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FULL PAPER

Detection of the *meq* gene in the T cell subsets from chickens infected with Marek's disease virus serotype 1

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Abstract

The *meq* gene was thought to be only detected in Marek's disease virus serotype 1 (MDV 1) including a very virulent strain, Md5, while L-*meq*, in which a 180-bp sequence is inserted into the *meq* open reading frame, is found in other strains of MDV 1, such as CVI 988/R6. However, both *meq* and L-*meq* were previously detected by PCR in chickens infected with MDV 1, suggesting that MDV 1 may consists of at least two subpopulations, one with *meq*, the other with L-*meq*. To further analyze these subpopulations, we analyzed the time course changes in distribution of these subpopulations among T cell subsets from chickens infected with MDV1. Both *meq* and L-*meq* were detected in $CD4^+$ and $CD8^+$ T cells infected with strain Md5 or CVI 988/R6. The shift in MDV subpopulations from one displaying *meq* to the other displaying L-*meq* and/or the conversion from *meq* to L-*meq* occurred mainly in the $CD8^+$ T cell subset from Md5-infected chickens. PCR products corresponding to L-*meq* rather than *meq* were frequently amplified from the $CD8^+$ T cell subset from CVI 988/R 6 -infected chickens. These results suggest that a dominant subpopulation of MDV 1 changes depending on the T cell subsets, and that L-*meq* is dominantly present in the $CD8^+$ T cells which play a role in the clearance of pathogenic agents.

KEY WORDS : Marek's disease virus, *meq*, L-*meq*, $CD4^+$ T cell, $CD8^+$ T cell

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Introduction

Marek's disease virus (MDV) is the causative agent of Marek's disease (MD), which is characterized by $CD4^+$ T cell lymphoma and nerve enlargement caused by infiltration of lymphocytes and/or lymphoma cells[1,6]. The pathogenesis of MD can be chronologically divided into three phases : early cytolytic infection ; latent infection ; and secondary cytolytic infection with immunosuppression and tumor development[1,14,15]. Early cytolytic infection occurs mainly in B cells, and latently infected lymphocytes are mainly activated T cells[19]. In addition, target cells for transformation by MDV are mainly $CD4^+$ T cells, suggesting that latent infection in this T cell subset could be intimately related to the subsequent transformation by MDV[18].

MDV strains are classified into three serotypes based on their pathogenecity, and only strains of MDV serotype 1 (MDV 1) are oncogenic. An MDV 1-specific gene, *meq*, has been identified, and its gene product, Meq, a basic leucine zipper (bZIP) oncoprotein, has been detected in all tumor samples and established MDV-transformed T-cell lines examined thus far, suggesting that Meq protein plays an important role in the transformation by MDV[9,10,11,16]. However, the role of the Meq protein in MDV transformation remains to be determined. In the genome of strains of vvMDV 1, the *meq* open reading frame (ORF) encoding a 339-amino-acid bZIP protein, is present,

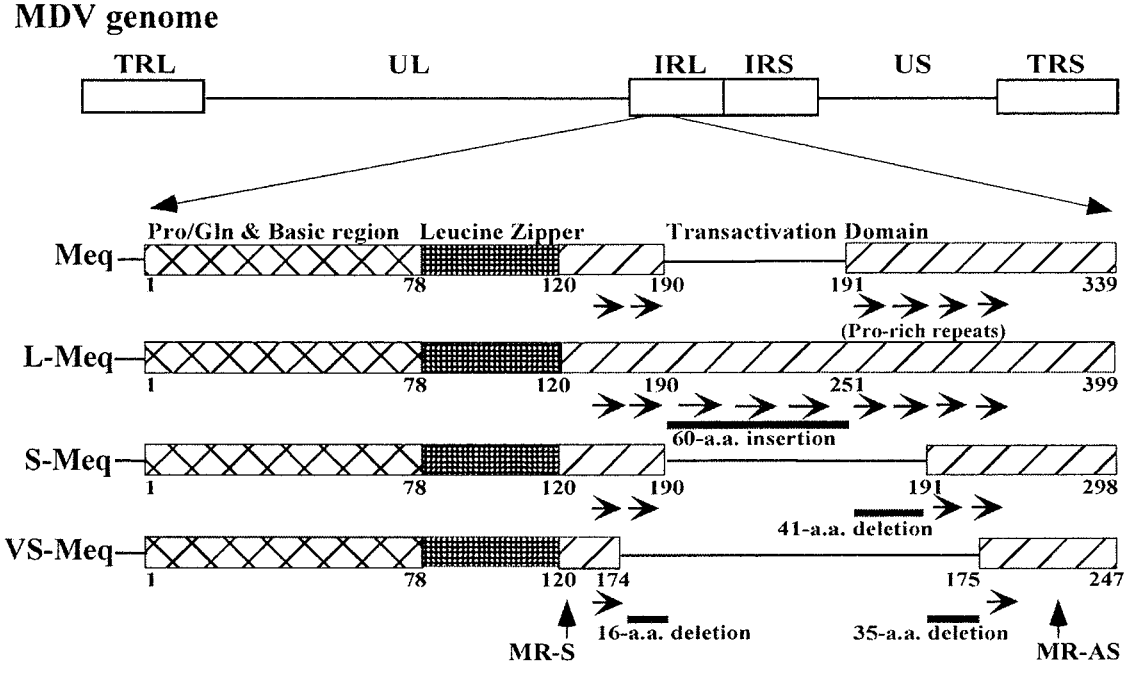

Fig. 1. Structures of the *meq* ORF in MDV1. The *meq* ORF of oncogenic MDV 1, Md5, encodes a 339 amino-acid protein consisting of an N-terminal proline-glutamine-rich region (Pro/Gln), the basic region, and the leucine zipper, as well as the transactivation domain. In the case of L-*meq* found in the MDV tumor cell lines, MSB 1 and MTB 1, and JM attenuated by serial passage, 60 amino acids (a 180-bp sequence) are inserted into the transactivation domain. In the case of S-Meq and VS-Meq, 92 amino acids (a 276-bp sequence) and 41 (a 123-bp sequence) are deleted in the transactivation domain of the Meq protein, respectively. The proline-rich repeat region is composed of 6 copies of the repeats in Meq, 9copies in L-Meq, 4 copies in S-Meq, and 2 copies in VS-Meq.

while a slightly longer *meq* ORF, termed L-*meq*, in which a 180-bp sequence is inserted into the meq ORF, is found in other strains of MDV 1, such as JM and CVI988/R6. Furthermore, short *meq* (S-*meq*) and very short *meq* (VS-*meq*), in which123 and 275-bp sequences are deleted in the *meq* ORF, have been also detected, respectively. This diversity in *meq* is due to the difference in the copy number of the proline-rich repeat region (Fig. 1)[5,7]. When the *meq* gene was monitored in chickens infected with vvMDV 1, both the *meq* and L-*meq* genes were detected, suggesting that MDV 1 consists of some subpopulations though the possibility of conversion from *meq* to L-*meq* can not be ruled out[3]. Since an MDV subpopulation displaying the L-*meq* gene is detected at the latent phase, the L-*meq* and its gene product, if any, might contribute to the maintenance of the MDV latency[3]. Though the shift in subpopulations and/or conversion of the *meq* genes have been observed in chickens infected with MDV 1, these changes in the T cell subsets have not been elucidated. Thus, in this study, to characterize the shift in subpopulations and/or conversion of the *meq* genes in T cell subsets, especially $CD4^+$ T cells, targets for transformation by MDV 1, we have analyzed the time course changes in the meq genes detected in T cell subsets from chickens infected with MDV 1. In addition, we have also compared this shifts/conversion in the T cell subsets from both MDV-sensitive and -resistant lines of SPF chickens.

Materials and methods

Chickens

Fertilized eggs of commercial chickens were purchased from Hokuren Co. Ltd. (Sapporo, Japan) and two lines of SPF chickens (MHC B^{19} B^{19} sensitive to MD: B19 and B^{21} B^{21} resistant to MD: B21)[13] were obtained from Nisseiken Co. Ltd. (Oume, Japan). These eggs were hatched in our laboratory, and chickens were raised in isolators.

Viruses and cells

A strains of very virulent MDV 1, Md5,[20] was obtained from chicken kidney cell culture from experimentally infected chickens. A vaccine strain of MDV 1, CVI 988/R6, was purchased from Gehn Corp. Inc. (Gifu, Japan). These viruses were propagated in chicken embryo fibroblasts (CEF). When cytopathic effects were confluent, the infected cells were harvested, and virus titers were measured by plaque assay. These infected CEFs were used for viral inoculation of chickens.

Inoculation of MDVs and sample collection from infected chickens

Groups of 3-day-old chickens were inoculated intraperitoneally with 2,000 plaque forming unit (PFU)/0.2ml of Md5, or 10,000 PFU/0.2ml of CVI 988/R6. We used B19 and B21 chickens for the inoculation of Md5 or CVI988/R6. The spleens were collected from 4 chickens from each group infected with MDV every week pi. for 10 weeks.

Isolation of $CD4^+$ and $CD8^+$ T cells

Peripheral blood mononuclear cells (PBMC) were obtained by homogenizing the spleens between two frostendend glass slides and subsequent centrifugation over Ficoll-Conray gradients to remove dead cells and red blood cells. PBMC (5×10^7) were incubated with 1 ml of monoclonal antibodies (mAbs), anti-chicken $CD4^+$ mAb ($CT4^+$)[2] or anti-chicken $CD8^+$ mAb (11-39)[12] (culture supernatant), for 30min at 4°C. After incubation, cells were washed twice with DMEM/F-12 (GIBCO BRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS: Filtron, Brooklyn, Australia), 5×10^{-5}

M 2-mercaptoethanol (2-ME), 100 IU/ml penicillin, and 100μg/ml streptomycin (complete DMEM/F-12). These cells were then resuspended in 500μl of complete DMEM/F-12 containing 2×10^7 of sheep anti-mouse IgG-magnetic beads (Dynabeads M-450, Dynal Inc., Oslo, Norway), and incubated at 4°C for 45min with gentle rotation. After incubation, cells attached to the beads were trapped by a magnetic field. To separate the cells from the beads, the cells binding to the beads were cultured in RPMI 1640 (GIBCO BRL, Grand Island, NY) containing 10% heat-inactivated FCS, 5×10^{-5} M 2-ME, 100IU/ml penicillin, and 100μg/ml streptomycin, at 40°C for 3.5 hours in a humidified atmosphere containing 5% CO_2. Flowcytometric analysis revealed that the purity of each T cell subset separated by this procedure ranged from 92 to 98%. The isolated $CD4^+$ and $CD8^+$ T cells were used for the extraction of total cellular DNA.

Extraction of DNA from T cell subsets

Total cellular DNA was extracted from $CD4^+$ and $CD8^+$ T cell subsets using SepaGene (Sankoujunyaku Co., Japan). The DNA was treated with RNase and re-extracted with phenol-chloroform-isoamyl alcohol (25 : 24 : 1), precipitated with ethanol, and dissolved in distilled water.

Nested polymerase chain reaction (PCR)

Total cellular DNA samples extracted from each of T cell subsets of infected chickens were used as a template for nested PCR amplification to detect the *meq* gene. The first round of PCR was performed with a primer set, M-S and M-AS, to amplify the 1.0-kb *meq* or 1.2-kb L-*meq* gene fragments as described by Lee *et al*[8]. After the amplification, 1 μl of the reaction was used for the second round of PCR. The second round of PCR was performed in the 20μl reaction mixture containing 1.5mM $MgCl_2$, and a primer set, 10pM of MR-S (5'-AGTTGGCTTGTCATGAGCCAG-3') and MR-AS (5'-TGTTCGGGATCCTCGGTAAGA-3'), to amplify a 0.6-kb *meq* or 0.8-kb L-*meq* fragment. Amplification was carried out over 35 cycles of 94°C for 45sec, 55°C for 45sec, and 72°C for 1.5min. The amplified fragments were separated on an agarose gel (1.5%) and visualized by staining with ethidium bromide.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNAs were extracted from $CD4^+$ and $CD8^+$ T cell subsets using the Trizol reagent (Invitrogen, CA). Lymphocytes separated by immuno-magnetic beads were resuspended in 1 ml of Trizol reagent, and 200μl of chloroform was added. After centrifugation at 12,000×g for 10min at 4°C, 500μl of isopropanol was added to the resultant supernatants. Total cellular RNA was precipitated by centrifugation at 12,000μg for 10min at 4°C after incubation at room temperature for 10 min. The resultant RNA was dissolved in diethylpyrocarbonate-treated water, and used as template to synthesize cDNA by using RAV 2 reverse transcriptase (Takara, Japan). The cDNA samples were used to amplify the *meq* transcripts by PCR using the M-S and M-AS primer set and MR-S and MR-AS primer set as described above.

Results

Detection of the meq gene in the T cell subsets from chickens infected with vvMDV 1,Md5

When the *meq* gene was monitored in the T cell subsets from chickens infected with Md 5, the *meq* gene was detected in $CD4^+$ and $CD8^+$ T cells from the infected chickens throughout the 10 weeks though the intensities of the bands corresponding to the *meq* gene detected were variable (Figs. 2-4). The

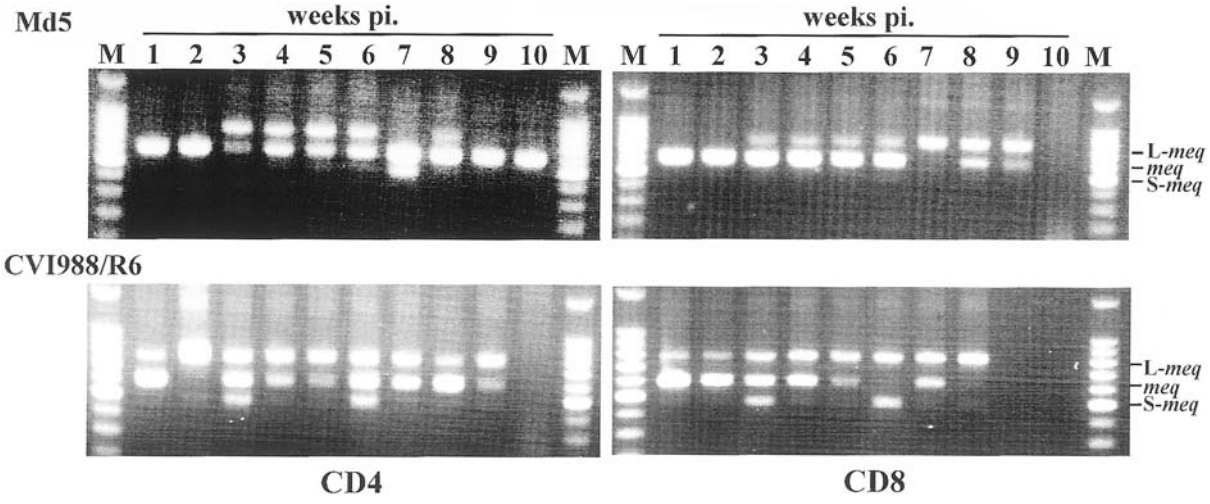

Fig. 2. Nested PCR amplification of the *meq* genes from T cell subsets of chickens infected with 2,000 PFU/chicken of strain Md5, or 10,000 PFU/chicken of strain CVI988/R6. DNA samples were collected at every week for 10 weeks pi. M : molecular size marker (100-bp DNA ladder).

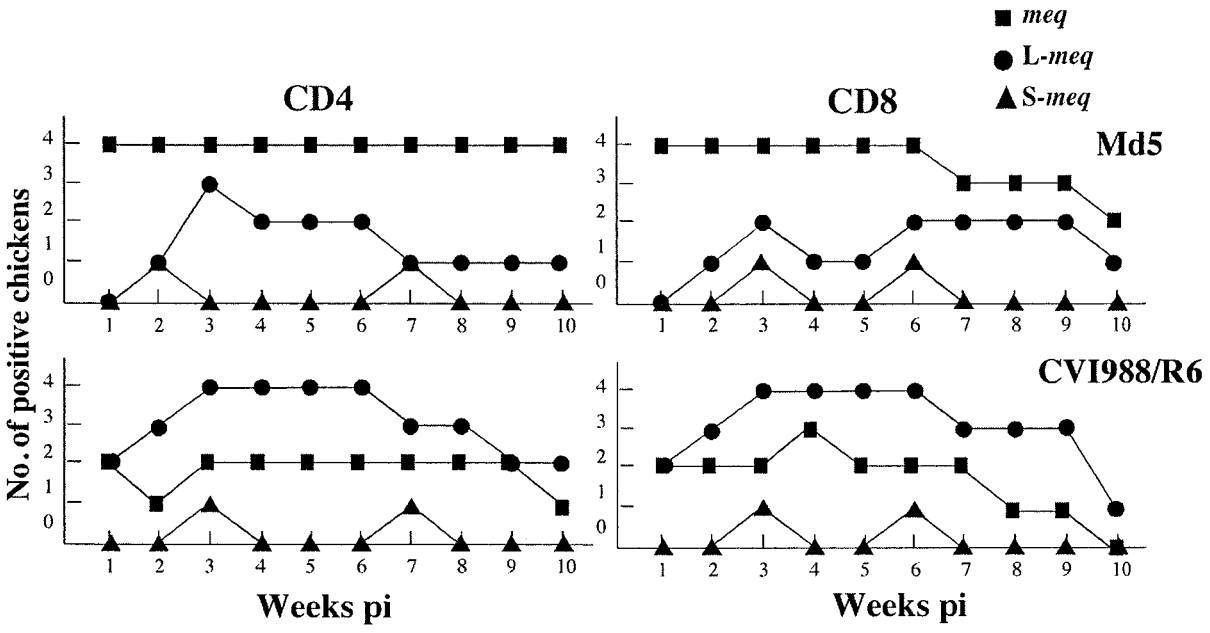

Fig. 3. Detection of the *meq* genes in the T cell subsets from the B19 chickens infected with MDV 1. DNA samples were collected from 4 chickens/group at each week pi.

L-*meq* gene was also detected in both the $CD4^+$ and $CD8^+$ T cells after 3 weeks pi. Interestingly, the frequency of the chickens from which the L-*meq* gene was transiently increased in the $CD4^+$ T cell from Md5-infected chickens at 3 to 6 weeks pi, presumably the latent phase of the MDV infection, and then reduced after 7 weeks pi (Figs. 3-4). However, this frequency was lower in the case of $CD8^+$ T cells at 3 to 6 weeks pi, while higher after 7 weeks pi. In addition, 0.5kb-short *meq* (S-*meq*) was occasionally detected at 2 to 3 and 6 to 7 weeks pi. These changes were observed both in the B19 and B21 chickens, but the de-

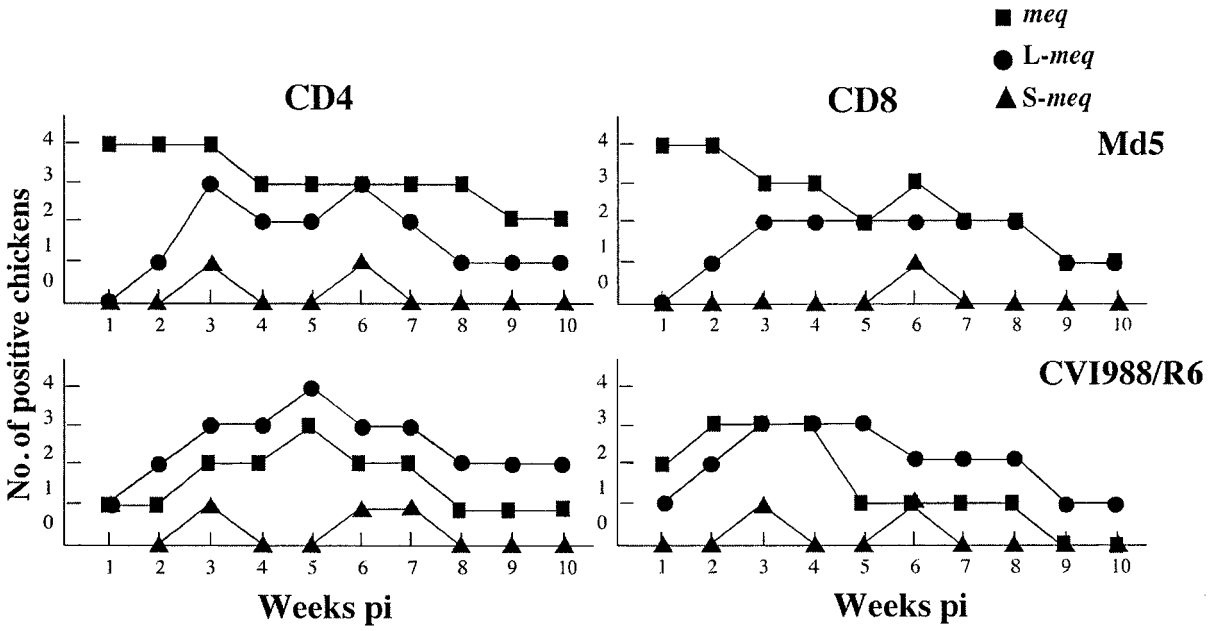

Fig. 4. Detection of the *meq* genes in the T cell subsets from the B21 chickens infected with MDV1. DNA samples were collected from 4 chickens/group at each week pi.

tection frequency of the *meq* genes was decreased faster in MD-resistant B21 chickens than in MD-sensitive B19 chickens (Figs. 3-4).

Detection of the meq gene in the T cell subsets from chickens infected with CVI 988/R 6

When the *meq* genes were monitored in chickens infected with CVI 988/R 6 in which the L-*meq* gene was already identified (11, 18), the L-*meq* gene was consistently detected in both $CD4^+$ and $CD8^+$ T cells from the infected chickens throughout the experimental period. The *meq* gene was also detected in the $CD4^+$ and $CD8^+$ T cells, though the frequency of the chickens from which the *meq* gene was detected was lower than that from which the L-*meq* gene was detected (Figs. 2-4). These frequencies were gradually decreased after 7 weeks pi. In addition, 0.5kb-S-*meq* was detected temporarily at 2 to 3 or 6 to 8 weeks pi. These changes were observed both in B19 and in B21 chickens. The detection frequency of the *meq* genes was lower in B21 than B19 chickens throughout the experimental period (Figs. 3-4).

Expression of the meq genes in the T cell subsets from chickens infected withMDV 1

To determine whether the *meq* genes present in T cell subsets were transcribed, RT-PCR analysis was performed. As shown in Fig. 5, when RNA samples were analyzed from infected chickens at 6 weeks pi, the *meq* gene was abundantly transcribed in the $CD4^+$ T cells from Md5-infected chickens from which both the *meq* and L-*meq* genes were detected. On the other hand, both the meq and L-*meq* genes were transcribed in the $CD8^+$ T cells from these chickens. Only the *meq* transcript was detected in $CD4^+$ and $CD8^+$ T cells from chickens from which the *meq* gene was detected (data not shown).

In the case of CVI 988/R 6-infected chickens, the L-*meq* gene was transcribed in both the $CD4^+$ and $CD8^+$ T cells though the *meq* transcript was also detected in the $CD4^+$ T cells. Only the L-*meq* transcript was detected

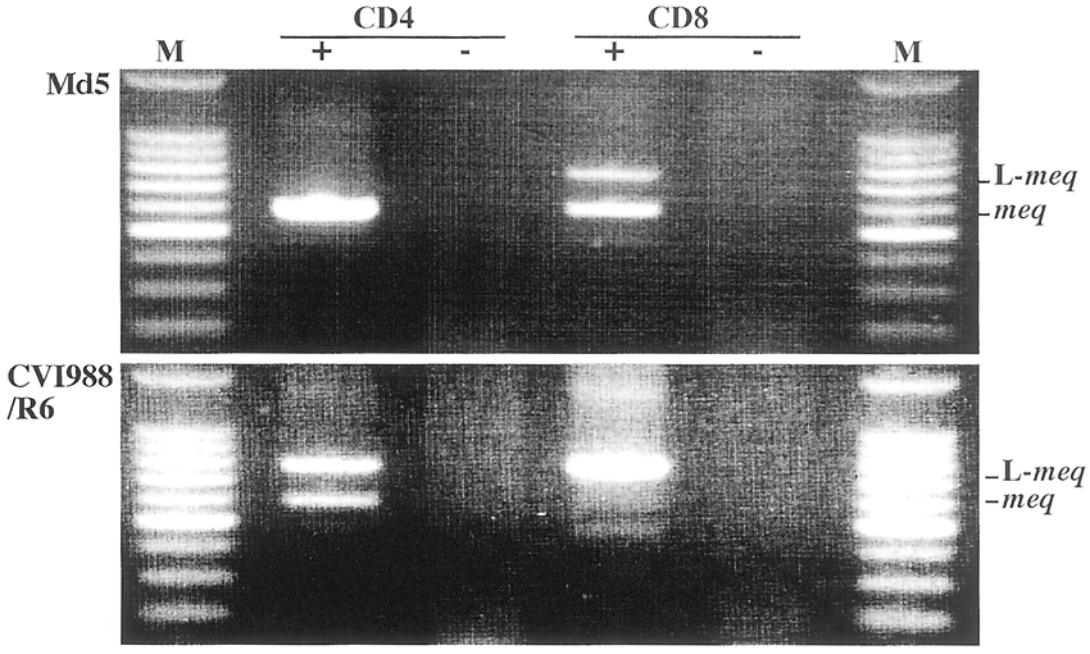

Fig. 5. RT-PCR detection of the *meq* transcripts from T cell subsets of chickens infected with 2,000 PFU/chicken of strain Md5 or 10,000 PFU/chicken of strain CVI988/R6. RNA samples, collected from chickens from which both the *meq* and L-*meq* genes were detected at 6 weeks pi., were used for RT-PCR (lane +), or directly used for PCR to examine the contamination of genomic DNA (lane −). M: molecular size marker (100-bp DNA ladder, Promega).

in $CD4^+$ and $CD8^+$ T cells from chickens from which the L-*meq* gene was detected (data not shown).

Discussion

There is evidence that the *meq* gene and its gene product, Meq, play important roles in transformation of $CD4^+$ T cells by MDV[19]. The Meq protein is consistently expressed in the vast majority of MD-transformed cell lines and $CD4^+$ T cells obtained from lymphomas[17]. MDV 1 mutants lacking a functional *meq* failed to cause tumors in chickens[16]. Polymorphism of the *meq* genes in the attenuated MDV 1, MDV-tumor cell lines and chickens infected with MDV 1 has been observed[5]. This polymorphism suggested that MDV 1 consists of at least 3 subpopulations, virus displaying the *meq*, L-*meq* and S-*meq* genes, respectively. During the MDV 1 infection, a dynamic shift from one subpopulation of MDV 1 to another may occur due to some selective pressures by host chickens. In addition, the L-*meq* gene product suppressed the transactivation activity of the Meq protein of oncogenic MDV 1, and replication of vvMDV 1 carrying the *meq* gene was partially suppressed in cells transfected with the L-*meq* gene[4].

In this study, we monitored the *meq* gene in the T cell subsets from chickens infected with strains of oncogenic and nononcogenic MDV 1 for 10 weeks pi. Surprisingly, both the *meq* and L-*meq* genes were detected in the T cell subsets from infected chickens during experimental periods. In addition, S-*meq* has also been detected temporarily though biological significance of the S-*meq* gene and its gene product remains unknown. It is of interest that, in chickens infected with vvMDV 1, Md5, the L-*meq* gene was detected during the

latent phase (3 - 6 weeks pi.) in $CD4^+$ T cells which are the target cells for latent infection and subsequent transformation by oncogenic MDV 1, though the *meq* gene was consistently detected throughout the experimental period. In the case of chickens infected with CVI 988/R6, the L-*meq* but not *meq* gene was consistently detected in both the $CD4^+$ and $CD8^+$ T cells. The detection frequency of the *meq* genes in the chickens was decreased more rapidly in the $CD8^+$ than in the $CD4^+$ T cells. These results suggest that a major subpopulation displaying the *meq* gene in the $CD4^+$ T cells would be important for the transformation by Md5, and that the presence of a subpopulation displaying the L-*meq* gene may contribute to the maintenance of MDV latency. Furthermore, the presence of the major subpopulation with the L-*meq* gene in the $CD8^+$ T cells, as detected in CVI988/R 6 (vaccine) - inoculated chickens, suggest that the L-*meq* gene product recognized by $CD8^+$ T cells could play a role in the cell-mediated immunity against MDV infection. It should be noted that the L-*meq* rather than *meq* gene is dominantly detected in MD tumor cell lines[5].

The shift in subpopulations and/or conversion of the *meq* genes mentioned above was observed in both the B19 chickens sensitive to MD, and the B21 chickens resistant to MD. Interestingly, detection frequency of the meq gene was decreased faster in both the $CD4^+$ and $CD8^+$ T cells from B21 chickens than from B19 chickens. The reason for this observation remains unknown, but these differences observed between two lines of chickens may be intimately related to the resistance to tumor formation by MDV 1.

Thus, MDV 1 could potentially express not only the *meq* but also L-*meq* gene in each of the T cell subsets from the infected chickens dependent upon the phase of MDV 1 infection. In the future, it will be necessary to analyze the roles of the L-*meq* and S-*meq* gene and/or their gene products in viral transcription and transformation and immune responses to MDV infection.

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