Contribution of Matrix, Fusion, Hemagglutinin, and Large Protein Genes of the CAM-70 Measles Virus Vaccine Strain to Efficient Growth in Chicken Embryonic Fibroblasts[▽]

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Attenuated live vaccines of measles virus (MV) have been developed from clinical isolates by serial propagation in heterologous cells, mainly chicken embryonic cells. The safety and effectiveness of these vaccines have been well established. However, the molecular mechanism of their attenuation remains a subject of investigation. The CAM-70 MV vaccine strain was developed from the Tanabe strain by serial propagation in chicken embryonic cells. In the present study, we assessed the contribution of each gene in the CAM-70 strain to efficient growth in chicken embryonic fibroblasts (CEF). We used a cloned MV IC323 based on the wild-type IC-B strain and generated a series of IC323s that possess one or more of the CAM-70 genes. Then, we examined the infection of CEF and CEF expressing human signaling lymphocyte activation molecule with the recombinant MVs. Our results demonstrated that MV needs to adapt to CEF at both the entry and postentry steps and that the CAM-70 matrix protein gene plays an important role in adaptation to CEF at the early stage of the virus replication cycle. The CAM-70 large protein gene was responsible for the efficient transcription and replication in CEF, and the CAM-70 hemagglutinin and fusion protein genes were responsible for efficient entry. Investigations focusing on these genes might elucidate unknown molecular mechanisms underlying the attenuation of MV.

Measles is an extremely contagious disease and has been a major killer of children in developing countries. The widespread use of effective live attenuated measles vaccines has resulted in the control of measles in many industrialized countries. Moreover, mortality due to measles was reduced from 750,000 deaths to 197,000 deaths worldwide between 2000 and 2007 through mass vaccination in developing countries (10, 46). In Japan, measles vaccines were introduced in 1978 as part of routine immunization schedules and the number of measles cases has decreased dramatically, although relatively large outbreaks still occur (9, 24, 28).

One of the attenuated live vaccines for measles virus (MV), the CAM-70 strain, was originally developed in Japan from the Tanabe strain by serial propagation in amniotic and chorioallantoic membranes of chicken embryos and chicken embryonic fibroblasts (CEF) (44, 45). Other attenuated live vaccines of MV have been developed from clinical isolates (e.g., the Edmonston strain) by serial propagation in heterologous cells and tissues, mainly chicken embryos and CEF (16). The safety and effectiveness of CAM-70 and the other measles vaccine strains have been well established (16, 29, 30). However, the molecular mechanism of their attenuation remains a subject of investigation. Understanding the molecular basis of the MV adaptation to heterologous cells, especially chicken

embryonic cells, would help to elucidate the mechanism of the MV attenuation.

MV, an enveloped virus with a nonsegmented negative-strand RNA genome, belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*. The MV genome of 15,894 nucleotides (nt) consists of six tandem-linked genes that encode the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The P gene, in addition to the P protein, encodes two accessory proteins, C and V proteins. The C protein is synthesized by an alternative translational initiation in a different reading frame, and the V protein is translated from the mRNA edited by a cotranscriptional insertion of a single nontemplated G residue. The N protein encapsidates the viral genomic RNA, and the N protein-encapsidated viral genome is associated with a viral RNA-dependent RNA polymerase composed of the P and L proteins, forming a helical ribonucleoprotein complex (RNP). In infected cells, the viral RNA-dependent RNA polymerase transcribes the genomic RNA into mRNAs and also replicates the viral genome (16). The H and F proteins are integral membrane glycoproteins. The H protein recognizes the cellular receptor, and its binding to the receptor leads to activation of the F protein, resulting in fusion between the viral envelope and the cellular membrane and entry of the RNP into the cytoplasm (21). The cellular receptors for MV include CD46 and signaling lymphocyte activation molecule (SLAM; also called CD150) (12, 13, 18, 26, 43). CD46 is expressed on all nucleated human cells (23) and is recognized by both vaccine and laboratory-adapted strains but generally not by wild-type MV strains (48). SLAM is expressed on cells of the immune

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system (2, 11, 33) and is a receptor for all MV strains, including wild-type strains (48). In addition to CD46 and SLAM, an unidentified receptor for MV on the basolateral side of the epithelium has been reported (22, 38, 41). The M protein interacts with the RNP and the cytoplasmic tails of the H and F proteins and plays an important role in the assembly and budding of the viral particles (6, 7, 16, 17, 34, 37). The M protein also inhibits viral transcription and replication (32, 35).

In the present study, we tried to identify the genes that are responsible for efficient growth of the MV CAM-70 strain in CEF. For this purpose, we used a cloned MV IC323 that can be rescued from the full-length genomic cDNA of the wild-type IC-B strain (39) and replaced each of the IC323 genes with the corresponding region of the CAM-70 strain singly and in various combinations. We then examined infection of CEF and CEF expressing human SLAM with the recombinant MVs and found that M, F, H, and L genes contribute to efficient growth of the CAM-70 strain in CEF.

MATERIALS AND METHODS

Cells and viruses. CEF were prepared from 10-day-old chicken embryos and cultured in Dulbecco modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (DMEM–10% FBS). The adherent marmoset B-cell line B95a (20) was incubated in RPMI 1640 medium (RPMI; Nissui Pharmaceutical) containing 5% FBS (RPMI–5% FBS). Chinese hamster ovary (CHO) cells expressing human SLAM (CHO/SLAM) (43) and 293FT cells (Invitrogen, Carlsbad, CA) were cultured in DMEM–10% FBS supplemented with 500 µg of G418 (Geneticin; Nacalai Tesque, Tokyo, Japan) per ml. The vaccine strain CAM-70 was derived from a commercially available vaccine vial (The Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan) and propagated in B95a cells once or twice at 37°C to prepare virus stocks. Recombinant MVs were generated from cDNAs by using CHO/SLAM cells and the vaccinia virus carrying T7 RNA polymerase, vTF7-3, according to a previously reported procedure (40). Infectivity titers of virus stocks were determined by measuring the 50% tissue culture infectious dose (TCID₅₀) in B95a cells.

Cloning of human SLAM gene and transduction of CEF. Human peripheral blood lymphocytes (PBL) were prepared as described previously (27). PBL were cultured in RPMI containing 10% FBS supplemented with 2.5 µg of PHA-L (Sigma, St. Louis, MO) per ml for 2 days, and then total RNA was isolated from the activated PBL by using an RNeasy mini kit (Qiagen, Hilden, Germany). To easily subclone the cDNA of human SLAM mRNA into a pFB retroviral vector (Stratagene, La Jolla, CA), restriction enzyme recognition sequences were added to the 5' ends of the specific sequences in the primers for reverse transcription-PCR (RT-PCR) of human SLAM gene. We used the forward primer 5'-CCGG AATTCCAGACAGCCTCTGCTGCATGAC-3' (the EcoRI recognition site is underlined) and the reverse primer 5'-CCGCTCAGAGCCTTCAGAAAGTCCC TTGTGG-3' (the XhoI recognition site is underlined). Total RNA was reverse transcribed into cDNA by using the reverse primer and the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was amplified by 30 PCR cycles under the following conditions: 30 s at 94°C, 30 s at 60°C, and 120 s at 68°C by using the Platinum *Pfx* DNA polymerase (Invitrogen) with the forward and reverse primers. The amplified PCR product was cloned into the pCR-Blunt-II-TOPO vector (Invitrogen), and the cDNA was sequenced by using the Big Dye terminator cycle sequencing kit and an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). The cDNA sequence was the same as the previously reported sequence of the human SLAM gene (GenBank accession number U33017). The cDNA of human SLAM was subcloned into a pFB retroviral vector by using the EcoRI and XhoI sites, and the generated plasmid was designated pFB-hSLAM.

293FT cells in 60-mm tissue culture plates were transfected with 2 µg each of pVPack-VSV-G, pVPack-GP (Stratagene), and pFB-hSLAM by using the FuGENE HD transfection reagent (Roche Diagnostics, Indianapolis, IN). The medium was replaced with 3 ml of fresh DMEM–10% FBS after 24 h. The retrovirus-containing supernatant was harvested at 48 h posttransfection. pFB-Neo (Stratagene), which contains the neomycin-resistant (Neo^r) gene, was also transfected into 293FT cells with pVPack-VSV-G and pVPack-GP to generate retrovirus expressing Neo^r gene. CEF in 25-cm² flasks were infected with 2 ml of

each retrovirus-containing supernatant, followed 3 h later by the addition of 2 ml of DMEM–10% FBS. At 48 h postinfection (p.i.), the expression of human SLAM on transduced CEF was examined by flow cytometry, and the cells were used for infection experiments.

Flow cytometry analysis. CEF transduced with each of the retroviruses expressing human SLAM or the Neo^r gene were stained with phycoerythrin (PE)-conjugated anti-CD150 monoclonal antibody (MAb) (clone A12) or PE-conjugated mouse immunoglobulin G1 (IgG1) isotype-control antibody (BD Biosciences, San Jose, CA). Stained cells were analyzed on a FACS Calibur (BD Biosciences).

Cloning of genes of the CAM-70 strain. B95a cells cultured in 75-cm² flasks were infected with the MV CAM-70 strain at a multiplicity of infection (MOI) of 0.01 TCID₅₀/cell, and the infected cells were harvested 2 days p.i. Total RNA was then isolated from the infected cells and reverse transcribed into cDNA by using each of the specific sense primers for the MV genes by following the protocol for the generation of the human SLAM-gene cDNA (described above). The cDNA was amplified by 30 PCR cycles under the following conditions: 30 s at 94°C, 30 s at 55°C, and 120 s at 68°C by using the Platinum *Pfx* DNA polymerase with each set of MV-specific sense and antisense primers. The sets of sense and antisense primers used for RT-PCR were 5'-⁸⁴AGAGCAGGATTAGGGATATCCGA G¹⁰⁷-3' and 5'-²¹⁸⁹GAGTCAGCATCTTGGATTC²¹⁷¹-3' for the N gene, 5'-¹⁶⁹⁰GAGAGGCCGAGGACCAGAACA¹⁷¹¹-3' and 5'-³⁴⁶⁵ACTTGTCGAAG TCGTGGATCTCTGTCATTG³⁴³⁶-3' for the P gene, 5'-³¹⁷⁸CCTGCATCAGC CAGTGAATCC³¹⁹⁹-3' and 5'-⁶⁷³⁴TTCACCTCGACTACCGGGCA⁶⁷¹⁵-3' for the M gene, 5'-⁴⁵⁹²CAGCACAGAACGCCCTGACACA⁴⁶¹⁵-3' and 5'-⁷²⁷³CATTGTGGATGATCTTGCACCCTA⁷²⁵⁰-3' for the F gene, and 5'-⁹¹⁵⁹G ATACGAGGCATACCCACTAGTGTG⁹¹⁸³-3' and 5'-¹⁵⁷⁸⁰CCCTAATCAGA GCGCTGTATCCGAC¹⁵⁷⁵⁶-3' for the L gene. The amplified PCR product was cloned into pCR-Blunt-II-TOPO vector, and three clones of the cDNA were sequenced. The consensus sequence of the N, P, F, and L genes of the CAM-70 strain was the same as the previously reported sequence (GenBank accession no. DQ345723). The nucleotide at position 4111 was G in the M gene of the CAM-70 strain that we used, while the nucleotide at this position is T in the previously reported sequence (GenBank accession no. DQ345723). This difference results in an arginine, instead of a methionine, at amino acid position 225 of the M protein. Because the nucleotide G at position 4111 in the M gene is shared by IC-B (GenBank accession no. NC_001498), the Edmonston-lineage vaccine strains (31), and the Tanabe strain from which the CAM-70 strain is derived (GenBank accession no. AB506683), we used our sequence of the CAM-70 M cDNA in the present study.

Plasmid construction. All plasmids encoding the mutant MV genome were based on p(+)MV323, which encodes the antigenomic full-length cDNA of the wild-type IC-B strain of MV (39). By using the appropriate restriction enzymes, we replaced each of the N, P, M, F, and L genes of p(+)MV323 with the corresponding regions of cloned cDNAs encoding the CAM-70 genes. The BspI-PstI fragment between nt 128 and 1737 of p(+)MV323 was replaced with the corresponding region of the CAM-70 N gene, generating full-length genome plasmids termed p(+)MV323-CAM N (Fig. 1A). Similarly, each of the SspI-SalI (nt 1808 to 3364), SspI-EcoRI (nt 3364 to 4383), BsmBI-PacI (nt 5481 to 7242), and SpeI-AfeI (nt 9175 to 15767) fragments was replaced with the corresponding region of the CAM-70 genes, generating p(+)MV323-CAM P, p(+)MV323-CAM M, p(+)MV323-CAM F, and p(+)MV323-CAM L, respectively (Fig. 1A). Although the nucleotide at position 122 in the coding region of the N gene of p(+)MV323-CAM N and the nucleotides at positions 15767, 15770, and 15782 in the coding region of the L gene of p(+)MV323-CAM L remained the same as the sequence derived from p(+)MV323, these nucleotide differences did not result in differences in the deduced amino-acid sequences between the IC-B and CAM-70 strains. Each nucleotide sequence in the coding region of the P gene of p(+)MV323-CAM P, the M gene of p(+)MV323-CAM M, and the F gene of p(+)MV323-CAM F was the same as that in the corresponding region of the CAM-70 strain. However, p(+)MV323-CAM F did not possess ATG (nt 5449 to 5451) which is found in the CAM-70 F gene. p(+)MV323-CAM H, in which the H gene region of p(+)MV323 was replaced with the corresponding region of the CAM-70 strain, was generated previously (19).

The genes of p(+)MV323 were also replaced with genes of the CAM-70 strain in combination, by using the same restriction enzymes as described above. Both the SspI-SalI (nt 1808 to 3364) and SpeI-AfeI (nt 9175 to 15767) fragments of p(+)MV323-CAM P were replaced with the corresponding fragments of the CAM-70 cDNAs, generating p(+)MV323-CAM NPL. Then, the SspI-EcoRI (nt 3364 to 4383) fragment of p(+)MV323-CAM NPL was replaced with that of the CAM-70 cDNA, generating p(+)MV323-CAM NPL+M. Finally, the BsmBI-SpeI (nt 5481 to 9175) fragment of p(+)MV323-CAM NPL+M was replaced with that of the CAM-70 cDNA, generating p(+)MV323/CAM in which all of

FIG. 1. Infection of CEF with recombinant MVs possessing the CAM-70 genes. (A) Schematic diagram of the recombinant MVs. Wide boxes indicate open reading frames of the MV N, P, M, F, H, and L proteins. Narrow boxes indicate untranslated regions, and vertical lines in untranslated regions indicate intergenic trinucleotides. Narrow boxes at the 3' and 5' genome termini are leader and trailer sequences. Regions derived from the CAM-70 strain are shaded, and regions from IC323 are in white. Restriction enzyme sites used to replace the regions of IC323 with the corresponding regions of the CAM-70 strain are indicated. (B) Growth kinetics of recombinant MVs in CEF. CEF were infected with each of the recombinant MVs or the CAM-70 strain at an MOI of 0.05 TCID₅₀/cell. Infected culture medium and cells were harvested together every day up to 5 days p.i. After freezing and thawing, infectivity titers were determined by measuring TCID₅₀ in B95a cells. The means ± the standard deviations for three independent experiments are shown.

the genes of p(+)MV323 were replaced with genes derived from the CAM-70 strain (Fig. 1A).

The *PacI*-*SpeI* (nt 7242 to 9175) fragment of p(+)MV323-CAM F was also replaced with the corresponding region of the CAM-70 cDNA, generating p(+)MV323-CAM FH (Fig. 1A). Similarly, each of the *BsmBI*-*PacI* (nt 5481 to 7242), *PacI*-*SpeI* (nt 7242 to 9175), and *BsmBI*-*SpeI* (nt 5481 to 9175) fragments of p(+)MV323-CAM M was replaced with the corresponding region of the CAM-70 cDNA, generating p(+)MV323-CAM MF, p(+)MV323-CAM MH, and p(+)MV323-CAM MFH, respectively (Fig. 3A).

Plasmids encoding a mutant MV genome based on p(+)MV323/CAM were also generated (Fig. 4A). Each of the *BspI*-*PsiI* (nt 128 to 1737), *SapI*-*SalI* (nt 1808 to 3364), *SalI*-*EcoRI* (nt 3364 to 4383), and *SpeI*-*AfeI* (nt 9175 to 15767) fragments of p(+)MV323/CAM was replaced with the corresponding fragment of p(+)MV323, generating p(+)MV323/CAM-IC N, p(+)MV323/CAM-IC P, p(+)MV323/CAM-IC M, and p(+)MV323/CAM-IC L (Fig. 4A).

Growth kinetics of MV. CEF were seeded at a density of 10⁵ cells per well in 48-well cluster plates the day before infection. Cells were incubated with MV at an MOI of 0.05 TCID₅₀/cell for 1 h at 37°C. After two washes with DMEM, the cells were incubated in 500 µl of DMEM–10% FBS/well at 37°C. For infection of CEF transduced with retroviruses expressing human SLAM or the Neo^r gene, cells were seeded at 24 h posttransduction (p.t.) and infected with MV at 48 h p.t. Infected culture medium and cells were harvested together every day up to 5 days p.i. After freezing and thawing, infectivity titers were determined by measuring TCID₅₀ in B95a cells.

Quantification of MV antigenome and mRNAs by RT-PCR. CEF or CEF transduced with retroviruses were seeded at a density of 2 × 10⁵ cells per well in 24-well cluster plates and infected with MV at an MOI of 1 TCID₅₀/cell, following the procedure for growth kinetics (described above). At 16 h p.i., total RNA was isolated from the infected cells and then reverse-transcribed into cDNA by using oligo(dT)₂₀ primer and following the protocol for the generation of human

FIG. 2. Infection of CEF/Neo and CEF/SLAM with recombinant MVs. (A) Expression of human SLAM on CEF/Neo and CEF/SLAM. CEF were transduced with retroviruses expressing the Neo^r gene or human SLAM. After 48 h, the cells were stained with PE-conjugated anti-CD150 MAb (filled profile) or PE-conjugated mouse IgG1 isotype-control antibody (gray line). Stained cells were analyzed by flow cytometry. The mean percentages $\pm$ the standard deviations of SLAM-positive cells for three independent experiments are shown. (B) Quantification of MV antigenome and mRNAs. CEF/Neo and CEF/SLAM were infected with recombinant MVs at an MOI of 1 TCID₅₀/cell. At 16 h p.i., total RNAs were isolated from the infected cells, and the amounts of MV antigenome and N and P mRNAs were quantified by real-time RT-PCR. The mean expression level in CEF/Neo infected with IC/CAM was set to 1. The means $\pm$ the standard deviations and *P* values using Student *t* test for three independent experiments are shown. (C) Accumulation of MV N and P proteins. CEF/Neo and CEF/SLAM were infected with recombinant MVs at an MOI of 1 TCID₅₀/cell, and cells were lysed at 16 h p.i. The cell lysates were resolved by SDS-PAGE under reducing conditions, transferred onto PVDF membrane, and immunoblotted for MV N and P proteins. (D) Growth kinetics of recombinant MVs in CEF/Neo and CEF/SLAM. The growth kinetics of IC323, IC-CAM M, IC-CAM NPL, and IC-CAM NPL+M in CEF/Neo and CEF/SLAM were examined as described in Fig. 1B. The means $\pm$ the standard deviations for three independent experiments are shown.

FIG. 2—Continued.

SLAM-gene cDNA (described above). For quantification of the MV antigenome, a specific antisense primer 5'-15894^{ACCAGACAAAGCTGGGAATA} GAA¹⁵⁸⁷²-3' was used for RT. The PCR was performed in triplicate with Fast SYBR green master mix (Applied Biosystems) in a 7500 Fast Real-Time PCR system (Applied Biosystems). Thermal cycling conditions were 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The primers were 5'-307^{CCAACTAACAGGGGCACTA}326-3' and 5'-403^{ATGCTAACGTCAG} GTGCATC³⁸⁴-3' for the MV N mRNA, 5'-2250^{TGAAAACAGCGATGTGGA} TA²²⁶⁹-3' and 5'-2414^{AAGTTGTTGCCTCTGGATTG}2395-3' for the MV P mRNA, and 5'-14689^{CTGGTCAAAGGGAATTAGCA}14708-3' and 5'-14754^{CTA} CTCCATTCTGTGTTCTGA¹⁴⁷³⁴-3' for the MV antigenome. Chicken GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was also quantified with the primers 5'-GCTGAATGGGAAGCTTACTG-3' and 5'-GACAACCTGGT CCTCTGTGT-3' as an internal control. As a standard, total RNA isolated from IC/CAM-infected CEF was serially diluted, reverse transcribed, and subjected to real-time PCR. The data were analyzed with the 7500 software version 2.0.1 (Applied Biosystems).

Western blotting. CEF or CEF transduced with retroviruses were infected with MV at an MOI of 1 TCID₅₀/cell. At 16 h p.i., cells were harvested and homogenized in lysis buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris buffer [pH 8.0]) containing 1 mM phenylmethylsulfonyl fluoride. The lysates were resolved by SDS-10% polyacrylamide gel electrophoresis (PAGE) and then transferred onto polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA). Non-specific binding was blocked with dried skim milk powder in phosphate-buffered saline containing 0.1% Tween 20, and then the membrane was incubated with anti-MV N (clone 3E1) and anti-MV P (clone 9H4) MAbs (Abcam, Cambridge, United Kingdom). The membranes were then washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (MBL, Nagoya, Japan), followed by the enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, United Kingdom).

RESULTS

Infection of CEF with recombinant chimeric MVs. To identify the genes responsible for efficient growth of the MV CAM-70 strain in CEF, we used a cloned MV IC323 that can be rescued from the full-length genomic cDNA of the wild-

type IC-B strain (39). There are amino acid differences in all of the proteins encoded by the viral genes between the IC-B (GenBank accession no. NC_001498) and the CAM-70 (accession no. DQ345723) strains. Therefore, individual genes of IC323 were replaced with the corresponding sequence derived from the CAM-70 strain, and IC323s possessing each of the CAM-70 N, P, M, F, and L genes were rescued successfully. The rescued recombinant MVs were designated IC-CAM N, IC-CAM P, IC-CAM M, IC-CAM F, and IC-CAM L, respectively (Fig. 1A). IC323 possessing the CAM-70 H gene (IC-CAM H) was generated previously (19) (Fig. 1A). All of these recombinant MVs grew efficiently in B95a cells (data not shown). As expected, the CAM-70 strain grew efficiently in CEF (Fig. 1B). However, none of the recombinant MVs grew well in CEF (Fig. 1B). This result indicates that no single gene derived from the CAM-70 strain is sufficient to allow IC323 to grow well in CEF.

Then, we generated recombinant MVs possessing different combinations of the CAM-70 genes (Fig. 1A). IC323 possessing all of the genes derived from the CAM-70 strain (IC/CAM) grew as efficiently as the CAM-70 strain in CEF (Fig. 1B). IC323 possessing the N, P, M, and L genes of the CAM-70 strain instead of its own (IC-CAM NPL+M) also grew in CEF, although the efficiency was less than that of IC/CAM (Fig. 1B). However, IC323 possessing the CAM-70 F and H genes (IC-CAM FH) and IC323 possessing the CAM-70 N, P, and L genes (IC-CAM NPL) did not grow well in CEF (Fig. 1B). The N, P, and L proteins form the RNP and conduct transcription of the viral genes and replication of the viral genome (16). Moreover, the M protein plays crucial roles in the assembly and budding of the viral particles and also regulates viral transcription and replication (16, 32, 35). Therefore, we assumed that MV needs to adapt to CEF at the transcription and replication steps and/or the assembly and budding steps.

Infection of CEF expressing human SLAM with recombinant chimeric MVs. Next, we sought to verify that MV needs to adapt to CEF at the transcription and replication steps and to identify the viral components that contribute to this adaptation, the M protein or the N, P, and L proteins forming the RNP. For this purpose, we expressed human SLAM on CEF with a retroviral expression vector, so that the recombinant MVs could use human SLAM to efficiently enter CEF. CEF were also transduced with the retrovirus expressing the Neo^r gene as a control. Expression of human SLAM on CEF was confirmed by staining with a PE-conjugated anti-SLAM MAb and performing flow cytometry, and 30% of the cells were SLAM-positive in CEF transduced with the retrovirus expressing human SLAM (Fig. 2A). CEF expressing human SLAM (CEF/SLAM) or the Neo^r gene (CEF/Neo) were infected with IC323, IC-CAM M, IC-CAM NPL, or IC-CAM NPL+M at an MOI of 1 TCID₅₀/cell. Cells were harvested at 16 h p.i., and the synthesis of viral mRNAs and antigenome within one round of virus replication was examined (Fig. 2B). In CEF/SLAM, all of the recombinant MVs produced larger amounts of the viral mRNAs than they did in CEF/Neo (Fig. 2B), indicating that the recombinant MVs entered CEF/SLAM more efficiently than CEF/Neo and that the entry step is a step where MV needs to adapt to CEF. In CEF/Neo and CEF/SLAM, IC-CAM M and IC-CAM NPL+M, but not IC-CAM NPL, synthesized viral mRNAs more efficiently than IC323 (Fig. 2B).

FIG. 3. Contribution of the CAM-70 H and F genes to the entry into CEF. (A) Schematic diagram of the recombinant MVs. (Elements are depicted as described in Fig. 1A.) (B) Quantification of MV mRNAs. CEF were infected with recombinant MVs at an MOI of 1 TCID₅₀/cell. At 16 h p.i., total RNAs were isolated from the infected cells, and the amounts of MV N and P mRNAs were quantified by real-time RT-PCR. The mean expression level in CEF infected with IC/CAM was set to 1. The means $\pm$ the standard deviations and *P* values using Student *t* test for three independent experiments are shown. (C) Accumulation of MV N and P proteins. CEF were infected with recombinant MVs at an MOI of 1 TCID₅₀/cell, and cells were lysed at 16 h p.i. The cell lysates were resolved by SDS-PAGE under reducing conditions, transferred onto PVDF membrane, and immunoblotted for MV N and P proteins. (D) Growth kinetics of recombinant MVs in CEF. Growth kinetics of IC-CAM M, IC-CAM MF, IC-CAM MH, IC-CAM MFH, IC/CAM, and IC323 in CEF were examined as described in Fig. 1B. The means $\pm$ the standard deviations for three independent experiments are shown.

The transcription and genome replication of IC-CAM NPL+M were more efficient than those of IC-CAM M in both cell types (Fig. 2B). Northern blot analysis of CEF infected with IC/CAM revealed that monocistronic mRNAs of the N, P, M, F, and H genes were the major bands detected and that the amounts of bicistronic mRNAs were much less than those of monocistronic mRNAs (data not shown). This indicated that

the amounts of read-through in CEF are similar to those in HeLa cells infected with MV (8) and in CEF infected with mumps virus (1).

Consistent with the results of transcription of the N and P genes, small amounts of the N and P proteins accumulated in CEF/Neo infected with IC-CAM NPL+M, but the levels of these proteins were less than the detection limits in the cells

FIG. 4. Infection of CEF with recombinant MVs based on IC/CAM. (A) Schematic diagram of the recombinant MVs. (Elements are depicted as described in Fig. 1A.) (B) Quantification of MV antigenome and mRNAs. CEF were infected with recombinant MVs at an MOI of 1 TCID₅₀/cell. At 16 h p.i., total RNAs were isolated from the infected cells and the amounts of MV antigenome and N and P mRNAs were quantified by real-time RT-PCR. The mean expression level in CEF infected with IC/CAM was set to 1. The means $\pm$ the standard deviations and *P* values using Student *t* test for three independent experiments are shown. (C) Growth kinetics of recombinant MVs in CEF. Growth kinetics of IC/CAM, IC/CAM-IC N, IC/CAM-IC P, IC/CAM-IC M, and IC/CAM-IC L were examined as described in Fig. 1B. The means $\pm$ the standard deviations for three independent experiments are shown.

infected with the other recombinant MVs (Fig. 2C). Reflecting the mRNA production in CEF/SLAM, cells infected with IC-CAM M accumulated detectable amounts of the N and P proteins, and IC-CAM NPL+M produced larger amounts of the viral proteins than IC-CAM M (Fig. 2C). However, accu-

mulation of the viral proteins in CEF/SLAM infected with IC323 or IC-CAM NPL was marginal or less than the detection limits (Fig. 2C). These results suggested that the CAM-70 M protein enhances viral propagation at the early stage of the virus replication cycle and that the RNP derived from the

CAM-70 strain is advantageous to transcription and replication in CEF.

We examined the growth kinetics of IC323, IC-CAM M, IC-CAM NPL, and IC-CAM NPL+M in CEF/Neo and CEF/SLAM (Fig. 2D). In CEF/Neo, only IC-CAM NPL+M grew, while the infectious virus production of the other recombinant MVs was marginal or less than the detection limit (Fig. 2D). In CEF/SLAM, IC-CAM M and IC-CAM NPL+M grew more efficiently than in CEF/Neo, and the growth of IC-CAM NPL+M was more efficient than that of IC-CAM M (Fig. 2D). However, infectious virus production of IC323 and IC-CAM NPL in CEF/SLAM was still marginal or less than the detection limit (Fig. 2D). Although these results most likely reflect the different efficiencies of transcription and replication described above, the possibility of the CAM-70 M protein being advantageous to the efficient assembly and budding of the viral particles in CEF cannot be excluded.

The H and F proteins derived from the CAM-70 strain are advantageous to efficient entry into CEF. We next examined the contribution of the CAM-70 H and F proteins to the efficient entry into CEF. Since transcription of IC-CAM M in CEF/SLAM was considerably efficient (Fig. 2B), we generated IC323s possessing the CAM-70 M and F genes (IC-CAM MF), the CAM-70 M and H genes (IC-CAM MH), and the CAM-70 M, F, and H genes (IC-CAM MFH) (Fig. 3A) and then examined the entry efficiencies of these recombinant MVs by quantifying the amounts of viral mRNAs synthesized after the entry of the recombinant MVs into CEF (Fig. 3B). CEF were infected with each of the recombinant MVs at an MOI of 1 TCID₅₀/cell, and cells were harvested at 16 h p.i. The amount of viral mRNA synthesized in CEF infected with IC-CAM MFH was five times more than the amount in cells infected with IC-CAM M (Fig. 3B). However, IC-CAM MF and IC-CAM MH synthesized only slightly more viral mRNAs than IC-CAM M (Fig. 3B). These results suggest that IC-CAM MF and IC-CAM MH entered CEF only slightly more efficiently than IC-CAM M and that IC-CAM MFH entered CEF with a much greater efficiency. Therefore, the CAM-70 H and F proteins are necessary for efficient entry into CEF. IC/CAM synthesized three times more viral mRNAs than IC-CAM MFH, confirming the advantage provided by RNP derived from the CAM-70 strain to efficient transcription in CEF (Fig. 3B). Consistent with the result of viral mRNA production, immunofluorescence assay using an anti-MV N MAbs detected nine times more N-protein-positive cells in CEF infected with IC-CAM MFH than in cells infected with IC-CAM M (data not shown). However, there were only twice more N-protein-positive cells in CEF infected with IC-CAM MF or IC-CAM MH, compared to cells infected with IC-CAM M (data not shown). Moreover, immunoblot analysis revealed that IC-CAM MFH accumulated detectable amounts of the N and P proteins but that the accumulation of viral proteins was less than the detection limits in cells infected with IC-CAM M, IC-CAM MF, or IC-CAM MH (Fig. 3C). IC/CAM accumulated greater amounts of viral proteins than IC-CAM MFH (Fig. 3C). The molecular weight of the CAM-70 P protein was slightly larger than that of the IC-B P protein (Fig. 2C and Fig. 3C). This is similar to the previous observation that the molecular weight of the CAM-70 V protein is larger than that of other MV strains (15).

We examined the growth kinetics of these recombinant MVs in CEF. IC-CAM MF and IC-CAM MH grew more efficiently than IC-CAM M, and IC-CAM MFH grew more efficiently than IC-CAM MF and IC-CAM MH (Fig. 3D). This result most likely reflects the difference in entry efficiencies of the recombinant MVs and confirms that both of the CAM-70 H and F proteins are necessary for efficient growth in CEF. IC/CAM grew more efficiently than IC-CAM MFH (Fig. 3D), reconfirming the advantage of the CAM-70 RNP to the efficient transcription and replication in CEF.

The CAM-70 L protein plays an important role in efficient transcription and replication in CEF. Our experiments suggested that the proteins forming the RNP contributed to the efficient transcription and replication in CEF. We next tried to identify the RNP-forming protein (N, P, or L protein) derived from the CAM-70 strain that is the most important for the efficient growth in CEF. We replaced each of the N, P, and L genes of IC/CAM with the corresponding gene of the IC-B strain, generating IC/CAM-IC N, IC/CAM-IC P, and IC/CAM-IC L (Fig. 4A). We also generated IC/CAM-IC M, which possesses the IC-B M gene instead of the M gene derived from the CAM-70 strain (Fig. 4A). CEF were infected with each of the recombinant MVs at an MOI of 1 TCID₅₀/cell, and cells were harvested at 16 h p.i. Although the transcription of the viral mRNAs and genome replication of IC/CAM-IC N and IC/CAM-IC P were not impaired compared to IC/CAM, the transcription and replication were prominently impaired in CEF infected with IC/CAM-IC L (Fig. 4B). Thus, the CAM-70 L protein plays an important role in the efficient viral transcription and replication in CEF. As expected, the transcription and replication of IC/CAM-IC M was drastically impaired (Fig. 4B). This result confirms the important role of the CAM-70 M protein in adaptation to CEF at the early stage of the virus replication cycle.

Reflecting the results of transcription and replication, infectious virus production of IC/CAM-IC L in CEF was less efficient than that of IC/CAM, while that of IC/CAM-IC N was comparable to that of IC/CAM (Fig. 4C). Moreover, infectious virus production of IC/CAM-IC M was more than one log₁₀ lower than that of IC/CAM (Fig. 4C), again confirming the important role of the CAM-70 M protein in efficient virus growth in CEF. IC/CAM-IC P grew slightly less efficiently than IC/CAM. This may be attributed to inefficient function of one or more of the gene products of the IC-B P gene at the late stage of the virus replication cycle.

DISCUSSION

In this study, we showed that MV needs to adapt to CEF at the steps of transcription and viral genome replication to grow efficiently. In CEF/SLAM, IC-CAM M transcribed viral genes with considerable efficiency, but the transcription of IC-CAM NPL was quite poor. In the presence of the CAM-70 M protein, the RNP formed by the CAM-70 N, P, and L proteins worked efficiently. Therefore, the CAM-70 M protein plays a central role in adaptation to CEF at the early stage of the virus replication cycle. Among the proteins that form the RNP, the CAM-70 L protein was responsible for efficient transcription and replication in CEF. We also found that MV needs to adapt to CEF at the entry step, in addition to the transcription and

replication steps, and that the CAM-70 H and F proteins play roles in the efficient entry.

The CAM-70 M protein functions in the adaptation to CEF at the early stage of the virus replication cycle. The MV M protein interacts with the RNP and inhibits viral transcription and replication (17, 32, 35). One hypothesis to explain the mechanism that causes the difference in the function in CEF between the CAM-70 and wild-type IC-B M proteins might be that the wild-type IC-B M protein inhibits the viral transcription and replication in CEF too strongly for the virus to replicate efficiently. However, the inhibition by the CAM-70 M protein may be weaker than that by the IC-B M protein and, as a result, the MVs possessing the CAM-70 M gene may replicate more efficiently. We are currently investigating how the two types of M proteins function differently in CEF at the early stage of the virus replication cycle. Since virus growth of IC-CAM M in B95a cells was not more efficient than that of IC323 (data not shown), the effect of the CAM-70 M protein appears to be cell type or species dependent.

Vaccine strains but not wild-type MV can grow efficiently in SLAM-negative, CD46-positive Vero cells. Tahara et al. have shown that the M gene derived from the Edmonston vaccine strain can confer wild-type IC323 with the ability to grow in Vero cells and that two specific amino acid substitutions, P64S and E89K, are responsible for this ability (36). Moreover, Tahara et al. have demonstrated that the substitutions allow for a strong interaction between the M protein and the cytoplasmic tail of the H protein and result in the efficient assembly of infectious virus particles in Vero cells (37). The CAM-70 M protein also possesses the P64S and E89K substitutions. Therefore, it is possible that the CAM-70 M protein plays a role in the adaptation to CEF at the assembly step in addition to its role at the early stage of the virus replication cycle.

The RNP derived from the CAM-70 strain is advantageous to transcription and replication in CEF. Among the viral proteins forming the RNP, the CAM-70 L protein is responsible for efficient transcription and replication. Reported data on the RNA polymerase activity of the MV vaccine strains compared to that of the wild-type strains are confusing. A minigenome reporter assay has revealed that the L proteins of the MV vaccine strains (including the CAM-70 strain) exhibit higher transcription and replication activity than the MV wild-type L proteins in monkey kidney-derived CV-1 cells (3). The L gene of the Edmonston strain confers wild-type IC323 with the ability to grow in African green monkey kidney-derived Vero cells, suggesting that the RNA polymerase activity of the Edmonston L protein in Vero cells is more efficient than that of the wild-type IC-B L protein (36). However, IC323 possessing the Edmonston L gene exhibits attenuated gene expression and virus replication in various cell types, including CV-1 and Vero cells that express human SLAM (42). Since the virus growth of IC/CAM-IC L in marmoset B-cell-derived B95a cells was not less efficient than that of IC/CAM (data not shown), the difference in activity of the RNA polymerase between the CAM-70 and wild-type IC-B strains appears to be cell type or species dependent. The cellular protein tubulin interacts with the MV L protein and is necessary for the efficient function of the viral RNA polymerase (25). Thus, the CAM-70 RNA polymerase may interact with such host factors in CEF more effi-

ciently than the wild-type polymerase and, as a result, may act more efficiently in CEF.

Bankamp et al. repeatedly passaged the wild-type MV strain (D87-wt) in CEF to recreate the process of attenuation and obtained a CEF-adapted virus, D-CEF (4). Sequence analysis of D-CEF has revealed that adaptation of D87-wt to CEF resulted in four amino acid substitutions: V102A in the C protein, Y110H and V120A in the common N-terminal domain of the P and V proteins, and T84I in the M protein (4). This observation is in agreement with our results showing the adaptation of MV to CEF at the postentry steps. However, the presence of amino-acid substitutions in the P, V, and C proteins but not in the L protein may suggest that there are alternative ways for MV to adapt to CEF other than that demonstrated with the CAM-70 strain.

We demonstrated that MV needs to adapt to CEF at the entry step to grow efficiently. This result is consistent with a previous report that used the Edmonston-derived vaccine strain, Schwarz (14). Moreover, both the CAM-70 F and the H proteins were involved in efficient entry into CEF. Since CEF do not express the identified cellular receptors for MV, human SLAM and CD46 (47), MV must enter CEF via an unidentified pathway. An unidentified cellular receptor for MV on CEF was previously suggested (14). The F and H proteins derived from the CAM-70 strain appear to utilize the unidentified entry pathway on CEF more efficiently than wild-type F and H proteins.

The adaptation to heterologous cells, including CEF, has been thought to result in the attenuation of MV, and substitutions induced by such adaptation must be responsible for the attenuation. According to this point of view, the substitutions Y110H and V120A in the common N-terminal domain of the P and V proteins of D-CEF reduce the ability of these proteins to inhibit type I and II interferon signaling (5). In the present study, we demonstrated that the M, F, H, and L genes are responsible for the efficient growth of the CAM-70 vaccine strain in CEF. Investigations focusing on these genes might elucidate unknown molecular mechanisms underlying the attenuation of MV.

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