Long Untranslated Regions of the Measles Virus M and F Genes
Control Virus Replication and Cytopathogenicity

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Measles is a highly contagious disease characterized by high fever, cough, and maculopapular rash. The causative agent, *measles virus* (MeV), belongs to the genus *Morbillivirus* of the family *Paramyxoviridae*. One feature of the morbillivirus genomes is that the M and F genes have long untranslated regions (UTRs). However, the M and F mRNAs of morbilliviruses are transcribed by nontranscribed intergenic (IG) trinucleotides, flanked by a linked genes (some paramyxoviruses have more genes) separated by nontranscribed intergenic (IG) trinucleotides, flanked by a short leader (Le) and a short trailer (Tr) sequence at the 3′ and 5′ end of the genome, respectively (18). The six genes encode nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins, respectively. The P gene, exceptionally, encodes additional accessory proteins, V and C proteins, by a process of RNA editing of a cotranscriptional insertion of a single nontemplated G residue and by an alternative translational initiation in a different reading frame, respectively (18, 30). The viral RNA-dependent RNA polymerase is thought to bind the Le sequence at the 3′ terminus of the genome and transcribe respective genes in a sequential and polar manner, by recognizing gene start (GS) and gene end (GE) sequences at each gene boundary (29).

In paramyxoviruses, mRNAs generally contain open reading frames (ORFs) with short 5′ and 3′ untranslated regions (UTRs). However, the M and F mRNAs of morbilliviruses have unusually long 5′ and 3′ UTRs, respectively (1, 2, 6, 7, 9, 14, 15, 19, 23, 32, 33, 39, 45). One exception is the canine distemper virus (CDV) F gene. The sequence originally considered as long 5′ UTR was later shown to encode an atypically long leader peptide that modulates the F protein function (47). In MeV, the length of the 3′ UTR of the M mRNA (M 3′ UTR) is 426 nt and that of the 5′ UTR of F mRNA (F 5′ UTR) is 583 nt (6, 39, 42). In the genome, the regions that are transcribed into the M 3′ UTR and F 5′ UTR, combined with the IG trinucleotide, constitute a long stretch of the UTRs of ~1 kb in length between M and F ORFs (Fig. 1A). In this paper, we indicate the genomic regions by the corresponding regions of mRNA, into which they are transcribed.) Although most morbilliviruses have these long UTRs, the nucleotide sequences are unique among different virus species, and no conserved motifs are found. The only common feature of these long UTRs is the high GC content, and they were suggested to form extensive secondary structures, which may regulate the translation or localization of mRNAs (31, 48). It is also suggested that the high GC content is due to the action of adenosine deaminase, which is implicated in the development of subacute sclerosing panencephalitis (SSPE) by introducing the biased hypermutation in the M gene of MeV (4, 5).

Using the F protein expression systems from cDNA, the F 5′ UTRs of morbilliviruses have been shown to modulate the translation efficiency of the F protein. Some studies reported that the long F 5′ UTRs of MeV, rinderpest virus, and CDV inhibit the production of the F protein (11, 13, 16, 20), whereas others showed that they enhance it (3, 16). To analyze the role of the long F 5′ UTR of MeV in virus infection, Radecke et al. generated a recombinant MeV that has a 504-nt deletion in the F 5′ UTR, using a reverse genetics system based on the Edmonston (Ed) B vaccine strain (38). They showed that there was little difference in the growth in cultured cells between the Ed virus having the
504-nt deletion (∆5F-Ed) and the parental virus, suggesting that the long F 5′ UTR has no significant role in MeV replication in cultured cells (38). Valsamakis et al. analyzed the growth of the ∆5F-Ed virus in human thymus/liver implants that were engrafted into SCID mice (SCID-hu thy/liv model) (46). Their results thus suggested some role in the long F 5′ UTR in virus replication in vivo, but little information exists on the possible role for the long M 3′ UTR. Wong et al. showed that, using M protein expression plasmids, at least 146 nt of the M 3′ UTR of the Ed strain could be deleted without affecting the production of the M protein (48).

Although the Ed strain is the most well-characterized MeV strain, it is known to have lost the original highly pathogenic nature of MeV during the adaptation to grow in unnatural host cells, accumulating significant mutations in the genome (42, 50). Therefore, using a reverse genetics system based on the virulent wild-type (WT) IC-B strain of MeV (41, 43), we generated a series of recombinant MeVs containing altered M 3′ UTR and F 5′ UTR. We show here that these long UTRs of the WT MeV modulate gene expression and protein production, controlling virus replication and cytopathogenicity.

### MATERIALS AND METHODS

**Viruses and cells.** Vero cells constitutively expressing human signaling lymphocyte activation molecule (SLAM) (Vero/hSLAM) (35) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (ICN Biomedicals, Aurora, Ohio) supplemented with 7.5% fetal bovine serum (FBS) and 500 μg of Geneticin (G418; Nakalai Tesque, Tokyo, Japan) per ml. CHO cells constitutively expressing human SLAM (CHO/hSLAM) (44) were maintained in RPMI medium (ICN Biomedicals) supplemented with 7.5% FBS and 500 μg of G418 per ml. Recombinant MeVs were generated by the procedure reported recently (41). Briefly, CHO/hSLAM cells were infected with the vaccinia virus encoding T7 RNA polymerase, vTF7-3 (a gift from B. Moss), and then transfected with the full-length plasmid encoding the antigenic epitope of MeV and three support plasmids, pCAG-T7-IC-N, pCAG-T7-IC-PAC, and pGEMCR-9301B-L (41). On the following day, the CHO/hSLAM cells were cocultured with B95a cells to amplify the recombinant MeV rescued from the transfected full-length genome plasmid.

**Construction of plasmids.** All full-length genome plasmids were derived from the p(+)MV323 (43) that encodes the antigenic full-length cDNA of the virulent IC-B strain of MeV (25, 26). The p(+)MV323-EGFP plasmid that has an additional transcriptional unit of the enhanced green fluorescent protein (EGFP) was reported previously (21). Since no available antibody against the MeV F protein was suitable for the quantification of the F protein, the influenza virus hemagglutinin (HA) epitope tag sequence (amino acid sequence YYVDVPDYA) was added to the p(+)MV323-EGFP plasmid at the cytoplasmic region of the F protein with a flexible linker sequence (amino acid sequence PPEELGGP) by standard molecular cloning procedures, generating p(+)MV323-EGFPtagF plasmid. All recombinant viruses used in this study possessed this modification in the F gene. Then the entire region of the long UTRs between M and F ORFs in the p(+)MV323-EGFPtagF was replaced by the counterpart short UTRs between N and P (N-P), P and M (P-M), and F and H (F-H), or H and L (H-L) ORFs, generating the first set of full-length plasmids, p(+)MV-EGFPtagF-N-P, p(+)MV-EGFPtagF-P-M, and p(+)MV-EGFPtagF-H-L, respectively (Table 1). To keep the genome length in multiples of 6, 3, 5, and 3 nt in the long UTRs remained undeleted for the generation of the p(+)MV-EGFPtagF-N-P, p(+)MV-EGFPtagF-P-M, and p(+)MV-EGFPtagF-H-L, respectively, but the hexamer position of the initiating A residue of the F mRNA in the WT genome is 3, while those of the UTR replacement mutant N-P, P-M, F-H, and H-L virus genomes were 5, 2, 2, and 6, respectively. In the second set of the full-length genome plasmids, either or both of the 360 nt in the M 3′ UTR at nt positions 4447 through 4806 and the 540 nt in the F 5′ UTR at nt positions 4910 through 5449 were deleted from the p(+)MV-EGFPtagF-N-P, p(+)MV-EGFPtagF-P-M, and p(+)MV-EGFPtagF-H-L, respectively. A detailed cloning procedure will be provided upon request.

Individually M and F genes were also cloned into the eukaryotic expression plasmids. The pCA7-IC-tagF plasmid was generated by cloning the entire 5′ long UTR and coding region of the IC-B F gene (nt positions 4875 through 7110) with the HA tag sequence into the pCA7 plasmid. The pCA7-IC-tagF plasmid was generated by deleting the 579 nt (nt positions 4875 through 5453) of the 5′ long UTR from the pCA7-IC-tagF plasmid. The pCMV-IC-M+UTR plasmid was generated by cloning the entire sequence of the IC-B M gene (nt positions 3406 through 4870) between the human cytomegalovirus major immediate early enhancer.

### TABLE 1. Construction of the UTR replacement mutants

<table>
<thead>
<tr>
<th>Full-length plasmid</th>
<th>Fragment removed*</th>
<th>Fragment inserted*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p(+)MV-EGFPtagF-N-P</td>
<td>4449-5457</td>
<td>1686-1806</td>
</tr>
<tr>
<td>p(+)MV-EGFPtagF-P-M</td>
<td>4451-5457</td>
<td>3331-3437</td>
</tr>
<tr>
<td>p(+)MV-EGFPtagF-F-H</td>
<td>4446-5457</td>
<td>7111-7270</td>
</tr>
<tr>
<td>p(+)MV-EGFPtagF-H-L</td>
<td>4449-5457</td>
<td>9125-9233</td>
</tr>
</tbody>
</table>

* Shown are nucleotide positions defining the fragments removed or inserted to construct full-length plasmids.

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**FIG. 1. Rescue of MeVs in which the long UTRs between M and F ORFs have been replaced with the counterpart short UTRs between other ORFs.** (A) A diagram showing construction of mutant virus genomes. The entire region of the long UTRs between M and F ORFs (M-F) was replaced with the counterpart short UTRs between other ORFs (N-P, P-M, F-H, and H-L), as indicated by arrows. The filled areas indicate ORFs, which are encoded with white characters. The open areas indicate UTRs. M 3′ UTR and F 5′ UTR indicate the genomic regions corresponding to the 3′ UTR of M mRNA and 5′ UTR of F mRNA, respectively, when they are transcribed. The vertical lines in the UTRs indicate IG trinucleotides. (B) Plaques produced by the rescued viruses on Vero/hSLAM cells. Images were obtained with a light and a fluorescence microscope and merged by using Axio Vision software. The region used for the replacement in each mutant is shown at the bottom.
promoter and SV40 early mRNA polyadenylation signal sequence obtained from the pRRESg3 plasmid (Clontech, Palo Alto, Calif.). The pCMV-IC-ΔUTR plasmid was generated by deleting the 360 nt (positions 4447 through 4806) of the 3′ long UTR from the pCMV-IC-M+UTR.

**Virus titration.** Monolayers of Vero/hSLAM cells on 6-well cluster plates were infected with serially diluted virus samples, and incubated for 1 h at 37°C. The inoculum was then removed, and the cells were washed with phosphate-buffered saline (PBS). The cells were overlaid with DMEM containing 5% FBS and 1% agarose. At 3 days postinfection (p.i.), PFU was determined by counting the number of plaques under a fluorescence microscope. Monolayers of Vero/hSLAM cells on 24-well cluster plates were infected with 50 µl of serially diluted virus samples, and incubated for 1 h at 37°C. After 1-h incubation, 150 µl of DMEM supplemented with 7.5% FBS and 100 µg of the fusion block peptide (Z-D-Phe-Phc-Gly) (Pep tide Institute Inc., Osaka, Japan) (40) per ml was added to each well to inhibit the second round of infection by progeny virions. At 30 h p.i., the number of EGFP-expressing cells was counted under a fluorescence microscope. The number was expressed as cell infectious unit (CIU). CIU was essentially comparable to PFU.

**Northern blotting.** Total RNA was extracted from virus-infected cells with the TRIzol reagent (Life Technologies, Gaithersburg, MD). From the total RNA, mRNA was purified with the Oligo(dT)30 mRNA purification kit (TaKaRa Bio Inc., Shiga, Japan). Two µg of the purified mRNA was electrophoresed, transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-N, Amersham Biosciences, Piscataway, N.J.), and hybridized with 32P-labeled gene specific cDNA probes synthesized with Prime-it II random primer labeling kit (Strategene, La Jolla, Calif.) and gene specific cDNA fragments. The gene specific cDNA fragments were used as nt positions 1,134 through 1,680 for N, 1,829 through 2,074 for P, 2,461 through 4,462 for M, 6,224 through 6,415 for F, and 7,261 through 7,586 for H, and 11,001 through 11,216 for L gene, respectively. The membranes were stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe used as an internal control. Radioactivity was analyzed and quantified with a Fuji BioImager 1000 and MacOS Bas excursion radioactivity.

**Quantification of mRNAs by PCR.** Purified mRNAs from virus-infected cells were reverse transcribed into cDNAs with Script select cDNA synthesis kit (Promega). PCR (40 to 60 cycles of 95°C for 5 s and 60°C for 20 s, or 95°C for 5 s, 55°C for 20 s, and 72°C for 20 s) was then performed with SYBR Premix Ex Taq (TaKaRa Bio Inc.) in capillary tubes. Primers used for this assay were 5′-GAATCTGGTATCACGTCG3′ and 5′-TCTGTTGACCTTCCTTC3′ for the N mRNA, 5′-TCCAGAGGAACTACCCCTTC3′ and 5′-GAGTGGTCCAGGCTGAC3′ for the M mRNA, 5′-GGAAGTCGCTTACAGTTCAGAC3′ and 5′-GAGGCTTCAATGACACC3′ for the F mRNA, 5′-GAAAGTCGCTTCAATGACACC3′ and 5′-GAGGCTTCAATGACACC3′ for the M mRNA, 5′-CAATATCTGAGAGCAAGCTG3′ and 5′-TGAGCAATTTGAGCCCTAGC3′ for the F mRNA, 5′-TGGAGAATTTGCCTGC3′ and 5′-TCGATTCACCTTCGAG3′ for the N mRNA, and 5′-CACTTGTGCATATTGAAGG3′. The quantity or antigenome RNAs, total RNA from virus-infected cells were reverse transcribed with the specific primers for the 3′ genome or antigenome termini, 5′-ACCAAAACAGTTGGTGAAC3′ and 5′-ACGACAAAGCGTGGAAGA3′, respectively. PCR was then performed with SYBR Premix Ex Taq (TaKaRa Bio Inc.) with the specific primer pairs that amplify the genome or antigenome ORFs. The H-L virus caused much stronger cytopathic effect (CPE) than the WT virus. In the H-L virus-infected cells, syncytia developed and spread more rapidly, accompanied with strong cell lysis (Fig. 2A). The H-L virus replicated more efficiently than the WT virus, although the peak virus titer was essentially comparable to PFU.

**Characterization of the mutant H-L virus.** We analyzed in detail of each of the mutants, the H-L virus (Fig. 1), in which the entire region of the long UTRs between M and F ORFs was replaced with the counterpart short UTRs. In the MeV genome, the M 3′ UTR and F 5′ UTR are combined with the IG trinucleotide, composing a long stretch of UTRs of 1,012 nt in length between M and F ORFs (Fig. 1A). To test if these long UTRs have an essential role in MeV replication, we replaced the entire region of the long UTRs between M and F ORFs with the counterpart short UTRs of 107–160 nt in length present between the other pairs of ORFs (Fig. 1A). To follow the “rule of six” (10, 27) each replacement was done so as to keep the genome length in multiples of six, while the hexamer position of the initiating A residue of the F mRNA was altered by the replacement. We tested all possible ways of replacement (N-P, P-M, F-H, and H-L) (Fig. 1A), and could rescue all four viruses with efficiency similar to that obtained with the parental WT virus rescued from pMV322-EGFPtagF. Notably, all four viruses were viable and even produced bigger plaques than the WT virus (Fig. 1B). These results indicate that the long UTRs between M and F ORFs per se are not indispensable for MeV replication. Also, these data suggested that the hexamer position of the initiating A residue was not critical for MeV gene expression, as found with simian virus 5 (22).

**RESULTS**

**Rescue of recombinant MeVs in which the long stretch of UTRs between M and F ORFs was replaced with the counterpart short UTRs.** In the MeV genome, the M 3′ UTR and F 5′ UTR are combined with the IG trinucleotide, composing a long stretch of UTRs of 1,012 nt in length between M and F ORFs (Fig. 1A). To test if these long UTRs have an essential role in MeV replication, we replaced the entire region of the long UTRs between M and F ORFs with the counterpart short UTRs of 107–160 nt in length present between the other pairs of ORFs (Fig. 1A). To follow the “rule of six” (10, 27) each replacement was done so as to keep the genome length in multiples of six, while the hexamer position of the initiating A residue of the F mRNA was altered by the replacement. We tested all possible ways of replacement (N-P, P-M, F-H, and H-L) (Fig. 1A), and could rescue all four viruses with efficiency similar to that obtained with the parental WT virus rescued from pMV322-EGFPtagF. Notably, all four viruses were viable and even produced bigger plaques than the WT virus (Fig. 1B). These results indicate that the long UTRs between M and F ORFs per se are not indispensable for MeV replication. Also, these data suggested that the hexamer position of the initiating A residue was not critical for MeV gene expression, as found with simian virus 5 (22).
virus-infected cells quantified by RT-QPCR were 2.4-, 2.9-, 4.0-, and 4.6-fold higher, respectively, than those in the WT virus-infected cells (Fig. 2D). Since the relative levels of the H and L mRNAs increased more than those of the N and P mRNAs, the reinitiation rate of transcription at the short M-F gene boundary of the H-L virus genome may be more efficient than that of the original M-F gene boundary having the long UTRs. More importantly, the increase in transcript levels was even more pronounced for the M and F mRNAs (7.6- and 7.5-fold higher than WT levels, respectively) in the H-L virus-infected cells. Amounts of the genome and antigenome were 1.9- and 1.7-fold higher, respectively, in the H-L virus-infected cells than in the WT virus-infected cells (Fig. 2D).

As reported previously (12), the amounts of bicistronic mRNAs were generally much less than those of monocistronic mRNAs (Fig. 2C). The ratios of bicistronic mRNAs to monocistronic ones were not significantly altered in the H-L virus-infected cells, as compared with the WT virus-infected cells. It was notable that the amount of the bicistronic mRNA through the F and H gene boundary (FH) was apparently greater than that of the M-F bicistronic mRNA (MF) (Fig. 2C). However, this was observed not only in the H-L virus-infected cells, but also in the WT virus-infected cells (Fig. 2C) (12). Thus, these data suggest that the long UTRs have no significant role in the M-F read-through transcription.

Next, the amounts of viral proteins in the H-L virus-infected cells were compared with those in the WT virus-infected cells (Fig. 2E). The amounts of the N and F proteins in the H-L virus-infected cells were 2.1- and 16.4-fold higher, respectively, than those in the WT virus-infected cells. The 2.1-fold increase in the N protein production could be attributed to the 2.4-fold increase in the amount of the N mRNA in the H-L virus-infected cells (Fig. 2D and 2E). However, the 16.4-fold increase in the F protein production in the H-L virus-infected cells could be only partially explained by the 7.5-fold increase in the amount of the F mRNA (Fig. 2D and 2E). These results, therefore, suggest that the increased production of the F protein in the H-L virus-infected cells was due to the increased amount as well as more efficient translation of the F mRNA.
missing the long 5′ UTR, as previous reports have suggested (11, 13, 20).

Rescue and characterization of recombinant MeVs missing either or both of the long M 3′ UTR and F 5′ UTR. To analyze the respective roles of the F 5′ UTR and M 3′ UTR, another set of three mutants was generated. These mutants contain either a 360-nt deletion in the M 3′ UTR, a 540-nt deletion in the F 5′ UTR, or both of these deletions (Fig. 3A). All three mutant viruses could be rescued with efficiency similar to that of the WT virus and were viable in cultured cells. Thus, neither the long M 3′ UTR nor long F 5′ UTR proved essential for MeV replication, consistent with results shown in Fig. 1A and B. We compared CPE of the WT and mutant viruses in A549/hSLAM, Vero/hSLAM, and B95a cells (Fig. 3B). In these cells, CPE induced by the mutant virus with the 360-nt deletion in the M 3′ UTR (M ΔUTR virus) was similar to that induced by the WT virus (Fig. 3B). By contrast, the mutant virus with the 540-nt deletion in the F 5′ UTR (F ΔUTR virus) induced much stronger CPE in all three cell lines. In the F ΔUTR virus-infected cells, syncytia developed and spread more rapidly, and cells were lysed more quickly than in the WT or the M ΔUTR virus-infected cells (Fig. 3B). CPE induced by the mutant with both deletions (M ΔUTR + F ΔUTR virus) was similar to that induced by the F ΔUTR virus in all cell lines (Fig. 3B). In addition, plaques produced by the F ΔUTR or M ΔUTR + F ΔUTR virus were bigger than those produced by the WT or M ΔUTR viruses (data not shown). The first four mutant viruses having the UTR replacements also produced bigger plaques than the WT virus (Fig. 1B). These results indicate that the long F 5′ UTR, but not the long M 3′ UTR, moderates CPE, and reduces the size of the plaques produced.

Next, we compared replication kinetics of the WT, M ΔUTR, F ΔUTR, and M ΔUTR + F ΔUTR viruses in A549/hSLAM, Vero/hSLAM, and B95a cells (Fig. 3C). The long F 5′ UTR appeared to inhibit virus replication, as the F ΔUTR virus replicated more efficiently in all cell lines than the WT virus (Fig. 3C). The M ΔUTR + F ΔUTR virus also replicated more efficiently than the M ΔUTR virus (Fig. 3C). By contrast, the long M 3′ UTR seemed to promote virus replication, as the
M ΔUTR virus replicated less efficiently than the WT virus, and the M ΔUTR + F ΔUTR virus grew less efficiently than the F ΔUTR virus (Fig. 3C). The WT virus replicated as efficiently as the M ΔUTR+F ΔUTR virus, but with greatly reduced cytopathogenicity (Fig. 3B and 3C).

**Gene expression of the WT, M ΔUTR, F ΔUTR, and M ΔUTR + F ΔUTR viruses.** We compared the levels of viral mRNAs and proteins in the WT, M ΔUTR, F ΔUTR, and M ΔUTR+F ΔUTR virus-infected cells. At 24 h p.i., the amounts of the viral mRNAs in the WT, M ΔUTR, F ΔUTR, and M ΔUTR+F ΔUTR virus-infected cells were analyzed by RT-QPCR. Figure 4A shows the relative amounts of N, P, M, F, H, and L mRNAs in the M ΔUTR, F ΔUTR, or M ΔUTR+F ΔUTR virus-infected cells compared with the amounts of these mRNAs in WT virus-infected cells (M ΔUTR/WT, F ΔUTR/WT, or M ΔUTR+F ΔUTR/WT, respectively). Similarly, M ΔUTR+F ΔUTR/F ΔUTR shows the relative amounts of the viral mRNAs in the M ΔUTR+F ΔUTR virus-infected cells compared with the amounts of these mRNAs in the F ΔUTR virus-infected cells. In all comparisons (M ΔUTR/WT, F ΔUTR/WT, M ΔUTR+F ΔUTR/WT, or M ΔUTR+F ΔUTR/F ΔUTR), the relative amounts of the N, P, H, and L mRNAs were more or less constant (Fig. 4A). These results suggest that neither the long M 3′ UTR nor long F 5′ UTR modulates the reinitiation rate of transcription at the M-F gene boundary. By contrast, the relative amounts of the M mRNAs increased ∼1.5-fold compared with those of other mRNAs when the virus possessed the 360-nt deletion in the M ΔUTR (Fig. 3C). The WT virus replicated as efficiently as the M ΔUTR+F ΔUTR virus-infected cells, even when they were normalized with the amounts of the N protein (Fig. 4B). These results suggest that the long F 5′ UTR inhibits the F protein production, consistent with the results of the H-L virus (Fig. 2E). By contrast, the long M 3′ UTR appeared to enhance the M protein production, as the amount of the M protein in the M ΔUTR virus-infected cells normalized with the amount of the N protein was about half of that detected in the WT virus-infected cells (Fig. 4B). Similarly, the amount of the M protein in the M ΔUTR+F ΔUTR virus-infected cells was about three times less than that detected in the F ΔUTR virus-infected cells, while the amounts of the N and F proteins were comparable in those cells (Fig. 4B). These results were confirmed by a time course experiment (Fig. 4C). Vero/hSLAM cells were infected with the WT and M ΔUTR viruses, and the amounts of the N and M proteins were then analyzed at various time intervals. The production of the M protein was reduced in the M ΔUTR virus-infected cells (Fig. 4C), while the level of the M mRNA in them was similar to that of the WT virus-infected cells (Fig. 4A, M ΔUTR/WT). Thus, our results suggest that the long M 3′ UTR is required for efficient M protein translation and production.
or not having the long 3′ UTR exhibited the similar levels of stability in relation to the cellular mRNAs.

**DISCUSSION**

Viruses are absolute parasites of living organisms, and may have evolved to minimize the sizes of their genomes, in which the coding capacities are expanded with a variety of the coding strategies. For example, the genomes of paramyxoviruses encode three unique polypeptides (the P, V, and C proteins) in the overlapping ORFs in the P gene (30). Considering such astonishing coding strategies of these viruses, it seems unlikely that a virus would retain wasteful genome regions throughout its evolutionary history. Nevertheless, morbillivirus genomes, unlike the genomes of other paramyxoviruses, have unusually long M 3′ UTRs and F 5′ UTRs (1, 2, 6, 7, 9, 14, 15, 19, 23, 32, 33, 39, 45).

In this study, we showed that these long UTRs per se were not essential for MeV replication, but that alteration or deletion of these long UTRs influenced virus replication and cytopathogenicity. Viruses with a 540-nt deletion in the F 5′ UTR had enhanced replication capacity, and caused much stronger CPE in the host cells. Therefore, one explanation for the maintenance of the long F 5′ UTR during the evolution of MeV may be that the long F 5′ UTR is required to moderate cytopathogenicity of MeV at the expense of replication capacity. On the other hand, viruses with a 360-nt deletion in the M 3′ UTR had a reduced replication phenotype, suggesting that the long M 3′ UTR promotes virus replication. Consequently, by having both the long M and F UTRs, MeV may be able to replicate efficiently with reduced cytopathogenicity.

How do the long UTRs modulate virus replication and cytopathogenicity? Our results showed that the long F 5′ UTR has the capacity to decrease the F protein production. The reduced cytopathogenicity is likely a direct effect of this decreased F protein production, because the F protein is the key molecule for the virus-induced cell-to-cell fusion. Inhibition of virus replication by the long F 5′ UTR may also result from the reduced cell-to-cell fusion, which causes less efficient cell-to-cell virus transmission. Furthermore, the reduced amount of the F protein, which is a major structural glycoprotein on the virus envelope, may also affect virus assembly, resulting in poor replication. On the other hand, the long M 3′ UTR has the ability to increase the M protein production. We speculate that the increased production of the M protein directly promotes virus assembly and budding of progeny virions.

Interestingly, it has been reported that Sendai virus (SeV) and human parainfluenza virus type 1 (hPIV1) also possess strategies to inhibit production of the F protein (8, 24). Kato et al. showed that SeV inhibits production of the F protein and its downstream H and L proteins by having a unique GS signal at the F gene and thus reducing the reinitiation rate of transcription at the M-F gene boundary (24). The mutant SeV, in which the GS signal at the F gene was replaced by the common GS signal found at the P, M, and HN genes, replicated more efficiently and showed higher virulence in mice than the parental SeV (24). In spite of the apparent disadvantage of the GS signal at the F gene for virus replication in both cultured cells and animals, the sequence of the GS signal at the F gene is conserved among all known
SeV strains. The authors therefore suggested that the moderation of transcription at the F gene might be relevant to viral fitness in nature (24). The F gene of hPIV1 possesses a long 5′ UTR of 264 nt in length. Bousse et al. showed that the long UTR contributed to the read-through transcription at the M-F gene boundary, inhibiting the expression of the F gene (8). CDV also moderates the virus-induced cell fusion by having the unique amino-terminal precursor sequence of the F protein encoded in the region originally considered as a 5′ long UTR of CDV F gene (47).

In the case of MeV studied here, the relative amounts of the M and F mRNAs increased ~1.5-fold with the 360- and 540-nt deletions in the M and F UTRs, respectively. However, comparison of the amounts of N, P, H, and L mRNAs of the deletion mutants indicated that neither the long M 3′ UTR nor the long F 5′ UTR of MeV altered the reinitiation rate of transcription at the M-F gene boundary. On the other hand, the small alteration of the reinitiation rate of transcription may have occurred in the H-L virus-infected cells. The cis-acting sequence(s) (such as, GE, IG, and GS) replaced in the H-L virus genome may be responsible for this observation, as found with SeV (24). The ~1.5-fold increase in the amounts of the F mRNA was likely due to the increase in the stability of the mRNA missing the long UTR. However, the change in the stability was not observed for the M mRNA using the plasmid-expression system. Thus, some specific mechanism(s) may operate in virus-infected cells, but not plasmid-transfected cells, which modulates the M mRNA stability or M protein production by acting on the M 3′ UTR. Alternatively, some properties, including terminal sequences, of the M mRNAs expressed from virus and plasmids may differ from each other. Further analysis is now in progress to clarify the mechanism of action of the M 3′ UTR.

In agreement with previous studies in which individual genes were expressed from plasmids (11, 13, 20), the long F 5′ UTR appeared to inhibit the F protein translation, as the ratio of the F protein production to the amount of the F mRNA was significantly higher in the absence of the long F 5′ UTR. On the other hand, our results suggested that in the virus-infected cells the long M 3′ UTR promotes the M protein translation, since the M protein production was severely restricted in the absence of the long M 3′ UTR. Lines of evidence have indicated that the 3′ and 5′ UTRs of viral and eukaryotic mRNAs have crucial roles in gene expression. They may control transcription of mRNA, and also function post-transcriptionally by determining the subcellular localization, stability, and translation efficiency of mRNAs (17, 28, 37). Our initial search using the UTR database failed to find any known motif in the MeV long UTRs (36). A detailed mapping of functional motifs in the MeV long UTRs and search for host factors that may interact with the motifs may reveal a novel strategy of MeV or cells to regulate gene expression.

In conclusion, we demonstrated that the long M 3′ UTR and long F 5′ UTR of MeV are nonessential for virus replication, but that they regulate MeV replication and cytopathogenicity by modulating the productions of the M and F proteins. By having both long M and F UTRs, MeV may replicate efficiently and minimize cytopathogenicity. We speculate that the reduction of cytopathogenicity may be advantageous for MeV fitness and survival in nature.

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