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Suppression of Transcription Activity of the MEQ Protein of Oncogenic Marek’s Disease Virus Serotype 1 (MDV1) by L-MEQ of Non-Oncogenic MDV1

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ABSTRACT. meq is one of the candidate oncogenes in the MDV1 genome. We previously reported a difference in the meq open reading frame (ORF) between oncogenic and non-oncogenic MDV1: L-meq, in which a 180-bp sequence is inserted into the meq ORF, is detected in non-oncogenic MDV1. To study the functions of a gene product of L-meq (L-MEQ), transactivation by L-MEQ was analyzed by dual luciferase assay using a reporter gene under the control of long (-1–-873 bp) and short (-1–-355 bp) meq promoter (LMP and SMP, respectively). LMP showed higher promoter function than SMP. L-MEQ transactivated the expression of the reporter gene, but less than MEQ did. In the presence of SMP or the cytomegalovirus immediate-early promoter, the same or slightly higher transactivation was observed in cells cotransfected with both meq and L-meq than cells transfected only with meq. However, in the presence of LMP, lower transactivation was observed in cells cotransfected with both meq and L-meq than cells transfected only with meq, suggesting that L-MEQ can be a transrepressor. Replication of vvMDV1 was enhanced in the cells with meq. Interestingly, however, replication of vvMDV1 was suppressed in the cells with L-meq or with both L-meq and meq, compared to untransfected cells. Thus, L-MEQ could suppress replication of vvMDV1 displaying the meq gene in cotransfected cells.

KEY WORDS: dual luciferase assay, L-meq, Marek’s disease virus, meq, transcription activity.


Marek’s disease virus (MDV), an avian alphaherpesvirus, is one of the most potent oncosogenic herpesviruses [2, 9, 16, 18]. MDV infection elicits a rapid onset of malignant T-cell lymphomas in chicken within several weeks after infection [3, 4]. Strains of MDV are classified into 3 serotypes based on their pathogeneticities, and only strains of MDV serotype 1 (MDV1) are oncogenic. Several candidate genes encoded by the BamHI-D, -H, -I2, -L and -Q2 fragments of the MDV genome have been implicated in oncogenesis by MDV [22]. Among them, the meq gene is most consistently detected in all tumor samples and cell lines [8]. The meq gene is only present in MDV1, and its gene product, MEQ, is an MDV1-specific 339-amino-acid protein with an N-terminal basic-leucine zipper (bZIP) [13, 15, 16, 21] (Fig. 1A). In addition, MEQ contains a C-terminal 33-amino acid proline-rich region which is a potent transcriptional transactivator [8, 13, 15, 19]. The MEQ protein can dimerize, with varying affinities, with c-Fos and c-Jun, cAMP-response-element-binding protein (CREB), camp-inducible transcription factors (ATF1 and ATF2) and with itself. Binding of MEQ with c-Jun is the strongest, and tetradecanoly-phorbol-13-acetate (TPA)-response element (TRE) and cAMP-response element (CRE) bind more avidly to MEQ-Jun heterodimers than to MEQ-MEQ homodimers and are transactivated more efficiently by the heterodimers [13–16, 21]. Interestingly, overexpression of MEQ induces morphological transformation of Rat cells and protect these cells from apoptosis [16]. In addition, transfection of anti-sense oligonucleotide to the meq gene results in inhibition of the growth of an MDV-transformed cell line [25]. These results strongly suggest that, as a viral oncogene, meq plays a significant role in transformation and oncogenesis by MDV1.

Oncogenic MDV1 can be attenuated by a prolonged passage in vitro, and attenuated strains of MDV1 such as a vaccine strain, CVI988, are non-oncogenic. Several changes in the viral genome during this attenuation have been reported including expansion of the 132-bp direct repeats in the BamHI-H region [10] and a 200-bp deletion in the BamHI-L region [23]. In addition, we have recently identified a change in the meq open reading frame (ORF) of a non-oncogenic MDV1 vaccine strain, CVI988: a 180-bp nucleotide sequence is inserted into the region encoding the transactivation domain in the meq gene, and this gene is termed as L-meq [11, 12]. Since this insertion results in increase in the number of proline-rich repeats (PRR) of the transactivator domain, the L-meq gene product may be different in transactivator activity from MEQ and, subsequently the presence of L-meq might be the reason why CVI988 is not oncogenic. Therefore, in this study, transcription activities of MEQ and L-MEQ were analyzed by dual luciferase assay using reporter genes under the control of meq promoter. Moreover, replication of MDV1 in cells transfected with L-meq gene was also examined.

MATERIALS AND METHODS

Cells and viruses: A human liver cancer cell line, HepG2 and a mouse embryo fibroblast cell line, Swiss3T3 cells, were obtained from Riken cell bank (Tsukuba, Japan), and maintained in Dulbecco’s Modified Eagle Medium (DMEM, GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). Chicken embryo fibroblast (CEF), prepared from 11-day-old embryonated eggs, was maintained in Eagle’s Modified Essential Medium (EMEM, Nissui-sieyaku, Tokyo, Japan) supplemented with 0.6% tryptose phos-
gene fragments as described by Lee et al. [11, 12]. Primer pairs specific for the long and short meq promoters (LMP: -1–-873 and SMP: -1–-355, respectively), MP1 and MP3, and MP2 and MP3, respectively, were designed from the reported nucleotide sequence of the meq gene [8]. The PCR was performed in a 50 µl reaction mixture containing 1.5 mM MgCl₂, 0.2 µM of each primers, 500 ng of total cellular DNA, 0.2 mM dNTPs, and 0.2 unit of Taq DNA polymerase. Amplification was carried out over 35 cycles of 94°C for 45 sec, 53°C for 45 sec, and 72°C for 1.5 min. The amplified fragments were separated on agarose gel (1.5%), and visualized by staining with ethidium bromide. The amplified bands were excised from the gel, purified using the Geneclean kit (Bio101, La Jolla, CA), and cloned into pGEM-T easy vector (Promega, Madison, WI).

Construction of the expression plasmids: The long and short meq promoters were amplified by PCR using primer sets in which the HindIII sites were added to the 5’ ends (Table 1). The amplified LMP and SMP sequences were subcloned into the pGEM-T vector (Promega, Madison, WI), and digested with HindIII. The digested fragments were inserted into the HindIII site, immediately upstream of the chimeric intron and Renilla luciferase reporter gene of the pRL-null vector (Promega), which contains no enhancer and promoter elements. These resultant vectors, designated pRL-SMP and pRL-LMP were used as control reporter vectors for cotransfection with any experimental reporter vector.

To construct vectors to express the meq genes in mammalian cells, the ORFs of the meq and L-meq genes were amplified by PCR using primer sets in which either the XhoI or MluI site was added to the 5’ end for cloning (Table 1). The amplified meq and L-meq ORFs were subcloned into the pGEM-T vector (Promega, Madison, WI), and digested with XhoI and MluI. The digested XhoI-MluI fragments containing meq and L-meq ORFs were cloned into the XhoI and MluI sites of the pCI-neo vectors (Promega, Madison, WI) to construct pCI-MEQ and pCI-LMEQ plasmids, respectively.

Cotransfection and Renilla luciferase assay: In order to analyze transactivation of L-MEQ, the pCI-MEQ and/or pCI-LMEQ plasmids were cotransfected with either the pRL-SMP or pRL-LMP plasmids into HepG2 and Swiss 3T3 cell lines. In addition, pRL-CMV, in which the cytomegalovirus enhancer and immediate-early promoter had been inserted into the upstream of the chimeric intron and Renilla luciferase reporter gene, was also used as a reporter plasmid. Transfection of HepG2 and Swiss 3T3 cells (2 × 10⁶/ml cells) was carried out in a 12-well plate culture format using the FuGENE™ 6 Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer’s protocol. For each well, a total amount of 1.5 µg of the vectors were used: 0.5 µg of the reporter plasmid and 1.0 µg of pCI-neo or 0.5 µg each of the two expression plasmids, either pCI-MEQ and pCI-neo, pCI-LMEQ and pCI-neo, or pCI-MEQ and pCI-LMEQ. After incubation of the transfected cells at 37°C for 36 hr, the cells were lysed with Passive Lysis Buffer (PLB), and luciferase activity was measured using Dual-Luciferase reporter assay system (Promega, WI, USA), and a Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan). The luciferase activities presented were representative of four different experiments.

Replication of MDV in CEF transfected with the meq or L-meq: In order to study the effect of the presence of L-meq on the replication of MDV1 in cell culture, the pCI-MEQ and/or...
pCI-LMEQ plasmids were transfected into CEF, and transfected CEF were then infected with strains of MDV1. Transfection of CEF (2 × 10⁵ cells/ml) with 0.5 µg of pCI-MEQ and/or L-MEQ plasmids was carried out in a 12-well plate culture format using the FuGENETM 6 transfection reagent (Roche) following the manufacturer’s protocol. The cells were infected with 100 plaque forming units (PFU)/50 µl of the Md5 or attenuated JM strain. The numbers of plaques were calculated after 7-day-incubation.

RT-PCR detection of the meq and L-meq transcripts in transfected cells: mRNAs were extracted from the cells transfected with the plasmids using the QuickPrep Micro mRNA purification kit (Amersham Pharmacia biotech, Uppsala, Sweden) following the manufacturer’s protocol. cDNA was synthesized from the mRNA using RAV-2 reverse transcriptase (Takara, Kyoto, Japan), and the DNA fragment corresponding to the part of the meq or L-meq gene was amplified by PCR using M and MR primer sets (Table 1).

Statistics: All values in figures were expressed as means ± S.E.M. Statistical comparison was done by analysis of variance, followed by unpaired two group t-test.

RESULTS

Promoter function of LMP and SMP: To confirm the promoter function of the meq promoter, reporter plasmids expressing Renilla luciferase reporter gene under the control of long and short meq promoters (LMP and SMP) were constructed, and their promoter activities were measured in HepG2 and Swiss 3T3 cells. SMP includes the REL binding site (REL), AP1-like motif (TRE/CRE), TATA box (TATA), and heat-shock protein binding site (HSP), while LMP contains SMP and an extra 500-bp 5’ region (Fig. 1). In this assay, pRL-null vector containing no promoters, and pRL-CMV containing CMV immediate-early promoter, were used as negative and positive controls, respectively. As shown in Fig. 2, both LMP and SMP showed the promoter function in HepG2 and Swiss 3T3 cells. The promoter activity of LMP was higher than that of SMP (p<0.001, Fig. 2), and the promoter activity of LMP was comparable to CMV immediate-early promoter (data not shown).

Transactivation by MEQ and L-MEQ: Transactivation through the meq promoter by MEQ has already been reported [20]. In order to determine transactivation activity of L-MEQ, we performed Renilla luciferase assay using mammalian expression vector to express MEQ or L-MEQ and reporter vectors under the control of the LMP, SMP, or CMV immediate-early promoter. The transfection efficacy, which was measured by firefly luciferase activity, was identical in this study. When these vectors were transfected into HepG2 and Swiss 3T3 cells, meq or L-meq mRNA were detected at 2 days after transfection, as determined by RT-PCR (data not shown). In the presence of MEQ, luciferase expression under the control of LMP, SMP or CMV immediate-early promoter was increased 2–3.5 times in the HepG2 and Swiss 3T3 cells, compared to that in the presence of the negative control, pCI-neo vector without inserts (Fig. 3). Luciferase expression was also increased 1.5–2.5 times in cells co-transfected with the L-meq expression vector, indicating that L-MEQ can function as a transactivator. However, the transactivation by L-MEQ was lower than that by MEQ (Fig. 3).

To study the transactivation in the presence of both MEQ and L-MEQ, HepG2 and Swiss 3T3 cells were cotransfected with both meq and L-meq expression plasmids, in combination with the luciferase reporter plasmid under the control of the 3 promoters as described above, and the expression of luciferase was measured (Fig. 3). Under the control of SMP or CMV promoter, luciferase expression was slightly higher in cells cotransfected with both meq and L-meq expression plasmids, compared to the cells transfected with only meq expression plasmid (p<0.01). On the other hand, under the control of the LMP, luciferase expression was decreased in the cells cotransfected with both meq and L-meq expression plasmids compared to the cells transfected with only meq expression plasmid (p<0.004, Fig. 3). These results suggest that transrepression could be induced when both MEQ and L-MEQ are present in cells.

Effect of MEQ and L-MEQ on the replication of MDV1 in vitro: The L-meq gene has been first identified in an attenuated vaccine strain of MDV1, CVI988 [11, 12], suggesting that L-MEQ might contribute to the protection mechanism induced by the vaccine. Thus, the effects of the presence of L-MEQ on the replication of MDV1 were studied using two strains, Md5 in which the meq gene is dominantly present and attenuated JM in which the L-meq gene is dominantly present [12]. The replication of the oncogenic Md5 strain was increased in the CEF transfected with the meq expression plasmid, but decreased in cells with L-meq expression plasmid, compared to the control pCI-neo-transfected cells (Fig. 4). The sizes of plaques were slightly smaller in cells transfected with the L-meq expression plasmid than cells transfected with either the meq expression plasmid or pCIneo (data not shown).

Expected sizes of amplified fragments in the meq gene (L-meq gene).

Table 1. Sequences of MDV-specific primers used in PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location of primer</th>
<th>Size of fragment</th>
<th>Name of gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP1-S</td>
<td>5’-CCGACGTCTAGTCTGTGCGCCGAT-3’</td>
<td>-853~873</td>
<td>873</td>
<td>LMP</td>
</tr>
<tr>
<td>MP2-S</td>
<td>5’-CGGACGTCTAGTCTGTGCGCCGAT-3’</td>
<td>-335~355</td>
<td>355</td>
<td>SMP</td>
</tr>
<tr>
<td>MP3-AS</td>
<td>5’-CGCAAGCTTTTATGACGACGAT-3’</td>
<td>-1~21</td>
<td>21</td>
<td>MR</td>
</tr>
<tr>
<td>M-S</td>
<td>5’-CGCCCTGAGATGTCTCAGGAGCCAGAGCCGGCT-3’</td>
<td>1~26</td>
<td>26</td>
<td>meq</td>
</tr>
<tr>
<td>M-AS</td>
<td>5’-CGACGCCTGAGATGTCTCAGGAGCCAGAGCCGGCT-3’</td>
<td>-1038~1162</td>
<td>1162</td>
<td>L-meq</td>
</tr>
<tr>
<td>MR-S</td>
<td>5’-AGTGGCTGTACGTAGCGCCAG-3’</td>
<td>347~367</td>
<td>367</td>
<td>MR</td>
</tr>
<tr>
<td>MR-AS</td>
<td>5’-TGTTCGGAATTCCTGAGGAA-3’</td>
<td>909~929</td>
<td>929</td>
<td>L-MR</td>
</tr>
</tbody>
</table>

Statistics: All values in figures were expressed as means ± S.E.M.
not shown). The replication of the Md5 strain was slightly increased in cells with both meq and L-meq expression plasmids (Fig. 4). The replication of attenuated MDV1, the JM strain in which the L-meq gene was detected, was increased in cells with meq expression plasmid, and slightly increased in the cells with L-meq expression plasmid, compared to the control pCIneo-transfected cells. The replication of the JM strain was not altered in cells with both meq and L-meq expression plasmids, compared to that in cells with L-meq expression plasmid (Fig. 4). These results indicate that MEQ promotes the replication of the Md5 and JM strains in vitro, while L-MEQ can suppress the replication of the Md5 in vitro.

DISCUSSION

A basic-leucine zipper (bZIP) gene, meq, is located in the Bam12-BamQ2/EcoQ fragment within the terminal and inverted repeat long regions (TRL and IRL, respectively) of the MDV genome [8, 17]. L-meq gene was initially identified in the genome of an attenuated vaccine strain of MDV1, CVI988 [11, 12]. One distinctive feature of MEQ is the presence of bZIP domain [8], common to many cellular transcription factors. This ZIP part is known to form a coiled-coil structure and has the propensity to form homodimers with cellular repression factors, such as CBP, and induce transrepression [7]. Similarly, by using dual luciferase assay in this study, both MEQ and L-MEQ were found to function as transactivators though transactivation by L-MEQ was lower than that by MEQ. On the other hand, transactivation by MEQ was suppressed when both MEQ and L-MEQ were expressed, showing that L-MEQ can function as a transrepressor. The extra portion of MEQ is proline-rich, contains two and half proline-rich repeats (PRR) with several SH3 binding motifs, and is rich in PPPP motifs [8]. At least one copy of the PRR is required for full transactivation activity of MEQ [13]. In addition, the proline-rich domain is known to be associated with both transactivation or transrepression functions. Although no cellular homologue has been defined for the transactivation domain, the high proline content of this domain is reminiscent of the transrepression domain of WT-1 [1]. Liu et al. [15, 16] reported that the high PRR structure displays transrepression properties if expressed as an isolated form. As mentioned, MEQ had 6 PRR, while L-MEQ had 9 PRR, in which 3 PRR were added [11, 12]. Thus, similar to MEQ, L-MEQ may also have both transactivating and transrepressing functions, depending on the phosphorylation status, the interacting partners, or other factors that modulate the conformation of MEQ or L-MEQ as suggested by Liu et al. [13, 14]. Furthermore, the increased number of the PRR may convert L-MEQ into a repression-dominant transcriptional factor. Transactivation and transrepression by L-MEQ as well as MEQ was higher when the reporter gene was used under the control of LMP than SMP, suggesting that the 5‘ upstream region of the meq promoter (-355 to -873) is important for the function of L-MEQ. However, the exact molecular mechanism for transrepression by L-MEQ is still unknown. L-MEQ may bind to cellular repression factors, such as CBP, and induce transrepression [7].

The replication of an oncogenic Md5 strain was increased in the cells with the meq gene, and the replication of the attenuated JM strain was increased in the cells with either the meq or L-meq gene. Interestingly, however, the replication of Md5 was suppressed in the cells with L-meq or with both L-meq and meq genes. These results suggest that L-MEQ can specifically inhibit the replication of oncogenic but not non-oncogenic or attenuated MDV1 in which the L-meq gene is present. Although the exact mechanism for this inhibition
remains to be elucidated, L-MEQ may interfere or modulate the binding of MEQ/MEQ homodimer to regulate viral replication, or bind to specific promoters to regulate the growth of MDV.

REFERENCES


**Fig. 4.** Replication of the Md5 or attenuated JM strain in CEF transfected with pCI-LMEQ, -MEQ, or -MEQ and L-MEQ. The CEF transfected with pCI-LMEQ, pCI-MEQ, or control pCIneo were infected with 100 PFU of Md5 or JM strain. The numbers of plaques were calculated at 7 days after infection. Results are shown as relative ratios of plaque numbers observed in untransfected cells infected with Md5 or JM. Vertical bars represent mean ± SEM.