Reciprocal Complementation of Bovine Parainfluenza Virus Type 3 Lacking Either the Membrane or Fusion Gene

Marina Takada[a], Ryosuke Matsuura[a], Takehiro Kokuho[b], Takamitsu Tsuboi[c], Ken-ichiro Kameyama[c], and Kaoru Takeuchi[a]

[a] Laboratory of Environmental Microbiology, Faculty of Medicine, University of Tsukuba, Ibaraki 305-8575, Japan

[b] Biologics Production, Center for Animal Disease Control and Prevention, National Institute of Animal Health, NARO, Tsukuba, Ibaraki 305-0856, Japan

[c] Viral Diseases and Epidemiology Research Division, National Institute of Animal Health, NARO, Tsukuba, Ibaraki 305-0856, Japan

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Address correspondence to Kaoru Takeuchi, ktakeuch@md.tsukuba.ac.jp

23 **ABSTRACT**

24 Two defective bovine parainfluenza virus type 3 (BPIV3) strains were generated, one
25 lacking the membrane (M) protein gene and expressing EGFP (ΔM-EGFP) and the
26 other lacking the fusion (F) protein gene and expressing mStrawberry (ΔF-mSB), by
27 supplying deficient proteins *in trans*. When Madin-Darby bovine kidney (MDBK) cells
28 were co-infected with ΔM-EGFP and ΔF-mSB at a multiplicity of infection (MOI) of
29 0.1, complemented viruses were easily obtained. Complemented viruses grew as
30 efficiently as wild-type BPIV3 and could be passaged in MDBK cell cultures even at
31 an MOI of 0.01, possibly due to multiploid virus particles containing genomes of both
32 ΔM-EGFP and ΔF-mSB. This reciprocal complementation method using two defective
33 viruses would be useful to express large or multiple proteins in cell cultures using
34 paramyxovirus vectors.

35

36 **1. Introduction**

37 Bovine parainfluenza virus type 3 (BPIV3), a member of the genus *Respirovirus* in the
38 family *Paramyxoviridae* in the order *Mononegavirales*, is an enveloped virus with a
39 non-segmented negative-sense RNA genome (Karron and Collins, 2013). The BPIV3
40 genome encodes six structural proteins: nucleocapsid (N), phospho (P), matrix (M),
41 fusion (F), hemagglutinin-neuraminidase (HN) and large (L) proteins. The M protein is
42 a nonintegral, membrane-associated protein localizing under the lipid bilayer of virus
43 particles. The M protein is a key driver of virus particle formation. The F protein is an
44 integral membrane protein on virus particles and essential for virus-cell fusion (Karron

and Collins, 2013).

Reverse genetics systems of *Mononegavirales* were first achieved for rabies virus in 1994 (Schnell et al., 1994). Since then, many reverse genetics systems have been established for *Mononegavirales*, and generated recombinant viruses were utilized to study the function of viral proteins or viral genome sequences. Reverse genetics systems of *Mononegavirales* have also been used to generate novel vaccines expressing foreign proteins as antigens (Le Bayon et al., 2013; Sato et al., 2011) or novel medicines such as oncolytic viruses (Pfaller et al., 2015). Recently, recombinant paramyxoviruses are used to establish induced pluripotent stem (iPS) cells (Ban et al., 2011; Nishimura et al., 2011). However, the insertion of multiple or long transcription cassettes of foreign genes into paramyxovirus genomes could reduce viral growth (Bukreyev et al., 2006). To increase the capacity of paramyxovirus vectors to carry multiple or long extra gene units, paramyxoviruses containing segmented genomes were generated (Gao et al., 2008; Takeda et al., 2006).

In this study, we generated two defective BPIV3 strains, one lacking the M protein gene and the other lacking the F protein gene, to increase the capacity of BPIV3 vectors and propagated them by co-infection.

2. Materials and methods

2.1. Cells and viruses

MDBK, HeLa and Vero monolayer cell cultures were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum

67 (FBS), 100 units/ml of penicillin G and 100 μg/ml of streptomycin. The modified
68 vaccinia virus Ankara (MVA-T7), which expresses the phage T7 RNA polymerase
69 (Wyatt et al., 1995), was grown in chicken embryonic fibroblasts.
70
71 2.2. Cloning of the M and F genes of BPIV3 into a pCAGGS plasmid vector
72 To construct the M-expressing plasmid, the open reading frame of the M gene was
73 synthesized from p(+)BPIV3-EGFP (Ohkura et al., 2015) by PCR using the primers
74 5'-CGCGCTCGAGATGAGCATTACCAACTCTGC-3' (*Xho*I site is underlined) and
75 5'-CGCGCGATATCTTACTGTCTGATTTTCCCGA-3' (*Eco*RV site is underlined) and
76 ligated between *Xho*I and *Eco*RV sites of the pCAGGS plasmid (Niwa et al., 1991),
77 resulting in pCAGGS-M. To construct the F-expressing plasmid, the open reading
78 frame of the F gene was synthesized from p(+)BPIV3-EGFP (Ohkura et al., 2015) by
79 PCR using the primers 5'-GCGCCTCGAGCATGATCATCACAAACACAAT-3' (*Xho*I
80 site is underlined) and 5'-GCGCGGATATCTCATTGTCTACTTGTTAGTA-3' (*Eco*RV
81 site is underlined) and ligated between *Xho*I and *Eco*RV sites of pCAGGS plasmid,
82 resulting in pCAGGS-F.
83
84 2.3. Construction of BPIV3 cDNA deficient of either the M or F gene
85 To construct BPIV3 cDNA deficient of the M gene, two fragments were synthesized
86 from p(+)BPIV3-EGFP by PCR. The first fragment covering the *Xho*I site in the P
87 gene to the noncoding region between the gene start sequence for the M gene and M
88 gene open reading frame was amplified using primers

5'-GCAGCTCAGATAGTAGAGCT-3' and 5'-GCGAAGCTTCGGAGGATGGATTGATACTT-3' (*Hin*dIII site is underlined) and digested with *Xho*I and *Hin*dIII. The second fragment covering the noncoding region between the gene start sequence for the F gene and F gene open reading frame and the *Nhe*I site in the HN gene was amplified using primers 5'-GCGAAGCTTCCAATACATAGATCACAGGA-3' (*Hin*dIII site is underlined) and 5'-GCGGCTAGCCTGATTGCAGTCTCTCTGTG-3' (*Nhe*I site is underlined) and digested with *Hin*dIII and *Nhe*I. The two PCR fragments were then ligated between *Xho*I and *Nhe*I sites of p(+)BPIV3-EGFP, resulting in p(+)BPIV3ΔM-EGFP. To construct BPIV3 cDNA deficient of the F gene, two fragments were synthesized from p(+)BPIV3-EGFP by PCR. The first fragment covering the *Xho*I site in the P gene to the noncoding region between the gene start sequence for the F gene and F gene open reading frame was amplified using primers 5'-GCAGCTCAGATAGTAGAGCT-3' and 5'-GCGAAGCTTTAACTGTTGCTCGGAGTTTG-3' (*Hin*dIII site is underlined) and digested with *Xho*I and *Hin*dIII. The second fragment covering the noncoding region between the gene start sequence for the HN gene and HN gene open reading frame and the *Nhe*I site in the HN gene was amplified using primers 5'-CGGAAGCTTAGAGACGACACCAAATTCAA-3' (*Hin*dIII site is underlined) and 5'-GCGGCTAGCCTGATTGCAGTCTCTCTGTG-3' (*Nhe*I site is underlined) and digested with *Hin*dIII and *Nhe*I. The two PCR fragments were then ligated between *Xho*I and *Nhe*I sites of p(+)BPIV3-EGFP, resulting in p(+)BPIV3ΔF-EGFP. The mStrawberry (mSB) gene was synthesized from pmStrawberry (Clontech, Mountain

111 View, CA) by PCR using primers
112 5'-CCCGTCGACCACCATGGTGAGCAAGGGCGAG-3' (*Sal*I site is underlined) and
113 5'-CCCACGCGTTTACTTGTACAGCTCGTCCATGCC-3' (*Mlu*I site is underlined)
114 and digested with *Sal*I and *Mlu*I. The EGFP gene in p(+)BPIV3ΔF-EGFP was removed
115 by digesting with *Sal*I and *Mlu*I and replaced with the mSB gene digested with *Sal*I
116 and *Mlu*I, resulting in p(+)BPIV3ΔF-mSB. All plasmids were prepared in Stbl2 cells
117 (Life Technologies, Grand Island, NY) at 30˚C.
118
119 2.4. Rescue of infectious viruses from p(+)BPIV3ΔM-EGFP or p(+)BPIV3ΔF-mSB
120 plasmids
121 HeLa cells in a 6-well plate (80% confluent) were infected with vaccinia virus
122 MVA-T7 at an MOI of 1. One hour post-infection, the p(+)BPIV3ΔM-EGFP or
123 p(+)BPIV3ΔF-mSB plasmid (4 μg) was transfected into the MVA-T7-infected HeLa
124 cells together with pCAGGS-M or pCAGGS-F in addition to supporting plasmids
125 (pGEM-N, pGEM-P and pGEM-L) in the presence of 10 μl of Lipofectamine 2000
126 (Life Technologies) in 250 μl of Opti-MEM (Life Technologies). After 6 hours
127 incubation, media were replaced with DMEM supplemented with 10% FBS and
128 antibiotics. Three days post-transfection, the supernatants of p(+)BPIV3ΔM-EGFP- or
129 p(+)BPIV3ΔF-mSB-transfected cells were harvested and transferred onto Vero cells
130 transfected with pCAGGS-M or pCAGGS-F, respectively. After incubation for 3 days,
131 rBPIV3ΔM-EGFP or rBPIV3ΔF-mSB were recovered from p(+)BPIV3ΔM-EGFP- or
132 p(+)BPIV3ΔF-mSB-transfected cells, respectively.

133

134 2.5. Reciprocal complementation between M- and F-deficient BPIV3 strains

135 Monolayer cultures of MDBK cells in 6-well cluster plates were infected with
136 rBPIV3ΔM-EGFP and rBPIV3ΔF-mSB at an MOI of 0.1 50% tissue culture infective
137 dose ($TCID_{50}$)/cell and incubated at 37˚C for 3 days.

138

139 2.6. Microscopic detection of EGFP and mSB fluorescence

140 EGFP and mSB fluorescence in infected cells was photographed using a fluorescence
141 microscope (TS100, Nikon, Tokyo, Japan) equipped with a charge-coupled device
142 (CCD) camera (DS-Fi1, Nikon).

143

144 2.7. Growth curves

145 Monolayer cultures of MDBK cells in 24-well cluster plates were infected with the
146 rBPIV3ΔM-EGFP, rBPIV3ΔF-mSB or reciprocally complemented viruses and
147 incubated at 37˚C. At various time points, media were harvested and the infectious titer
148 was determined by the $TCID_{50}$ in MDBK cells under a fluorescent microscope.

149

150 2.8. Western blotting

151 Cells were lysed in SDS loading buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and
152 were disrupted by sonication for 10 min. After centrifugation, the lysates were
153 electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE).
154 The proteins in the gel were transferred to a polyvinylidene difluoride membrane

155 (Millipore, Bedford, Mass). M and F proteins were detected using rabbit sera against
156 synthetic peptides corresponding to the M protein (CRSKDRYGSVSDLDDDPS) and
157 the F protein (IQGKNQNDKNSEPYVLTSRQ), respectively.
158
159 2.9. Recover of viruses from plaques
160 Viruses in plaques were recovered with agarose gel overlay using sterilized Pasteur
161 pipets under a fluorescent microscope and were suspended in the medium. After brief
162 centrifugation, viruses were inoculated into MDBK cells.
163
164 **3. Results**
165 3.1. Generation of an M or F gene-deficient BPIV3 strain
166 To construct M or F gene-deficient infectious BPIV3 cDNA, the M or F gene was
167 deleted from p(+)BPIV3-EGFP (Ohkura et al., 2015) using mutant primers and
168 RT-PCR, resulting in the generation of p(+)BPIV3ΔM-EGFP or the
169 p(+)BPIV3ΔF-EGFP, respectively. To discriminate M- and F-deficient viruses, the
170 EGFP gene of p(+)BPIV3ΔF-EGFP was replaced with mSB gene, resulting in
171 p(+)BPIV3ΔF-mSB (Fig. 1A). The M-deficient virus (ΔM-EGFP) and the F-deficient
172 virus (ΔF-mSB) were recovered from p(+)BPIV3ΔM-EGFP and p(+)BPIV3ΔF-mSB
173 using reverse genetics for BPIV3 (Ohkura et al., 2015) by supplying the M protein
174 from pCAGGS-M plasmid or the F protein from pCAGGS-F plasmid, respectively.
175 ΔM-EGFP and ΔF-mSB were propagated in Vero cells transfected with pCAGGS-M
176 and pCAGGS-F, respectively. Immunofluorescence assay and western blotting

177 confirmed that ΔM-EGFP and ΔF-mSB expressed EGFP and mSB, respectively (Fig.
178 1B), and not M protein and F protein, in infected MDBK cells (Fig. 1C). Interestingly,
179 ΔM-EGFP occasionally induced EGFP-expressing satellite cells at late stage (Fig. 1B).
180 ΔM-EGFP may have enhanced cell-cell fusion activity as reported for other M-less
181 paramyxoviruses (Cathomen et al., 1998).
182
183 3.2. Generation of complemented viruses
184 When MDBK cells were singly infected with ΔM-EGFP or ΔF-mSB at a multiplicity
185 of infection (MOI) of 0.1, infectious virus was not detected in culture supernatant as
186 expected (Fig. 2A). However, when MDBK cells were co-infected with ΔM-EGFP and
187 ΔF-mSB at an MOI of 0.1, large amounts of infectious viruses expressing EGFP or
188 mSB were produced in the culture supernatant, and EGFP and mSB fluorescence was
189 observed throughout the entire area of culture dishes at day 3 post-infection (Fig. 2B).
190 In contrast, at an MOI of 0.01, EGFP and mSB fluorescence did not spread even at day
191 3 post-infection. These results suggest that when cells were co-infected with ΔM-EGFP
192 and ΔF-mSB, the F and M proteins reciprocally complemented the growth of
193 ΔM-EGFP and ΔF-mSB in infected cells.
194
195 3.3. Complemented viruses contain multiploid virus particles containing genomes of
196 both ΔM-EGFP and ΔF-mSB
197 To analyze the nature of complemented viruses, MDBK cells were infected with
198 complemented viruses and overlaid with agarose containing culture medium. When

199 infected cells were observed under a fluorescent microscope at 2 days post-infection,
200 three types of infected cells were observed; isolated single cells expressing EGFP,
201 isolated single cells expressing mSB and plaques expressing both EGFP and mSB.
202 Plaques co-expressing EGFP and mSB were observed at low MOI, suggesting that
203 these plaques were induced by infection of multiploid virus particles containing
204 genomes of both ΔM-EGFP and ΔF-mSB. Approximately one-quarter of
205 complemented virus particles were multiploid virus particles containing genomes of
206 both ΔM-EGFP and ΔF-mSB (Table 1). To rule out the possibility that these plaques
207 were induced by recombinant viruses between ΔM-EGFP and ΔF-mSB, viruses in
208 plaques were recovered and inoculated into MDBK cells. Again, isolated single cells
209 expressing EGFP or mSB and cells expressing both EGFP and mSB were observed
210 (Fig. 3A), indicating that plaques were induced by multiploid virus particles containing
211 genomes of both ΔM-EGFP and ΔF-mSB. Complemented viruses spread efficiently in
212 culture dishes even at an MOI of 0.001 (Fig. 3B) and grew as efficiently as wild-type
213 virus (Ohkura et al., 2015) (Fig. 3C). The EGFP and mSB fluorescence were stable,
214 and the ratio of ΔM-EGFP and ΔF-mSB was not changed during passages (data not
215 shown).
216
217 **4. Discussion**
218 In this study, we generated two defective BPIV3 strains deficient for either the M or F
219 gene and found that these BPIV3 strains could be easily rescued and propagated by
220 co-infection. As each defective virus absolutely requires another defective virus to

221 replicate, extra genes inserted in both defective virus genomes could be maintained in
222 successive culture. In paramyxovirus vectors, the addition of several extra transcription
223 units or longer transcriptional units into genomes usually reduces viral replication
224 (Bukreyev et al., 2006). It is generally believed that short genomes replicate rapidly,
225 and long genomes replicate slowly. In our system, the M or F gene was deleted from
226 the BPIV3 genome, resulting in a 1,164 base or 1,902 base reduction in the total
227 genome length of BPIV3, respectively. These reductions could expand the coding
228 capacity of the BPIV3 vector. According to the polar effect of transcription in
229 paramyxoviruses (Bukreyev et al., 2006), extra transcriptional units should be inserted
230 in the 3'-proximal region of paramyxoviruses to obtain the highest expression level.
231 According to this requirement, two extra transcriptional units can be inserted in the
232 nearly 3'-proximal region between the N and P genes in our system. Another advantage
233 of using deleted genomes was increased rescue efficiencies; more infectious viruses
234 were recovered from deleted genomes compared to complete genomes (data not
235 shown).

236 We found that complemented viruses produced plaques co-expressing EGFP and
237 mSB (Fig. 3A and Table 1). Previous studies indicated that paramyxoviruses particles
238 are pleomorphic and contain multiple genomes (Dahlberg and Simon, 1969; Goff et al.,
239 2012; Granoff, 1959; Hosaka et al., 1966; Loney et al., 2009; Lund et al., 1984; Rager
240 et al., 2002; Terrier et al., 2009). Thus, it is reasonable that multiploid virus particles in
241 our complemented viruses contributed to the efficient spread of viruses in cell culture.
242 We found that 23% of complemented viruses produced plaques co-expressing EGFP

243 and mSB. This frequency agrees with previous results obtained for Newcastle disease
244 virus (NDV) (Goff et al., 2012), which showed that approximately 25% of NDV
245 particles were multiploid.

246 Although several groups reported natural recombination between NDV strains
247 (Chong et al., 2010; Han et al., 2008; Qin et al., 2008; Satharasinghe et al., 2016), there
248 was no evidence of recombination between ΔM-EGFP and ΔF-mSB (Fig. 3A). These
249 results agree with previous results obtained for human respiratory syncytial virus
250 (RSV) (Spann et al., 2003), which showed that genetic recombination between two
251 RSV strains was an extremely rare event.

252 In our system, multiploid virus particles are constantly generated and maintained in
253 cell culture in contrast to the vesicular stomatitis virus system (Chattopadhyay and
254 Rose, 2011), and there is a possibility that multiploid virus particles might be generated
255 *in vivo*.

256 In summary, we found that two paramyxovirus strains deficient for different genes
257 could be easily rescued by co-infection. This complementation method using two
258 defective viruses may be useful to express large or multiple proteins in cell cultures.

259

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Conflict of interest

The authors have declared that no conflicts of interest to declare.

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371

372 **Figure legends**

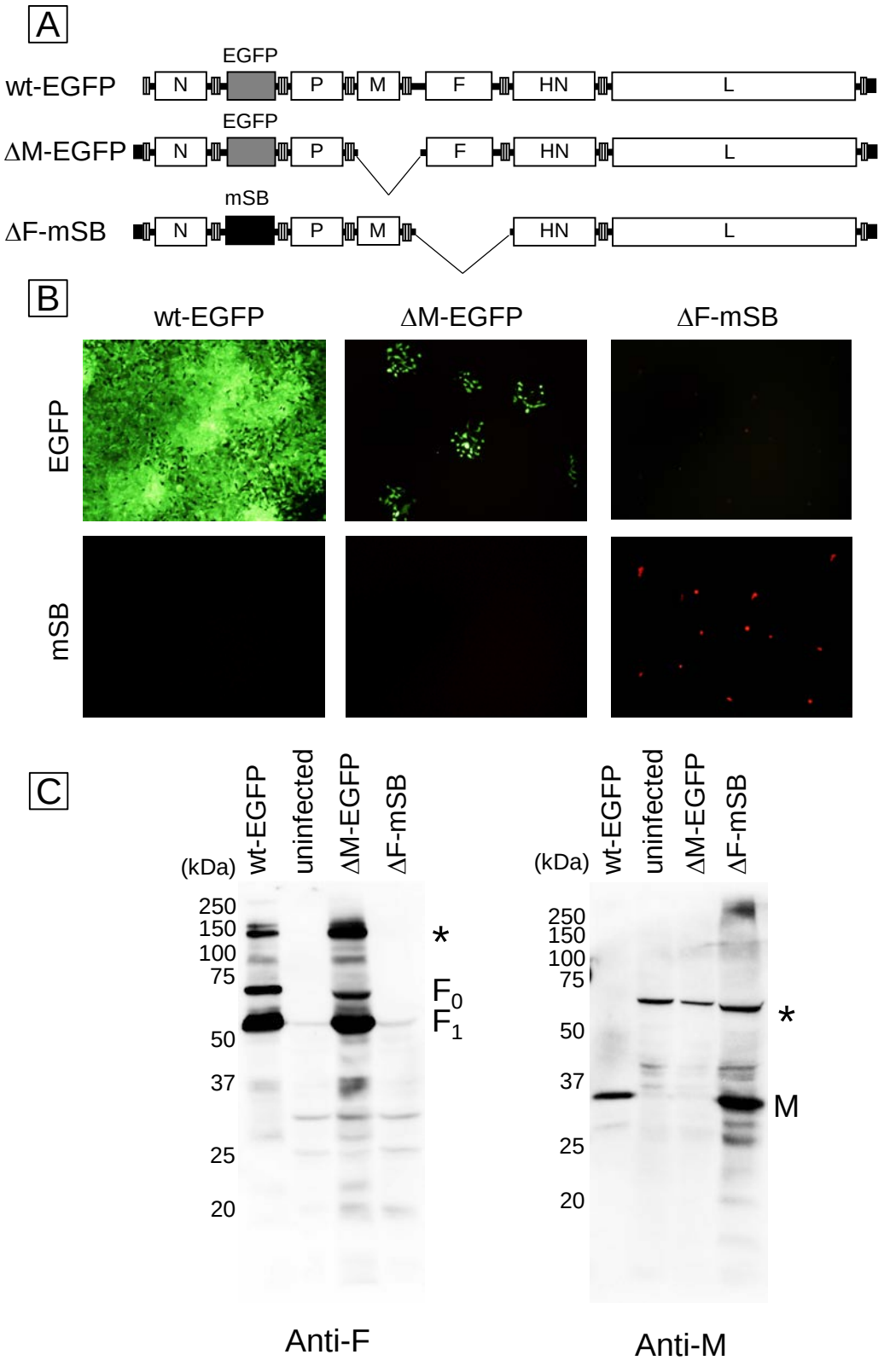
373 **Fig. 1. Construction of the BPIV3 strains deficient in either the M or F gene**

374 (A) Schematic diagram of the genomic organization of wt-EGFP, ΔM-EGFP and

375 ΔF-mSB. (B) MDBK cells were infected with wt-EGFP, ΔM-EGFP or ΔF-mSB at an
376 MOI of 0.01. Infected cells were visualized with autofluorescence of EGFP or mSB
377 without fixation. (C) MDBK cells were infected with ΔM-EGFP, ΔF-mSB or
378 uninfected. At day 2 post-infection, cells were lysed in SDS loading buffer and
379 subjected to SDS-PAGE. M and F proteins were detected using rabbit sera against
380 synthetic peptides corresponding to the M protein and the F protein, respectively. The
381 asterisk in the left panel may be the F_1 protein trimer. The asterisk in the right panel
382 indicates nonspecific signal.
383
384 **Fig. 2. Recovery of complemented viruses**
385 (A) Replication kinetics of ΔM-EGFP (triangles), ΔF-mSB (squares) and the mixture
386 of ΔM-EGFP and ΔF-mSB (circles). MDBK cells were infected with ΔM-EGFP or
387 ΔF-mSB at an MOI of 0.1 or infected with the mixture of ΔM-EGFP and ΔF-mSB at
388 an MOI of 0.1 for each virus. Media were harvested at 0, 12, 24, 36, 48, 60 and 72 h
389 post-infection, and infectious titers were assessed as $TCID_{50}$ using MDBK cells. Data
390 are presented as the mean ± standard deviations of triplicate samples. (B) MDBK cells
391 were co-infected with ΔM-EGFP and ΔF-mSB at an MOI of 0.1 and 0.01. Infected
392 cells were visualized with autofluorescence of EGFP or mSB at days 1 and 3
393 post-infection. Photomicrographs of EGFP and mSB fluorescence were merged.
394
395 **Fig. 3. Growth and spread of complemented viruses in MDBK cells.**
396 (A) Isolation of viruses from a plaque. MDBK cells were infected with complemented

viruses at an MOI of 0.01 and overlaid with agarose. Plaques expressing both EGFP and mSB were observed using a fluorescent microscope. Viruses in a plaque were recovered from agarose and inoculated into MDBK cells. Infected cells were visualized with autofluorescence of EGFP and mSB at day 1 post-infection. Photomicrographs of EGFP and mSB fluorescence were merged. The white arrow indicates cells expressing both EGFP and mSB. (B) Spread of complemented viruses in MDBK cells. MDBK cells were infected with complemented viruses at an MOI of 0.1, 0.01 and 0.001. Infected cells were visualized with autofluorescence of EGFP or mSB at days 1 and 3 post-infection. Photomicrographs of EGFP and mSB fluorescence were merged. (C) Replication kinetics of complemented viruses in MDBK cells. MDBK cells were infected with complemented viruses at an MOI of 0.1 and 0.01. Media were harvested at 0, 12, 24, 36, 48, 60 and 72 h post-infection, and infectious titers were assessed as $TCID_{50}$ using MDBK cells.

FIG.1


A
EGFP
wt-EGFP
N
P
M
F
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EGFP
ΔM-EGFP
N
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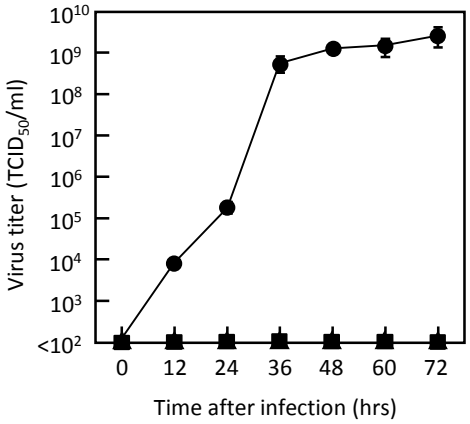
L
mSB
ΔF-mSB
N
P
M
HN
L
B
wt-EGFP
ΔM-EGFP
ΔF-mSB
EGFP
mSB
C
(kDa)
wt-EGFP
uninfected
ΔM-EGFP
ΔF-mSB
250
150
100
75
50
37
25
20
*
F0
F1
Anti-F
(kDa)
wt-EGFP
uninfected
ΔM-EGFP
ΔF-mSB
250
150
100
75
50
37
25
20
*
M
Anti-M

FIG.2

A


Virus titer (TCID50/ml)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
<10^2
0
12
24
36
48
60
72
Time after infection (hrs)

B

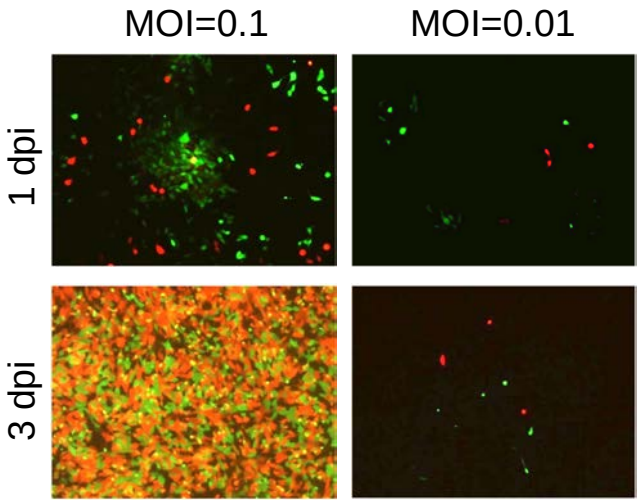
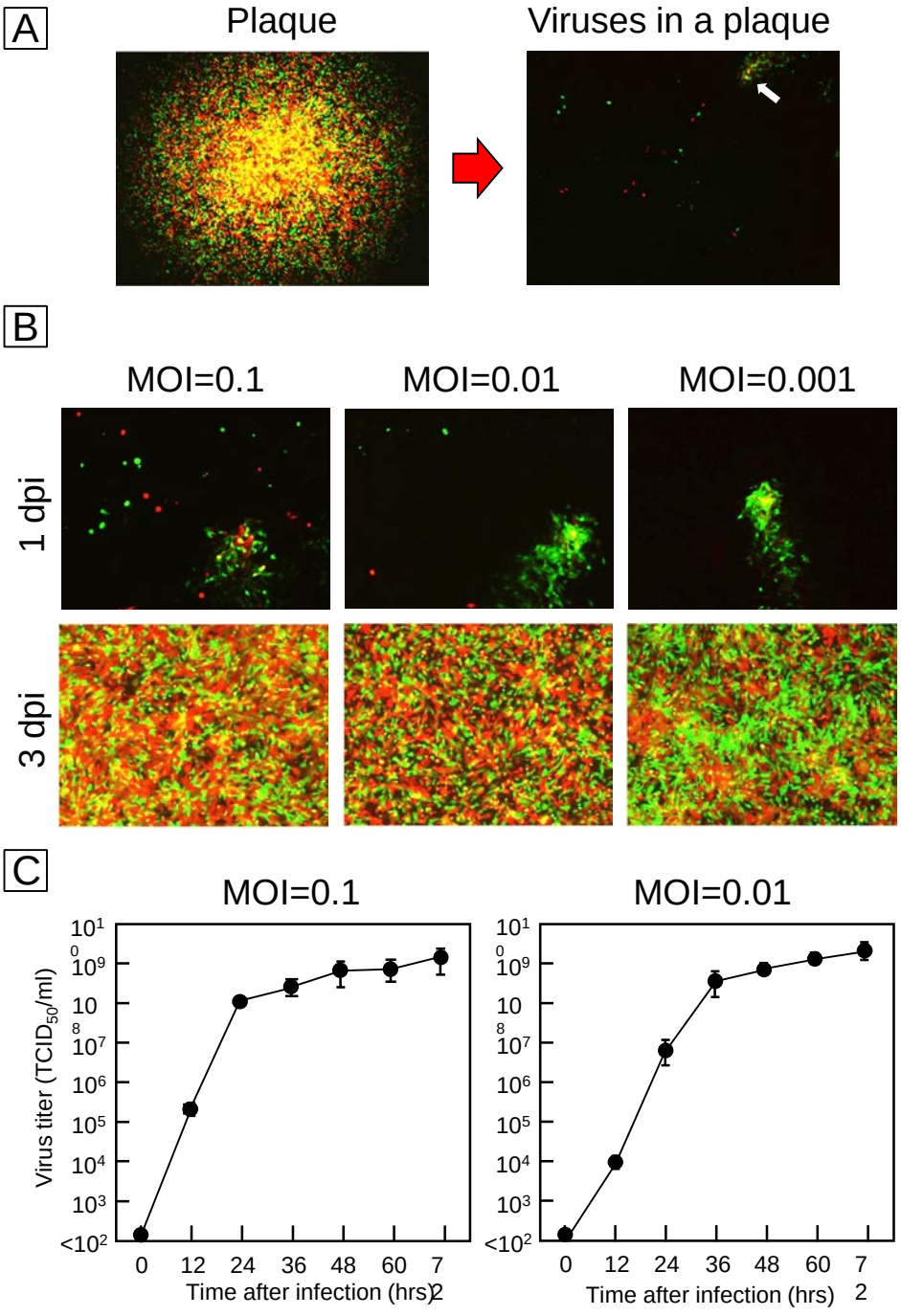

MOI=0.1
MOI=0.01
1 dpi
3 dpi

FIG.3


A
Plaque
Viruses in a plaque
B
MOI=0.1
MOI=0.01
MOI=0.001
1 dpi
3 dpi
C
MOI=0.1
MOI=0.01
Virus titer (TCID50/ml)
Time after infection (hrs)
0 12 24 36 48 60 72