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Characterization of Meq proteins from field isolates of Marek's disease virus in Japan

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Abstract

Serotype 1 strains of Marek's disease virus (MDV-1) cause malignant lymphomas in chickens (Marek's disease; MD). Although MD has been controlled by vaccination, field isolates of MDV-1 have tended to increase in virulence and cause MD even in vaccinated chickens. Meq, a putative MDV-1 oncoprotein, resembles the Jun/Fos family of basic leucine zipper (bZIP) transcription factors and can regulate the expression of viral and cellular genes as a homodimer or as a heterodimer with a variety of bZIP family proteins. Sequencing analysis of some of the viral genes of various MDV-1 strains revealed a distinct diversity of and point mutations in Meq, which may contribute to changes in the transcriptional activities of Meq and, consequently, to increases in MDV-1 oncogenicity. However, few reports have characterized MDV-1 strains isolated in Japan. In this study, we established the amino acid sequences of MDV-1 field isolates from Japan in order to determine whether they display a distinct diversity of and point mutations in Meq. In addition, we analyzed the transactivation activities of the Meq proteins in order to evaluate whether the observed mutations affect their functions. Japanese MDV-1 isolates displayed the distinct mutations in basic region 2 (BR2) and proline-rich repeats (PRRs) of the Meq proteins as well as some unique mutations. Reporter assays revealed that the amino acid substitutions in BR2 and the PRRs affected the Meq transactivation activity. These results suggest that the distinct mutations are also present in the Meq proteins of MDV-1 isolates from Japan and affect their transactivation activities.

Keywords: Marek's disease virus, Meq, distinct diversity, transactivation activity

1. Introduction

Marek's disease virus (MDV: family, *Herpesviridae*; subfamily, *α-Herpesvirinae*; genus, *Mardivirus*; species, *Gallid herpesvirus* 2) is the causative agent of Marek's disease (MD), which manifests as malignant lymphomas in infected chickens (Calnek and Witter, 1997). MDV strains had been previously classified as serotypes but are currently reclassified as species: GaHV-2 (MDV serotype 1 (MDV-1)), *Gallid herpesvirus* 3 (MDV serotype 2), and *Meleagrid herpesvirus* 1 (MDV serotype 3 or herpesvirus of turkeys). It is the MDV-1 strains, except for the attenuated vaccine strains, that cause MD in chickens (Calnek and Witter, 1997). Although MD formerly caused serious economic losses to the poultry industry, it is currently well controlled by vaccination (Schat, 1987). Attenuated strains of MDV-1 and the naturally non-oncogenic serotypes 2 and 3 have been used as monovalent or bivalent vaccines; an attenuated MDV-1 strain, CVI988, is considered to be the most protective vaccine currently available and has been introduced in many countries (Witter, 1998). However, MDV-1 field isolates have tended to increase in virulence in some countries (Barrow and Venugopal, 1999; Raja et al., 2009; Sung, 2002; Witter, 1997). Pathogenic MDV-1 strains have been generally classified as mild (mMDV), virulent (vMDV), very virulent (vvMDV) and very virulent + (vv+MDV) MDV based on their abilities to resist immunities induced by different types of vaccines (Witter, 1997). Therefore, highly virulent MDV-1 strains could potentially cause future outbreaks despite vaccination (Witter, 1997). While MD cases are sporadically reported in Japan, the pathogenicity and virulence of Japanese MDV-1 field isolates have not been characterized.

The molecular mechanisms of MDV-1 oncogenicity and pathogenicity have been difficult to elucidate due to the lack of appropriate chicken T cell transformation systems. The search for viral factors related to MDV-1-induced latency and tumorigenicity identified the viral protein Meq as a putative oncoprotein (Jones et al., 1992). Meq is present only in MDV-1 strains and is consistently expressed in MDV-1-transformed cell lines and tumor samples (Peng and Shirazi, 1996; Peng et al., 1995; Qian et al., 1995; Ross et al., 1997). The *meq* gene encodes a 339-amino acid protein with an N-terminal basic region leucine zipper (bZIP) domain and a C-terminal transactivation domain (Fig. 1) (Liu and Kung, 2000). The bZIP domain, similar to that of the Jun/Fos family of oncoproteins, consists of 2 stretches of basic residues (basic regions 1 and 2 (BR1 and BR2)) and a leucine zipper (Liu and Kung, 2000). The transactivation domain is characterized by 2.5 proline-rich repeats (PRRs), which contain several SH3-binding motifs (Liu and Kung, 2000). The overexpression of Meq in rodent and chicken fibroblast cell lines produced morphological changes and protected the expressing cells from apoptosis (Ajithdoss et al., 2009; Levy et al., 2005; Liu et al., 1998). Microarray analysis determined that the genes induced by v-Jun transformation, including JTAP1, JAC, and HB-EGF, were also upregulated by the expression of Meq in transformed DF-1 cells (Levy et al., 2005). In addition, Meq also upregulated the expression of Bcl-2 and Ski and downregulated the expression of DAP5 and Fas, a pattern consistent with an anti-apoptotic effect (Levy et al., 2005; Liu et al., 1998). Moreover, a *meq*-null recombinant mutant virus was completely nononcogenic (Lupiani et al., 2004). These observations strongly suggest that Meq plays a key role in the oncogenicity of MDV-1.

Several factors that could contribute to the enhanced virulence of MDV-1 field isolates have been identified (Chang et al., 2002; Lee et al., 2000; Shamblin et al., 2004). The most important of these findings were the distinct diversity of and mutations in the Meq proteins of highly virulent MDV-1 strains, especially in basic region 2 (BR2) and the proline rich repeats (PRRs) in the transactivation domain. The PRRs exhibit a transrepression effect, whereas the BR2 is involved in the nuclear and nucleolar localization and the DNA binding of Meq (Liu and Kung, 2000). Therefore, the mutations in BR2 and the PRRs may influence the transcriptional activity of Meq. Indeed, we recently showed that a proline-to-alanine substitution at position 217 of Meq, which is in the PRRs, enhanced Meq transactivation activity (Murata et al., 2011). Therefore, the distinct diversity of and point mutations in the Meq proteins could contribute to their transactivation activities and, consequently, may be responsible for the increased oncogenicity of these MDV-1 strains.

In this study, we investigated the amino acid sequences of the Meq proteins of MDV-1 field isolates collected in Japan from the early 1980s–2010. Comparison of the amino acid sequences of old and new isolates showed that the distinct diversity and point mutations were present in BR2 and the PRRs of the Meq proteins of field isolates in Japan. In addition, we evaluated the transactivation activities of these Meq proteins using dual luciferase reporter assays in order to determine whether the mutations affect Meq function. Our reporter assays revealed that the amino acid substitutions in BR2 and the PRRs contribute to the enhanced transactivation activities of the Meq proteins, and influence of the amino acid substitutions in BR2 seems to be dominant compared with those in the PRRs. These results suggest that the

distinct diversity and point mutations are present in the Meq proteins of field isolates from Japan and affect their transactivation activities.

2. Materials and Methods

2.1. Sample collection

Samples were collected from the early 1980s–2010 from poultry farms in Japan at which some chickens had developed MD symptoms. Samples from internal organs and/or feather tips of MDV-infected chickens were used for the detection and sequencing analysis of the *meq* genes.

2.2. DNA sequencing

Total cellular DNA samples were extracted from feather tips as previously described (Murata et al., 2007) and from internal organs by using SepaGene (Sankojunyaku, Tokyo, Japan) according to the manufacturer's instructions. Total cellular DNA samples were used as templates for PCR using *meq*-specific primer sets. PCR was performed with the primer set M-S (5′-ATGTCTCAGGAGCCAGAGCCGGGCGCT-3′) and M-AS (5′-GGGGCATAGACGATGTGCTGCTGAG-3′). Amplification was performed using 35 cycles of 94°C for 45 sec, 57°C for 45 sec, and 72°C for 1.5 min. The nucleotide sequences of the amplified *meq* genes were determined. The PCR products were gel-purified and cloned into the pGEM-T easy vector (Promega, Madison, WI). The plasmid containing the insert was purified by a standard mini-prep method and sequenced using the CEQ 2000 Dye

Terminator Cycle Sequencing method and the Quick Start kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. The obtained sequences were analyzed using the CEQ 2000 DNA analysis system (Beckman Coulter, Fullerton, CA).

2.3. Construction of expression plasmids

To construct Meq expression plasmids, total cellular DNA was extracted from each field sample or from chicken embryo fibroblasts infected with the vv MDV-1 strain RB1B (Schat et al., 1982), and the open reading frames (ORFs) of the *meq* genes were amplified from these samples by PCR using primers M-S- *Eco*RI and M-AS- *Not*I, which have the same sequences as M-S and M-AS but contain an *Eco*RI and a *Not*I site, respectively, at their 5′ ends. The amplified *meq* ORFs were digested with *Eco*RI and *Not*I. The digested fragments were cloned into the *Eco*RI and *Not*I sites of the pCI-neo vector (Promega, Madison, WI) to construct expression plasmids for wild type Meq from RB1B and field samples. In addition, a c-Jun expression plasmid was constructed as previously described (Okada et al., 2007). In brief, the chicken *c-jun* transcript was also amplified by PCR using primers cJ-S- *Xho*I (5′-CTCGAGAAGATGGAGCCTACTTTCTACGA-3′) and cJ-AS (5′-GTTTGGTTATACCACAACATCACAG-3′). The amplified fragments were subcloned into the pGEM-T easy vector (Promega, Madison, WI) and digested with *Xho*I and *Not*I. The digested fragments were cloned into the *Xho*I and *Not*I sites of the pCI-neo vector.

2.4. Introduction of point mutations into Meq expression plasmids

All point mutations were introduced into plasmids expressing wild type Meq from RB1B using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers used to introduce the point mutations are shown in Table 1.

2.5. Dual luciferase reporter assay

Reporter plasmids were constructed by inserting the Meq promoter region upstream of the firefly luciferase-coding region of the pGL3-Basic vector (Promega, Madison, WI). DF-1 cells were seeded in 24-well plates at 2.0×10^5 cells per well in 0.5 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and incubated at 39°C under 5% CO_2 for 24 h. The cells in each well were transfected with 500 ng of Meq expression plasmid, 500 ng of reporter plasmid, and 10 ng of control pRL-TK *Renilla* luciferase plasmid (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In the c-Jun co-transfection experiments, cells were transfected with 300 ng of Meq expression plasmid, 200 ng of c-Jun expression plasmid, 500 ng of reporter plasmid, and 10 ng of control plasmid pRL-TK. Cell lysates were prepared 24 h post-transfection with 1× Passive Lysis Buffer (Promega, Madison, WI). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) and a Luminescencer-JNR AB-2100 (Atto, Tokyo, Japan). The luminescence intensity of firefly luciferase was normalized to that of *Renilla* luciferase. Three independent experiments were performed.

2.6. Statistics

All values in figures are expressed as mean ± standard deviation. Statistical comparison was performed by analysis of variance followed by paired 2-group *t* test.

3. Results

3.1. Distinct diversity of the Meq proteins from MDV-1 field isolates in Japan

To determine whether the Meq proteins of MDV-1 field samples from Japan exhibit distinct diversity and point mutations, we obtained their nucleotide sequences and established their deduced amino acid sequences. Upon aligning the deduced amino acid sequences, we found the distinct mutations at each position in the Meq protein reported by Shamblin et al. (2004), including the distinct mutations at positions 77 and 80 in BR2, position 115, and position 217 in the PRRs (Table 2). In addition, some samples also contained unique mutations at positions 174, 176, and 193: a threonine-to-asparagine substitution at position 174, a proline-to-serine/leucine substitution at position 176, and a proline-to-serine substitution at position 193. The amino acid sequences of the Meq proteins analyzed in this study were classified into 6 groups (Table 3). The Meq proteins detected in relatively old isolates (from the early 1980s) had serine at position 193, whereas the Meq proteins detected in most of the recent field samples had proline at position 193. In contrast to the older isolates, the Meq proteins of some recent samples had lysine at position 77 and aspartate at position 80 in BR2 or serine/leucine at position 176 in the PRRs.

3.2. Transactivation activities of the Meq proteins from field samples

The observed substitutions at positions 174, 176, 193, and 217 of Meq were located in the PRRs of the transactivation domain. We recently reported that a proline-to-alanine substitution at position 217 in the PRR could affect the transactivation function of the Meq protein (Murata et al., 2011). Therefore, the substitutions observed in the PRRs may affect the transactivation activities of the Meq proteins. In addition, we confirmed that the amino acid substitutions at positions 77 and 80 in BR2 affected the transactivation activities of the Meq proteins (in preparation for the submission). To determine whether the observed substitutions in the Meq proteins affect their transactivation activities, the transactivation activity of each Meq was analyzed by a dual luciferase reporter assay using corresponding mutant Meq expression plasmids. In this study, we used the Meq promoter to evaluate the transactivation activities of the Meq proteins and performed co-transfections with Meq and c-Jun expression plasmids because these proteins can form a heterodimer that activates the Meq promoter (Levy et al., 2003). The transactivation activities of RB1B- and Nig-c1-Meq were almost the same, which was expected as they have the same amino acid sequence (Fig. 2). The transactivation activity of Tkc-c1-Meq was higher than that of RB1B-Meq, indicating that a proline-to-alanine substitution at position 217 could enhance transactivation activity (Fig. 2). The transactivation activities of the Meq proteins of Isk-c1, Gf-c1, MD239, and MD242 were very similar to but lower than that of RB1B-Meq (Fig. 2). Although the distinct mutations at positions 174, 176, or 193 were present in the PRRs of the Meq proteins

of Isk-c1, Gf-c1, MD239, and MD242, these proteins displayed no differences in transactivation activity (Fig. 2). Thus, the transactivation activities of the Meq proteins were affected by the amino acid substitution at position 217 in the PRRs when the amino acid residues were lysine, aspartate, and valine at positions 77, 80, and 115, respectively. On the other hand, the transactivation activities of the Meq proteins were similar when the amino acid residues were glutamic acid, tyrosine, and alanine at positions 77, 80, and 115, respectively. Therefore, the influence of the amino acid substitutions at position 115 or in BR2 on the functions of the Meq proteins may be dominant compared with those at positions 174, 176 and 193 in the PRRs.

3.3. Transactivation activities of RB1B-Meq proteins with point mutations

To determine whether the difference observed in the transactivation activities between RB1B-Meq and the Meq proteins of Isk-c1, Gf-c1, MD239, and MD242 depend on the amino acid substitutions at position 115 or in BR2, we constructed mutant RB1B-Meq expression plasmids and analyzed the transactivation activities of the resulting proteins. The transactivation activities of wild type RB1B-Meq and mutant RB1B-Meq with a valine-to-alanine substitution at position 115 (RB1B-Meq (V115A)) were almost the same, indicating that the transactivation activity of Meq was unaffected by the amino acid substitution at position 115 (Fig. 3). In order to determine whether the amino acid substitutions in BR2 affect the transactivation activity, we compared the transactivation activities of wild type RB1B-Meq, a mutant RB1B-Meq protein with a proline-to-leucine

substitution at position 176 (RB1B-Meq (P176L)), another Meq protein with a lysine-to-glutamic acid substitution, an aspartate-to-tyrosine substitution, and a proline-to-leucine substitution at positions 77, 80, and 176, respectively (RB1B-Meq (K77E, D80Y, P176L)), and Gf-c1-Meq (Fig. 4). The transactivation activity of RB1B-Meq (K77E, D80Y, P176L) was lower than that of wild type RB1B-Meq or RB1B-Meq (P176L) and similar to that of Gf-c1-Meq. No difference in the transactivation activities of wild type RB1B-Meq and RB1B-Meq (P176L) was observed, suggesting that a proline-to-leucine substitution at position 176 could not affect the transactivation activity. In addition, no difference in the transactivation activities of RB1B-Meq (K77E, D80Y, P176L) and Gf-c1-Meq was observed, although Gf-c1-Meq had a proline-to-alanine substitution at position 217. These results suggest that the amino acid substitutions in BR2 and the PRRs contribute to the enhanced transactivation activities of the Meq proteins, and the distinct mutations in the BR2 may predominantly affect the transactivation activity.

4. Discussion

MDV-1 is one of the potent oncogenic herpesviruses (Calnek and Witter, 1997). MDV-1 field isolates in various countries have tended to increase in virulence over time, but MDV-1 isolates from Japan had not previously been characterized. To date, several viral factors that could relate to the enhanced virulence of MDV-1 have been identified. Among them, one notable finding was that the distinct diversity of and point mutations in the Meq protein, which is the most important molecule in MDV-1 oncogenicity, correlate with the level of

virulence (Chang et al., 2002, Lee et al., 2000, Shamblin et al., 2004). Meq is associated with latent infection and involved in the transformation of lymphocytes but is not essential for cytolytic infection in the lymphoid organs and the feather follicular epithelium (Lupiani et al., 2004). Therefore, the distinct mutations in the Meq proteins may affect MDV-1 oncogenicity and thereby correlate with MDV-1 virulence. In this study, we established the deduced amino acid sequences of the Meq proteins of MDV-1 field isolates from Japan. In addition, since Meq contributes to MDV-1-mediated transformation by altering the expression of various cellular genes (Brown et al., 2009; Levy et al., 2003, 2005; Shchodolski et al., 2009, 2010), we analyzed the transcriptional functions of the Meq proteins of field isolates from Japan.

Previous studies in which the deduced amino acid sequences of the Meq proteins were aligned have noted some trends with respect to mutations and virulence (Shamblin et al., 2004). Lower-virulence MDV-1 strains showed point mutations at positions 77 and 80 in BR2. In addition, some low-virulence MDV-1 strains tended to include an amino acid insertion in the transactivation domain and an increased number of PRRs. Higher-virulence MDV-1 strains had point mutations at positions 153, 176, and 217 in the PRRs that interrupted stretches of four prolines at position 2. In this study, we found that some MDV-1 strains recently isolated in Japan had the same substitutions in BR2 and the PRRs, whereas the Meq variant including the amino acid deletion or insertion was not detected. Some field isolates contained the previously reported substitutions in BR2: a glutamic acid-to-lysine substitution at position 77 and a tyrosine-to-aspartate substitution at 80. In addition, some of

the Meq proteins also had the substitutions in the PRRs: a proline-to-leucine/serine substitution at position 176 and a proline-to-alanine substitution at 217. However, to date the substitutions at position 176 have only been detected in recent Japanese isolates. According to a recent report, a proline-to-arginine substitution at 176 was found in MDV-1 strains isolated in China (Zhang et al., 2011). Therefore, the patterns of the substitutions in the Meq proteins of field isolates seem to vary among countries, although the positions at which the substitutions were observed were almost the same. These observations suggest that different MDV-1 strains are prevalent in each country.

Among recent field isolates from Japan, the amino acid substitutions were observed in BR2 of the Meq proteins of Nig-c1, Kgw-c1, and Tkc-c1. We confirmed that the substitutions at positions 77 and 80 in BR2 could affect the transactivation activity and transformation capability (in preparation for the submission). In this study, the Meq proteins of RB1B, Tkc-c1, and Nig-c1 (K and D at positions 77 and 80) exhibited higher transactivation activities than other Meq proteins (E and Y at positions 77 and 80). These results indicate that the substitutions in BR2 are also found in the Meq proteins of recent field isolates from Japan and could affect the transactivation activity. On the other hand, the BR2 amino acid substitutions were not found in the old field isolates analyzed in this study. Therefore, MDV-1 field isolates from Japan may also have increased in virulence due to enhanced Meq-mediated oncogenicity.

We recently reported that a proline-to-alanine substitution at position 217 could affect Meq transactivation activity when the combination of amino acid residues at positions 283

and 320 was alanine and isoleucine, or valine and threonine, respectively (Murata et al., 2011). Indeed, the transactivation activity of Tkc-c1-Meq (A, V, T at positions 217, 283, 320) was higher than that of RB1B (P, A, I at positions 217, 283, 320) in this study (Fig. 2). However, no difference in transactivation activity was observed between mutated RB1B-Meq (K77E, D80Y, and P176L) and Gifu-c1-Meq, although the amino acid residue at position 217 of Gf-c1-Meq was alanine (Fig. 4). These results suggest that the influence of the amino acid substitutions in BR2 on the functions of the Meq proteins may be dominant compared with that at position 217 in the PRRs. On the other hand, no difference was observed in the transactivation activities between RB1B (P176L) and RB1B-Meq (Fig. 4), although the combination of the amino acid residues at positions 77 and 80 was lysine and aspartate. Therefore, the amino acid substitution at position 176 may not affect the transactivation activity. In addition, we found that the amino acid substitutions at position 193 in the PRRs may not affect the transactivation activity when the amino acid residues at positions 77 and 80 were glutamic acid and tyrosine, respectively, as no difference in the transactivation activity was observed among the Meq proteins of Isk-c1, Gf-c1, MD239, and MD242 (Fig. 2). Therefore, the amino acid substitutions in the PRRs may affect the transactivation activity only in combination with substitutions in BR2, although further studies are needed to clarify the influence of the combination of amino acid substitutions in BR2 and the PRRs on the transactivation activity of the Meq proteins. However, the amino acid residue at position 193 was proline in recent field isolates but serine in old isolates; in addition, the proline-to-serine/leucine substitution at position 176 was only observed in most of Meq

proteins from recent field isolates. Therefore, comparison of the Meq proteins of old and recent isolates revealed obvious trends in the substitutions at positions 176 and 193 in the PRRs, and these substitutions may therefore correlate with the transformation capabilities of the Meq proteins. Further studies are required to determine whether these substitutions affect the transformation ability. In addition, *in vivo* studies using molecular clones would be required to confirm whether these substitutions affect the virulence of MDV-1.

We previously reported that a 59 or 60-amino acid insertion was found in the PRRs of the Meq proteins encoded by CVI988 and CVI988/R6 (Lee et al., 2000), and this insertion increased the copy number of the PRRs. This longer Meq protein, termed L-Meq, was not detected in vvMDV and vv+MDV strains. whereas L-Meq-encoding strains, such as CU-2, BC-1, and JM, are classified as low-virulence m or vMDV (Shamblin et al., 2004). The transactivation activity of L-Meq was significantly lower than that of Meq (Chang et al., 2002). In this study, we found that the amino acid substitutions in BR2 and the PRRs contribute to the enhanced transactivation activities of the Meq proteins, and the distinct mutations in the BR2 could predominantly affect the transactivation activity compared with those in the PRRs. Since the amino acid residues at positions 77 and 80 in BR of CVI988-L-Meq were glutamic acid and tyrosine (Shamblin et al., 2004), the amino acid substitutions in BR may also contribute to the reduced transactivation activity. However, we could not deny a possibility that the amino acid insertion in PRRs of L-Meq affect the transactivation activity and transformation capability. Therefore, further studies are needed whether the insertion of the PRRs or the amino acid substitutions in BR could be responsible

for the reduced transactivation activity observed in L-Meq.

5. Conclusion

In the present study, we characterized Meq proteins from field isolates in Japan, and as previously reported, we found that the distinct diversity exists in the Meq proteins of MDV-1 strains recently isolated in Japan, and some of these mutations enhanced Meq transactivation activity. In addition, we also found the obvious trends in substitutions at positions 176 and 193, and these substitutions were unique to Japanese isolates. Therefore, the recent MDV-1 strains prevalent in Japan are different from old strains in Japan and those of other countries, and some mutations in the Meq proteins from recent isolates seem to affect their transcriptional activities.

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Figure captions

Fig. 1. Structure of the Meq protein. Meq encodes a 339-amino-acid-preotein consisting of an N-terminal proline-glitamine-rich (Pro/Gln), the basic region (BR1 and BR2), and the leucine zipper, as well as the transactivation domain. The transactivation domain is characterized by 2.5 proline-rich repeats (PRRs). The distinct mutations are present in BR and the PRRs.

Fig. 2. Comparison of the transactivation activities of the Meq proteins from RB1B and field isolates. The panel shows the Meq promoter-driven luciferase activities measured during transient expression of the Meq protein constructs from RB1B or field samples in transfected DF-1 cells. DF-1 cells were transfected with 500 ng of reporter plasmid combined with 10 ng of pRL-TK internal control plasmid. Meq expression plasmids were co-transfected with 300 ng of pCI-neo (indicated as "vector") or each Meq expression plasmid and 200 ng of pCI-c-Jun or pCI-neo. Luciferase activities were analyzed 24 h post-transfection as described in the Materials & Methods. Firefly luciferase activity is expressed relative to the mean basal activity in the presence of pCI-neo after normalization to *Renilla* luciferase activity. The lower panel indicates the amino acid residues at each position in the Meq proteins from RB1B and field samples. Three independent experiments were performed in triplicate. Error bars indicate standard deviations.

Fig. 3. Transactivation activity of a mutant RB1B-Meq protein that carries a

valine-to-alanine substitution at position 115. A Meq expression plasmid with a valine-to-alanine substitution at position 115 was constructed by site-directed mutagenesis. DF-1 cells were co-transfected with 500 ng of reporter plasmid, 10 ng of pRL-TK internal control plasmid, 300 ng of wild-type or mutant RB1B-Meq expression plasmid, and 200 ng of pCI-c-Jun expression plasmid. Luciferase activities were analyzed 24 h post-transfection, and firefly luciferase activity was expressed relative to the mean basal activity in the presence of pCI-neo after normalization to *Renilla* luciferase activity. Three independent experiments were performed in triplicate. Error bars indicate standard deviations.

Fig. 4. Transactivation activities of mutant RB1B-Meq proteins with amino acid substitutions in BRs and the PRRs. The expression plasmids used in this experiment were constructed by site-directed mutagenesis. DF-1 cells were co-transfected with 500 ng of reporter plasmid, 10 ng of pRL-TK internal control plasmid, 300 ng of wild-type RB1B- or Gifu- or mutant RB1B-Meq expression plasmid, and 200 ng of pCI-c-Jun expression plasmid. Luciferase activities were analyzed 24 h post-transfection, and firefly luciferase activity was expressed relative to the mean basal activity in the presence of pCI-neo after normalization to *Renilla* luciferase activity. The lower panel indicates the amino acid residues at each position in the Meq proteins. Three independent experiments were performed in triplicate. Error bars indicate standard deviations.

Table 1

Primers used for the construction of mutant Meq expression plasmids.

Name indicated in figures	Amino acid substitution (position)	Type	Sequence (5′-3′)
V115A	Valine-to-alanine (115)	FW	5′-CACGTCCCTGCGTGCACAGTTGGCTTGTC-3′
		RV	5′-GACAAGCCAACTGTGCACGCAGGGACGTG-3′
P176L	Proline-to-leucine (176)	FW	5′-ATCTGTACCCCCCTTCCTCCCGATA-3′
		RV	5′-TATCGGGAGGAAGGGGGGTACAGAT-3′
K77E	Lysine-to-glutamate (77)	FW	5′-GAGAAGACGCAGGGAGCAGACGGACTA-3′
		RV	5′-TAGTCCGTCTGCTCCCTGCGTCTTCTC-3′
D80Y	Aspartate-to-tyrosine (80)	FW	5′-CAGGAAGCAGACGTACTATGTAGACAA-3′
		RV	5′-TTGTCTACATAGTACGTCTGCTTCCTG-3′

Table 2

Amino acid substitutions in the Meq proteins of field isolates of MDV from Japan.

Year	Strain	Basic region					Transactivation domain							
		71	77	80	115	119[a]	153[b] PPPP	174	176[b] PPPP	193	217/276[b, d] PPPP	283/342[d]	320/379[d]	326/385[d]
Early	MD239	A	E	Y	A	C	P	N	P	S	A	A	I	T
1980s	MD242	A	E	Y	A	C	P	T	P	S	A	A	I	T
	MD245	A	E	Y	A	C	P	T	P	S	A	A	I	T
1997	Isk-c1	A	E	Y	A	C	P	T	S	P	A	A	I	T
2003	Isk-c2	A	E	Y	A	C	P	T	S	P	A	A	I	T
2004	Nr-c1	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Me-c1	A	E	Y	A	C	P	T	L	P	A	A	I	T
	Nig-c1	A	K	D	V	C	P	T	P	P	P	A	I	T
	Tkc-c1	A	K	D	V	C	P	T	P	P	A	V	T	T
2008	Gf-c1	A	E	Y	A	C	P	T	L	P	A	A	I	T
	Fks-c1[c]	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Fks-c2[c]	A	E	Y	A	C	P	N	P	S	A	A	I	T
2010	Aic-c1	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Aic-c2	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Gf-c2	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Gf-c3	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Gf-c4	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Gf-c5	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Hug-c1	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Kgw-c1	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Me-c2	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Oky-c1	A	E	Y	A	C	P	T	S	P	A	A	I	T
	Hrs-c1	A	E	Y	A	C	P	T	S	P	A	A	I	T
	RB1B (vvMDV)	A	K	D	V	C	P	T	P	P	P	A	I	T
	CVI988 (attenuated vaccine strain)	S	E	D	V	C	P	T	P	P	P	P	I	I

a) Putative retinoblastoma binding domain “LXCXE.”

b) Interruptions at position 2 of the direct proline repeats.

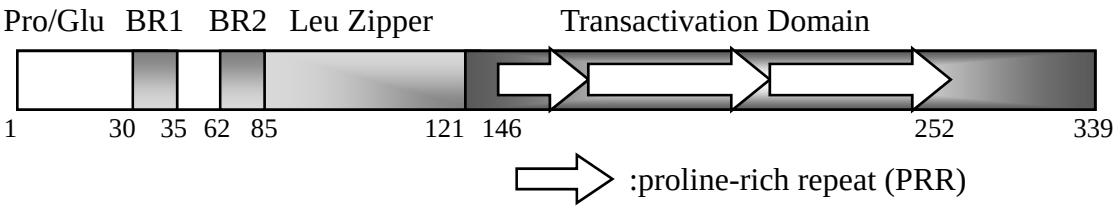
c) These isolates were derived from the same poultry farm.

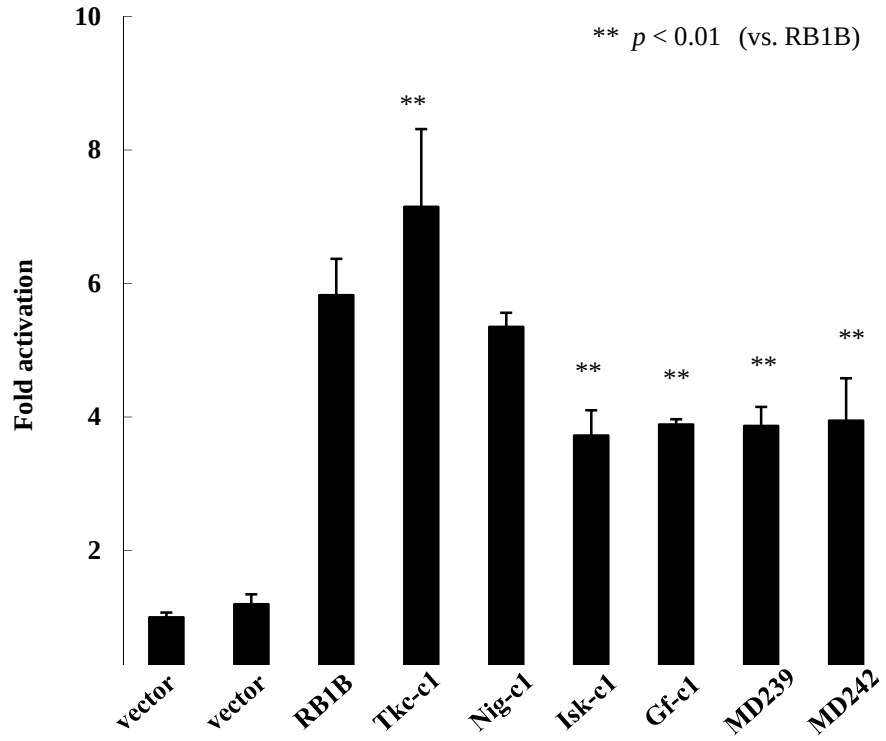
d) CVI988 contains 59 a.a. proline-rich amplification.

Table 3

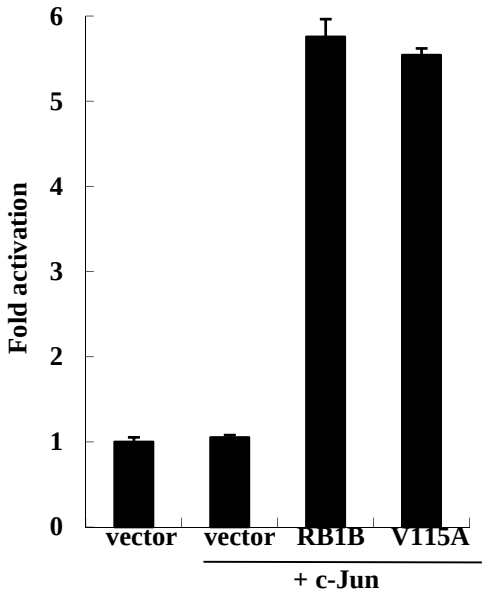
Comparison of the Meq protein amino acid sequences among field isolates from Japan.

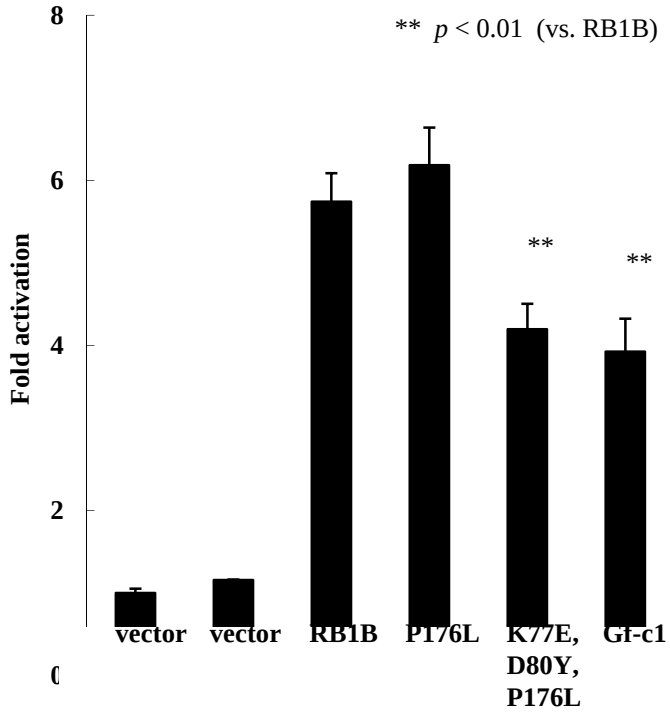
Amino acid sequence							Field samples from Japan
77	80	115	174	176	193	217	
E	Y	A	N	P	S	A	MD239, Fks-c2
E	Y	A	T	P	S	A	MD242, MD245
E	Y	A	T	S	P	A	Isk-c1, c2, Nr-c1, others
E	Y	A	T	L	P	A	Me-c1, Gf-c1
K	D	V	T	P	P	P	Nig-c1, Kgw-c1, (RB1B)
K	D	V	T	P	P	A	Tkc-c1


Pro/Glu
BR1
BR2
Leu Zipper
Transactivation Domain
1
30
35
62
85
121
146
252
339
:proline-rich repeat (PRR)



c-Jun	-	+	+	+	+	+	+	+	+	
77	-	-	K	K	K	E	E	E	E	BR
80	-	-	D	D	D	Y	Y	Y	Y	
115	-	-	V	V	V	A	A	A	A	Leu Zip
174	-	-	T	T	T	T	T	N	T	PRRs
176	-	-	P	P	P	S	L	P	P	
193	-	-	P	P	P	P	P	S	S	
217	-	-	P	A	P	A	A	A	A	


Fold activation
6
5
4
3
2
1
0
vector
vector
RB1B
V115A
+ c-Jun



c-Jun	-	+	+	+	D80Y, P176L +	+	
77	-	-	K	K	E	E	BR
80	-	-	D	D	Y	Y	
115	-	-	V	V	V	A	Leu Zip
174	-	-	T	T	T	T	PRRs
176	-	-	P	L	L	L	
193	-	-	P	P	P	P	
217	-	-	P	P	P	A	