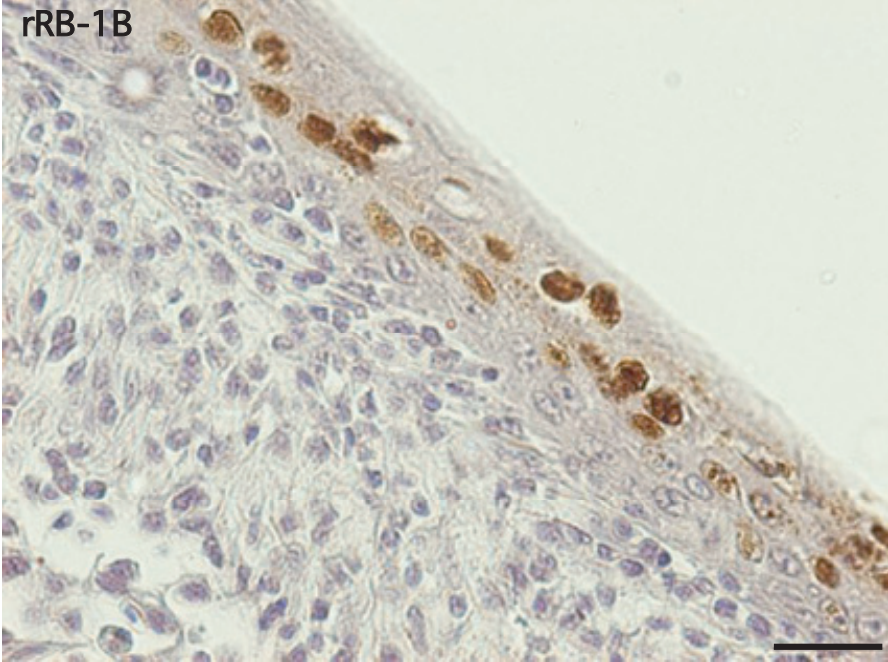
A _{rRB-1B}



RB-1B strain



B



lack of pUL13) is common to both viruses and only affects spreading from T-cells to skin cells but does not affect the transformation process.

We were unable to determine whether the rRB-1B-derived viruses express pUL13 and/or a pUL13 protein kinase activity because of the lack of pUL13 antibodies. The absence of pUL13 kinase activity is predictable owing to the deletion of the conserved DFG (DFS in UL13 MDV) motif, which is present in almost all kinases and considered necessary for the transfer of phosphate from ATP to the substrate. We cannot, however, exclude that the truncated (176 aa) MDV pUL13 product has another function besides kinase activity. For instance, such an alternative function has been identified for the rOka Δ C mutant encoding for a 268-aa long ORF47 product [2]. It is also possible that the truncated MDV pUL13 product is not expressed like for the VZV ROka47 considered as an ORF47 null mutant, i.e. not expressing a shortened protein product encoded by ORF47 (165 codons) in infected cells [9].

In this study we showed that MDV structural proteins are present in the feather tips of chickens infected with rRB-1B demonstrating that pUL13 is not required for MDV targeting to feathers and replication in this tissue. However, the cell types expressing MDV antigens within the feather follicle remain to be determined. In the absence of double staining with a cellular marker, we cannot formally exclude that the cells expressing MDV antigens were infected-T cells. This result also shows that the presence of late MDV antigens in the feathers might not be associated with horizontal dissemination of the virus. One hypothesis is that, although rRB-1B is present in feather follicles, virus morphogenesis may not be entirely complete, which in consequence would not allow the formation of enveloped and fully infectious virions as is the case after infection

with the parental RB-1B strain. This experiment was originally designed to study the impact of the UL13 gene in MDV transmission in vivo. By successfully constructing a pRB-1B-derived virus harboring a full-length UL13 ORF, we demonstrated that the integrity of the UL13 ORF is still not sufficient to restore MDV horizontal dissemination. Therefore, this phenotype may be attributable to other mutations elsewhere in the pRB-1B sequence.

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REFERENCES

- [1] Baigent S.J., Davison F., Marek's disease virus: biology and life cycle, in: Davison F., Nair V.K. (Eds.), Marek's disease. An evolving problem, Elsevier Academic Press, Compton, UK, 2004, Biology of animal infections, pp. 62–77.
- [2] Besser J., Sommer M.H., Zerboni L., Bagowski C.P., Ito H., Moffat J., Ku C.C., Arvin A.M., Differentiation of varicella-zoster virus ORF47 protein kinase and IE62 protein binding domains and their contributions to replication in human skin xenografts in the SCID-hu mouse, *J. Virol.* (2003) 77:5964–5974.
- [3] Besser J., Ikoma M., Fabel K., Sommer M.H., Zerboni L., Grose C., Arvin A.M., Differential requirement for cell fusion and virion formation in the pathogenesis of varicella-zoster virus infection in skin and T cells, *J. Virol.* (2004) 78:13293–13305.
- [4] Calnek B.W., Adldinger H.K., Kahn D.E., Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease, *Avian Dis.* (1970) 14:219–233.

- [5] Coulter L.J., Moss H.W., Lang J., McGeoch D.J., A mutant of herpes simplex virus type 1 in which the UL13 protein kinase gene is disrupted, *J. Gen. Virol.* (1993) 74:387–395.
- [6] Cunningham C., Davison A.J., Dolan A., Frame M.C., McGeoch D.J., Meredith D.M., Moss H.W., Orr A.C., The UL13 virion protein of herpes simplex virus type 1 is phosphorylated by a novel virus-induced protein kinase, *J. Gen. Virol.* (1992) 73:303–311.
- [7] Dorange F., El Mehdaoui S., Pichon C., Coursaget P., Vautherot J.F., Marek's disease virus (MDV) homologues of herpes simplex virus type 1 UL49 (VP22) and UL48 (VP16) genes: high-level expression and characterization of MDV-1 VP22 and VP16, *J. Gen. Virol.* (2000) 81:2219–2230.
- [8] Dorange F., Tischer B.K., Vautherot J.F., Osterrieder N., Characterization of Marek's disease virus serotype 1 (MDV-1) deletion mutants that lack UL46 to UL49 genes: MDV-1 UL49, encoding VP22, is indispensable for virus growth, *J. Virol.* (2002) 76:1959–1970.
- [9] Heineman T.C., Cohen J.I., The varicella-zoster virus (VZV) open reading frame 47 (ORF47) protein kinase is dispensable for viral replication and is not required for phosphorylation of ORF63 protein, the VZV homolog of herpes simplex virus ICP22, *J. Virol.* (1995) 69:7367–7370.
- [10] Jarosinski K.W., Osterrieder N., Nair V.K., Schat K.A., Attenuation of Marek's disease virus by deletion of open reading frame RLORF4 but not RLORF5a, *J. Virol.* (2005) 79:11647–11659.
- [11] Kamil J.P., Tischer B.K., Trapp S., Nair V.K., Osterrieder N., Kung H.J., vLIP, a viral lipase homologue, is a virulence factor of Marek's disease virus, *J. Virol.* (2005) 79:6984–6996.
- [12] Kato A., Yamamoto M., Ohno T., Tanaka M., Sata T., Nishiyama Y., Kawaguchi Y., Herpes simplex virus 1-encoded protein kinase UL13 phosphorylates viral Us3 protein kinase and regulates nuclear localization of viral envelopment factors UL34 and UL31, *J. Virol.* (2006) 31:1476–1486.
- [13] Kawaguchi Y., Van Sant C., Roizman B., Eukaryotic elongation factor 1delta is hyperphosphorylated by the protein kinase encoded by the U(L)13 gene of herpes simplex virus 1, *J. Virol.* (1998) 72:1731–1736.
- [14] Kingston E., Introduction of DNA into mammalian cells, in: Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K. (Eds.), *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., Boston, USA, 2001, *Current Protocols*, Vol. 1, pp. 9.0.1–9.1.11.
- [15] Klopffleisch R., Klupp B.G., Fuchs W., Kopp M., Teifke J.P., Mettenleiter T.C., Influence of pseudorabies virus proteins on neuroinvasion and neurovirulence in mice, *J. Virol.* (2006) 80:5571–5576.
- [16] Ku C.C., Besser J., Abendroth A., Grose C., Arvin A.M., Varicella-Zoster virus pathogenesis and immunobiology: new concepts emerging from investigations with the SCIDhu mouse model, *J. Virol.* (2005) 79:2651–2658.
- [17] Lee L.F., Wu P., Sui D., Ren D., Kamil J., Kung H.J., Witter R.L., The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus, *Proc. Natl. Acad. Sci. USA* (2000) 97:6091–6096.
- [18] Moffat J.F., Zerboni L., Sommer M.H., Heineman T.C., Cohen J.I., Kaneshima H., Arvin A.M., The ORF47 and ORF66 putative protein kinases of varicella-zoster virus determine tropism for human T cells and skin in the SCID-hu mouse, *Proc. Natl. Acad. Sci. USA* (1998) 95:11969–11974.
- [19] Ng T.I., Ogle W.O., Roizman B., UL13 protein kinase of herpes simplex virus 1 complexes with glycoprotein E and mediates the phosphorylation of the viral Fc receptor: glycoproteins E and I, *Virology* (1998) 241:37–48.
- [20] Ogle W.O., Ng T.I., Carter K.L., Roizman B., The UL13 protein kinase and the infected cell type are determinants of posttranslational modification of ICPO, *Virology* (1997) 235:406–413.
- [21] Petherbridge L., Brown A.C., Baigent S.J., Howes K., Sacco M.A., Osterrieder N., Nair V.K., Oncogenicity of virulent Marek's disease virus cloned as bacterial artificial chromosomes, *J. Virol.* (2004) 78:13376–13380.
- [22] Purves F.C., Roizman B., The UL13 gene of herpes simplex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein alpha 22, *Proc. Natl. Acad. Sci. USA* (1992) 89:7310–7314.
- [23] Reddy S.M., Cox E., Iofin I., Soong W., Cohen J.I., Varicella-zoster virus (VZV) ORF32 encodes a phosphoprotein that is posttranslationally modified by the VZV ORF47 protein kinase, *J. Virol.* (1998) 72:8083–8088.

- [24] Reddy S.M., Sui D., Wu P., Lee L., Identification and structural analysis of a MDV gene encoding a protein kinase, *Acta Virol.* (1999) 43:174–180.
- [25] Ronfort C., Afanassieff M., Chebloune Y., Dambrine G., Nigon V.M., Verdier G., Identification and structure analysis of endogenous proviral sequences in a Brown Leghorn chicken strain, *Poult. Sci.* (1991) 70:2161–2175.
- [26] Schumacher D., Tischer B.K., Fuchs W., Osterrieder N., Reconstitution of Marek's disease virus serotype 1 (MDV-1) from DNA cloned as a bacterial artificial chromosome and characterization of a glycoprotein B-negative MDV-1 mutant, *J. Virol.* (2000) 74:11088–11098.
- [27] Sinzger C., Knapp J., Schmidt K., Kahl M., Jahn G., A simple and rapid method for preparation of viral DNA from cell associated cytomegalovirus, *J. Virol. Methods* (1999) 81:115–122.
- [28] Soong W., Schultz J.C., Patera A.C., Sommer M.H., Cohen J.I., Infection of human T lymphocytes with varicella-zoster virus: an analysis with viral mutants and clinical isolates, *J. Virol.* (2000) 74:1864–1870.
- [29] Spengler M., Niesen N., Grose C., Ruyechan W.T., Hay J., Interactions among structural proteins of varicella zoster virus, *Arch. Virol. Suppl.* (2001) 71–79.
- [30] Tanaka M., Nishiyama Y., Sata T., Kawaguchi Y., The role of protein kinase activity expressed by the UL13 gene of herpes simplex virus 1: the activity is not essential for optimal expression of UL41 and ICP0, *Virology* (2005) 341:301–312.
- [31] Tischer B.K., von Einem J., Kaufer B., Osterrieder N., Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*, *Biotechniques* (2006) 40:191–197.
- [32] Tulman E.R., Afonso C.L., Lu Z., Zsak L., Rock D.L., Kutish G.F., The genome of a very virulent Marek's disease virus, *J. Virol.* (2000) 74:7980–7988.