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Generation of recombinant influenza A virus without M2 ion-channel protein by introduction of a point mutation at the 5' end of the viral intron

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The aim of this study was to inhibit influenza virus M2 protein expression by mutating the splicing signal of the M gene. Mutations were introduced into the GU dinucleotide sequence at the 5'-proximal splicing site of the M gene (corresponding to nt 52–53 of M cRNA). Transfected cells expressing mutated M viral ribonucleoproteins failed to generate M2 mRNA. Interestingly, recombinant viruses with mutations at the dinucleotide sequence were viable, albeit attenuated, in cell culture. These recombinants failed to express M2 mRNA and M2 protein. These observations demonstrated that the GU invariant dinucleotide sequence at the 5'-proximal splicing site of M gene is essential for M2 mRNA synthesis. These results also indicated that the M2 ion-channel protein is critical, but not essential, for virus replication in cell culture. This approach may provide a new way of producing attenuated influenza A virus.

INTRODUCTION

For cellular mRNA maturation, group II introns of RNA polymerase II transcripts are excised by splicing. This event occurs co-transcriptionally and recent evidence suggests that the C-terminal heptad-repeat domain of RNA polymerase II recruits factors to facilitate this biological process (McCracken *et al.*, 1997; Hirose & Manley, 1998). For transcripts that undergo the U2-type (or major-class) splicing pathway, the accuracy and efficiency of splicing have been attributed to several *cis*-acting sequence elements and *trans*-acting factors. The *trans*-acting factors include U1, U2 and U4–6 small nuclear ribonucleoproteins (snRNPs) and splicing factors that interact specifically with the pre-mRNA *cis* elements. The *cis*-acting elements include consensus RNA sequences at the 5' and 3' splice sites, a conserved branch point, a polypyrimidine tract and a splicing enhancer and silencer (Miriami *et al.*, 2002). Of these sequences, the invariant GU and AG dinucleotides at the 5' and 3' ends, respectively, of introns are known to be critical for splicing (Nissim-Rafinia & Kerem, 2002). Mutations at these highly conserved sequences impair proper splicing, although less-conserved consensus motifs, adjacent to both donor and acceptor sites, might also be involved in the process (Hastings & Krainer, 2001).

The genome of influenza A virus contains eight RNA segments (vRNA) of negative polarity. Each segment associates with the nucleoprotein (NP) and polymerase subunits (i.e. PB2, PB1 and PA) to form a viral ribonucleoprotein (vRNP) complex. Influenza A virus uses a different strategy from

eukaryotes for mRNA synthesis. The viral mRNA is generated exclusively by the vRNP (Huang *et al.*, 1990; Fodor *et al.*, 1994; Poon *et al.*, 1999; Leahy *et al.*, 2001). With a genome of less than 14 kb, the virus has developed several strategies to expand its genome-coding capacity (Lamb & Takeda, 2001). These strategies include translation of unspliced, spliced (NS segment) and alternatively spliced (M segment) mRNAs and bicistronic mRNAs (PB1 segment). Of these eight vRNA segments, the M1 mRNA generated from the M vRNP can further undergo alternative splicing to generate two different spliced viral mRNA molecules (Inglis & Brown, 1981). The influenza A virus M gene has two alternative 5' splice sites: a proximal 5' splice site (corresponding to nt 52–53 of M cRNA), producing M2 mRNA, and a distal 5' splice site (corresponding to nt 12–13 of M cRNA), producing M3 mRNA (Shih *et al.*, 1995). It has been noted that M3 mRNA has almost no coding capacity, whereas M1 and M2 mRNAs encode structural and ion-channel proteins, respectively (Valcarcel *et al.*, 1991). The choice of alternative splicing sites is suggested to be controlled by the viral polymerase (Shih *et al.*, 1995) and related to a host splicing factor (Shih & Krug, 1996).

The splicing of influenza A virus mRNA has been studied via both *in vitro* and *in vivo* assays (Lamb & Lai, 1984; Plotch & Krug, 1986; Agris *et al.*, 1989; Alonso-Caplen & Krug, 1991; Valcarcel *et al.*, 1991, 1993; Nemeroff *et al.*, 1992; Shih *et al.*, 1995; Shih & Krug, 1996). However, the majority of these studies were based on transcripts that were not synthesized by vRNPs. Sequence analyses of the

influenza M and NS genes have shown that the viral introns possess typical features of U2-type splicing sequences, suggesting that the viral introns can be excised by U2-type spliceosomes. In particular, the introns of the M and NS genes contain invariant GU and AG dinucleotides at the 5' and 3' ends, respectively (Lamb & Lai, 1980). *In vitro* experiments have shown that small nuclear RNPs can bind to viral-like mRNA transcripts to form U2-type spliceosomes (Agris *et al.*, 1989). In addition, M mRNA has also been found to contain an enhancer sequence to facilitate the binding of cellular splicing factors (Shih & Krug, 1996).

In this study, we tried to inhibit M2 protein expression by introducing mutations at the 5'-proximal splicing site of the M gene. In addition, we investigated the effect of mutations on the invariant GU dinucleotide at the 5'-proximal splicing site of the M gene. Rather than using classical *in vitro* splicing systems or viral-like RNA transcripts in our study, the viral transcripts we studied were generated entirely from recombinant vRNPs by using *in vivo* systems. We found that mutation of the dinucleotide sequence abolished splicing for M2 mRNA production. Furthermore, recombinant viruses with mutations at this unique sequence were attenuated in cell culture and failed to express M2 ion-channel protein.

METHODS

Cell culture and viruses. 293T human embryonic kidney cells and Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin and streptomycin (P/S) at 37 °C in 5% CO₂. Influenza A/WSN/33 (H1N1) virus and its derivatives were propagated routinely in MDCK cells. For virus-stock preparation, MDCK cells were infected with virus at an m.o.i. of 0.01. Progeny virus was harvested at 3 days post-infection. Routine haemagglutinin (HA) tests and plaque assays were used to measure the viral titre.

Construction of M vRNA expression plasmids with mutations at the invariant 5' GU at the 5'-proximal splicing site. Plasmids pPOLI-M-RT (Fodor *et al.*, 1999, 2002) and pHW187-M (Hoffmann *et al.*, 2000) were used to express M vRNA of influenza A/WSN/33 virus. Mutations at the 5'-proximal GU splicing sequence were introduced into these plasmids by inverse PCR techniques and all mutated sequences were confirmed by DNA sequencing.

Expression of M vRNP in transfected cells. Plasmids pcDNA-PB2, pcDNA-PB1, pcDNA-PA, pcDNA-NP and pPOLI-M-RT were used to express M vRNP of influenza A/WSN/33 virus in transfected cells (Fodor *et al.*, 1999, 2002). Briefly, 1 µg of each of the plasmids was transfected into 293T cells by using Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. The medium of transfected cells was replaced by MEM with 10% FCS and 1% P/S at 24 h post-transfection.

Generation of recombinant influenza viruses. To generate recombinant influenza A/WSN/33 virus, eight plasmids (pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, pHW187-M and pHW188-NS) were used in this study (Hoffmann *et al.*, 2000). Transfection experiments were performed as described by Hoffmann *et al.* (2000).

RNA extraction and cDNA synthesis. Total RNA from transfected or infected cells was harvested by using an RNeasy Mini kit

(Qiagen). In a typical reverse-transcription reaction, 3 µg total RNA was reverse-transcribed into cDNA by SuperScript II reverse transcriptase (Invitrogen). For the detection of vRNA, 400 ng vRNA-specific primer complementary to the 3' end of vRNA (Hoffmann *et al.*, 2001) was used in reverse-transcription reactions. By contrast, for the detection of M mRNA molecules, 20 pmol M mRNA-specific primer (5'-TTTTTTTTTTTTTACTC-3'; underlined sequence corresponds to nt 23–26 of the M vRNA) was used in reverse-transcription reactions.

Quantitative RT-PCR assays for vRNA and mRNA. For quantification of M vRNA and mRNA, a SYBR green-based real-time PCR method was employed. Real-time PCR experiments were performed by using a LightCycler (Roche). Plasmids containing the target sequence were used as positive controls. Primers for the M vRNA and M1 mRNA were 5'-GACCAATCCTGTACCTC-3' (corresponding to nt 171–188 of the M cRNA) and 5'-GATCTCCGT-TCCCATTAAGAG-3' (corresponding to nt 735–755 of the M vRNA). For M2 and M3 mRNA detection, intron-spanning primers were used. Primers for the M2 RNA were 5'-GAGGTCGAAAC-GCCTAT-3' (underlined sequence corresponds to nt 41–51 of the M cRNA, italic sequence corresponds to nt 740–745 of the M cRNA) and 5'-CTCCAGCTCTATGTTGACAAA-3' (corresponding to nt 24–44 of the M vRNA). Primers for the M3 mRNA were 5'-CATAGCAAAGCAGGCCTA-3' (underlined sequence corresponds to the first 11 nt of the M cRNA, italic sequence corresponds to nt 740–744 of the M cRNA) and 5'-CATAGACTCTGGCACTCC-3' (underlined sequence corresponds to nt 102–116 of the M vRNA). The amplification program started with one cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 5 s (56 °C for M3 mRNA detection) and 72 °C for 15 s. The specificity of the assay was confirmed by melting-curve analysis at the end of each run (65–95 °C, 0.1 °C s⁻¹). Amplified products were further analysed by agarose-gel electrophoresis and sequencing.

Indirect immunostaining of M2 protein. Confluent monolayers of MDCK cells grown on glass coverslips were washed with PBS and infected at an m.o.i. of 1 with influenza A viruses. Infected cells were harvested at 8 h post-infection, washed twice in PBS and fixed in 4% paraformaldehyde in PBS. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 30 min, followed by two washes in PBS. Cells were then incubated with 14C2 mouse anti-M2 IgG monoclonal antibody (mAb) (diluted 1:1000; Affinity Bioreagents) overnight at 4 °C. After two washes in PBS with 0.01% Triton X-100, cells were incubated with rabbit anti-mouse antibody conjugated with fluorescein isothiocyanate (diluted 1:100; Zymed Laboratories) for 1 h at room temperature. Samples were washed twice in PBS containing 0.01% Triton X-100 and counterstained for 1 min with propidium iodide solution (BD Biosciences Pharmingen) for DNA staining. After two more washes with PBS, coverslips were mounted by using ProLong anti-fading reagent (Molecular Probes). The surface fluorescence of cells was observed with a Zeiss Axiophot Microscope and images were taken by using a Leica CCD camera.

Virus purification and Western blot analysis. MDCK cells were infected with wild-type or mutant virus at an m.o.i. of 0.01. Virus was harvested at 3 days post-infection and purified by using sucrose gradients as described previously (Poon *et al.*, 2000). Purified virus was lysed in lysis buffer [0.6 M KCl, 50 mM Tris/HCl (pH 7.5), 0.5% Triton X-100]. Viral lysates were analysed by 12% SDS-PAGE and transferred to a PVDF membrane (Amersham Biosciences). Transferred membrane was first blocked for 1 h at room temperature with PBS containing 5% skimmed milk and 0.1% Tween 20 (Sigma, ICI Americas) followed by overnight incubation with 14C2 mouse anti-M2 IgG mAb (Affinity Bioreagents). The membrane was washed three times with PBS containing 0.1% Tween 20 and then incubated for 1 h at room temperature with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibody

(Zymed Laboratories). Signals were detected by using the Western Immunoblot ECL Plus detection system, following the manufacturer's instructions (Amersham Biosciences).

RESULTS

Mutated M vRNA fails to produce M2 mRNA in transfected cells

In this study, we first used a plasmid-based reverse-genetics system in our initial investigation (Fodor *et al.*, 1999). To determine whether the GU dinucleotide at the 5'-proximal splicing site of M gene was essential for the generation of M2 mRNA, mutated vRNA expression plasmids with all possible combinations of dinucleotide sequences were generated. The mutated M vRNA expression plasmid and four viral protein expression plasmids encoding PB2, PB1, PA and NP were co-transfected into 293T cells to generate M vRNPs. At 3 days post-transfection, vRNA and M1–M3 mRNA generated from the transfected cells were detected by corresponding RT-PCR assays. As shown in Fig. 1, M vRNA could be found in the wild-type and all mutants, showing that the introduced mutations did not alter vRNA expression. M1 mRNA was detected in cells transfected with wild-type or mutated constructs (Fig. 1, row 2). In the presence of recombinant RNP expression, wild-type M1 mRNA could be spliced into M2 or M3 mRNA as expected (Fig. 1, rows 3 and 4) (Shih *et al.*, 1995; Shih & Krug, 1996). By contrast, M2 mRNA molecules could not be detected in cells transfected with the mutated constructs (Fig. 1, row 3). All of the mutated constructs were able to yield M3 mRNA (Fig. 1, row 4). These results suggested that mutations at the 5'-proximal splicing site specifically inhibited splicing for M2 mRNA but not for M3 mRNA.

Generation of recombinant viruses with mutations at the 5'-proximal splicing site

To investigate whether our studied mutations would be detrimental to the viability of influenza A virus, we used a reverse-genetics system to generate recombinant viruses with the above mutations. Of 15 possible mutations, only infectious recombinant viruses with AU, CU and UU dinucleotide sequences could be generated. Interestingly, these three mutations were all silent for M1 protein expression. By contrast, all of the other mutations altered aa 9 or 10 of the M1 protein, suggesting that these amino acid residues are critical for influenza virus viability.

From our transfection experiments (Fig. 1), we demonstrated that mutations at the 5'-proximal splicing site inhibited M2 mRNA synthesis. It was therefore of interest to test whether these recombinant viruses also failed to produce M2 mRNA. Two of the mutated viruses (AU and CU) were selected for further analysis. MDCK cells were infected by wild-type or mutants at an m.o.i. of 2. Total RNA from infected cells was harvested at various time points post-infection. The amounts of M1, M2 and M3 mRNA in these samples were quantified by real-time RT-PCR. Consistent with the observations deduced from the transfection experiments, M vRNA (not shown), M1 mRNA and M3 mRNA were detectable in all samples (Fig. 2a), indicating that these mutated vRNA segments were templates for viral transcription and replication in infected MDCK cells. By contrast, M2 mRNA could only be detected in MDCK cells infected with wild-type virus, but not with the mutants.

With the quantitative data deduced from the real-time PCR assays, we also estimated the relative proportions of M1–M3 mRNA species in infected cells. In agreement with

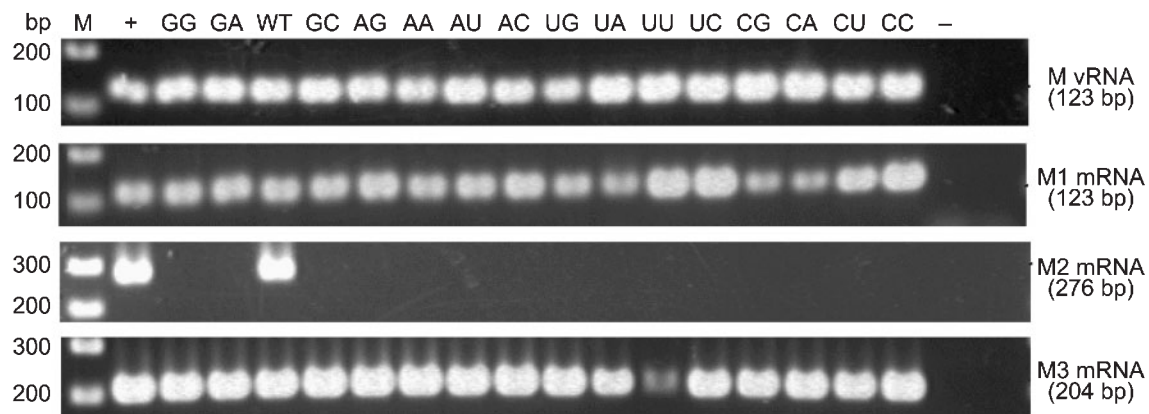


Fig. 1. Detection of M vRNA and mRNA in transfected cells. M vRNA (123 bp), M1 mRNA (123 bp) and M3 mRNA (204 bp) signals were observed in all samples. By contrast, M2 mRNA (276 bp) was only detected in the wild-type (WT) sample (GU). Mutations introduced into the M vRNA expression plasmid are indicated. M, DNA markers; +, cDNA from virus-infected cells; -, negative water control. The faint M3 mRNA signal from the mutant UU was due to a sample-loading problem.

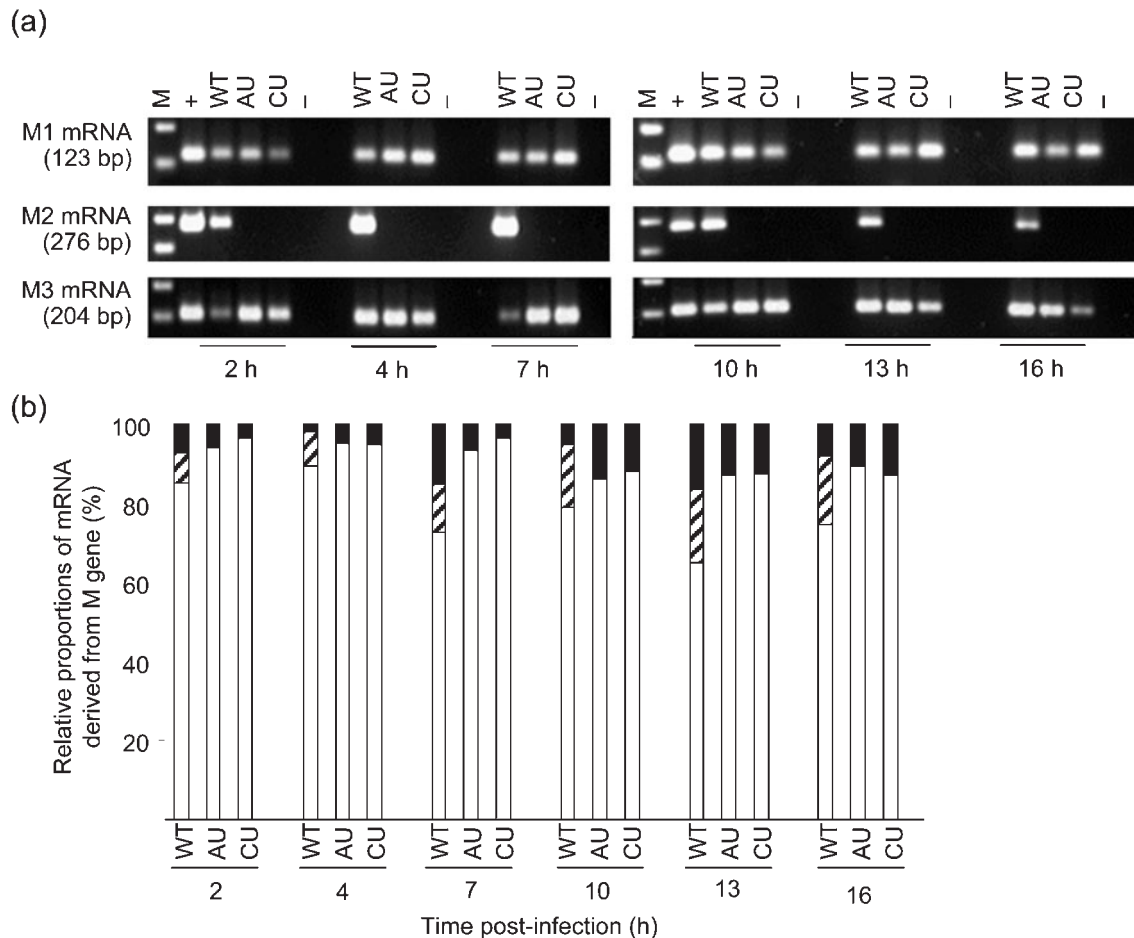


Fig. 2. Detection of M mRNA in infected cells. (a) Detection of M1–M3 mRNA in infected cells by RT-PCR. Total RNA from cells infected by wild-type (WT) or mutants (AU or CU) was harvested at the indicated time points post-infection. Signals for the PCR products were captured by using different exposure time and thus do not correlate to the amount of targeted RNA in the sample. M, DNA markers; +, cDNA from virus-infected cells; –, negative water control. (b) Relative proportions of M1 (empty bars), M2 (hatched bars) and M3 (filled bars) mRNA derived from the wild-type (WT) or mutated (AU and CU) M vRNA. Data were deduced from two or more independent experiments.

previous studies (Valcarcel *et al.*, 1991), the amount of M2 generated from wild-type-infected cells increased with time (Fig. 2b). At 2 h post-infection, M2 mRNA comprised about 8% of mRNA generated from the M vRNP (Fig. 2b). At 16 h post-infection, 17% of mRNA derived from M vRNA was M2 mRNA (Fig. 2b).

Recombinant viruses with mutations at the 5'-proximal splicing site are attenuated

Viruses with mutations at the 5'-proximal splicing site failed to synthesize spliced M2 mRNA. Although these mutants were able to replicate in cell culture, they could only produce pinpoint plaques when grown on MDCK cells. The plaque sizes of the mutants (~0.5 mm diameter) were three to four times smaller than those of the wild-type (Fig. 3a). To characterize the growth properties of these mutants in detail, MDCK cells were infected with the mutant viruses at an m.o.i. of 0.01. At various time points

post-infection, the number of infectious progeny viral particles released into the medium was determined by plaque assay. The maximum viral titres of these mutants were about 2 log units lower than that of the wild-type (Fig. 3b). In addition, we determined the stability of these mutations by passaging the mutants in MDCK cells 10 times. The desired mutations were all retained, indicating that the introduced mutations were stable in routine cell culture. These results suggested that, at least under cell-culture conditions, M2 protein might not be absolutely essential for virus replication. However, our results also indicated that lack of M2 protein expression severely affected growth of the virus.

M2 mutants fail to produce M2 ion-channel protein

The splicing mutants failed to produce detectable M2 mRNA in infected cells, suggesting that these mutants

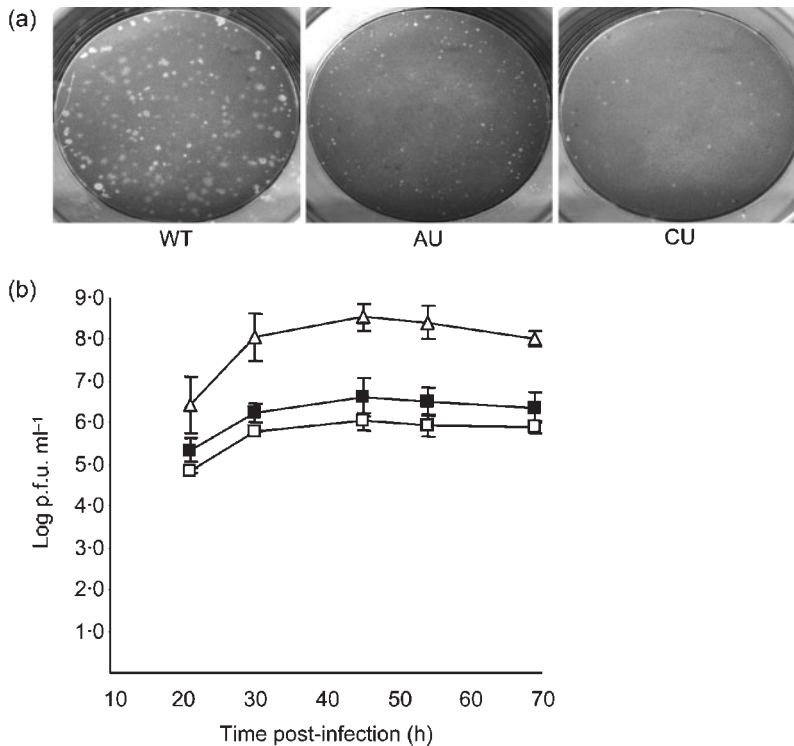


Fig. 3. Attenuation of splicing mutants in MDCK cells. (a) Plaque morphology of wild-type (WT) and mutants (AU and CU) in MDCK cells. (b) Growth curves of wild-type (WT, Δ) and mutants (AU, \square , and CU, \blacksquare). The amount of progeny viral particles generated from infected cells at the indicated time points post-infection was quantified by plaque assay. Results are shown as means \pm SD from three independent experiments.

might not be able to synthesize M2 ion-channel protein. To test this possibility, we examined M2 protein expression in infected cells by indirect immunofluorescence. By using an M2-specific mAb, a pattern of immunofluorescence signals similar to that described by others (Hughey *et al.*, 1992) was observed in wild-type-infected cells (Fig. 4a). However, none of the splicing mutants was found to express M2 protein in infected cells (Fig. 4a). We also used purified viral particles to confirm the above observations by Western blot analysis. As shown in Fig. 4(b, lower panel), M2 protein was only detected in the purified wild-type virion and not in the mutants. By contrast, the amount of M1 protein detected in these viruses was similar (Fig. 4b, upper panel), indicating that similar amounts of virion were used in the analysis.

To validate these findings further, we also attempted to detect M2 protein expressed from recombinant M vRNPs in transfected cells (i.e. the cells used in Fig. 1). Equal amounts of cell lysates (Fig. 4c, upper panel) were analysed by Western blotting as described above. Similar to the results observed in virus-infected cells, M2 protein could only be detected in cells transfected with the wild-type construct (Fig. 4c, lower panel).

Virus morphology

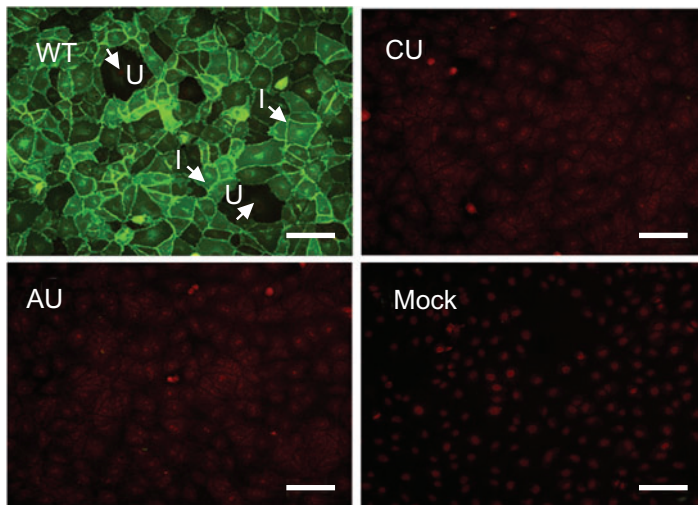
M2 protein has been reported to be associated with virus morphology (Roberts *et al.*, 1998). We examined the morphology of the splicing mutants by electron microscopy. Both splicing mutants were spherical in shape and morphologically indistinguishable from the wild-type (data

not shown). The sizes of the AU (138.83 ± 15.70 nm, $n=100$) and CU (134.98 ± 14.42 nm, $n=100$) splicing mutants were not statistically significantly different from that of the wild-type (139.60 ± 20.20 nm, $n=100$) ($P > 0.05$, Student's *t*-test).

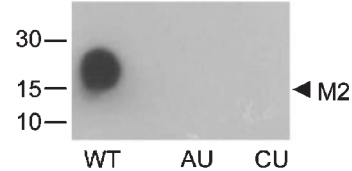
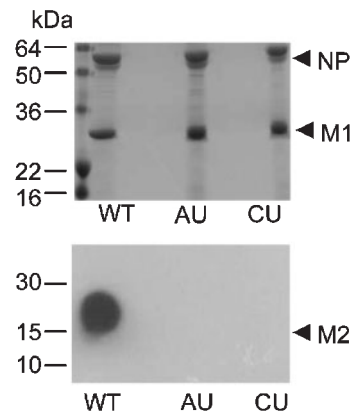
DISCUSSION

In this study, we inhibited M2 protein expression by introducing point mutations at the 5' end of the viral intron. Sequence analysis of data available from the Influenza Sequence Database (<http://www.flu.lanl.gov>) indicated that this invariant dinucleotide sequence is conserved in all M gene sequences. Thus, this GU sequence might be critical for splicing of influenza transcripts. Indeed, none of our mutants in this study was capable of generating M2 mRNA. This inhibitory effect was highly specific and these mutations did not affect the alternative splicing pathway for M3 mRNA production. In eukaryotes, the majority of cellular group II introns contain the invariant GU dinucleotide at their 5' ends and are processed by the U2 splicing pathway (Burset *et al.*, 2000). This GU dinucleotide sequence is essential for the binding of U1 snRNP, a critical event for U2-type spliceosome formation (Weber & Aebi, 1988). Mutating this cellular GU dinucleotide sequence often results in abnormal splicing processes and gene expression (Aebi *et al.*, 1986; Mount, 2000). Thus, inhibition of M2 mRNA synthesis in the splicing mutants might be due to disruption of the interaction between the 5'-proximal splicing site and U1 snRNP.

(a) Infected cells



(b) Virions



(c) Transfected cells

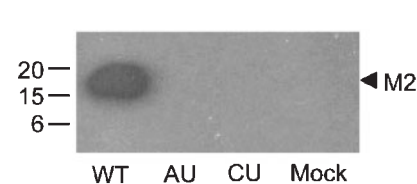
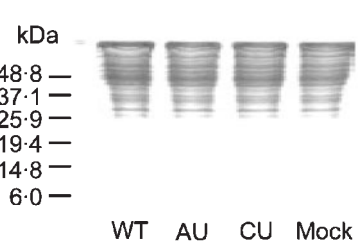


Fig. 4. Splicing mutants fail to produce M2 protein. (a) Indirect immunostaining of M2 protein in infected cells. Cells infected with wild-type (WT) or mutants (AU or CU) were harvested at 8 h post-infection and stained for M2 protein expression. Propidium iodide was used as a counterstain and is shown in red. Mock, Mock infection; I, infected cells; U, uninfected cells. Bars, 30 μ m. (b) Upper panel: Coomassie blue-stained SDS gel loaded with purified virions. The positions of M1 and NP are indicated. Lower panel: detection of M2 protein in purified virions by Western blot analysis. The positive signal for the M2 protein is indicated. An equal amount (10 μ g) of purified virions was added in each lane. (c) Upper panel: Coomassie blue-stained gel loaded with lysates from transfected cells. Lower panel: detection of M2 protein in 293T cells expressing M vRNPs by Western blot analysis. The positive signal for the M2 protein is indicated. An equal amount (25 μ g) of lysate was added in each lane.

None of the mutants was capable of synthesizing M2 ion-channel protein (Fig. 4a, b). The lack of M2 protein expression in these mutants was validated by transfection experiments (Fig. 4c). In addition, using a highly sensitive quantitative RT-PCR assay with a detection limit of 10 copies per reaction (data not shown), we failed to detect M2 mRNA from these mutants. Thus, it is highly unlikely that these mutants expressed a low level of M2 protein in infected cells. Although these mutants might have been expected to revert to the wild-type by altering a single nucleotide, they were found to be stable for at least 10 passages.

The p.f.u./HA ratios of our studied viruses were similar (data not shown), suggesting that the lack of M2 expression did not affect the formation of infectious viral particles. Several previous findings have suggested that M2 protein is not absolutely essential for influenza virus. First, recombinant viruses with defective M2 proteins have been generated by two independent groups (Watanabe *et al.*, 2001; Takeda *et al.*, 2002). In addition, M2 protein has been

found not to be required for viral-like particle formation (Mena *et al.*, 1996), indicating that M2 protein is not essential for the budding process. Here, we further demonstrated that M2 protein expression is not required for influenza virus to grow in cell culture. Interestingly, unlike the M2 truncated mutants described by Watanabe *et al.* (2001), our M2 splicing mutants showed similar growth kinetics to the wild-type. This discrepancy might be partly due to the different viral strains used in the studies. Nonetheless, the recombinant viruses generated by us and others (Takeda *et al.*, 2002; Watanabe *et al.*, 2002) were all attenuated in animals or in cell culture, indicating that the M2 protein plays an important role in efficient virus replication.

In this study, we did not investigate the exact mechanism of attenuation of the mutants. The ion-channel activity of the M2 protein is essential for modulating the pH of the *trans*-Golgi of virus-infected cells and the virion interior. In the early phase of viral infection, the dissociation of vRNPs from M1 protein is triggered by the proton influx from

endosomes to virion interiors via the M2 ion channel (Bui *et al.*, 1996). Using ion-channel inhibitors (e.g. amantadine) to block the ion-channel activity could inhibit the dissociation of M1 protein from vRNPs during viral entry (Kemler *et al.*, 1994). For some influenza virus subtypes, these inhibitors could also induce conformational changes of cleaved HA in the *trans*-Golgi network (Ciampor *et al.*, 1992; Takeuchi & Lamb, 1994). As the HA of our parental strain (i.e. A/WSN/33) is not cleaved by endogenous proteinase (Goto & Kawaoka, 1998, 2000), it would maintain its native structure within the *trans*-Golgi network. Thus, the absence of M2 expression in our mutants would have little impact on the HA conformation in infected cells. A more plausible hypothesis is that, in the absence of M2 ion-channel protein, vRNPs within virions cannot be acidified by endosomes in the early stage of viral infection. As a result, the dissociation of M1 protein from vRNPs would be inhibited, thereby affecting normal virus transcription and replication. Alternatively, the lack of M2 ion-channel protein might affect other biological processes of the virus. Further work, such as studies of transcription and replication kinetics and the nuclear importing of vRNP of these mutants during early viral infection, is required to elucidate the underlying reason for the attenuation of these mutants.

Of the 15 splicing mutants, only those viruses containing a GU to AU, CU or UU mutation could be rescued. Interestingly, these three mutations did not alter the M1 protein-coding sequence. By contrast, all of the other mutations resulted in changes to aa 9 (Thr) or 10 (Tyr) of the M1 protein. Our results thus suggest that these two amino acid residues are critical for the virus. These two residues are located at the first helical structure of the M1 protein and are involved in lipid-membrane binding (Sha & Luo, 1997). Further investigation is required to elucidate the precise effect of these mutations on the M1 protein.

Our results have demonstrated a novel method for generating attenuated influenza viruses. Sequence analyses of influenza M mRNA indicated that the invariant dinucleotide sequence is absolutely conserved (data not shown). Thus, this approach can be used to generate attenuated viruses with other genetic backgrounds. However, one should be aware that this strategy might not be applicable to strains that encode an intracellularly cleavable HA (Sugrue *et al.*, 1990; Takeuchi & Lamb, 1994).

Finally, the splicing mutants in this study might be used to study other biological processes of influenza A virus. For example, it has been suggested that the M1 and M2 proteins encoded by the M gene segment contribute to filamentous virus morphology (Hughey *et al.*, 1995; Roberts *et al.*, 1998; Bourmakina & García-Sastre, 2003). The virus strain used in this study (A/WSN/33) displayed a spherical morphology and we did not observe any changes in virus morphology in our mutants by electron microscopic examination. However, it would be possible to delineate the roles of the M1 and M2 genes in virus morphology by

applying similar mutations to strains that are predominantly filamentous in shape (Bourmakina & García-Sastre, 2003; Elleman & Barclay, 2004).

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