Open reading frame L1 of Marek’s disease herpesvirus is not essential for in vitro and in vivo virus replication and establishment of latency

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Introduction

Marek’s disease (MD), a lymphoma of thymus-derived lymphocytes in chickens, is caused by Marek’s disease herpesvirus (MDV), which has been classified as an alphaherpesvirus (Buckmaster et al., 1988). MDV DNA consists of a unique long sequence (UL) and a unique short sequence (US) both flanked by a set of inverted repeats (terminal repeat long region, TRL; inverted repeat long region, IRL; inverted repeat short region, IRS; and terminal repeat short region, TRS) (Fig. 1a) (Fukuchi et al., 1984). The process of induction and maintenance of latency and transformation have not yet been elucidated despite the fact that a number of MDV genes have recently been cloned and sequenced. There is limited gene transcription in MD lymphoblastoid chicken cell (MDCC) lines (Maray et al., 1988; Schat et al., 1989; Sugaya et al., 1990), while many transcripts from almost the complete MDV genome have been described in lytically infected cells (Silver et al., 1979; Schat et al., 1989). Most of the transcripts in MDCC cell lines have been mapped to the repeat areas and were classified as immediate early or early genes (Schat et al., 1989).

Fig. 1. (a) Genomic structure of MDV. UL, unique long region; US, unique short region; IRL, inverted repeat long region; IRS, inverted repeat short region; TRL, terminal repeat long region; TRS, terminal repeat short region. The approximate location of US10 is indicated. (b) Locations of the BamHI-H, -I2, -Q2, and -L regions. The location of the XbaI site in the BamHI-L region is indicated (X). (c) Location of ORF L1 in relation to the BamHI-Q2 and -L regions. The locations of the start and stop codons are indicated by an open and closed arrowhead, respectively. The location of the poly(A) signal is at the end of the arrow. Relevant restriction enzyme sites are indicated as follows: B, BamHI; C, CiaI; E, EcoRI; N, NdeI. (d) Insertion of the lacZ cassette between the NdeI and EcoRI sites of ORF L1. The SV40 promoter is shaded, while the ORF for lacZ is indicated by the open box. The ORF L1 fragments are shown as black boxes. Relevant restriction enzyme sites, location of the start and stop codons of ORF L1 are indicated as in (c). The approximate locations of primers 1, 2, 3 and 4 are indicated by arrows.

Two mutant CVI988 Marek’s disease virus (MDV) strains were developed in which a part of ORF L1 was replaced by lacZ with the SV40 early promoter. These mutant strains, CVIL1LacZ-A and -B, were inoculated into chickens to test the hypothesis that ORF L1 is involved in the induction and/or maintenance of latency. Mutant virus could be reisolated from lymphocytes obtained from chickens during both the lytic and latent phase of infection, indicating that ORF L1 is not essential for the induction and/or maintenance of latency or the reactivation from latency. β-Galactosidase-positive lymphocytes were detected during the latent infection demonstrating that the SV40 early promoter can be active in recombinant MDV strains during latent infection. Although the insertion of lacZ was stable in cell culture, recombination within lacZ and the BamHI-L fragment was observed during in vivo infection.

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Recently, Ohashi et al. (1994) cloned and sequenced a cDNA derived from MDCC-CU41. The cDNA contains a small open reading frame (ORF), L1, encoding 107 amino acids. This ORF is located in the Q2 and L fragments of the BamHI MDV DNA library (Fig. 1b, c). Northern blot analysis showed that the cDNA hybridized to three transcripts of 2.5, 0.8 and 0.6 kb, which are more abundantly expressed in MDCCs than in lytically infected cells. Peng et al. (1995) described an identical ORF as part of a bicistronic mRNA in this region, which they identified as ORF 2. Interestingly, the 0.6 kb transcript can also be detected by RT-PCR in a reticuloendotheliosis virus-transformed chicken cell line (RECC) which had been superinfected with MDV (Ohashi et al., 1994). This cell line, RECC-CU210, does not express any MDV-specific proteins, but two MDV-specific transcripts are expressed (Pratt et al., 1992). This finding suggests that ORF L1 could play a role in the induction and/or maintenance of latency of MDV. To elucidate the role of ORF L1 during the lytic and latent infection cycle of MDV, a CVI988 strain (Rispens et al., 1997) lacking a functional ORF L1 was developed. This mutant virus was able to establish lytic and latent infections in vivo, suggesting that ORF L1 is not essential for virus replication and establishment of latency.

Methods

**Experimental chickens and holding conditions.** Specific-pathogen-free chickens were obtained from SPAFAS (Storrs, CT, USA) for experiment 1 or from the genetically MDV-susceptible P2a (B6B6B6) line (Weinstock & Schat, 1987) for experiment 2. One-day-old chicks were wing-banded and placed in stainless-steel isolators with feed and water freely available.

**Virus strains and cell cultures.** MDV strain CVI988 was propagated in secondary chicken embryo fibroblasts (CEF) following established procedures. Primary CEF were prepared from chicken embryos free of endogenous avian leukosis virus (line ev0) (Astrin et al., 1979).

**Preparation of virus DNA.** Virus DNA together with cellular DNA was extracted from CEF infected with CVI988 passage 33 (p33) using a procedure adapted from Pignatti et al. (1979). Briefly, CEF showing confluent cytopathic effect (CPE) were incubated in TET buffer (0.25% Triton X-100, 10 mM Tris pH 8.0 and 10 mM EDTA) for 10 min at room temperature with moderate shaking. Cells were harvested using a rubber policeman, and 1/10 vol 2 M NaCl was added. The supernatant fluid was discarded after centrifugation at 1000 g for 10 min and the pellet was incubated for 1 h at 37 °C in digestion buffer (0.5% SDS, 100 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA) containing 20 µg/ml RNase A. After 1 h 0.2 mg/ml proteinase K was added and the mixture was incubated for an additional 16 h at 37 °C. DNA was extracted once with phenol, twice with chloroform–isoamylalcohol (24:1), precipitated with 1/10 vol 3 M NaCl and 2 vol ethanol, and resuspended in TE buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA). In addition, DNA was extracted from the MD lymphoblastoid cell line MDCC-CU41 as previously described (Schat et al., 1989).

**Plasmids.** The following plasmids were obtained from K. Ohashi, Cornell University, NY, USA. (i) pBluescript (pBS) pBSLQ, containing the Clal–EcoRI fragment of the BamHI-Q2 region, which consists of the 5’ part of ORF L1 until the EcoRI restriction site (240 bp) and the flanking region (730 bp) upstream from the start codon (Fig. 1a). (ii) pBS2.1 containing the 101 bp EcoRI–BamHI fragment of the Q2 region of the BamHI library of MDV DNA. This fragment includes the remainder of ORF L1. The MDV fragments in plasmids pBSLQ and pBS2.1 were obtained from the RB-1B strain of MDV (Ohashi et al., 1994). (iii) pBSLQ, which contains the L fragment of the BamHI MDV DNA library prepared from the GA strain (Fukuchi et al., 1984). The lacZ gene under control of the SV40 early gene promoter was obtained by digestion of pCH110 (Pharmacia) with HindIII and BamHI and blunt-ended. pMA7 contains ORF US10, which was derived from an EcoRI fragment of BamHI-A of the MDV DNA library (Fukuchi et al., 1984).

**Insertion of the lacZ cassette into ORF L1 and US10.** pBSLQ was digested with EcoRI and BamHI, gel-purified and ligated to the 101 bp EcoRI–BamHI fragment of pBS2.1 (Fig. 2). The resulting plasmid pBSLQ lacZ was digested with NdeI and EcoRI to remove 200 bp of ORF L1, gel-purified, blunt-ended and ligated to the lacZ cassette. The orientation of the lacZ cassette in pBSLQ lacZ was verified by restriction enzyme analysis. The 2 kb BamHI–XbaI fragment obtained from pBSLQ (Fig. 2) was inserted in the BamHI site of pBSLQ lacZ to provide an MDV-specific flanking sequence downstream of lacZ. The final plasmid, pBSL lacZ, contained the lacZ cassette in forward orientation in ORF L1 (Figs 1d and 2).

The plasmid containing the lacZ cassette in ORF US10 was constructed by digestion of pMA7 with Msl. The lacZ cassette was inserted into ORF US10 at the MscI site. All reactions were carried out according to standard procedures (Sambrook et al., 1989).

**Construction of MDV mutant strains.** Prior to transfection, pBSL lacZ and pUS10lacZ were digested with ApaI [located in the multiple cloning site (MCS) of pBS 5’ to the Clal site] and XhoI, and with PstI, respectively, phenol extracted, precipitated and resuspended in TE buffer (pH 7.4). Four µg MDV DNA was co-transfected with 0.1, 0.2, 0.4 or 0.8 µg pBSL lacZ or 0.2 and 0.5 µg pUS10lacZ using the CaCl2 precipitation method (Graham & van der Eb, 1973) with some modifications. MDV DNA and plasmid DNA were diluted in a total volume of 500 µl H2O containing 63 µl 2 M CaCl2. The mixture was incubated overnight at 4 °C and placed at room temperature the next morning. Five hundred µl filter-sterilized HEPES-buffered saline (pH 7.05) was added drop by drop to the DNA mixture with gentle mixing. This mixture was added immediately to a cell pellet of 106 freshly trypsinized secondary CEF using a cut-off 1 ml pipette tip with gentle mixing. The mixture of DNA and CEF was digested for 10 min at room temperature. Ten ml growth medium was added, the cells were seeded into two 60 mm tissue culture dishes, and incubated at 38 °C in 5% CO2. After 5 h, the media was removed, the cultures were washed once with PBS, and 1.5 ml 15% glycerol in HEPES-buffered saline (pH 7.05) was added. After 2 min the glycerol was removed, the cultures were washed once with PBS, 5 ml growth medium was added, and the cultures were incubated at 38 °C in 5% CO2. Four days after transfection MDV plaques were examined for the expression of lacZ by the addition of 0.2 mg/ml Bluegal (Pharmacia) (stock solution of 20 mg/ml Bluegal in dimethylformamide). Blue-staining MDV plaques were selected, trypsinized and co-cultivated with secondary CEF. This procedure was repeated until 100% of the MDV plaques stained blue.

**PCR.** Virus DNA was extracted as described above from CEF infected with CVI1 LacZ or from CEF infected with selected plaques reisolated from chickens infected with CVI1 LacZ recombinant strains. Primers 1 (nt 742–761, 5’ TATACAGCCACAGGACCTCCA 3’) and 2 (nt 1149–1130, 5’ GTACAACAGTGCCACACATC 3’) (Fig. 1d) were used
MDV ORF L1 is not essential for latency

Fig. 2. Construction of pBSL1LacZ. The MCS was removed from pBSI2Q as an EcoRI–BamHI fragment and the 0–10 kb EcoRI–BamHI ORF 1 fragment of pBS2.1 was cloned into pBSI2Q to generate pBSQ2L1. The NdeI–EcoRI fragment was removed from pBSQ2L1 and the plasmid was blunt-ended. The lacZ cassette was removed from pCH110 by digestion with ThhII-I and BamHI, blunt-ended and cloned into pBSQ2L1. The MCS was removed from this plasmid as a BamHI–XbaI fragment and the 2 kb BamHI–XbaI fragment of the BamHI-L pBSL1 clone was ligated into the plasmid to generate pBSL1LacZ. The numbers in parentheses indicate the nucleotide position of the MDV sequence based on Ohashi et al. (1994).

to verify the deletion of ORF L1. These primers are located outside the deleted NdeI–EcoRI fragment of 193 bp (Ohashi et al., 1994). lacZ-specific primers (primer 3, 5’ TTATGGCCCACACCATGTCG 3’ , and primer 4, 5’ CTGGAATTCCGCCGATACTG 3’) (Fig. 1d), located at nt 3105–3124 and nt 3294–3275 of lacZ in plasmid pCH110, respectively, were used to amplify an internal fragment near the 3’ end of the lacZ cassette.

‘Hot-start’ PCR was used for primers 1 and 2, followed by 5 min at 94°C and 35 cycles of 2 min at 60°C, 5 min at 72°C and 1 min at 94°C. For primers 3 and 4, PCR conditions were 5 min at 94°C, followed by 35 cycles of 2 min at 45°C, 1 min at 72°C and 1 min at 94°C. All samples were held after the 35 cycles at 72°C for 10 min followed by 4°C until removal from the thermocycler. PCR products were separated by electrophoresis in 1% TAE-agarose gels and analysed by ethidium bromide staining and Southern blot hybridizations. Southern blots were prepared according to standard methods (Sambrook et al., 1989). The Southern blots were hybridized with the gel-purified NdeI–EcoRI fragment of ORF L1 or a lacZ probe prepared by PCR amplification of a 189 bp fragment of pCH110 using primers 3 and 4. All probes were labelled with [α-32P]dCTP using the Rediprime random-labelling kit (Amersham).

■ In vivo pathogenesis experiments. To examine the importance of ORF L1 for lytic and latent infections, 7-day-old chicks were inoculated intra-abdominally with p44 or p45 of CVI988, CVILacZ-A, CVILacZ-B, or CVIUsl0LacZ in two experiments. In experiment 1 an additional group was inoculated with CVI988 p33. The actual numbers of chickens used in experiments 1 and 2 and the number of plaque-forming units (p.f.u.) of virus inoculated are presented in Tables 1 and 2. Birds were euthanized at 4, 5, 6, 12 and 19 days post-inoculation (p.i.) in experiment 1 and at 5, 20 and 21 days p.i. in experiment 2. Sera were obtained just prior to euthanasia. Spleens were harvested aseptically and divided into halves. One half of each spleen was used to prepare a single cell suspension (Schat et al., 1985), which was used for virus isolation and determination of expression of virus proteins and β-galactosidase in lymphocytes. The other half was snap-frozen together with a thymus fragment and the bursa of Fabricius in experiment 1, or alone in experiment 2.

■ Virus isolations. For virus isolations, 5 × 10^5 spleen cells were plated onto secondary CEF in 35 mm culture dishes. All virus isolations were carried out in duplicate. Plaques were counted at 4 days p.i. and all
cultures were stained for β-galactosidase activity. Results are reported as number of p.f.u. per 10^3 lymphocytes. All white plaques isolated from birds inoculated with recombinant strains were individually propagated in CEF and DNA was extracted after two to four additional passages. In addition, a few blue plaques were also propagated in CEF for DNA extraction. DNA obtained from white and blue plaques was analysed by Southern hybridization and PCR for the presence of complete ORF L1 and/or lacZ.

Detection of MDV antigens and β-galactosidase. Frozen sections of the lymphoid organs were examined for the presence of virus antigens by a direct fluorescent antibody (FA) test using fluorescein isothiocyanate (FITC)-conjugated antisera obtained from MDV-infected chickens. Intensity of infection was numerically scored from a rarely occurring positive cell (score 1) to widespread involvement of the tissue (score 4) (Calnek & Hitchner, 1969).

The direct FA test for the detection of virus antigens in lymphocytes was carried out as described by Calnek et al. (1981). Briefly, 2 x 10^6 spleen lymphocytes were spotted on slides, air-dried, fixed in acetone for 10 min and stained with FITC-conjugated anti-MDV serum. Positive cells were enumerated and the results are expressed as the number of MDV-positive cells per 200,000 cells (see Table 3).

To enumerate the number of lymphocytes expressing β-galactosidase slides were prepared as above. Cells were fixed for 5 min in 0.05 M phosphate buffer pH 7.4 containing 0.2% (v/v) glutaraldehyde, 2% (v/v) formaldehyde and 2 mM MgCl_2 followed by three 5 min washes with a solution of 2 mM MgCl_2 and 0.02% NP40 in 0.05 M phosphate buffer pH 7.4. The cells were incubated overnight at 37 °C in a solution containing 0.5 mg/ml X-Gal (stock solution 20 mg/ml X-Gal DMSO), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM MgCl_2 in 0.05 M phosphate buffer pH 7.4 (Brazolet et al., 1991).

Detection of antibodies against MDV and β-galactosidase. The presence of MDV antibodies was examined using an immunoperoxidase monolayer assay essentially as described by Peeters et al. (1992). Briefly, CEF were seeded into 96-well plates and inoculated with CVI988 p33. At 3 days p.i., monolayers were washed with PBS, dried for 60 min at 37 °C and stored at -20 °C until use. The plates were fixed prior to use with cold 4% (w/v) paraformaldehyde in PBS for 5 min at room temperature, washed three times with PBS, and incubated for 60 min at 37 °C with chicken serum diluted in PBS containing 1% BSA and 0.01% Tween 80. Horseradish peroxidase-conjugated mouse anti-chicken Ig light chain MAb, diluted at 1:500 in the same buffer, was used in the second incubation for 60 min at 37 °C. The plates were washed three times with PBS/0.05% Tween 80 after each incubation. Peroxidase activity was visualized by the addition of 2 mg/ml 3-aminoo-9-ethylcarbazole (Sigma) in 0.05 M sodium acetate (pH 5.0) containing 0.01% hydrogen peroxide.

Antibodies against β-galactosidase were detected using a similar assay with the following modifications. CEF transfected with pCH110 and fixed 2 days post-transfection as described above were used as antigen. The chicken sera were diluted in PBS containing 0.05% Tween 80 and 4% horse serum.

Statistical analysis. Where appropriate, the Wilcoxon test for unpaired samples was used (Snedecor & Cochran, 1967).

Results

Development of recombinant MDV strains

The different concentrations of the ApoI–XhoI fragment of pBSL_LacZ were co-transfected with 4 µg CVI988 DNA p34 resulting in 251/1504 (17%) plaques staining for β-galactosidase over a 24 h staining period. Thirty-four plaques were selected for passage based on staining within a 4 h period. These plaques were obtained with all levels of plasmid DNA. Four additional screening cycles were needed before two mutant strains, CVIL_LacZ-A and CVIL_LacZ-B, were obtained with 100% blue-staining plaques. Both mutant strains were derived from the co-transfection using 0.4 µg plasmid. These strains were propagated in CEF and frozen at p45. Virus titres were determined in CEF and dilutions with approximately 100 plaques per culture were stained for β-galactosidase activity. Only blue plaques were present based on counting 287 and 268 plaques for CVIL_LacZ-A and -B, respectively. In vitro replication of the recombinant strains and development of cpe were comparable to those features of the parent strain at the same passage level based on microscopic examination of plaques and the time required for the development of plaques.

DNA prepared from p44 of both recombinant strains was used for PCR analysis to determine if both copies of ORF L1 were deleted, using primers 1 and 2 for the amplification of a 407 bp fragment of ORF L1. In addition, the same DNA was used to amplify a 189 bp fragment of lacZ using primers 3 and 4. DNA extracted from CVI988 was used as a control for ORF L1 and a plasmid containing lacZ was used as a control for the lacZ amplification. Recombinant strains CVIL_LacZ-A and -B were negative by ethidium bromide staining and Southern blot hybridization for the 407 bp fragment, indicating that recombination had occurred in the TRL and IRL. Both mutant strains were positive for the 189 bp fragment of lacZ (Fig. 3).

The mutant strain CVIUS10LacZ was developed in a similar way. Stocks of this virus were frozen at p45. Virus titrations were verified for the presence of the lacZ insert by staining for β-galactosidase activity. All plaques stained blue suggesting that this passage contained the insert. Restriction enzyme analysis and hybridization showed that the lacZ cassette was present within US10 (data not shown).

Pathogenesis of infection with deletion mutant strains

The data for experiment 1 are summarized in Table 1. Although the inocula varied over a wide range from 362 to 5012 p.f.u., this was not a major factor due to the cell-associated nature of MDV. Virus could be reisolated from all virus-infected groups between 4 and 6 days p.i. (lytic phase of the infection). Likewise, virus antigens were detected in the lymphoid organs independent of the virus strain used. In general, there was good correlation between the presence of virus antigens in the lymphoid organs and virus isolation. At 12 and 19 days p.i. virus could be reisolated from all groups but most of the birds did not express virus antigen in the lymphoid organs. All plaques were positive for β-galactosidase activity except one of the plaques isolated at 19 days p.i. from one bird inoculated with CVIL_LacZ-B. This plaque (I-116) was passaged twice in CEF and DNA was extracted for PCR analysis (see below). Spleen cells were tested at 12 and 19 days...
RF DNA. (7, CVI988; 8, H2O control. (52x336)
3, CEF; 4, blank; 5, CVI988; 6, H2O control; 7, CVIL1LacZ-A p44, CVIL1LacZ-B p44, CVI988 p36 or DNA isolated from recombinant strains (Table 2). All plaques isolated from the inoculated cultures. Three (II-90, II-130, II-134) and two (II-98, II-136) white plaques were obtained from cultures inoculated with spleen cells from infected with CVIL, LacZ-A and -B, respectively. DNA was isolated from all white plaques after two and five additional passages in CEF. Spleen lymphocytes obtained from some chickens infected with the recombinant viruses were positive for β-galactosidase activity in the absence of virus antigens (Table 4).

Fig. 3. PCR analysis of MDV DNA isolated from CEF inoculated with CVIL, LacZ-A p44, CVIL, LacZ-B p44, CVI988 p36 or DNA isolated from control cells. Primers 3 and 4, and 1 and 2 were used to amplify the lacZ fragment (a) and the 407 bp fragment of ORF L1 (b), respectively. The PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide (a, b). The DNA was transferred to a nylon filter and hybridized to the 189 bp lacZ fragment (c) and the Adel-EcoRI fragment of ORF L1 (d). (a) Lanes 1, HaeIII-digested PhiX174 RF DNA; 2, blank; 3, CVIL, LacZ-A; 4, CVIL, LacZ-B; 5, CEF; 6, blank; 7, CVI988; 8, H2O control. (b) Lanes 1, CVIL, LacZ-A; 2, CVIL, LacZ-B; 3, CEF; 4, blank; 5, CVI988; 6, H2O control; 7, HaeIII-digested PhiX174 RF DNA. (c, d) Lanes 1, CVIL, LacZ-A; 2, CVIL, LacZ-B; 3, CEF; 4, blank; 5, CVI988; 6, H2O control.

p.i. for β-galactosidase activity: 1/8, 3/6 and 4/8 birds inoculated with CVIL, LacZ-A, CVIL, LacZ-B, and CVIUS10LacZ were positive, respectively.

In experiment 2 virus could be isolated from all four groups at 5 days p.i., but there was a significant difference in the rate of virus isolation between CVI988 p45 and the three recombinant strains (Table 2). All plaques isolated from the recombinant strains were positive for β-galactosidase activity with the exception of two plaques (birds II-35 and II-52), which were obtained from one chicken infected with CVIL, LacZ-A and one chicken infected with CVIL, LacZ-B. However, other plaques isolated from these two birds were positive for β-galactosidase activity. DNA was obtained from these plaques after two additional passages in CEF for PCR analysis (see below).

Spleen cells from chickens inoculated with CVI988 p45 were positive for virus antigen by direct FA test on frozen sections and individual spleen cell examination. The expression of virus antigens in tissues from chickens infected with CVIL, LacZ-B was comparable to that in tissues from birds infected with CVI988 p45 (Table 3). In contrast, tissues from CVIL, LacZ-A- and CVIUS10LacZ-infected birds had significantly lower levels of virus antigen-positive cells (P < 0.01). Lymphocytes obtained from chickens infected with CVIL, LacZ-A or -B or CVIUS10LacZ expressed β-galactosidase activity. The level of expression and the number of positive birds were highest in the group inoculated with CVIL, LacZ-B. The difference in number of positive cells was significant, comparing CVIL, LacZ-A and -B (P < 0.01) (Table 3). Lymphocytes obtained from the two birds yielding a white plaque on virus isolation were positive for β-galactosidase activity.

The effect of the deletion in ORF L1 on the latent phase of infection was investigated at 20 and 21 days p.i. The data obtained for both days were similar and were pooled for both days. Virus antigens were not detected by direct FA assays in spleen sections and duplicate smears of 200,000 lymphocytes per bird independent of the challenge virus (data not shown), suggesting that latent infections had been established. Virus was reisolated from all groups, but not from all birds (Table 4). The level of virus infection was low (1 to 2 virus-positive lymphocytes per 500,000 cells) in all groups, and positive chickens were occasionally identified after a blind passage of the inoculated cultures. Three (II-90, II-130, II-134) and two (II-98, II-136) white plaques were obtained from cultures inoculated with spleen cells from infected with CVIL, LacZ-A and -B, respectively. DNA was isolated from all white plaques after two and five additional passages in CEF. Spleen lymphocytes obtained from some chickens infected with the recombinant viruses were positive for β-galactosidase activity in the absence of virus antigens (Table 4).

Antibodies against MDV were detected in variable numbers of chickens infected with all viruses. The group infected with CVI988 p45 had the highest number of positive sera and the highest mean titre (Table 4). Antibodies against β-galactosidase were present in 4/18 (titres 1:20) and 2/18 (titres 1:20 and 1:40) chickens inoculated with CVIL, LacZ-A and -B, respectively. Sera from chickens inoculated with CVIUS10LacZ were negative for these antibodies.

Analysis of virus DNA isolated from infected chickens

The isolation of white plaques from lymphocytes infected with CVIL, LacZ-A or -B suggested that in vivo recombination occurred or that a minor population of wild-type virus could be detected only after in vivo amplification. All white plaques and selected blue plaques were analysed by PCR assays to determine if intact ORF L1 could be detected. In the first series of PCR assays, DNA from three blue plaques, three white plaques, and CVI988 p34 was amplified. The expected fragment for intact L1 (407 bp) was only detected in DNA amplified from CVI988 p34, but not in DNA obtained from the white or blue plaques. A 189 bp fragment was amplified from...
Table 1. Effect of deletion of 200 bp in ORF L1 on the pathogenesis of infection of MDV strain CVI988 (experiment 1)

Numbers of positive birds out of the total at each day p.i. are shown.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Virus isolation*</th>
<th>Virus antigen in lymphoid organs†</th>
<th>MDV antibodies‡</th>
<th>No. positive birds/total for all tests</th>
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* Two 35 mm CEF cultures were inoculated with 500 000 spleen cells per bird. Data for 4, 5 and 6 days p.i. (lytic phase of infection) were pooled.
† Pooled data for bursa of Fabricius, spleen and thymus. Frozen sections were examined by direct immunofluorescence assay for the presence of virus antigens using FITC-conjugated chicken anti-MDV serum.
‡ The presence of antibodies was determined using an immunoperoxidase monolayer assay. Sera were examined at a dilution of 1:20.

Table 2. Effect of deletion of 200 bp in ORF L1 on the pathogenesis of infection of MDV strain CVI988 (CVI) at 5 days p.i. Virus isolation (experiment 2)

| Inoculum          | Virus isolation* | No. of Percentage of No. p.f.u. per 5 × 10⁵ cells |
|-------------------|------------------|-----------------------------------------------|-----------------------------------------------|
|                   | Virus           | No. of birds | Percentage of birds positive | Median† | Range |
|                   | Virus           | No. p.f.u. |           |               |       |
|                   | Virus           |           |           |               |       |
| None              | –               |           |           |               | 0     | –    |
| CVI p45           | 340             | 17       | 94        | 26³          | 0–136 |
| CVII,LacZ-A       | 640             | 17       | 67        | 1³           | 0–5   |
| CVII,LacZ-B       | 607             | 17       | 82        | 3³           | 0–16  |
| CVIII10LacZ       | 380             | 17       | 59        | 1³           | 0–44  |

* Two 35 mm CEF cultures were inoculated with 500 000 spleen cells per bird.
† Median values followed by a different superscript upper case letter are significantly different at P < 0.01.

the DNA obtained from blue plaques using the lacZ-specific primers (data not shown).

In a second series of PCR reactions, DNA was examined from additional white and blue plaques, and DNA obtained from uninfected CEF, using primers 1 and 2. A fragment of approximately 4·4 kb was amplified from pBSL,LacZ (Fig. 4a, lane 9), which is the expected size for the lacZ cassette flanked by the two ORF L1 fragments (Fig. 1d). A similar sized fragment was amplified using DNA from four blue plaques (I-54, I-64, I-77 and I-81), but this band was not obtained with DNA from the white plaque I-116. The 407 bp fragment representing the L1 fragment was only amplified from p34 MDV (Fig. 4a). Additional bands were observed but these were considered to be non-specific since they were also present after amplification of DNA from non-infected, control CEF. DNA from all white plaques obtained from experiment 2 was also analysed using these PCR conditions. Bands of 4·4 kbp representing the complete lacZ insert or 407 bp representing L1 were not detected using DNA from the white plaques (Fig. 4b). In a third series of PCR reactions DNA was amplified from uninfected CEF, wild-type CVI988 p36, two blue plaque viruses (I-64 and I-81), three white plaque viruses (II-90, II-98 and II-134), and DNA extracted from the MD lymphoblastoid cell line MDCC-CU41 (CU41) (an additional, positive control for MDV DNA) using primers 1 and 2. The amplified products were analysed by gel electrophoresis and Southern hybridizations. Only the wild-type CVI988 and CU41 were positive using the NdeI–EcoRI probe, confirming the absence of this fragment in all recombinant white plaque and blue plaque viruses (Fig. 4c). When the same DNA was amplified with
Table 3. Effect of deletion of 200 bp in ORF L1 on the pathogenesis of infection of MDV strain CVI988 (CVI) at 5 days p.i. Expression of virus antigen and β-galactosidase activity in spleen lymphocytes (experiment 2)

See Table 2 for virus dose and number of chickens per group. Six controls were negative for all assays.

<table>
<thead>
<tr>
<th>Virus inoculum</th>
<th>Frozen sections*</th>
<th>Spleen cells†</th>
<th>β-Galactosidase staining in spleen cells‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage</td>
<td>Average Score</td>
<td>Percentage</td>
</tr>
<tr>
<td>CVI p45</td>
<td>88</td>
<td>1.0</td>
<td>94</td>
</tr>
<tr>
<td>CVIL_LacZ-A</td>
<td>59</td>
<td>0.6</td>
<td>59</td>
</tr>
<tr>
<td>CVIL_LacZ-B</td>
<td>94</td>
<td>1.1</td>
<td>76</td>
</tr>
<tr>
<td>CVIU510LacZ</td>
<td>76</td>
<td>0.9</td>
<td>47</td>
</tr>
</tbody>
</table>

NT, Not tested.
* Sections of spleens were examined by immunofluorescence assay using FITC-conjugated chicken anti-MDV serum. The scoring system is detailed in Methods.
† Duplicate smears of 200000 spleen cells per bird were examined for the presence of virus antigens by immunofluorescence using FITC-conjugated chicken anti-MDV serum. Median values followed by a different superscript upper case letter are significantly different.
‡ Duplicate smears of 200000 spleen cells per bird were stained for β-galactosidase activity. Median values followed by a different superscript upper case letter are significantly different.

Table 4. Effect of deletion of 200 bp in ORF L1 on the pathogenesis of MDV strain CVI988 (CVI) at 20 and 21 days p.i. (experiment 2)

See Table 2 for virus dose. Six control chickens were negative for all tests.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of birds</th>
<th>Virus isolation*</th>
<th>β-galactosidase†</th>
<th>MDV Ab‡</th>
<th>Mean MDV Ab titre</th>
<th>Total percentage of birds positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVI p45</td>
<td>18</td>
<td>78</td>
<td>0</td>
<td>83</td>
<td>53</td>
<td>94</td>
</tr>
<tr>
<td>CVIL_LacZ-A</td>
<td>18</td>
<td>33</td>
<td>6</td>
<td>17</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>CVIL_LacZ-B</td>
<td>18</td>
<td>39</td>
<td>22</td>
<td>44</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>CVIU510LacZ</td>
<td>17</td>
<td>18</td>
<td>18</td>
<td>29</td>
<td>10</td>
<td>53</td>
</tr>
</tbody>
</table>

* Two 35 mm CEF cultures were inoculated with 500000 spleen cells per bird.
† Duplicate smears of 200000 cells per bird were examined for β-galactosidase activity.
‡ The titres of MDV antibodies were determined using an immunoperoxidase monolayer assay.

Discussion

Ohashi et al. (1994) and shortly afterwards Peng et al. (1995) described a small ORF (ORF L1) as part of a bicistronic mRNA, which also contains the ORF for meq. The function of this ORF is currently unknown. However, it is of interest to note that Pratt et al. (1992) reported the presence of only two MDV-specific transcripts in RECC-CU210, a reticuloendotheliosis virus-transformed cell line superinfected with MDV. This cell line does not express any MDV proteins detectable by antisera obtained from MDV-infected chickens. The 0.6 kb transcript was mapped to the L fragment of the BamH-I library of MDV DNA. Ohashi et al. (1994) later found that this transcript was identical to ORF L1. Based on these findings it was suggested that ORF L1 could be involved in the induction and/or maintenance of latency, or, as an alternative explanation, that ORF L1 is important for oncogenesis. In this communication it was suggested that ORF L1 could be involved in the induction and/or maintenance of latency, or, as an alternative explanation, that ORF L1 is important for oncogenesis. In this communication the hypothesis that ORF L1 is important for latency was tested by creating a deletion mutant in the CVI988 strain of MDV. Two mutant strains were obtained in which a
193 bp fragment of ORF L1 was replaced by a lacZ cassette in the TRL and IRL. Although p34 of CVI988 is no longer oncoenic, this strain was selected because earlier studies (B. J. L. Hooft van Iddekinge, unpublished data) suggested that mutant MDV CVI988 strains could be generated with relative ease. The reisolation of CVIL1LacZ-A and -B from inoculated chickens during the lytic infection (4–6 days p.i.) and latent infection (12–21 days p.i.) periods clearly indicates that ORF L1 is not essential for either of these phases of infection or for virus reactivation. It will be important to construct oncoenic MDV strains lacking ORF L1 to determine if this ORF is essential for oncogenesis. Attempts to construct these mutants using the vvMDV strain RB-1B are currently in progress. It is of interest to note that deletions in the meq ORF resulted in a marked decrease in virus replication (Kent et al., 1997).

The isolation of some white plaques from chickens inoculated with CVIL1LacZ-A and -B while other plaques obtained from the same birds were blue was unexpected based on the inability to detect intact ORF L1 by PCR and Southern hybridizations in the two mutant strains used to inoculate chickens (Fig. 3). The detection of white plaques could be caused by the presence of wild-type virus at levels below the detection limits of the PCR. This explanation is highly unlikely because the deleted NdeI–EcoRI fragment of ORF L1 could not be demonstrated in DNA obtained from the white plaques. The absence of intact ORF L1 in white plaque viruses confirms that the reisolation of mutant virus from lymphocytes was not caused by a contamination of CVIL1LacZ-A or -B with wild-type CVI988 containing a functional ORF L1. The inability to detect either the 4.4 kb fragment of ORF L1 with the lacZ insert using primers 1 and 2 or the 189 bp fragment of lacZ using primers 3 and 4 (Figs 2 and 4) suggests that some type of rearrangement occurred during in vivo replication. The nature of these changes has not been determined.

In contrast with CVIL1LacZ-A and -B, white plaques were not obtained after reisolation of CVIUS10LacZ. Earlier studies using lacZ insertions in gC and US10 had also demonstrated that genes inserted in these locations were stable (Sakaguchi et al., 1994). It is possible that in vivo recombinations occur in the repeats more frequently than in the unique sequences (Hirai et al., 1992).

Spleen cells were examined for expression of lacZ under control of the SV40 early promoter and for the expression of MDV proteins during the lytic and latent phases of infection. lacZ and MDV proteins were expressed during the lytic infection. Interestingly, the number of lacZ-expressing lymphocytes was often higher than the number of lymphocytes expressing MDV proteins with all three mutant strains (Table 3). Apparently, many virus-positive cells were already in the latent phase of infection for MDV, while β-galactosidase was still produced. Similarly, lacZ-positive cells were present in a small percentage of chickens at 20 and 21 days p.i. while MDV proteins could not be detected in lymphocytes from these birds (Table 4). Apparently, the SV40 promoter can be functional even in latent MDV infections.

Based on these results ORF L1 could be used to express foreign genes in recombinant MDV-based vaccines; in cell culture the insertion of lacZ appeared to be stable. On the other hand, in infected chickens rearrangements within the lacZ insertion may occur since white plaques were isolated from some lymphocytes. However, the majority of the chickens infected with CVIL1LacZ-B expressed lacZ during the lytic infection at the same or higher levels than chickens infected with CVIUS10LacZ, which might be the effect of duplication of the lacZ gene in the two repeats flanking UL in CVIL1LacZ-B. Only a single copy of the lacZ gene is present in CVIUS10LacZ with the insertion site being US10, a location which had been used for the development of recombinant vaccines (Sakaguchi et al., 1994). On the other hand, inoculation of CVIL1LacZ-A resulted in a lower level of expression of lacZ together with a significantly lower level of virus replication than inoculation of CVIL1LacZ-B (Table 4). Based on these observations it will be important to test a number of
recombinant strains expressing foreign genes to select those recombinants with a superior in vivo replication for vaccine development.

References


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