

# The Influenza Virus Variant A/FM/1/47-MA Possesses Single Amino Acid Replacements in the Hemagglutinin, Controlling Virulence, and in the Matrix Protein, Controlling Virulence as well as Growth

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Received 6 April 1993/Accepted 4 October 1993

**Genetic analysis of mouse-adapted influenza virus variant A/FM/1/47 (FM) MA has previously identified four genome segments, 4, 5, 7, and 8, that are statistically associated with virulence. On sequencing these genome segments, we found single amino acid replacements at amino acid 47 of the HA2 subunit of the hemagglutinin and at amino acid 139 of the matrix protein. Mutation was not detected in segments 5 and 8, obviating a role for these genes in FM-MA virulence. FM-MA replicates to higher titer than FM in MDCK cells and in mouse lung. FM X FM-MA reassortants were used to show that the M1 gene controlled replication in MDCK cells as well as in mouse lung.**

The ultimate goal of the study of pathogenesis is to determine the role of each viral gene in disease production. We have approached the identification of specific functions of individual genes in pathogenesis by characterizing mutations that increase virulence in a mouse-adapted variant of influenza virus. Although the mouse is not naturally infected with influenza virus, human strains can be adapted to the mouse by serial lung passage. The mouse model of influenza is a practical tool for the study of influenza virus pathogenesis since pneumonia in the mouse and pneumonia in the human are pathologically similar (29).

The human H1N1 prototype strain, A/FM/1/47 (FM), was adapted to the mouse by serial lung passage to produce a virulent variant, FM-MA, that had increased in virulence by  $10^{3.3}$ -fold as measured by 50% lethal dose ( $LD_{50}$ ). The FM-MA virus also acquired the property of interference that operates independently from virulence (3). By genetic analysis of reassortants, we previously identified four genome segments that were associated with virulence of FM-MA (2). Segments 4, 5, 7, and 8 (encoding HA, NP, M1 plus M2, and NS1 plus N2, respectively) were associated with virulence by Wilcoxon ranked sum analysis of FM-MA × A/HK/1/68 (HK) reassortants. Segments 4 and 7 are associated with virulence at the 0.1 and 1% levels of significance, whereas segments 5 and 8 are associated with virulence at the 5% level of significance. These four segments of FM-MA were then reintroduced in different combinations into the nonadapted FM parent to confirm their roles in virulence. This was done by backcrossing FM × HK with FM-MA × HK reassortants. The resulting reassortants differed from each other as a result of the substitution of FM-MA genome segments on an isogenic background of parental FM genome segments. Reassortants containing FM-MA segment 4 alone on an FM background had increased in virulence by  $10^{2.2}$ -fold. Reassortants containing FM-MA

segments 4, 7, and 8 were  $10^{1.2}$ -fold more virulent than the FM-MA segment 4-containing reassortants, indicating a role for segment 7 and/or 8. The relative contributions of segment 7 and 8 were obscured by reversion in backcrosses that introduced either of these segments alone (2). Here we have produced a reassortant, W29, resulting from a single backcross, that contained FM-MA segments 4 and 7. MDCK cells were coinfecting with E67, which contained FM-MA genome segments 4 and 7 on an HK genomic background, and G78, which contained segment 4 of HK on an FM genomic background (2). Reassortants containing FM-MA segments 4 and 7 on an FM genomic background were identified by RNA and protein electrophoresis of progeny obtained by plaque assay in the presence of anti-HK rabbit serum (2). The matrix protein of FM-MA is distinguishable from FM by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was not possible to reintroduce FM-MA segment 5 into FM virus to confirm its role in virulence because of a lack of specific reassortants for backcrossing. Table 1 shows the virulence of reassortants that contained combinations of segments 4, 7, and 8 from FM-MA on an FM background that were all produced by single backcrosses; the J and T reassortants had been produced previously (2). The  $LD_{50}$  is indicated as  $\log_{10}$  PFU and was determined by intranasal inoculation of groups of five anesthetized mice with graded doses of virus as described previously (2). Survival was monitored for 10 days. It appeared that both FM-MA segments 4 and 7 increase the virulence of FM by  $10^{2.2}$ - and  $10^{1.2}$ -fold, respectively, but that segment 8 did not affect virulence.

Sequence analysis was required to identify the mutations in segments 4, 5, 7, and 8. The FM-MA mutations responsible for acquired virulence were identified by comparing the nucleotide sequences of cDNA clones of FM and FM-MA segments 4, 5, 7, and 8. Double-stranded cDNA was synthesized and cloned essentially as described by Schmid et al. (26). Using DNA primers complementary to the conserved termini of genomic RNA (18), it was possible to clone full-length cDNA copies of all eight genome segments of both FM and FM-MA. The plus-sense primer FLUXBA (5'TCTAGAGCAAAAGCAGG3') and the minus-sense primer FLUT7 (5'TAATACGACT

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TABLE 1. Virulence due to the presence of segments 4, 7, and 8<sup>a</sup>

Virus	Origin of segment <sup>b</sup> :								LD <sub>50</sub> (log <sub>10</sub> PFU)	Increase in virulence (log <sub>10</sub> )
	1	2	3	4	5	6	7	8		
FM	f	f	f	f	f	f	f	f	6.5	
FM-MA	F	F	F	F	F	F	F	F	2.2	4.3
J9	f	f	f	F	f	f	f	f	4.3 ± 0.2 <sup>c</sup>	
J41	f	f	f	F	f	f	f	f	4.6	2.2 <sup>d</sup>
J52	f	f	f	F	f	f	f	f	4.1	
W29	f	f	f	F	f	f	F	f	3.1 ± 1 <sup>e</sup>	3.4
T-SR17	f	f	f	F	f	f	F	F	3.5 ± 0.8 <sup>c</sup>	
T142	f	f	f	F	f	f	F	F	3.7 ± 0.6 <sup>f</sup>	3.0 <sup>d</sup>
T154	f	f	f	F	f	f	F	F	3.2 ± 0.4 <sup>f</sup>	

<sup>a</sup> Reassortants were produced by back-crossing FM × HK with FM-MA × HK reassortants.

<sup>b</sup> FM and FM-MA segments are indicated in lowercase and uppercase, respectively.

<sup>c</sup> Geometric mean value from four LD<sub>50</sub> titrations.

<sup>d</sup> Geometric mean value.

<sup>e</sup> Geometric mean value from two LD<sub>50</sub> titrations.

<sup>f</sup> Geometric mean value from five LD<sub>50</sub> titrations.

CACTATAAGTAGAAACAAG) were used for cDNA synthesis; the virus complements are underlined. Double-stranded cDNA clones were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (25), using the Sequenase kit (U.S. Biochemical) and [<sup>35</sup>S]dATP. Mutations detected upon sequencing of cDNA clones were subsequently verified by direct sequencing of viral RNA as described by Geliebter (9). Mutations in the reassortants were confirmed for FM-MA genome segments 4 and 7 by direct sequencing of cDNA amplified by PCR. Viral RNA was reverse transcribed, amplified by PCR using pairs of primers specific for segment 4 (838+sense [GGTATGCTTTTCGCACTG] and 1275-sense [GAATTCTTTACCCACAGC]) or segment 7 (239+sense [GGAGGACTGCAGCGTAGA] and FLUT7), and then directly sequenced (33).

Sequence comparison between genome segments 4, 5, 7, and 8 of FM and FM-MA viruses detected three mutations that

resulted in two amino acid replacements (Fig. 1). Segments 5 and 8 had not acquired mutations upon mouse adaptation. Thus, sequence analysis has discredited the roles for genome segments 5 and 8 in the acquisition of virulence, consistent with their lesser statistical significance in the original genetic study (2). Segments 4 and 7 had one missense mutation each that was confirmed by direct sequencing of vRNA (data not shown). In segment 4 encoding the hemagglutinin, the mutation was found at amino acid 47 of the HA2 subunit, where a tryptophan (UGG) in FM was changed to a glycine (GGG) in FM-MA. Segment 7 encodes two overlapping genes, encoding M1 and M2, that are expressed by alternative mRNA splicing (19). In segment 7, the M1 protein was mutated at amino acid position 139 from a threonine (ACC) in FM to an alanine (GCC) in FM-MA. Thus, the capacity of each of the HA and M1 proteins to increase the virulence of FM-MA was due to single amino acid replacements. There was also a silent mutation found in segment 7 at amino acid position 32, coding for phenylalanine (TTT-TTC). The M2 gene was not affected by the mutations in segment 7.

In this study, nucleotide sequence analysis of the FM-MA HA gene predicts that amino acid replacement of a glycine for tryptophan at amino acid 47 of the HA2 subunit results in increased virulence. Amino acid 47 of HA2 has been observed to change in three amantadine HCl-resistant mutants of X31 and A/chicken/Germany/34 viruses (6), with a resultant increase in the pH optimum of HA-mediated fusion. It is interesting to note that some mutations that affect receptor binding also affect the pH of fusion (reviewed by Wiley and Skehel [32]), indicating that conformational changes associated with fusion are as far ranging as the receptor site at the distal tip of HA. Recently Yewdell et al. (36) have shown that mutations in or adjacent to the fusion peptide can mediate escape from neutralization with a monoclonal antibody that binds to the globular domain of HA at elevated temperature. This finding indicates that single amino acid replacements in the fusion peptide influence the flexibility of the globular domains of HA. FM-MA has become resistant to mouse α and β inhibitors of hemagglutination (data not shown), suggesting structural change in the hemagglutinin. Future studies will probe the possibility that FM-MA has altered properties that could affect pathogenesis in the lung such as receptor binding, cleavability, and pH of fusion. Histopathological studies are under way to determine the role of FM-MA HA in this aspect of pathogenesis.

Early studies of mouse-adapted stocks of human influenza virus indicated that they grow to higher yield in mouse lung than nonadapted virus does (1, 7, 14, 23, 31). The yield of FM-MA in mouse lung was compared with the yield of the parental FM strain by titration following intranasal infection of anesthetized, outbred Swiss Webster mice with 5 × 10<sup>3</sup> PFU of virus. The mice were sacrificed in groups of three, and the lungs were pooled for each time point. Mice were euthanized by using 90% CO<sub>2</sub> with 10% O<sub>2</sub> before lungs were removed for storage at -70°C in phosphate-buffered saline (PBS) supplemented with gelatin to 0.2%. For virus titration, the lungs were thawed and disrupted by sonication. Cell debris was cleared by low-speed centrifugation (3,000 rpm for 10 min) before plaque assays were performed. Growth experiments were done in triplicate, and the data were plotted graphically as geometric mean values ± 1 standard deviation. Figure 2A demonstrates the difference in viral growth kinetics between FM and FM-MA in the mouse lung. FM-MA grew faster and to higher titer in mouse lungs than the parental FM strain did. The FM-MA titer remained higher than the FM titer through 7 days postinfection. Extensive mortality occurred in FM-MA-

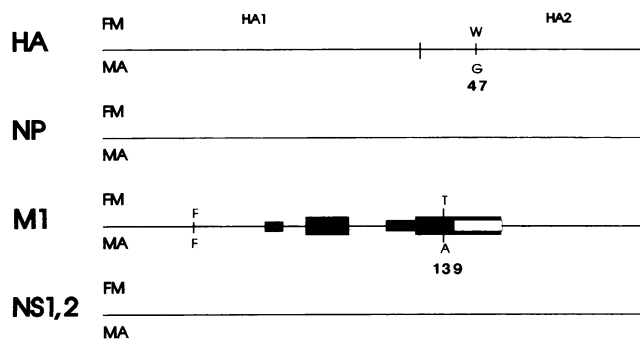


FIG. 1. Mutations detected in segments 4, 5, 7, and 8 of FM-MA relative to the parental FM strain. cDNA clones were sequenced and compared, and then viral RNA was directly sequenced to confirm mutations. Mutations were not detected in segments 5 and 8. For M1 protein, the locations of lipid binding regions are indicated by checked boxes (10) and the locations of regions that bind RNA and RNP are indicated by black boxes (34, 35). The location of a zinc finger motif is shown as an open box within one of the RNA-RNP binding sites (30).

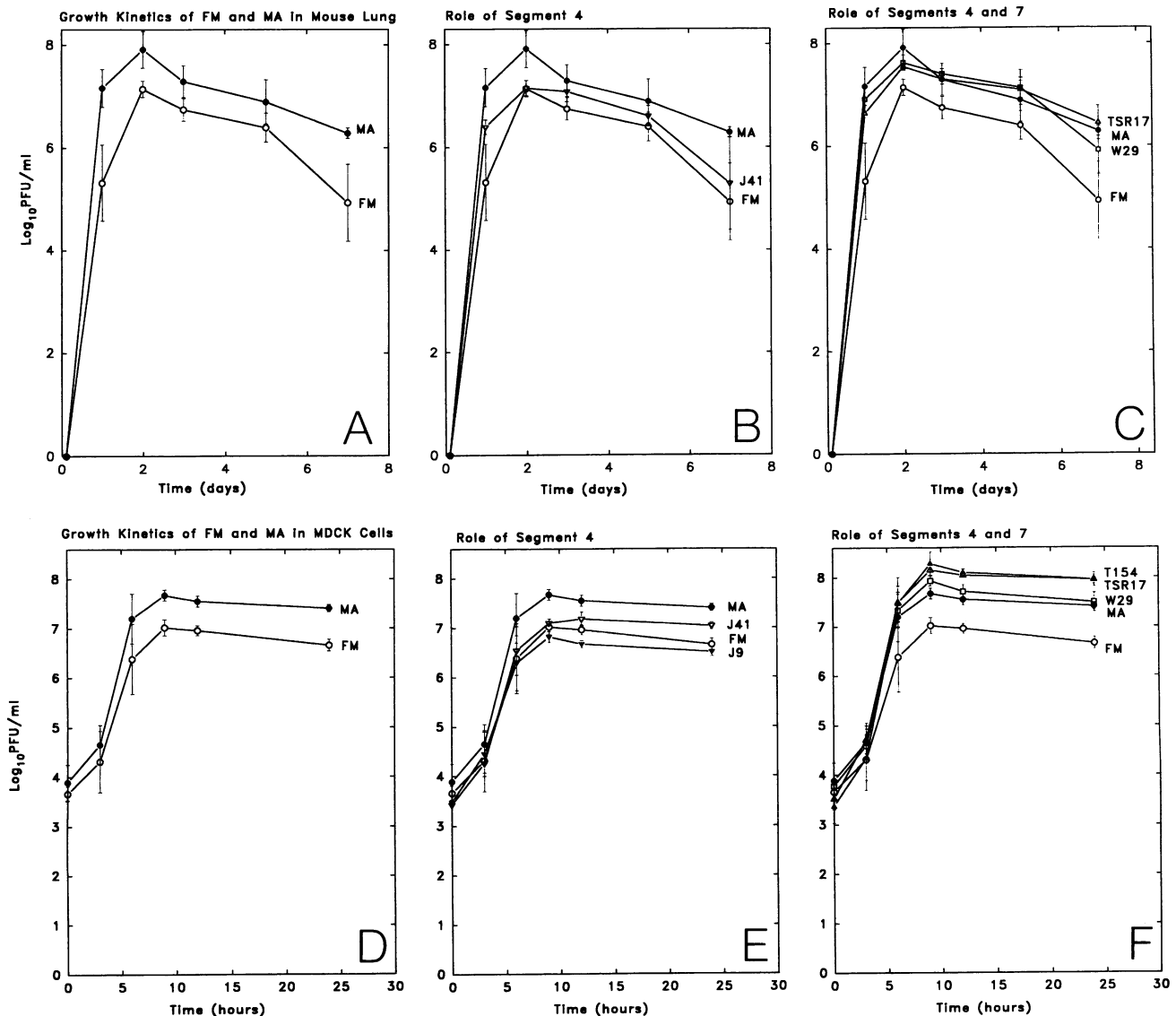


FIG. 2. Growth kinetics of FM, FM-MA, and FM  $\times$  FM-MA reassortants in mouse lung and MDCK cells. (A to C) Growth in mouse lung. Each point is the geometric mean titer of three pools of three lungs. Values are shown with error bars indicating  $\pm 1$  standard deviation. The following FM  $\times$  FM-MA reassortants contain FM-MA segments on an FM background: J41, segment 4; W29, segments 4 and 7; TSR17, segments 4, 7, and 8 (see Table 1). (D to F) Growth in MDCK cells. Each point is the geometric mean titer of three replicate experiments. Values are shown with error bars indicating  $\pm 1$  standard deviation. The following FM  $\times$  FM-MA reassortants contain FM-MA segments on an FM background: J9 and J41, segment 4; W29, segments 4 and 7; TSR17 and T154, segments 4, 7, and 8 (see Table 1).

infected mice beginning at day 5, whereas none occurred for FM-infected mice. Surviving mice clear both viruses by 9 or 10 days postinfection (data not shown).

We reasoned that the property of increased growth in lungs may be responsible at least in part for increased virulence and thus may be controlled by mutations in the HA or M1 gene. Analysis of the growth kinetics of reassortants that differ as a result of these mutations was done to determine whether they controlled growth in the lung. J41 differs from FM as a result of a single amino acid replacement in the hemagglutinin, whereas W29 and the T reassortants differ in virulence from the J viruses as a result of a single amino acid replacement in the matrix protein. The T series of reassortants also contain

FM-MA segment 8, but this segment has the same sequence as the FM segment 8. FM-MA also contains further unidentified mutation(s) that control increased virulence since it is more virulent than the W and T reassortants.

Growth kinetics were measured for reassortants J41, W29, and TSR17. In Fig. 2B, the growth kinetics of the J41 reassortant containing the HA of FM-MA on a background of FM is compared with the growth kinetics FM and FM-MA. The replication of J41 was slightly faster in the first day but was not statistically different for the remainder of the infection. However, the growth of reassortants containing FM-MA segment 4 as well as segment 7 (W29) or segments 7 and 8 (TSR17) was increased to the level of FM-MA in mouse lungs (Fig. 2C). The

addition of FM-MA segment 8 to this reassortant combination did not confer any further growth advantage, as expected since this genome segment was identical to the FM segment.

The increased yield of FM-MA in mouse lung may be due to its increased ability to replicate per se or an ability to circumvent host defenses or barriers to infection. We compared the growth kinetics of FM and FM-MA by measuring the yield of virus with respect to time over a 24-h period in MDCK cell culture. Confluent monolayers of MDCK cells in 35-mm-diameter dishes were washed twice with PBS before inoculation with 0.1 ml of virus diluted to produce a multiplicity of infection of 5. After adsorption at 37°C for 1 h, cultures were washed three times with PBS and then overlaid with 3 ml of minimal essential medium supplemented with bovine serum albumin to 0.2% (wt/vol), 1 µg of trypsin per ml, 100 µg of streptomycin per ml, and 100 U of penicillin per ml. Infected cells were incubated at 37°C, harvested at 3-h intervals, and stored at -70°C until a plaque assay was performed as described previously (2). Growth curves were the geometric means of three experiments. FM-MA grew to a higher titer than FM virus and therefore has an improved ability to replicate not only in mouse lung but also in MDCK cells (Fig. 2D). Growth was maximal by 9 h postinfection and gradually declined thereafter. FM-MA produced about sixfold more virus in MDCK cell culture than FM virus did.

Analysis of the growth kinetics of reassortants that differ as a result of mutations in HA (J9 and J41) and M1 (W29, TSR17, and T154) was done to determine whether these mutations controlled growth in MDCK cells. Segment 4 did not appear to play a role in enhanced growth in MDCK cells, since J41 and J9 were not statistically different from FM virus in peak growth (Fig. 2E). Reintroduction of the FM-MA segment 7 in addition to segment 4 into FM by reassortment resulted in high virus growth that was slightly higher than that of the FM-MA strain, as was seen for W29, T154, and TSR17 (Fig. 2F). Thus, we concluded that the matrix protein controlled the capacity of FM-MA virus to grow to high yield in MDCK cells as well as mouse lungs.

The FM-MA M1 gene has the capacity to increase growth and virulence as a result of a single amino acid replacement of a threonine with an alanine at position 139. Matrix protein lines the inner layer of the viral membrane and also contacts the ribonucleoprotein (RNP) core (24), and through these interactions, it controls both viral packaging and unpackaging events. The matrix protein binds nuclear RNP to promote export to the cytoplasmic membrane for assembly late in infection (20) but is released from RNP during uncoating after acidification of the inner virion (21), mediated by M2 proton channels (reviewed by Helenius [12]). A temperature-sensitive defect in M1 due to an amino acid replacement of Phe with Ser at position 79 results in defective nuclear-cytoplasmic transport of viral RNA (8). M1 binds to lipid vesicles, RNA, RNP, and other M1 protein to form oligomers. Using a combination of monoclonal antibody inhibition and peptide binding studies, Ye et al. (34, 35) have mapped the RNP and RNA binding of M1 to two regions, amino acids 90 to 108 and 128 to 164, in accord with findings of Hankins et al. (11). Placing the FM-MA M1 mutation on the current map of matrix protein functions (Fig. 1) indicates that this change may affect RNA binding or RNP binding and the concomitant inhibition of transcription (11, 13, 34, 35). It is possible that association with RNP or dissociation from RNP is perturbed by mutation at amino acid 139 and that this underlies the mechanism controlling virulence and/or growth. FM-MA M1 may bind RNP less strongly than FM M1, thus allowing genome replication to proceed to higher levels before being shut off by M1 binding which drives

assembly into virions. M1 protein tends to oligomerize (4, 30) and exists in the nucleus as large electron-dense structures (22). The partitioning of M1 between oligomers and RNP may be the mechanism of switching from transcription to assembly that would result in regulation of replication. Factors that favor oligomer formation would tend to increase yield, whereas factors that favor M1-RNP binding would tend to decrease yield. These interactions could be perturbed only within the limits allowable by the functional constraints of viral assembly and uncoating.

Other studies have identified segment 7 in growth control of influenza virus variants that produce high yields of virus in the allantoic cavity of chicken embryo (15, 27). Recently attempts were made to correlate specific amino acid positions of M1 and M2 with the property of high yield (17). FM-MA and FM viruses replicate to the same levels in the allantoic cavity of chicken embryo (data not shown), indicating that increased growth of FM-MA is a host-dependent phenomenon. Whereas growth may be a component of virulence, it is clear from the J series of reassortants that virulence is also mediated by mutations that are independent of growth.

Mouse adaption of A/FM/1/47 has specifically selected variants of increased virulence without a high background of mutation. This adaptation involved a relatively small number of serial passages (12 passages) in mouse lung. The number of passages may be important, since after 100 passages at reduced temperature, there were 11 coding changes involving the nonglycosylated proteins in the attenuated, cold-adapted variant A/Ann Arbor/6/60 (5, 28). Similarly, sequencing of the same group of proteins of cold-adapted variants of A/Leningrad/134/57 produced by 17 and 47 passages identified 8 and 11 amino acid replacements, respectively (16).

Future work will aim at elucidating the function and mechanism of action of mutant HA and M1 genes of FM-MA in specific stages of pathogenesis. In vitro mutagenesis will also be used to confirm the role of the single amino acid changes in HA and M1 in the control of virulence.

**Nucleotide sequence accession numbers.** The genome segment sequences in this report have been submitted to GenBank under accession numbers U02084 to U02087, U02463, and U02464.

This work was supported by the Medical Research Council of Canada.

We thank K. Dimock and K. Wright for critical review and D. Sheppard for aid in manuscript preparation.

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