Transcriptional activation of Mink Enteritis Virus VP2 by the C-terminal of MEV NS1 protein

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Abstract

Mink enteritis virus (MEV) NS1 is a multidomain and multifunctional protein containing origin binding, helicase and transactivation domains. In particular, parvoviral NS1 proteins are transactivators of the viral capsid protein promoter although the manner by which they exert these transactivation effects remained unclear. In this study, the region of the transactivation domain of the NS1 C-terminal was found located at aa 557 ~ 668 and any deletion within this region reduced the transactivation activity. A dominant negative mutation of the 63 aa deletion in the C-terminal of NS1 protein resulted in loss of ability to activate P38 and VP2-5’UTR in a dual-luciferase reporter assay system, a VP2 protein expression system, and within the whole MEV genome, independent of downstream genes. Additionally, a full-length MEV clone deficient in its NS1 C-terminal failed to rescue the virus, possibly due to the loss of integrity of DNA sequences interacting with NS1 protein, and expression of VP2 was also inhibited even when normal NS1 protein was supplied in trans.

1. Introduction

Mink enteritis virus (MEV) is a linear, single-stranded DNA virus belonging to the genus Parvovirus of the family Paroviridae. Its genome contains approximately 5000 bp arranged in two large open reading frames (ORFs). The left ORF encodes two nonstructural proteins, NS1 and NS2; the right encodes two capsid proteins, VP1 and VP2 [5, 10]. MEV infection is considered to be the most important infectious disease of mink and is found worldwide [13, 14, 17].

The parvoviral NS1 protein is a multifunctional and multidomain protein [1, 9, 12]. Its N-terminal contains an origin of replication binding (OBD) domain[7, 9], its central region contains a helicase domain [4, 8], and the C terminal includes an activation domain [7, 9]. The two DNA-interacting domains, the N-terminal and helicase domains, are necessary for viral genome replication and for the control of viral protein production, while the C terminal of NS1 is required for its transactivation function [9, 11].

As an essential non-structural protein, NS1 is required for effective viral replication and virion production. Many studies have implicated the NS1 protein in cellular apoptosis, cell cycle arrest, type I interferon responses and tumor suppression [1] and loss of the NS1 functional domain has an important impact on normal biological functions [7, 9, 15].

Parvoviral NS1 proteins are transactivators of the viral capsid protein promoter [9]. Besides the N-terminal domain, the transactivation activity of NS1 is dependent on the C terminus [3, 9], however, since the mechanism by which it exerts its transactivation effect remained unclear, we undertook the following investigation.

2. Materials And Methods

2.1 Cell culture
Feline kidney (F81) cells were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (CellMax) at 37°C in an atmosphere of 5% CO₂.

2.2 Plasmid construction

The genome of MEV strain L (MEV-L) had been constructed as an infectious viral clone pMEV in our laboratory (Yuan et al., 2014). Coding sequences of NS1 were amplified by PCR from pMEV and cloned into the BamHI and AgeI sites of plasmid pcDNA3.1-His/myc to generate plasmid pNS1. The pNS1-Δn20, pNS1-Δn40, pNS1-Δn49, pNS1-Δc20, pNS1-Δc40 and pNS1-Δc63 plasmids were mutated through amplified from pNS1 using the following primers (Table 1). pMEV-NS1-Δc63 was generated by deleting the 2128–2316 nt sequence of pMEV. Plasmids pMEVΔNS1 containing a premature stop codon was in the NS1 gene, pGL3-P38, pGL3-5'UTR and pGL3-P38 + 5'UTR used for luciferase activity assays have been described elsewhere [15].

Table 1
Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNS1-F</td>
<td>CGCGGATCCATGTCTGGCAACCAGTATACTGAGG</td>
</tr>
<tr>
<td>pNS1-R</td>
<td>CGGCACCGGTATCCAAATCTCCTCAAGTATGAGG</td>
</tr>
<tr>
<td>pNS1-Δc20-F</td>
<td>CGCGGATCCATGTCTGGCAACCAGTATACTGAGG</td>
</tr>
<tr>
<td>pNS1-Δc20-R</td>
<td>CGGCACCGGTGCTGCTCTCAAGTATGAGG</td>
</tr>
<tr>
<td>pNS1-Δc40-F</td>
<td>CGCGGATCCATGTCTGGCAACCAGTATACTGAGG</td>
</tr>
<tr>
<td>pNS1-Δc40-R</td>
<td>CGGCACCGGTGTTTTGATTTGGTTTGGATTTGCAGTTTCT</td>
</tr>
<tr>
<td>pNS1-Δc63-F</td>
<td>CGCGGATCCATGTCTGGCAACCAGTATACTGAGG</td>
</tr>
<tr>
<td>pNS1-Δc63-R</td>
<td>CGGCACCGGTACTACGTCCGGAGTGCAGAGG</td>
</tr>
<tr>
<td>pNS1-Δn20-F</td>
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</tr>
<tr>
<td>pMEV-NS1-Δc63-R</td>
<td>CGTACCTTACACTACGTCCGGAGTGCAGGAGTAG</td>
</tr>
</tbody>
</table>

2.3 Whole cell extracts and Western blotting
Transfected or infected F81 cells were collected and lysed in RIPA lysis buffer (Solarbio) containing 1 mM PMSF. The lysate was incubated on ice for 15 mins and then boiled for 10 mins after mixing with 6×SDS loading buffer, then separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked overnight with 5% milk at 4°C and then incubated with rabbit anti-MEV-VP2 polyclonal antibody (prepared in our laboratory) or rabbit anti-EGFP monoclonal antibody (MBL) followed by HRP-conjugated secondary antibodies (Proteintech). Protein bands were visualized by enhanced chemiluminescence (Thermo Scientific).

2.4 RNA extraction and quantitative real time PCR

F81 cells were added to 6-well plates and transfected with the corresponding plasmid. After 24 h, total RNA was extracted for analysis of mRNA expression. Untreated cells were included as a negative control. Total cellular RNA was extracted from cell samples using a Total RNA Purification kit (TIANGEN) according to the manufacturer’s guidelines and reverse transcription of RNA and PCR detection was carried out according to the method established by Yang et al. (Yang et al., 2018). The mRNA levels of target genes were normalized to that of β-actin.

2.5 Luciferase reporter assay

F81 cells were transfected in 24-well plates with 245 ng reporter plasmid pGL3, 10 ng pRL-TK and 245 ng pNS1 or pNS1-Δc63 using Lipofectamine 3000 (Invitrogen). Cells were collected at 24 h post-transfection and assayed with a dual luciferase kit (Promega) according to the manufacturer’s instructions.

2.6 Rescue of the recombinant virus

F81 cells were added to 6-well plates and transfected with pMEV or pMEV-NS1-Δc63 plasmids using Lipofectamine 3000 according to the manufacturer’s instructions. Five days after transfection, the cell pellets were collected (P0) and passaged in fresh cells, after freeze-thawing 3 times, to permit virus recovery.

2.7 Indirect immunofluorescence assay (IFA)

F81 cells were infected with 10% suspensions of sedimented P3 cells and unstimulated cells treated identically served as a negative control. After 24 h, the cells were washed 3 times with PBS and fixed in cold 4% paraformaldehyde for 10 min at 4°C. Cells were washed three times and incubated for 1 h at room temperature with anti-VP2 rabbit polyclonal antibody at 1:100, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (MBL: 1:100) for 1 h at room temperature. After 3 washes, images were observed by fluorescence microscopy.

2.8 Statistical analysis

Data were analyzed by Student’s t-test and reported as mean ± SD of three independent experiments. Analyses were performed using Prism 6.0 (GraphPad Software).

3. Results
3.1 Identification of activation sites in MEV NS1

Critical amino acid residues in the MEV transactivation domain were determined by alignment of amino acid sequences of the MEV NS1 protein and the well-studied AAV Rep protein. As illustrated in Fig. 1A, the origin-binding domain is located within aa 6 ~ 254 and the helicase domain within aa 340 ~ 556. Between them, there is also an oligomerization domain. The transactivation region of the MEV NS1 protein was identified at aa 557~668 (Fig. 1A). To further characterize transactivation activity, 6 truncation fragments of NS1 were constructed, as shown in Fig. 1B.

F81 cells were transfected with these truncated plasmids together with pMEV-ΔNS1 in which the NS1 expression had been stopped by introduction of a stop codon (Fig. 1C). Western blot analysis showed that transfection with any of the truncated fragments of the NS1 transactivation domain significantly reduced expression of the VP2 protein, and especially with pNS1-Δc63 (Fig. 1D). Detection of VP2 mRNA by qPCR showed that inhibition occurred at the transcription level (Fig. 1E).

3.2 NS1-Δc63 mutation lost its ability to activate transcription of P38 and VP2-5'UTR

The expression of VP2 is controlled by the P38 promoter and the 5' untranslated region (VP2-5'UTR) at the transcriptional level. We have previously shown that the expression of VP2 from a full-length infectious clone of MEV was abolished when its 5'UTR was deleted. To test whether deletion of 63 amino acids at the C-terminal of NS1 protein inhibited the transcriptional level of VP2, plasmid pP38 + 5'UTR was constructed with a pGL3 backbone in which the luciferase gene had been replaced by the MEV VP2 gene (Fig. 2A). Expression of VP2 protein in F81 cells harvested 24 h post transfection with either pNS1 or pNS1-Δc63 showed that VP2 protein was detectable by Western blot only in the presence of pNS1 (Fig. 2B) and the level of VP2 mRNA in the pNS1-Δc63 transfected group, as measured by qPCR, was significantly decreased (Fig. 2C).

The dual-luciferase reporter assay system was used to measure the ability of pNS1-Δc63 to activate the P38 and VP2-5'UTR. In this system, firefly luciferase was translated by P38 promoter and 5'UTR (pGL3-P38 + 5'UTR) together, by P38 promoter (pGL3-P38) alone, or by 5'UTR (pGL3-5'UTR) alone, and the Renilla luciferase was translated by pRL-TK as an internal reference. F81 cells were co-transfected with reporter plasmids, pRL-TK and pNS1 or pNS1-Δc63 (Fig. 2D), and the ratio of firefly luciferase: Renilla luciferase activity was measured to assess the transcriptional activity of the P38 promoter and 5'UTR.

Results showed that the VP2-5'UTR significantly enhanced transcriptional activity of the P38 promoter (Fig. 2E), and the highest transcript levels were observed in transfection with pNS1 and pFL3-P38 + 5'UTR together whereas pNS1-Δc63 significantly reduced the transcriptional activity of pP38 + 5'UTR (Fig. 2F). F81 cells were also transfected with plasmids containing either the P38 promoter or 5'UTR with pNS1 or pNS1-Δc63. Results showed that pNS1-Δc63 inhibited both P38 and 5'UTR transcriptional activities (Figs. 2G and H).
3.3 NS1-Δc63 failed to activate the transcriptional activity of P38 and VP2-5'UTR in the whole MEV genome

As described above, NS1 with a C-terminal 63 aa deletion failed to activate transcription of the P38 promoter and VP2-5'UTR in the dual-luciferase reporter assay system or the VP2 protein expression system controlled only by P38 and 5'UTR. To further determine whether the NS1 C-terminal 63 amino acids is necessary for the regulation of P38 and VP2-5'UTR in the MEV whole genome, a full-length infectious clone of MEV with the VP2 5'UTR (pMEV-Δ5'UTR) or the P38 promoter (pMEV-ΔP38) deletion was used. Since the VP2-5'UTR is indispensable for the translation of VP2 in MEV, the expression of VP2 protein was abolished when the VP2-5'UTR was deleted due to transcriptional repression of the VP2 protein itself. Instead, a plasmid with the VP2 gene replaced with EGFP (pMEV-5'UTR -EGFP) in which the EGFP protein was expressed under the control of only the P38 promoter (Fig. 3A) was used.

F81 cells were co-transfected with pMEV-ΔP38 or pMEV-5'UTR -EGFP and pNS1 or pNS1-Δc63, samples were harvested at 24 h and the expression of VP2 or EGFP was determined by Western blot analysis. Results showed that the 63 aa C-terminal deletion in the NS1 protein rendered it inadequate to promote the expression of VP2 (Fig. 3C) or EGFP (Fig. 3D). EGFP was also assayed by fluorescence and found to be expressed only in the presence of the intact NS1 protein (Fig. 3B). Additionally, detection of EGFP and VP2 mRNA by qPCR showed that inhibition occurred at the transcription level (Fig. 3E and F). These data support the conclusion that transcriptional activity of P38 and VP2-5'UTR within the MEV genome is abolished when the 63 aa of NS1 C-terminal are deleted.

Collectively, these results indicate that both P38 and VP2-5'UTR are required for efficient VP2 mRNA expression in both the truncated vector and in the full length cloned MEV, and that NS1 protein is critical for transcriptional activation of the VP2 gene, while a C-terminal deletion of 63 aa is sufficient to abolish transcriptional activation activity.

3.4 NS1-Δc63 failed to rescue the virus

Reverse genetics methodologies were used to determine the impact of the 63 amino acids of NS1 C-terminal on viral rescue and replication. Starting with the full-length MEV clone, pMEV [16], a deletion of 63 aa from the NS1 C-terminal (pMEV-NS1-Δc63) was constructed (Fig. 4A). F81 cells were transfected with pMEV-NS1-Δc63, with pMEV itself used as a positive control. When cultures were harvested after 5 days, cells transfected with the pMEV controls showed typical cytopathic changes, including cell detachment and elongation.

After 3 freeze-thaws, the cell lysates (passage 0, P0) were added to fresh F81 cell cultures and incubation was continued. No lesions were observed in the pMEV-NS1-Δc63 transfected cells (Fig. 4C). When P3 cell lysates were incubated with fresh cells and expression of VP2 protein was measured by IFA (Fig. 4C) and Western blot (Fig. 4D) 24 h later, results showed that VP2 protein was expressed only when the recombinant virus was rescued successfully, i.e., via pMEV, and no VP2 expression was detected following transfection with pMEV-NS1-Δc63, and VP2 mRNA in the pMEV-NS1-Δc63 transfected cells...
was significantly reduced as measured by qPCR (Fig. 4E). It can therefore be concluded that viruses with the pMEV-NS1-Δc63 mutation did not yield infectious virus, and that the NS1 protein C-terminal was critical for virus rescue.

3.5 NS1-Δc63 affects the integrity of DNA sequences that interact with the NS1 protein

The above results showed that deletion of 63 aa from the C-terminal of NS1 suppressed P38 promoter and VP2-5'UTR transcriptional activity, leading to inhibition of VP2 protein expression and virus rescue. This posed the question as to whether transcriptional activity could be restored after providing normal NS1 protein. F81 cells were therefore co-transfected with pMEV-NS1-Δc63 and pNS1 or pNS1-Δc63 (Fig. 5A), and harvested after 24 h. Western blots showed that the VP2 protein was not detected in the pMEV-NS1-Δc63 transfected cells although pNS1 was co-transfected (Fig. 5B), thereby demonstrating the need for an intact NS1 C-terminal for production of infectious virus.

4. Discussion

It has previously been noted that the C-terminal sequence of NS1 is sufficient to constitute a transcription activating domain, since the 126 C-terminal amino acids of MVM NS1 have been shown to have activating activity [6]. Transactivation is an essential function of the parvoviral non-structural protein NS1, required for regulation of the promoter activity of the viral protein.

The transactivation domain of the C-terminal of MEV NS1 protein was found located at aa 557 ~ 668 and to localize more precisely the functional transcriptional region 6 truncations of the C-terminal of the NS1 protein were constructed and tested. Tests with these mutants showed that any deletion within this region reduced the transactivation activity. This is partly consistent with reports of other parvoviruses in which a truncated NS1 protein lacking the 67 C-terminal amino acids, was found impaired with regard to its ability to transactivate promoter P38 [3, 9]. Since the expression of MEV VP2 protein requires its 5'UTR to promote VP2 gene expression at both transcriptional and translational levels [15]. We wished to determine whether the transcriptional inhibition caused by the NS1-Δc63 mutation affected the P38 promoter or the VP2-5'UTR. We found that an NS1-Δc63 mutation lost its ability to activate transcription of VP2 under control of the P38 promoter and VP2-5'UTR. Analysis using a dual-luciferase reporter assay system found that the NS1-Δc63 mutation lost its ability to activate transcription of firefly luciferase gene in which expression was also under control of the P38 promoter and VP2-5'UTR and in the whole cloned MEV genome, the NS1-Δc63 mutation also lost the ability to activate transcription of P38 and VP2-5'UTR. The NS1-Δc63 mutation therefore represses transcriptional activity independent of downstream genes but dependent on the P38 promoter and VP2-5'UTR; i.e., the NS1-Δc63 mutation has lost the ability to activate transcription of P38 and VP2-5'UTR.

Since the NS1-Δc63 mutation inhibited the expression of VP2, the 63 aa C-terminal deletion of NS1 in the full-length MEV clone resulted in the failure to rescue the virus, which is in agreement with previous
studies on CPV that dc67 mutation inhibited the virion production [9]. Even with a normal NS1 protein supplied in trans, expression of VP2 by the full-length MEV clone following the 63 aa deletion within the VP2-5'UTR was blocked. NS1 can specifically bind efficiently to many internal sites, including the transactivation region [2]. Since the 5'UTR is indispensable for the translation of VP2 in MEV [15] it may be that the deletion affected the integrity of the viral DNA, preventing the NS1 protein from binding to the VP2-5'UTR in the whole MEV genome and thereby inhibiting activation of transcription. All the evidence provided here supports the conclusion that the 63 aa region of the NS1 C-terminal is essential for the transcription of VP2 in the MEV genome.

Declarations

Author contributions WL, JX, JW and QX conceived and designed experiments. Material preparation, experiments, and data collection were performed by QX, YL, CG and JW. The first draft of the manuscript was written by QX and revised JS, JX and WL. All authors read and approved the final manuscript.

Conflicts of interest The authors declare that they have no conflict of interest.

References


Figures

Figure 1
Deletion analysis of the C-terminal of NS1 protein revealed a critical regulatory region. (A) Genetic map of MEV. The positions of NS1 and VP2 proteins and their regulatory promoters are shown. (B) 6 truncations of NS1 C-terminal were constructed within the pNS1 backbone. F81 cells were transfected with the truncated plasmids together with pMEV-ΔNS1. (C) Expression of VP2 protein was detected by Western blot analysis 24h post co-transfection (D). (E) VP2 mRNA in the co-transfection with pNS1-Δc63 and pMEV-ΔNS1 was analyzed by qPCR, **** P<0.0001 (Student’s t-test).

**Figure 2**

NS1-Δc63 mutation loses its ability to activate transcription of P38 and VP2-5'UTR. (A) Expression of VP2 protein in a P38 promoter and 5' UTR directed monocistronic system. The P38 promoter and VP2-5'UTR fragments were inserted upstream of VP2 CDS sequence. F81 cells were transfected with NS1 or NS1-Δc63 in an expression vector. Samples were harvested for Western blot analysis of VP2 protein levels (B) or for qPCR analysis (C). (D) The dual-luciferase reporter assay system was used to test the transcriptional activity of the P38 promoter and VP2-5'UTR. P38 promoter or VP2-5'UTR fragments or both were inserted into pGL3-Basic to generate a series of constructs. Transcriptional activity is defined as the ratio between firefly luciferase and Renilla luciferase (relative luciferase activity). (E, F, G and H) Luciferase activity analyses in the dual-luciferase reporter assay system. Transcriptional activity of both P38 promoter and VP2-5'UTR (F), or P38 promoter (G) or VP2-5'UTR (H) alone were analyzed. **, P<0.005; ***, P<0.0005; ****, P<0.0001; ns, not significant (Student's t-test).

**Figure 3**

Activity analysis of the P38 promoter and VP2-5'UTR in the MEV genome. (A) The VP2 gene was replaced by the EGFP gene in the 5'UTR deletion mutant. F81 cells transfected with pMEV-Δ5'UTR-EGFP and pMEV-ΔP38-VP2 were co-transfected with pNS1 or pNS1-Δc63. 24 h post-transfection, fluorescence was analyzed for P38 promoter activity (B), and the expression of VP2 (C) and EGFP (D) protein were determined by Western blot. The mRNA levels of EGFP (E) and VP2 (F) were analyzed by qPCR. **, P<0.005; ***, P<0.0005; ****, P<0.0001 (Student's t-test).

**Figure 4**

Virus rescue. (A) Cytopathic changes observed in F81 cells transfected with pMEV or pMEV-NS1-Δc63 5 d post-transfection. Expression of VP2 in F81 cells incubated with P3 cell lysates for 24 h, observed by IFA (B) and Western blot (C); (D) VP2 mRNA was determined by qPCR analysis. ****, P<0.0001 (Student’s t-test).
Figure 5

Deletion of 63 aa in the C-terminal of NS1 protein affects the integrity of the DNA sequence in the MEV full-length clone. (A) F81 cells transfected with pMEV-NS1-Δc63 were co-transfected with pNS1; (B) Expression of VP2 protein determined at 24 h by Western blot.