Marek's disease virus-encoded Meq gene is involved in transformation of lymphocytes but is dispensable for replication

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Marek's disease virus (MDV) causes an acute lymphoproliferative disease in chickens, resulting in T cell lymphomas in visceral organs and peripheral nerves. Earlier studies have determined that the repeat regions of oncogenic serotype 1 MDV encode a basic leucine zipper protein, Meq, which structurally resembles the Jun/Fos family of transcriptional activators. Meq is consistently expressed in MDV-induced tumor cells and has been suggested as the MDV-associated oncogene. To study the function of Meq, we have generated an rMd5ΔMeq virus by deleting both copies of the *meq* gene from the genome of a very virulent strain of MDV. Growth curves in cultured fibroblasts indicated that Meq is dispensable for *in vitro* virus replication. *In vivo* replication in lymphoid organs and feather follicular epithelium was also not impaired, suggesting that Meq is dispensable for lytic infection in chickens. Reactivation of the rMd5ΔMeq virus from peripheral blood lymphocytes was reduced, suggesting that Meq is involved but not essential for latency. Pathogenesis experiments showed that the rMd5ΔMeq virus was fully attenuated in chickens because none of the infected chickens developed Marek's disease-associated lymphomas, suggesting that Meq is involved in lymphocyte transformation. A revertant virus that restored the expression of the *meq* gene, showed properties similar to those of the parental virus, confirming that Meq is involved in transformation but not in lytic replication in chickens.

Herpesviruses have evolved different strategies to persist in an often hostile cellular environment. One such strategy is to encode products that mimic cellular proteins that are able to interact with cellular pathways, altering the host environment to suit their own needs. Such molecular mimicries sometime go overboard, allowing viruses to overtake the cellular pathways resulting in oncogenic transformation. Marek's disease virus (MDV), a member of the *Alphaherpesvirinae* subfamily in the family *Herpesviridae*, is one such virus. Infection with MDV results in the induction of T cell lymphomas in chickens as early as 3–4 weeks after infection (1). On the basis of these observations, it was speculated that the virus is likely to encode an oncogene(s). To search for this possible oncogene(s), early studies focused on genes expressed in tumor cells. It was shown that transcriptional activity of MDV in tumor cells was confined to the repeat regions (2–4). Within these regions there are limited number of genes, including viral telomerase RNA (5), viral IL-8 (6, 7), *meq* (8), *pp38* (9), and *ICP4* (10). Of these genes, only Meq was consistently expressed in all MDV lymphoblast tumor cells (7, 8) and it is present in serotype 1 strains but not in nononcogenic serotypes 2 and 3 (11–13).

Meq is a 339-aa-long protein encoded within the MDV *Eco*RI Q fragment of serotype 1 MDV strains, and thus the name Meq (8). There are two copies of Meq in the MDV genome, one in each of the repeats flanking the unique long regions (TR_L and IR_L) (Fig. 1 and ref. 14). Meq contains domains for DNA binding, dimerization, and transactivation/repression activities (15, 16). The basic DNA binding and the leucine zipper regions at the N-terminal region of Meq are closely related to the Jun/Fos oncoproteins, whereas the C-terminal proline-rich domain structurally resembles the WT-1 tumor suppressor gene (17). The Meq protein also contains retinoblastoma binding and RNA-binding motifs, the functional significance of which is not fully understood (18, 19)

Due to the lack of an *in vitro* chicken T cell transformation model, biological properties of Meq have been studied in a rodent fibroblast (Rat-2) cell line. Rat-2 cells overexpressing Meq were highly resistant to apoptosis induced by tumor necrosis factor α, C2-ceramide, UV irradiation, and serum withdrawal (20). Overexpression of Meq in Rat-2 cells also lead to serum- and anchorage-independent growth, and was associated with morphological changes (20). This work was supported by Xie *et al.* (21), who showed that inhibition of *meq* transcripts in MDV-transformed tumor cells by antisense oligonucleotides to *meq* resulted in growth inhibition, suggesting that Meq is required for maintenance of the transformed state. Although the work cited above provides convincing evidence that, *in vitro*, Meq is a multifunctional protein involved in transactivation, antiapoptosis, and transformation, its role in MDV pathogenesis has not been shown due to the difficulty in generating *meq* deletion mutant.

In this report, we demonstrate the deletion of both copies of the *meq* gene from a very virulent strain of MDV by using overlapping cosmid clones (22). Pathogenesis studies in MDV-susceptible chicken showed that Meq is not essential for cytolytic infection in the lymphoid organs and in the feather follicular epithelium (FFE); however, Meq is involved in transformation of lymphocytes *in vivo*. In addition, inoculation of chickens with a revertant MDV virus expressing Meq resulted in tumor induction and pathogenesis similar to that of parental virus confirming that Meq is the protein responsible for MDV oncogenesis.

Materials and Methods

Cells and Viruses. Primary duck embryonic fibroblasts (DEFs) were used for virus propagation, virus reactivation assay, and DNA transfections. Growth curves were performed in primary chicken embryonic fibroblasts as described (23). Recombinant viruses were generated from cosmids derived from a very virulent MDV strain, Md5 (24).

Cosmids. MDV cosmid clones SN5, P89, SN16, A6, and B40, from the very virulent strain Md5 (24), encompassing the entire MDV

Abbreviations: MDV, Marek's disease virus; FFE, feather follicular epithelium; DEF, duck embryonic fibroblast; IFA, immunofluorescence assay; IHC, immunohistochemistry; pfu, plaque forming units.

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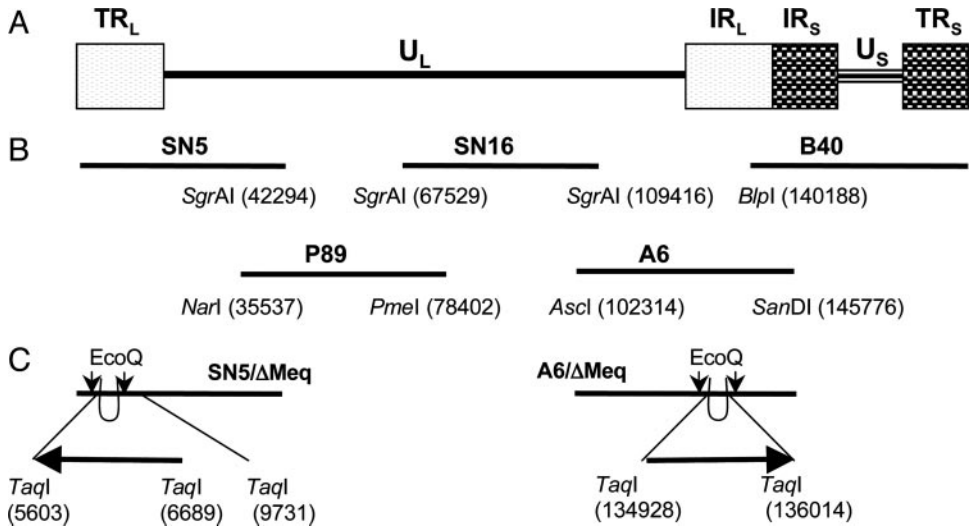

Fig. 1. Organization of the serotype 1 MDV genome. (*A*) The MDV genome consists of a unique long (U_L) region flanked by inverted repeats, terminal repeat long (TR_L), internal repeat long (IR_L), and a unique short region (U_S) also flanked by inverted repeats, internal repeat short (IR_S) and terminal repeat short (TR_S). (*B*) Schematic representation of the overlapping cosmid clones generated to reconstitute an infectious virus from a very virulent (vv) strain of MDV (Md5). The restriction enzymes used to generate the cosmid clones and their positions are indicated. (*C*) Location of *Taq*I restriction sites in cosmids SN5 and A6 used to delete the *meq* gene.

genome were used to generate recombinant Md5 viruses lacking the *meq* gene (ref. 22 and Fig. 1). Cosmids A6 and SN5, containing a copy of the complete coding sequence of the MDV unique gene *meq*, were used for the deletion of this gene by the RecA-assisted restriction endonuclease cleavage method (25). Briefly, two oligonucleotides, Meq Taq3′ (5′-TTT ATG TCA GTA AAT CGA TAA ATA ATG CCT TT-3′positions 5,589–5,620 and 136,000–136,031) and Meq Taq5′ (5′-ACG ATC CGT CCC CCC TCG ATC TTT CTC TCG GGT CG-3′ positions 6,673–6,707 and 134,913–134,947), located at both ends of the *meq* gene, were used to protect the *Taq*I sites (positions 5,603, 6,672, and 6,689 in SN5 cosmid and positions 134,928, 134,946, and 136,014 in the A6 cosmid) from methylation. The protected SN5 and A6 cosmids were methylated, by using *Taq*I methylase, denatured, digested with *Taq*I, religated, packaged and introduced into HB101 *Escherichia coli* cells. SN5 and A6 cosmid clones in which the *meq* gene had been deleted, SN5Δmeq and A6Δmeq, respectively, were identified by the loss of the 2,456-bp *Eco*Q fragment after digestion with *Eco*RI.

Transfections. To generate a mutant virus lacking the *meq* gene, rMd5Δmeq, 500 μg of *Not*I-digested and -purified cosmid DNA (P89, SN16, B40, SN5ΔMeq, and A6ΔMeq), along with 2 μg of sheared salmon sperm DNA, were used to transfect 1.2×10^6 DEF cells in 60-mm dishes by the calcium phosphate procedure as described (26). Five days after transfection, cells were trypsinized, seeded onto a 100-mm dish, and monitored for cytopathic effects. Viral stocks were subsequently made in DEFs for further analysis.

Revertant Virus. To generate a revertant virus from rMd5Δmeq containing the *meq* gene, rMd5ΔMeqR, Md5 EcoQ genomic DNA fragment was cotransfected into DEF cells with purified rMd5ΔMeq viral DNA. After viral plaques were evident, transfected cells were overlayed with 1.25% Bacto-Agar and harvested by trypsinization. Cells from each plaque were divided into two aliquots; one was used to reinfect a fresh 60-mm dish of DEFs, and the other was used for PCR analysis. Integration of the *meq* gene into the rMd5ΔMeq genome was detected by PCR using primers MeqG5498 (5′-GAG CCA ACA AAT CCC CTG AC-3′) and MeqL6910 (5′-CTT TCG GGT CTG TGG GTG T-3′) that would generate a 1,412- or 326-bp fragment in the rMd5ΔMeqR and rMd5ΔMeq, respectively.

Indirect Immunofluorescence Assay (IFA) and Immunohistochemistry (IHC). IFA of transfected DEF cells grown on 35-mm dishes was carried out as described (27). For IHC, lymphoid organs (thymus, spleen, and bursa of Fabricius), and feather follicles of infected and uninfected chickens were embedded in optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA), immediately frozen in liquid nitrogen and stored at −80°C until use. Four- to 8-μm-thick cryostat sections of tissue were prepared, fixed with cold ethanol for 5 min, and air-dried. Immunostaining was carried out by using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's instructions. For IFA staining, mAb H19, specific for MDV-unique protein pp38, was used at a working dilution of 1:300, and rabbit serum against Meq was used at a working dilution of 1:200. For IHC staining, monoclonal antibodies H19 and 1AN86 (specific for MDV gB protein) were used at a working dilution of 1:3,200 and 1:2,000, respectively.

Southern Blot. rMd5 and rMd5ΔMeq viral DNA were purified from nucleocapsids of infected cells. Five micrograms of each viral DNA were digested with *Eco*RI, separated on a 1% agarose TBE gel, and transferred to nylon membranes. The ^{32}P-dCTP-labeled probes representing the complete MDV genome (SN5, P89, SN16, A6 and B40 cosmid DNA fragments) or *Eco*Q fragment (2,456-bp fragment) were generated by random priming and were used to hybridize to viral DNA, using standard protocols.

Reactivation Assay. Buffy coats were obtained from heparinized blood by centrifugation at $500 \times g$ for 5 min. Lymphocytes were then counted and diluted to 10^6 cells per ml. For each chicken sample, duplicated 35-mm plates of freshly seeded DEF monolayers were inoculated with 10^5 and 10^6 lymphocytes, and viral plaques were counted 7 days after inoculation.

Pathogenesis Experiments. Chickens used in the studies were MDV-susceptible F_1 progeny (15×7) of the Avian Disease and Oncology Laboratory (U.S. Department of Agriculture, East

Lansing, MI) line $15I_5$ males and line 7_1 females. These chickens were free of maternal antibodies against MDV.

Experiment 1. To study the effect of *meq* deletion on cytolytic infection, chickens were randomly sorted into experimental groups and held in modified Horsfall–Bauer isolators for 2 weeks. One of the groups remained as a noninoculated control group, whereas the others were inoculated s.c. with 3,000 plaque forming units (pfu) of rMd5, rMd5Δmeq, or rMd5ΔMeqR at 1 day of age. Six days after inoculation, three randomly selected chickens from each group were killed, and lymphoid organs (thymus, bursa of Fabricius, and spleen) were collected and examined for viral antigen expression (pp38 and gB) by IHC. To examine *in vivo* virus replication during early cytolytic infection, two chickens from control group and five chickens each from rMd5- and rMd5Δmeq-inoculated groups were bled on day 8 after inoculation for viremia assay. To study the effect of Meq deletion on virus transmission, 14 days after inoculation, three randomly selected chickens from each group were killed, and the feather follicles were collected and examined for viral antigen (pp38) expression by IHC.

Experiment 2. To compare the pathogenic properties of rMd5, rMd5ΔMeq, and rMd5ΔMeqR, 17 susceptible chickens were inoculated with 1,500 pfu of rMd5, rMd5ΔMeq, or rMd5ΔMeqR viruses or were mock-infected at 1 day of age and raised in isolation for 8 weeks. Weekly mortality was recorded, and all chickens that died during the trial or were killed at the end of the experiment (8 weeks after inoculation) were necropsied and evaluated for MDV-specific gross tumors in the viscera and nerves. To examine establishment of latency and reactivation, five chickens from each experimental group were randomly selected and bled on days 19 and 42 after inoculation for viremia assay.

Results

Construction of Meq Deletion Mutant rMd5ΔMeq. By using overlapping cosmid clones, we have generated a mutant virus lacking both copies of the *meq* gene, rMd5ΔMeq. Plaques from recombinant rMd5ΔMeq and control rMd5 viruses were evident 10–12 days after transfection. To confirm the deletion of the *meq* gene, transfected cells showing plaques were examined by IFA with mAb H19 (anti-pp38) and rabbit anti-Meq polyclonal serum. As expected, rMd5 virus expressed both pp38 (data not shown) and Meq, whereas rMd5ΔMeq expressed pp38 but not Meq (Fig. 2). To verify that rMd5ΔMeq had the expected genome structure, *Eco*RI-digested genomic DNA from rMd5 and rMd5ΔMeq were examined by Southern blot. These viruses showed no detectable difference in the pattern of DNA fragments other than the Meq region (see below), suggesting that there were no gross rearrangements in the rMd5ΔMeq genome (Fig. 3, lanes 1 and 2). Probing of the *Eco*RI-digested DNA with a radiolabeled Md5 *Eco*Q fragment resulted in 2,456 bp in rMd5 and 1,370 bp in rMd5ΔMeq, due to the deletion of 1,086-bp fragment of the *meq* gene (Fig. 3, lanes 3 and 4). In addition, we found a 5,063-bp fragment in rMd5ΔMeq, which was unexpected (Fig. 3, lane 4). Further sequence analysis of the region surrounding the *meq* deletion indicated that in SN5ΔMeq cosmid there was deletion between two *Taq*I sites found at 5,603 and 9,731, instead of the expected from 5,603 to 6,689. This action resulted in the deletion of 4,128 bp instead of the expected 1,086 bp. This extended deletion resulted in the loss of *Eco*RI site corresponding to position 7,100, and therefore, the 1,370-bp *Eco*RI fragment from the TR_L migrated as a 5,063-bp fragment. Sequence analysis of this extended deletion indicated that no other large contiguous ORFs were deleted (14). However, because splicing and sense/antisense expression in this region is highly complex, it may affect gene expression. The size of *meq* deletion in the IR_L was as expected, thus, the extended deletion in the TR_L was not present on both copies of the repeats.

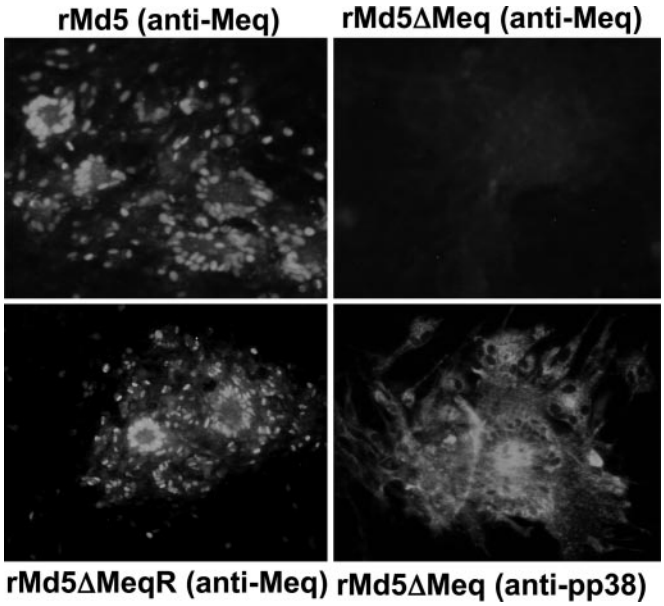

Fig. 2. Immunofluorescence analysis of DEF cells infected with recombinant viruses. Parental, rMd5, and revertant rMd5ΔMeqR express Meq protein, whereas the deletion mutant virus rMd5ΔMeq does not. The presence of rMd5ΔMeq virus was confirmed by staining of MDV-specific pp38 protein. Note the intranuclear expression of Meq and cytoplasmic expression of pp38.

***In Vitro* and *in Vivo* Replication of rMd5ΔMeq.** To determine whether the deletion of the *meq* gene had any effect on rMd5ΔMeq *in vitro* growth replication, the growth rate of rMd5ΔMeq virus was compared with that of rMd5 by single-step growth kinetics. Our results show that the growth characteristics of both viruses were similar at all time points tested (days 1, 2,

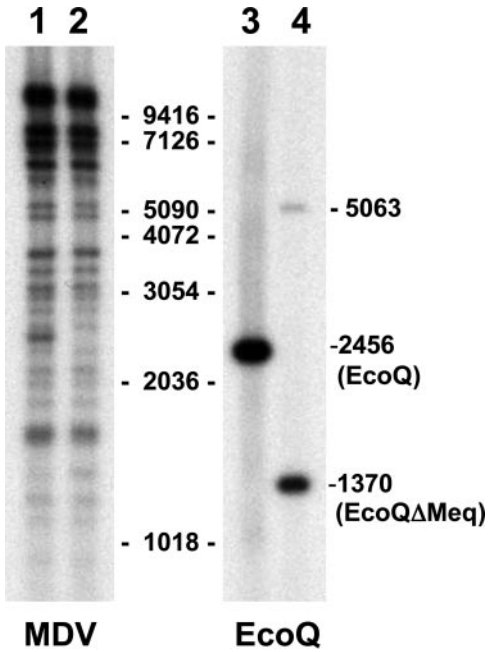

Fig. 3. Southern blot analysis of rMd5 and rMd5ΔMeq. DNA digested with *Eco*RI and probed with total viral MDV DNA (lanes 1 and 2) or an *Eco*Q (spanning *meq* gene)-specific probe (lanes 3 and 4) confirmed the deletion of a 1,085-bp fragment corresponding to the Meq gene (lane 4) and the absence of gross rearrangement in the viral genome (lanes 1 and 2). The band corresponding to the 5,063-bp fragment is explained in *Results*.

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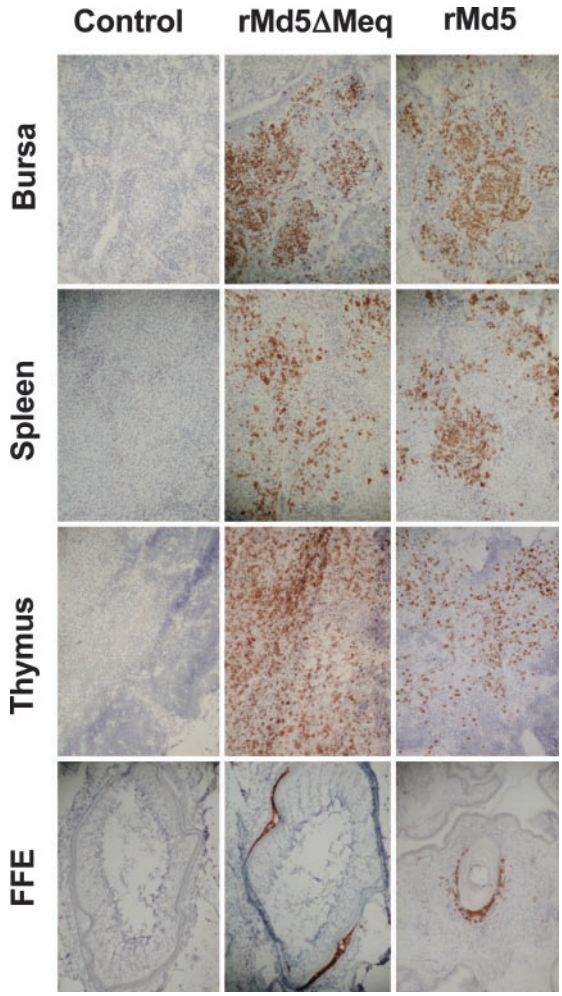

Fig. 4. Immunohistochemical analysis of lymphoid organs at 6 days (bursa, spleen, and thymus) and FFE at 14 days after inoculation of rMd5, rMd5Δmeq, and control chickens. Frozen sections were stained with pp38-specific mAb.

3, 4, and 5; data not shown) indicating that although Meq is expressed in MDV-infected cells, it is dispensable for *in vitro* growth.

To examine whether Meq plays a role in *in vivo* replication, MDV maternal antibody-negative chickens were inoculated with rMd5 or rMd5ΔMeq viruses at 1 day of age. Six days after inoculation, three randomly selected chickens from each group were killed, and lymphoid organs (thymus, bursa of Fabricius, and spleen) were collected and examined for viral antigen expression (pp38) by IHC. As seen in Fig. 4, there was a high level of expression of pp38 in the lymphoid organs of rMd5 and rMd5ΔMeq-inoculated chickens. To confirm that Meq deletion had no effect in viral replication, sections of lymphoid organs were also stained for gB, envelope glycoprotein expression. The results obtained confirm the data obtained with pp38 (data not shown) indicating that Meq is not essential for early cytolytic infection in lymphoid organs. However, virus isolation on day 8, from buffy coat cells isolated from rMd5 and rMd5ΔMeq-inoculated chickens indicated that there was a reduction of virus titer in rMd5ΔMeq group compared with parental rMd5 group (Table 1). An average of 300 pfu were recovered from the rMd5-inoculated group compared with 100 pfu from the rMd5ΔMeq-inoculated group when 10^6 lymphocytes were plated on DEFs. Virus titers at this early phase of infection are likely to reflect cytolytic infection in lymphocytes.

Table 1. Virus reactivation from peripheral blood lymphocytes

Virus	Day 8	Day 19	Day 42
rMd5	200, 245, 293, 407, 371	250, 268, 384, 470, 250	106, 232, 90*
rMd5ΔMeq	92, 85, 116, 117, 91	10, 21, 42, 194, 31	8, 2, 2, 1, 4
rMd5ΔMeqR	ND	312, 546, 225, 208, 310	91*
None	0, 0	0, 0, 0, 0, 0	0, 0

Reactivation assays were performed on days 8, 19, and 42 after inoculation. The numbers represent the average number of pfu observed on day 7, when 10^6 peripheral blood lymphocytes were cultured on DEF. Chickens assayed on day 8 are from experiment 1, which received 3,000 pfu, whereas chickens assayed on days 19 and 42 are from experiment 2, which received 1,500 pfu at 1 day of age.
*Tested fewer than five chickens per group because of mortality.

Transmission of rMd5ΔMeq. Transmission of MDV takes place after replication of virus in the FFE and release of infectious virus in the dander. The virus shed through the dander is extremely stable in the environment and serves as a source of infection (28). To examine whether Meq is necessary for virus replication in FFE, three randomly selected chickens from each group were killed, and the feather follicles were examined for viral antigen (pp38) expression by IHC. As shown in Fig. 4, both rMd5 and rMd5ΔMeq viruses had similar levels of pp38 expression in the feather follicles, indicating that Meq is not essential for secondary replication in the FFE, and presumably, for horizontal transmission.

Latency and Reactivation of rMd5ΔMeq. To examine whether Meq is involved in establishment of latency and reactivation, peripheral blood lymphocytes (buffy coat) isolated from chickens on days 19 and 42 were cocultivated with DEFs. As shown in Table 1, there was significantly lower virus recovery in rMd5ΔMeq compared with rMd5 virus at both time points tested. On day 19, an average of 60 pfu were recovered from rMd5ΔMeq compared with 303 pfu from rMd5-inoculated chickens (five per group). Similarly, on day 42, an average of only 3 pfu were recovered from rMd5ΔMeq compared with 143 pfu from rMd5-inoculated chickens (five per group). This reduced recovery of virus from peripheral blood lymphocytes from later stages of infection suggested that Meq might be involved in establishment of latency and/or reactivation.

Oncogenicity of rMd5ΔMeq. To determine whether the deletion of *meq* gene affects the pathogenic properties of MDV, chickens inoculated with rMd5 or rMd5ΔMeq were observed for mortality for a period of 8 weeks. All chickens that died during the experiment or at termination were examined for MDV-specific lesion, including gross tumors and nerve lesions. As indicated in Fig. 5, one chicken from mock-infected and rMd5ΔMeq groups died on day 8 due to nonspecific causes. MDV-associated mortality was observed in parental rMd5 group starting at 4 weeks after infection and only one chicken survived the duration of the experiment. There was no MDV-associated mortality in mock- or rMd5ΔMeq-inoculated groups. All the chickens in the rMd5 group had gross MDV-specific lesion, whereas none were observed in either rMd5ΔMeq- or mock-inoculated groups (Table 2). In addition, as in the mock-infected control group, no atrophy of the bursa of Fabricius and thymus was observed in rMd5ΔMeq group compared with massive atrophy in the rMd5 group (data not shown). It was interesting to note that even though there was robust early cytolytic infection in rMd5ΔMeq, the lymphoid organs were not atrophied. These data indicate

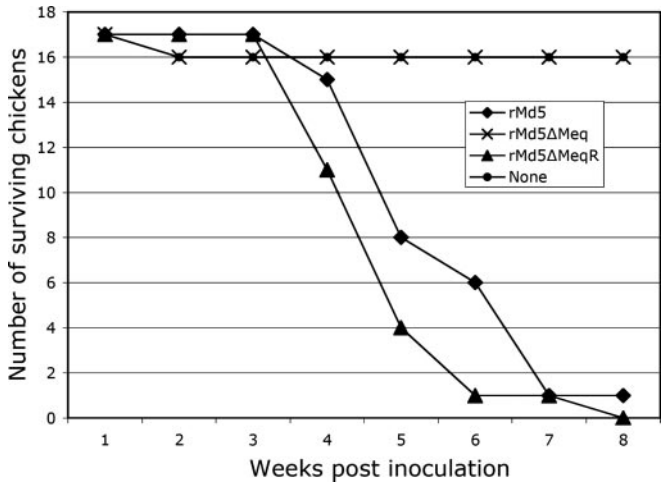

Fig. 5. Incidence of mortality in chickens inoculated with rMd5, rMd5ΔMeq, and rMd5ΔMeqR. Chickens were inoculated with 1,500 pfu of the indicated viruses at 1 day of age and maintained in isolation for 8 weeks. Uninoculated chickens served as negative controls. Weekly mortality was recorded. Chickens that died during the experiment were evaluated for MDV-specific gross lesions. One chicken from mock-infected and rMd5ΔMeq groups died on day 8 due to nonspecific causes.

that the deletion of the *meq* gene significantly decreases the virulence of the recombinant rMd5ΔMeq.

Construction and Biological Properties of Revertant Virus rMd5ΔMeqR. To verify that the phenotypic changes observed in the pathogenesis of rMd5ΔMeq were only due to the deletion of *meq*, we generated a revertant virus, rMd5ΔMeqR, by cotransfection of rMd5ΔMeq viral DNA with *Eco*Q fragment, containing the *meq* gene. Revertant viruses were selected by plaque purification and screened for the presence of the *meq* gene by PCR. In addition, expression of Meq in rMd5ΔMeqR-infected cells was confirmed by IFA (Fig. 2). As shown in Table 2, the pathogenic properties of the revertant rMd5ΔMeqR virus were fully restored, and tumors were observed in 100% of the inoculated chickens. The data indicate that the mortality in chickens started at 4 weeks and all of the chickens inoculated with rMd5ΔMeqR died before the end of the experiment (8 weeks; Fig. 5). These results confirm our observation that Meq plays an important role in MDV pathogenesis.

Discussion

MDV is a highly contagious herpesvirus, which elicits a rapid onset of malignant T cell lymphomas in chicken, usually within weeks after infection (1). Meq is the only protein persistently expressed in MDV tumors and MDV transformed T lymphoblast cell lines (8), and is thus likely to play a regulatory role in latency and transformation. Meq, a basic leucine zipper protein, associates with high affinity with a number of basic leucine zipper proteins like itself, c-Jun, JunB, ATF2, and Fos (29), and with less affinity to CREB, ATF1, ATF3, and C/EBP. The DNA-binding sequences of Meq homodimers and Meq/Jun heterodimers have been identified by cyclic amplification of selected targets (15) and confirmed by chromosomal immunoprecipitation techniques (30). Meq-binding sites are enriched in three regions of the MDV genome: the Meq promoter, ICP4 promoter, and the MDV origin of replication (or pp24/pp38 promoter). Transactivation studies with this DNA-recognition sites have shown that the Meq/Jun heterodimers transactivate the Meq promoter, whereas the Meq homodimers repress the pp14/pp38 promoter, indicating that Meq may play a role in latency and replication.

Table 2. Marek's disease in MDV maternal antibody-negative chickens

Virus*	No. of chickens that died/no. tested, %	No. of chickens with tumors†/no. tested, %
rMd5	16/17 (94.1)	17/17 (100)
rMd5ΔMeq	1/17 (5.9)‡	0/17 (0)
rMd5ΔMeqR	17/17 (100)	17/17 (100)
None	1/17 (5.9)‡	0/17 (0)

*All chickens were inoculated with 1,500 pfu of the indicated virus by subcutaneous route.
†Tumors indicate gross visible MDV-specific lymphoid tumors in visceral organs or nerves.
‡One chicken from rMd5ΔMeq and uninoculated groups died at day 8 due to nonspecific causes.

It is known that at 7–8 days after infection, or slightly later, MDV infection in the chicken lymphoid organs switches from productive to latent phase (1). Viremia, a latent infection of peripheral blood lymphocytes, can be detected by cocultivation of latently infected lymphocytes with fibroblasts. Therefore, viral titers measured beyond the 7–8 days after infection, are likely due to reactivation of virus from latently infected lymphocytes. Our *in vivo* experiments with the MDV Meq deletion virus showed that whereas the virus replicated at the parental rMd5 level during the early cytolytic infection (Fig. 4), virus reactivation measured at 8 and 19 days after infection was reduced by 3- and 5-fold respectively, compared with parental rMd5 levels (Table 1). These results indicate that Meq is dispensable for early cytolytic infection and supports previous hypothesis that Meq plays a role, but is not essential for latency and/or reactivation.

There is strong supportive evidence that Meq is an oncogene. Overexpression of Meq in a rodent fibroblast cell line (Rat-2) resulted in transformation, serum- and anchorage-independent growth, morphological changes, shortened G1 phase, and resistance to apoptosis-inducing factors (31). Overexpression of Meq in an immortalized chicken embryo fibroblast cell line (DF-1) gave similar results (A. M. Levy and H.-J.K., unpublished results), reinforcing the notion that Meq is an oncogene. *In vivo* experiments with the rMd5ΔMeq virus confirm that Meq is the MDV-associated oncogene. Whereas the rMd5ΔMeq virus did not induce any tumors in MDV-susceptible chickens, inoculation with parental or revertant viruses resulted in 100% Marek's disease. Histological examination of vagus and sciatic nerves (data not shown) confirmed the absence of MDV-associated tumor cells in rMd5ΔMeq virus-infected chickens, reaffirming that Meq is essential for transformation of lymphocytes. It has been documented that reduction or absence of early cytolytic infection correlates with absence or reduction of lymphomas (32–34). Because the rMd5ΔMeq virus was not impaired for early cytolytic infection, as observed by viral antigen expression (pp38 and gB) in lymphoid organs at 6 days after inoculation (Fig. 4), the loss of transformation observed is likely to be attributed to the transforming capability of Meq. Although it could be argued that the loss of transformation is a consequence of the reduction in viremia of the rMd5ΔMeq virus (Table 1), this is unlikely because pp38 (ref. 22 and S.M.R., unpublished results) and viral IL-8 (6, 35) null mutant viruses, which also present reduced viremia titers, are still able to induce transformation. Lack of oncogenicity of rMd5ΔMeq is reiterated by experiments with RB1B*meqlac*, a mutant independently constructed by the marker rescue technique in the background of the very virulent RB1B isolate. The RB1B*meqlac* mutant failed to cause tumors in chickens exposed to it by inoculation (0/31) or by contact (0/21), whereas the parent RB1B strain readily induced tumors in both inoculates (21/29) and contact-exposed chickens (11/22). Therefore, the observation that the

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rMd5ΔMeq and RB1B*meqlac* mutant viruses have no oncogenicity, argues strongly for the role of Meq in transformation.

It has recently been shown that Meq is able to transform immortalized chicken cells by activating the Jun/AP-1 pathway, and in MDV-transformed T cells, Meq and Jun are corecruited to the AP-1 site of the chicken IL-2 promoter, suggesting that a functional role of Meq is activating the IL-2 autocrine loop (30). These data, together with those presented here, suggest that Meq contributes to MDV oncogenesis by facilitating latency entry/reactivation and by mediating transformation of the target cells.

This report provides, to our knowledge, the first conclusive evidence that Meq is involved in MDV-associated transformation of lymphocytes in chickens. Future studies are needed to elucidate various functions of Meq by generating recombinant viruses with subtle mutations in the *meq* gene. The data presented here, as well as future studies with MDV Meq protein, may shed light on the role of basic leucine zipper proteins on the biology of Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus, two human herpesviruses of the *Gammaherpesvirinae* subfamily (36–38).

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