

Measles Viruses Possessing the Polymerase Protein Genes of the Edmonston Vaccine Strain Exhibit Attenuated Gene Expression and Growth in Cultured Cells and SLAM Knock-In Mice[∇]

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Live attenuated vaccines against measles have been developed through adaptation of clinical isolates of measles virus (MV) in various cultured cells. Analyses using recombinant MVs with chimeric genomes between wild-type and Edmonston vaccine strains indicated that viruses possessing the polymerase protein genes of the Edmonston strain exhibited attenuated viral gene expression and growth in cultured cells as well as in mice expressing an MV receptor, signaling lymphocyte activation molecule, regardless of whether the virus genome had the wild-type or vaccine-type promoter sequence. These data demonstrate that the polymerase protein genes of the Edmonston strain contribute to its attenuated phenotype.

Measles is a highly contagious disease associated with high morbidity and mortality. Measles virus (MV), the causative agent of the disease, is an enveloped virus with a nonsegmented negative-strand RNA genome of ~16 kb that is classified in the genus *Morbillivirus* in the family *Paramyxoviridae* (12). The genome is encapsidated by the nucleocapsid (N) protein and associated with a viral RNA-dependent RNA polymerase composed of two subunits, phosphoprotein (P protein) and large (L) protein. The genome and these proteins form a helical ribonucleoprotein complex that acts as the active template for transcription and replication (12). The incidence and mortality of measles have been remarkably reduced in many countries as the vaccine coverage rate increases (12).

Live attenuated MV vaccines have been generated via many rounds of passages of clinical isolates of MV in various cultured cells (12, 29). The Edmonston strain, isolated in 1954 (9), was used as a seed strain to generate live attenuated vaccines (17, 29). Although some of the molecular mechanisms by which Edmonston lineage vaccines have adapted to grow in various cultured cells have been elucidated (32, 33, 40), the mechanisms of their attenuation are poorly understood. In the present study, we generated various recombinant MVs with chimeric genomes of the Edmonston vaccine and wild-type strains of MV, and we found that viruses possessing the polymerase protein genes of the Edmonston strain exhibited attenuated MV gene expression and growth in cultured cells as well as in mice expressing a cellular receptor for MV, human signaling lymphocyte activation molecule (hSLAM) (21). These data provide a molecular basis for the avirulence of the Edmonston strain *in vivo*.

SLAM is a universal receptor for all MV strains, while CD46 functions as an additional receptor for vaccine strains but not for wild-type strains (41, 43). Thus, both wild-type and Edmonston strains replicate efficiently and produce high titers of progeny viruses in SLAM-expressing cells (10, 14). However, when plaque assays were performed, the Edmonston lineage strains produced smaller plaques than wild-type strains of MV (Fig. 1A). Wild-type strains of MV (JPN/50.98/1 and JPN/26.99/1) were isolated from patients with measles and passaged two or three times in B95a cells (15) to make stocks for experiments (kind gifts from H. Sakata). The Edmonston strain obtained from the American Type Culture Collection (ATCC) is designated the Ed-ATCC strain in this paper. Recombinant Edmonston tag (Ed-tag) (26) and wild-type IC323 (39) MV clones were recovered from cDNAs and grown in Vero/hSLAM cells (23). To clarify the genes responsible for the small-plaque-forming phenotype of the Edmonston strain, six recombinant MVs that each contained one of the Ed-tag strain genes in the backbone of the genome of the wild-type IC323 strain expressing enhanced green fluorescent protein (IC323-EGFP) (13) were prepared and designated IC/Ed-N-EGFP, IC/Ed-P-EGFP, IC/Ed-M-EGFP, IC/Ed-F-EGFP, IC/Ed-H-EGFP, and IC/Ed-L-EGFP. IC/Ed-N-EGFP, IC/Ed-P-EGFP, IC/Ed-F-EGFP, IC/Ed-H-EGFP, and IC/Ed-L-EGFP were reported previously and referred to as m2, m3, m7, m8, and m9, respectively (34). When the recombinant MVs were used to infect Vero/hSLAM cells, IC/Ed-P-EGFP and IC/Ed-L-EGFP produced smaller plaques than wild-type IC323-EGFP (Fig. 1B). These data indicate that the P and L genes of the Ed-tag strain contribute to the decreased plaque sizes produced by the Ed-tag strain. In addition to the P protein, the P gene encodes two accessory gene products, V and C (12). The V protein directly interferes with host interferon (IFN) induction and IFN signaling pathways, while the C protein modulates viral RNA synthesis to circumvent IFN induction (19). It should be noted that, unlike the V proteins of the Ed-ATCC and wild-

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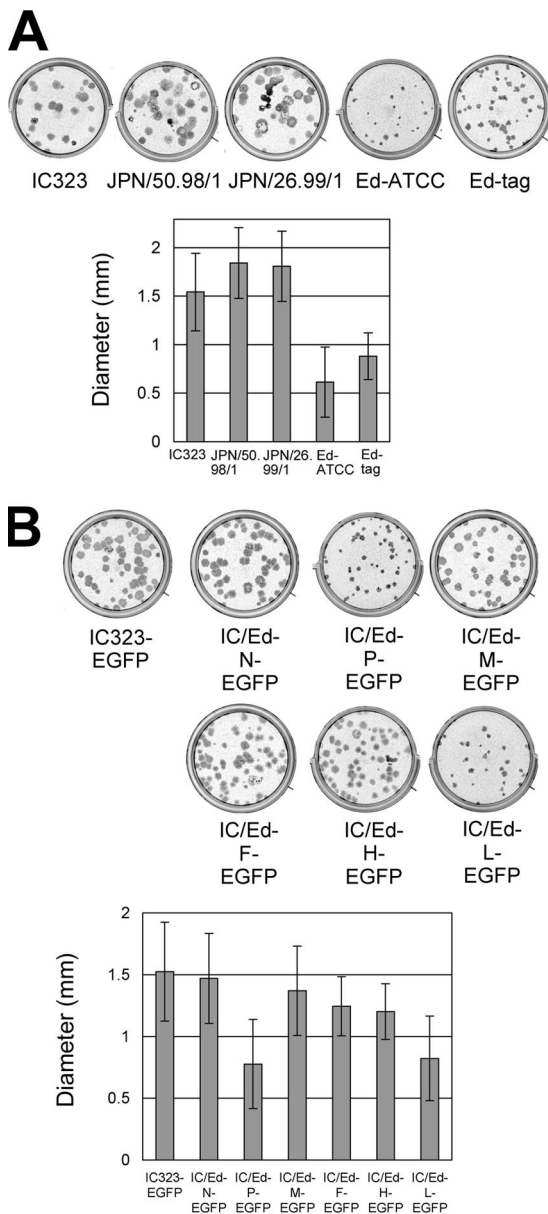


FIG. 1. Small plaque formed by the Edmonston strain and recombinant MVs with the P or L gene of the Edmonston strain on Vero/hSLAM cells. (A) Plaque assays were performed for various MV strains (three wild-type strains [IC323, JPN/50.98/1, and JPN/26.99/1] and two Edmonston lineage strains [Ed-ATCC and Ed-tag]). Monolayers of Vero/hSLAM cells on 12-well cluster plates were infected with 50 PFU of each virus and overlaid with Dulbecco's modified Eagle medium containing 2% fetal bovine serum and 1% methylcellulose. At 5 days p.i., the cells were stained with RTU Vectastain Elite ABC reagent (Vector Laboratories) using anti-MV H-protein monoclonal antibodies and a biotinylated secondary antibody. After high-resolution digital images were obtained, the sizes of all plaques were measured. The mean sizes \pm standard deviations are shown in the bar graph. (B) Plaque assays were performed for wild-type IC323-EGFP and six recombinant MVs (each containing one of the Ed-tag strain genes in the backbone of the IC323-EGFP genome) on Vero/hSLAM cells, as described for panel A.

type MV strains, the V protein of the Ed-tag strain is defective in counteracting IFN signaling pathways owing to tyrosine-to-histidine and cysteine-to-arginine substitutions at amino acid positions 110 and 272 (Y110H and C272R), respectively (5, 8,

11, 22). Substitutions at these positions are not unique to the Ed-tag strain and have been found in other vaccines and cultured cell-adapted MV strains, including the CAM-70 vaccine (4), the Changchun-47 vaccine (4), the chicken embryo fibroblast-adapted D-CEF strain (1), and the Vero cell-adapted 94YTV strain (containing the C272R substitution; our unpublished observation). These findings prompted us to carry out further experiments using the P gene of the Ed-tag strain.

To quantify the expression levels of MV genes, four recombinant MVs expressing *Renilla* luciferase instead of EGFP were generated. The wild-type IC323 strain expressing *Renilla* luciferase (IC323-Luci) was reported previously (38). The P and/or L genes of the Ed-tag strain were introduced into the IC323-Luci genome, thereby generating IC/Ed-P-Luci, IC/Ed-L-Luci, and IC/Ed-PL-Luci. The *Renilla* luciferase activities induced by these recombinant MVs were analyzed in various cell types expressing SLAM (Vero/hSLAM [23], CV1/hSLAM, HeLa/hSLAM, A549/hSLAM [36], CHO/hSLAM [41], B95a, MT2, Raji, Ramos, BJAB-B95-8, and C91/PL) or an unidentified MV receptor on epithelial cells (NCI-H358) (38). CV-1/hSLAM and HeLa/hSLAM cells were generated by cotransfecting CV1 and HeLa cells with the eukaryotic expression vector pCA7 (36), a derivative of pCAGGS (20) encoding human SLAM, and the selection vector pCXN2 (20), encoding a neomycin resistance gene, followed by selection in the presence of Geneticin (G418; Nacalai Tesque). Among all these cell lines, IC/Ed-P-Luci and IC/Ed-L-Luci showed reduced *Renilla* luciferase expression levels compared with wild-type IC323-Luci (Fig. 2A and data not shown). The luciferase activities induced by IC/Ed-PL-Luci were even lower than those induced by IC/Ed-P-Luci and IC/Ed-L-Luci among all the cell lines examined (Fig. 2A and data not shown). These data indicate that introduction of the P and L genes of the Ed-tag strain into recombinant virus genomes reduces viral gene expression and growth, regardless of the cell type. The viral mRNA levels in Vero/hSLAM cells infected with the recombinant MVs were also quantified. Confluent monolayers of Vero/hSLAM cells in 6-cm culture plates were infected with 1.0×10^4 PFU of IC323-Luci, IC/Ed-P-Luci, IC/Ed-L-Luci, or IC/Ed-PL-Luci. Multiple rounds of infection were blocked by a fusion-blocking peptide (Peptide Institute) (28). At 18 h postinfection (p.i.), mRNAs were purified from the cells using TRIzol reagent (Invitrogen) and an Oligotex-dT30 mRNA purification kit (TaKaRa Bio Inc.), reverse transcribed into cDNAs with an iScript cDNA synthesis kit (Promega), and subjected to PCR using SYBR Premix Ex *Taq* II (TaKaRa Bio Inc.). The primers used to amplify the MV genes were described previously (36). β -Actin mRNA was also quantified and used as an internal control (Fig. 2B). The levels of the viral transcripts (N, P, M, F, H, and L mRNAs, which show a transcriptional gradient [6, 7, 25]) were reduced \sim 10-fold in IC/Ed-P-Luci- and IC/Ed-L-Luci-infected cells, compared with the levels in IC323-Luci-infected cells (Fig. 2B). The viral transcript levels were even further reduced in IC/Ed-PL-Luci-infected cells, being about 100-fold lower than those in IC323-Luci-infected cells (Fig. 2B). These data show that the small-plaque-forming phenotype of the Edmonston strain is at least partly caused by reduced viral gene expression levels in cells. We also analyzed the activities of the P and L proteins using a minigenome assay (18, 19). All combinations of the P- and

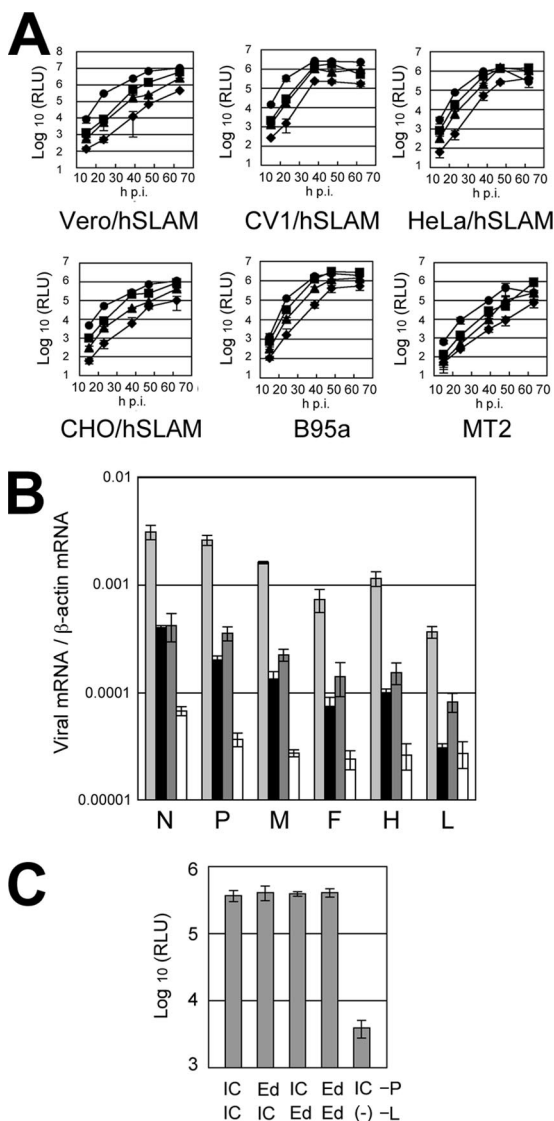


FIG. 2. Attenuated gene expression by recombinant MVs with the P and/or L genes of the Edmonston strain. (A) Confluent monolayers of various cell lines (Vero/hSLAM, CV1/hSLAM, HeLa/hSLAM, CHO/hSLAM, and B95a) and suspensions of nonadherent MT2 cells cultured in 24-well cluster plates were infected with 2.5×10^3 PFU of IC323-Luci (circles), IC/Ed-P-Luci (squares), IC/Ed-L-Luci (triangles), and IC/Ed-PL-Luci (diamonds). After various intervals, the *Renilla* luciferase activities were measured. Data are means \pm standard deviations for triplicate samples. RLU, relative light units. (B) Confluent monolayers of Vero/hSLAM cells in 6-cm culture plates were infected with 1.0×10^4 PFU of IC323-Luci (light gray bars), IC/Ed-P-Luci (black bars), IC/Ed-L-Luci (dark gray bars), and IC/Ed-PL-Luci (white bars) and cultured in the presence of a fusion-blocking peptide. At 18 h p.i., mRNAs were purified from the cells, and the levels of N, P, M, F, H, and L mRNAs were determined by reverse transcription-quantitative PCR. Data are means \pm standard deviations for triplicate samples. (C) Minigenome assays. The method was described in detail elsewhere (19). Monolayers of CHO/hSLAM cells cultured in Opti-MEM on 24-well plates were infected with $vTF7-3$ at a multiplicity of infection of 0.5 and then transfected with 0.2 μ g of p18MGLuc01-wt-Le, 0.2 μ g of pCAG-T7-IC-N, 0.3 μ g of a P-protein expression plasmid (pCAG-T7-IC-P Δ C or -Ed-P Δ C), and 0.2 μ g of an L-protein expression plasmid (pGEMCR-IC-L or -Ed-L) using Lipofectamine 2000 (Invitrogen). At 6 h p.i., the culture media were replaced with RPMI 1640 medium supplemented with 7.5% fetal bovine serum. At 48 h p.i., the firefly luciferase activities were measured. (-), L-protein expression plasmid was omitted. Data are means \pm standard deviations for triplicate samples.

L-protein-expressing plasmids of the wild-type IC323 and Ed-tag strains produced equivalent *Renilla* luciferase activities (Fig. 2C). The discrepancy between the data obtained with the minigenome assays and the data obtained using recombinant viruses may be attributed to differences in the gene expression systems. In minigenome assays, the components required for viral RNA synthesis (N, P, and L proteins) are continuously supplied in *trans* from expression plasmids, whereas in virus infections, MV has to synthesize these proteins by itself to continue viral RNA synthesis. Furthermore, in virus infections, but not in minigenome assays, MV produces the C, V, and matrix (M) proteins, which modulate viral RNA synthesis (3, 24, 27, 31, 42).

Compared with the genomes of wild-type MV strains, the genomes of the Edmonston vaccine strains have three nucleotide substitutions (uracil-to-adenine, uracil-to-guanine, and uracil-to-cytosine changes at nucleotide positions 26, 42, and 50, respectively) in the genomic promoter (leader [Le]) region (16, 37). Using minigenome assays, these substitutions have been shown to enhance the promoter activity (16). To analyze the effects of these substitutions on virus infection, they were introduced into the IC323-Luci genome, generating IC/Ed-Le-Luci. The substitutions were also introduced into the IC/Ed-P-Luci, IC/Ed-L-Luci, and IC/Ed-PL-Luci genomes, thereby generating IC/Ed-LeP-Luci, IC/Ed-LeL-Luci, and IC/Ed-LePL-Luci, respectively. The *Renilla* luciferase activities induced by IC323-Luci and IC/Ed-Le-Luci were compared in the 12 cell lines described above. No significant differences between the *Renilla* luciferase activities of IC323-Luci- and IC/Ed-Le-Luci-infected cells were observed for any of the 12 cell lines (Fig. 3A and data not shown). These data reveal that the three substitutions have neutral effects on the promoter function when introduced into the wild-type MV genome. Regardless of the presence or absence of these substitutions in the Le region, the P and L genes of the Ed-tag strain, especially when combined, reduced the plaque sizes of recombinant MVs (Fig. 3B).

It is now clear that the P and L genes of the Ed-tag strain attenuate MV gene expression when introduced into recombinant virus genomes. To exclude possible growth-inhibitory effects caused by the inability of the Ed-tag V protein to block IFN signaling due to the Y110H and C272R substitutions (5, 8, 11, 22), the histidine and arginine residues at these positions were replaced with the corresponding residues of wild-type MV (tyrosine and cysteine, respectively), generating Ed-tag- $V_{H110Y/R272C}$. An additional transcriptional unit for *Renilla* luciferase was also created in the Ed-tag strain genome, generating Ed-tag- $V_{H110Y/R272C}$ -Luci. Ed-tag- $V_{H110Y/R272C}$ -Luci still produced small plaques, with sizes equivalent to those of plaques produced by the original Ed-tag strain (Fig. 1A and 4A). The *Renilla* luciferase activities induced by Ed-tag- $V_{H110Y/R272C}$ -Luci were analyzed and compared with those induced by wild-type IC323-Luci in various cell lines described above (Fig. 4B and data not shown). The wild-type and Edmonston strains have been shown to enter SLAM-positive cells with similar efficiencies (41, 43). Nonetheless, Ed-tag- $V_{H110Y/R272C}$ -Luci induced 8- to 50-fold-lower *Renilla* luciferase activities in SLAM-positive cell lines than IC323-Luci (Fig. 4B). These data further confirmed the reduced gene expression levels induced by the Edmonston strain.

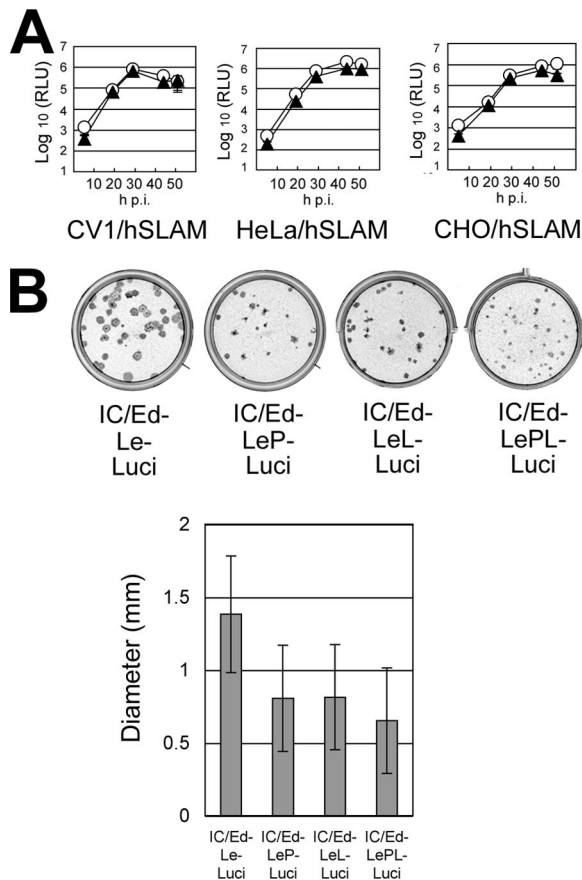


FIG. 3. Neutral effects of substitutions in the leader region of the Edmonston strain on MV gene expression levels and plaque sizes. (A) Confluent monolayers of CV1/hSLAM, HeLa/hSLAM, and CHO/hSLAM cells cultured in 24-well cluster plates were infected with 2.5×10^3 PFU of IC323-Luci (circles) and IC/Ed-Le-Luci (triangles). After various intervals, the *Renilla* luciferase activities were measured. Data are means \pm standard deviations for triplicate samples. RLU, relative light units. (B) Plaque assays were performed for the recombinant MVs, as described for Fig. 1A. The IC/Ed-Le-Luci, IC/Ed-LeP-Luci, IC/Ed-LeL-Luci, and IC/Ed-LePL-Luci genomes contain Ed-tag genome regions encoding Le alone, Le and P, Le and L, or Le, P, and L, respectively, in the backbone of the IC323-Luci genome.

Aliquots (1.0 ml) containing 1.5×10^5 PFU of recombinant MVs (IC323-Luci, IC/Ed-L-Luci, and Ed-tag- $V_{H110Y/R272C}$ -Luci) were injected into the peritoneal cavities of SLAM-knock-in mice crossed with mice lacking type I IFN receptor subunit 1 (IFNAR1^{-/-} SLAM-knock-in mice) (21). Eight mice were used for each recombinant MV. At 5 days p.i., the spleens were removed, homogenized using a BioMasher (Hi-Tech Inc.), and analyzed for their *Renilla* luciferase activities. The median *Renilla* luciferase activity in spleens from mice infected with Ed-tag- $V_{H110Y/R272C}$ -Luci was $\sim 10,000$ -fold lower than that in spleens from mice infected with wild-type IC323-Luci ($P < 0.001$; Fig. 4C). These data clearly indicate that the Edmonston strain is severely attenuated in IFNAR1^{-/-} SLAM-knock-in mice. The median *Renilla* luciferase activity in spleens from IC/Ed-L-Luci-infected mice was ~ 200 -fold lower than that in spleens from mice infected with wild-type IC323-Luci ($P < 0.001$; Fig. 4C). These data indicate that the L gene

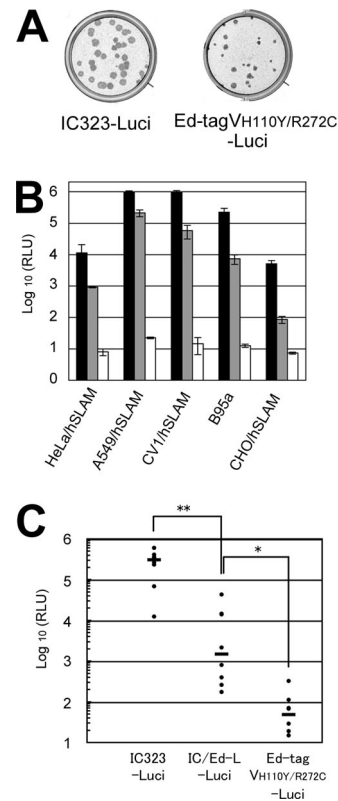


FIG. 4. Attenuated gene expression by the Edmonston strain in cultured cells and SLAM-knock-in mice. (A) Plaque assays were performed for IC323-Luci and Ed-tag- $V_{H110Y/R272C}$ -Luci on Vero/hSLAM cells, as described for Fig. 1A. (B) Confluent monolayers of HeLa/hSLAM, A549/hSLAM, CV1/hSLAM, B95a, and CHO/hSLAM cultured in 12-well cluster plates were infected with 2.5×10^3 PFU of IC323-Luci (black bars) and Ed-tag- $V_{H110Y/R272C}$ -Luci (gray bars). At 30 h p.i., the *Renilla* luciferase activities were measured. Data are means \pm standard deviations for triplicate samples. Mock-infected samples are shown by white bars. RLU, relative light units. (C) IC323-Luci, IC/Ed-L-Luci, and Ed-tag- $V_{H110Y/R272C}$ -Luci (1.5×10^5 PFU) were injected into the peritoneal cavities of IFNAR1^{-/-} SLAM-knock-in mice. At 5 days p.i., the spleens were removed and analyzed for their *Renilla* luciferase activities. Each symbol indicates the sample from a single mouse, and bars indicate the median values. RLU, relative light units. *, $P < 0.05$; **, $P < 0.001$.

of the Edmonston strain contributes to the in vivo attenuation of the Edmonston strain.

Many viruses can be adapted to various cultured cells by passaging in the cells, and it is empirically known that these adaptations often reduce virus virulence in natural host animals (15). Previous studies indicated that MV can adapt to grow in some cultured cells by acquiring specific substitutions in the receptor-binding hemagglutinin (H) and/or M proteins (43). Although some mechanisms by which these changes in the H and M proteins may cause attenuation have been proposed (30, 33), their contributions in vivo remain to be determined in animal models. Consistent with the present data, our previous study using a wild-type MV strain and its Vero cell-adapted strain suggested that substitutions introduced into the polymerase protein genes (L and P) during passages in Vero cells caused MV attenuation by reducing the transcriptional activities of viral polymerase (35). However, we observed at-

tenuated gene expression levels by the Edmonston strain only in virus infection analyses, not in minigenome assays. Bankamp et al. (2) also reported that the polymerase proteins of MV vaccine strains show higher transcriptional activities than those of wild-type MV strains when analyzed by minigenome assays. The detailed mechanisms of the attenuated gene expression induced by the polymerase protein genes of the Edmonston strain remain to be elucidated.

In conclusion, the present study demonstrates that the polymerase protein genes of the Edmonston strain contribute to its attenuated phenotype. Our data further show that assays using infectious recombinant viruses are crucial for understanding the contribution of each viral gene to virus replication and virulence and that the SLAM-knock-in mouse is a useful animal model for elucidating the attenuation mechanisms of MV vaccines.

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