Identification of *cis*-Acting Elements that Regulate Influenza A Virus

Segment 7 Differential mRNA Splicing

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Abstract

Influenza A virus segment 7 encodes the M1 matrix protein and M2 ion channel from an unspliced primary transcript mRNA1 and spliced mRNA2, respectively. Both proteins are essential for efficient virus replication, but the intrinsic sequences that determine segment 7 splicing balance are not fully defined. Here, we systematically investigated how conserved *cis*-acting splicing elements regulate 7 splicing. We found that while the splice acceptor sequence is optimal, both potential upstream polypyrimidine tracts regulate splicing, the 5' region acting positively and the 3' region negatively to maintain M1 expression. We could not unambiguously identify the mRNA2 branch point, but two closely spaced motifs can act redundantly. Saturation mutagenesis of the mRNA2 splice donor sequence showed that it is nearly optimal but that nucleotides 51 and 55 modestly downregulate splicing efficiency. Most introduced mutations that change splicing balance are nonsynonymous in M1. Nevertheless, mutations in the polypyrimidine tract and branch points that significantly perturbed M1:M2 balance resulted in viable viruses. In contrast, the splice donor sequence was intolerant of mutation. We conclude that M1:M2 balance is maintained via suboptimal splice donor and polypyrimidine tract splicing signals, with the latter allowing more evolutionary flexibility to vary the ratio and/or M1 sequence.

Introduction

Influenza A virus (IAV) is an enveloped negative-sense single-stranded RNA virus with eight genome segments. Ten proteins encoded by these segments are essential for virus fitness outside of the laboratory: segments 1-6 encode polymerase basic subunit 1 (PB1), polymerase basic subunit 2 (PB2), polymerase acidic subunit (PA), haemagglutinin (HA), nucleoprotein (NP), and neuraminidase (NA). Segments 7 and 8 encode more than one protein by alternative splicing: matrix protein (M1), matrix 2 ion channel protein (M2) from segment 7, nonstructural protein (NS1) and nuclear export protein (NEP) from segment 8 (1). The viral proteome is further elaborated through a variety of transcriptional and translational mechanisms to produce a virus strain-dependent suite of accessory protein species (2).

The M1 matrix protein of the virus has multiple functions. As well as forming a helical filament under the virus envelope, it recruits several other viral components to assemble at the site of budding (3, 4) and determines viral particle morphology (5, 6). M1 also binds to viral ribonucleoproteins (RNPs) in a regulated manner to control their nuclear import and export (7). The M2 protein is a 97 aa proton channel that is translated from the main spliced transcript of segment 7 mRNA and that mediates acidification of the interior of the virus particle in endosomes when the virus enters a cell (8, 9). It has also been proposed to mediate membrane scission of budding virions (10) as well as to block autophagosome fusion with lysosomes (11). Although both M1 and M2 are found in the virion, the copy number of M2 is two orders of magnitude lower than of M1 (12). Despite the markedly different demands of M1 and M2 for the virion particle, expression levels are more equal in cells, with M2 comprising between 10% to 60% of segment 7 protein production, depending on the cell and virus strain (13), implying M1 and M2 expression is regulated. Primarily, this is thought to occur at the level of mRNA splicing. Newly transcribed segment 7 mRNA

molecules accumulate in nuclear speckles for splicing at the earlier stages of infection and

although measurements of the ratio of mRNA2:mRNA1 varies according to time-point, virus

strain, host cell and assay method, the unspliced transcript tends to predominate (13-16).

Segment 7 mRNA splicing is more complex than simply producing mRNA2. At least

four spliced transcripts have been identified (17-20) in addition to unspliced mRNA1. The

spliced mRNAs use different splice donors (SD) but share the same splice acceptor site (SA).

Spliced mRNA2 encodes the M2 ion channel protein (8). Spliced mRNA3 is not required for

virus replication in tissue culture and no encoded protein has yet been identified (21).

mRNA4 encodes an M2 variant ("M42") with a distinct ectodomain but identical C-terminal

domain (22). All IAV strains produce unspliced mRNA1 and mRNA2, but the abundance of

mRNA3 is variable among strains and only a small minority of strains produce mRNA4 (19,

22).

Four *cis*-acting elements are required for cellular pre-mRNA splicing: an SD at the 5'-

end of the intron/exon boundary; and three separate but usually juxtaposed elements: a

branch point (BP) and polypyrimidine tract (PPT) at the 3'end of the intron and a 3'-SA

sequence overlapping the intron/exon boundary (Figure 1A). Most introns have highly

conserved ends: a GU dinucleotide at the 5'end and an AG dinucleotide at the 3' end (23-25).

These lie within broader consensus sequences for SD and SA sites, which are

MAG|GURAGU and YAG| respectively (26-28). The BP consensus (YUNAY, with the

underlined A being the actual branch point) is more loosely conserved, while the PPT is

usually a stretch that contains around 10 pyrimidines located 5-40 nucleotides upstream of

the SA (29). The SD sequence is recognised by U1 snRNP through RNA-RNA base pairing to

initiate splicing (26, 30). After cleaving at the 5' end of the intron, a guanine at the 5'-end

conjugates to the BP adenine to form a lariat structure (29). The two U2 auxiliary factor

(U2AF) subunits recognise the PPT and SA, respectively, recruiting U2 snRNP and

promoting spliceosome assembly (31, 32). After that, the two exons ligate and the intron in

lariat configuration is excised and degraded (29).

The cellular splicing machinery recognises the *cis*-acting splicing elements based on

their match to the conserved consensus sequences. Since the cellular spliceosome mediates

IAV segment 7 mRNA splicing (33-35), the splicing signals in IAV mRNA generally, though

imperfectly, resemble those of the host cells. However, in contrast to cellular mRNA, both

unspliced and spliced transcripts of segment 7 are translated to essential viral proteins.

Therefore, IAV must incompletely splice segment 7 to ensure correctly balanced M1 and M2

production. One possibility is that viral factors ensure incomplete splicing, and there is

evidence to support regulation by NS1 and/or the viral polymerase complex. The cellular

proteins NS1-BP, hnRNP and ASF/SF1 have been confirmed to regulate segment 7 mRNA

splicing (33, 34, 36). Also, the viral polymerase and NS1 may also be involved in segment 7

mRNA splicing (33, 34, 37, 38). However, segment 7 mRNA is incompletely spliced even

when expressed by cellular RNA polymerase II in the absence of other IAV proteins (37, 39,

40). The segment 7 mRNA2 splice sites are imperfect matches for the core cellular consensus

sequences (Figure 1) and from mutational studies of cellular splicing, these mismatches are

likely to impair the recognition of *trans*-acting elements (28), which may result in reduced

splicing efficiency. However, splice site efficiency is difficult to predict from sequence (28).

Other uncertainties are that the segment 7 BP has never been experimentally mapped, only

computationally predicted, while the extent and location of the PPT also remain unclear (41).

In this study, we set out to test the hypothesis that the primary balance between M1

and M2 mRNAs is set by the conserved *cis*-acting splice site elements of IAV segment 7

splicing, by systematically characterising the strength and location of the SD, BP, PPT and

SA motifs. We find that segment 7 has a strong SA and redundant BP sequences, and that the

 key suboptimal splicing signals that give the desired mRNA1/M1 and mRNA2/M2 ratios are

the SD and PPT signals.

Figure 1. Consensus alignments of cellular and IAV segment 7 *cis*-acting splice elements. Sequence

logos for (A) a selection of experimentally validated mammalian mRNAs (SD, PPT and SA motifs,

adapted with permission from (42, 43); BP motifs adapted from (44) under CC BY 4.0 permission) and (B)

IAV segment 7 sequences are shown, along with the cellular consensus (M indicates A or C and Y

indicates C or U/T). The sequence of the A/Puerto Rico/8/34 (PR8) virus strain is also shown. Note that

the sequence logos were generated from sequences downloaded from NCBI and thus follow the

convention of T rather than U for RNA. Red dashed lines indicate splice sites (boundary between intron

and exon). Boxes indicate splice site elements. SD: Splice donor; BP: Branch point; PPT: Polypyrimidine

Tract (following the cellular consensus location and length - the underline region from nucleotides 721-

727 indicates an alternative prediction (41)); SA: Splice Acceptor. Dashed boxes indicate that the location

of IAV segment 7 BP is unclear.

Materials and Methods

Bioinformatics analyses

To generate a consensus sequence for IAV segment 7 mRNA2 splice sites, 13,280 complete

IAV segment 7 sequences (all subtypes and host species) were downloaded from the National

Centre for Biotechnology Information (NCBI). The viral sequence logos were generated

using WebLogo (42, 43). Counterpart sequence logos representing the consensus SD, SA and

PPT sequences from experimentally verified human genes, originally abstracted from the

Exon-Intron database (45), were adapted with permission from

https://weblogo.berkeley.edu/examples.html. The consensus BP sequence from the bovine

genome was modified with permission (https://creativecommons.org/licenses/by/4.0/) from

reference (44). To predict potential splice site motifs in segment 7, the A/Puerto Rico/8/1934

(PR8) segment 7 sequence (GeneBank accession: EF467824) was truncated at the

polyadenylation signal (to mimic the mRNA sequence) and used as input for various

published prediction programmes: Alternative Splice Site Predictor (ASSP) (46), GENSCAN

(47), Human Splicing Finder (HSF) (48), NetGene2 (49, 50), NNSPLICE (51), Spliceator (52)

and SVM-BPFinder (53). Default parameters were used, other than for HSF, where HSF

scoring was used, and SVM-BP, where the maxlen parameter was increased to 1000 to scan

the entire segment. In addition, the output scores from SVM-BP were converted to

"confidence values" (0-1) using the maximum and minimum scores for cellular genes given

in (53) (approximately +5 to - 3.5 for BP sites and 0 to 31 for PPT tracts in introns of < 1000

ntds).

Cells, viruses and plasmids

Madin-Darby canine kidney (MDCK) and Human embryonic kidney cells (HEK 293T) were

cultured according to standard procedures (54). Cells were routinely tested for mycoplasma

contamination. Wild type (WT) and modified viruses were rescued by eight plasmid

transfections (55) into HEK 293T cells using Lipofectamine 2000 following the

manufacturer's instructions. Then, the virus was propagated in MDCK cells as previously

described (54). Site-directed mutagenesis of segment 7 was performed using Q5 Site-

Directed Mutagenesis Kits (New England Biolabs, E0554) following the manufacturer's

instructions. All constructs were confirmed by Sanger sequencing (GENEWIZ). To titre

viruses, confluent monolayers of MDCK cells were infected with serially-diluted viral

samples and incubated for one hour, followed by Avicel overlay and toluidine blue staining.

To quantify plaque size, fixed plates were scanned in an Epson Perfection V750 Pro Scanner.

at a resolution of 360 dpi. Images were converted to grey scale, contrast adjusted and the area

of each plaque determined in ImageJ 2. 5- 20 plaques were selected and measured per

experiment and normalised to the values from WT virus. For protein and mRNA analysis in

single- cycle infection, cells were infected at an MOI of 5.

Virion RNA extraction and sequencing

Viral genomic RNA was extracted from virus stocks using a QIAamp Viral RNA Kit

according to the manufacturer's instructions and DNase-treated using TURBO DNA-free kits

(Thermo Fisher Scientific). Reverse transcription was performed using Superscript IV

(Thermo Fisher Scientific) and Uni 12 primer (AGCAAAAGCAGG). cDNA was used as a

template for PCR using PrimeSTAR GXL Premix (Takara Bio Inc). Forward and reverse

primers AGCAAAAGCAGGTAGATATTG and AGTAGAAACAAGGTAGTTTTTTAC

respectively were used to amplify a segment 7 amplicon. PCR conditions were 98°C for 5

min, 35 cycles of 98°C for 10s, 55°C for 15s and 68°C for 2 min. PCR products were purified

by Monarch DNA Gel Extraction Kit (New England Biolabs) and analysed by Sanger

sequencing (GENEWIZ).

Minireplicon assay

HEK 293T cells were seeded in 24-well plates and transfected the next day with 50 ng of the

desired combinations of PR8 reverse genetics plasmids expressing combinations of PB2, PB1,

PA, NP, NS and M segments using lipofectamine 2000 (Thermo Fisher Scientific) following

the manufacturer's instructions. The empty vector plasmid containing bi-directional RNA

PolI and PolII promoters ("pDUAL") (55) was used as a negative control by replacing the

segment 7 plasmid. The medium was aspirated 48 hours after transfection, and cells were

lysed using Laemmli buffer (20% glycerol, 2% Sodium Dodecyl Sulfate (SDS), 100 mM

Dithiothreitol (DTT), 24 mM Tris pH 6.8, 0.016% bromophenol blue) for western blotting or

lysed for total RNA extraction as described below.

RT-PCR and splicing analysis

Total RNA was extracted from virus-infected or plasmid-transfected MDCK or 293T cells

using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. After

DNA treatment using a TURBO DNA-free Kit (Thermo Fisher Scientific), cDNA was

generated using Lunascript RT Master Mix Kit (New England Biolabs) and oligo dT (IDT) as

a primer. The cDNA was amplified using PrimeStar Max Premix (Takara Bio Inc.). M

segment PCR was performed with the following primers: forward primer:

GAGTCTTCTAACCGAGGTC for constructs with SD mutations or

TTCTAACCGAGGTCGAAAC for the rest of the analyses and a common reverse primer:

CCACAGCACTCTGCTGTTCCT. mRNA1 and mRNA2 were distinguished based on the

sizes of their PCR products: 939 or 934 bp for mRNA1 and 251 or 246 bp for mRNA2,

depending on the forward primer used. NP mRNA PCR was performed with forward primer:

ACGGAAAGTGGATGAGAGAACTCAT and reverse primer:

CAACCGACCCTCTCAATATGAGTGC. PCR conditions were 98 °C for 5 min, 23-30

cycles of 98 °C for 10s, 55 °C for 15s and 68 °C for 10s. PCR products were separated in 2%

agarose gels and imaged using an Odyssey Fc imaging system (Li-Cor Inc.) after staining

with SYBR™ Safe DNA Gel Stain (Thermo Fisher).

Western blotting analysis

SDS-PAGE followed by western blotting was performed using the Trans-Blot Transfer

System (BioRad) according to the manufacturer's instructions. Membranes were blocked

with phosphate-buffered saline (PBS)/0.1% Tween20/5% skimmed milk at room temperature

for one hour, washed with PBS/0.1% Tween20 and incubated with primary antibody diluted

in blocking media. Following overnight incubation at 4 °C, membranes were washed with

PBS/0.1% Tween20 and incubated with fluorophore-labelled secondary antibody diluted in

blocking media for one hour. Membranes were imaged using an Odyssey Fc imaging system

(Li-Cor Inc.). Image acquisition and analysis were performed using Image Studio Lite

software. Purchased monoclonal antibodies were against α-tubulin (Thermo Fisher Scientific,

Clone YL1/2), IAV M2 (Abcam, Clone 14C2), and IAV NP (Abcam, Clone C43). Purchased

polyclonal antibodies were against IAV M1 (GeneTex, GTX125928) and M2 (Thermo Fisher

Scientific, PA5-32233). In-house polyclonal rabbit anti-NP (antiserum A2915) and anti-NS1

(antiserum V29) have been previously described (56). Purchased secondary antibodies were

IRDye 800CW anti-Mouse (Li-Cor Inc, 925-32212), IRDye 680RD anti-Rat (Li-Cor Inc,

926-68076) and IRDye 800CW anti-Rabbit (Li-Cor Inc, 926-32213). Densitometric analysis

of western blots was done using the gel plot function in ImageJ 2 (57).

Statistical Analyses

All experiments that were statistically analysed were performed in at least biological

triplicates. All graphical data were plotted and analysed using GraphPad Prism 10. Statistical

tests used an ordinary One-way ANOVA test. Multiple comparisons were performed against

PR8 WT using Dunnett's post-test. **** = $P<0.0001$; *** = $P<0.001$; ** = $P<0.01$; * =

$P<0.05$; absence of significance symbols = $P>0.05$. Error bars indicate the standard error of

the mean.

Results

Bioinformatic analysis of segment 7 splicing *cis*-acting elements

Splicing of cellular mRNAs is directed by well-defined but degenerate consensus sequences

(28). Outside of the almost obligatory GU and AG nucleotides at either end of the intron,

individual pre-mRNA sequences are rarely complete matches to the consensus (Figure 1A)

and this can affect splice site selection (58). The SD sequence of IAV segment 7 mRNA2 is a

reasonable match to the cellular consensus, possessing the obligatory intron boundary

dinucleotide as well as matching at -3, +3 and +4, +5 positions (Figure 1B). The SA site is a

better match to consensus; perfect in many strains and varying only at the +1 position in a

minority of isolates. The viral PPT has been suggested to be composed of a stretch of 7

pyrimidines running from position 721 - 727 (41), but this would be both shorter and spaced

further away from the SA than the norm (59). The region immediately 5' to the SA site where

cellular PPTs are usually found is not especially pyrimidine-rich, having a run of 5 purines

upstream of three uracils (Figure 1B). A conserved adenosine at position 720 has been

predicted to be the segment 7 BP (41), based on its contextual match to the cellular consensus.

However, A713 is also in a similar context, only deviating from consensus at the 3'-end of the

motif. Thus overall, the segment 7 splice site motifs match the cellular consensus to varying

degrees.

Tool	SD	BP1 (A713)	BP2 (A720)	PPT	SA
ASSP	0.94				0.46
Genscan	X				X
HSF	0.81	0.83	X		0.79
NetGene2	0.0				X
NNSPLICE	0.98				0.24
Spliceator	0.94				0.92
SVM-BPFinder		0.42	0.39	0.61	

Table 1. Splice site prediction scores for PR8 segment 7. Scores are confidence values (0 to 1). Blanks

indicate that the tool does not predict a particular splice signal, crosses (X) indicate that the tool does, but

did not detect the one in IAV segment 7.

As a first approach to assessing the strength of the segment 7 splice signals, we used a

selection of splice site prediction tools. While the mRNA 2 SD site was predicted by the

majority of the bioinformatic tools with some confidence, identification of the SA was less

consistent and when found, often with low confidence (Table 1). Furthermore, none of the

tools picked either the SA or mRNA 2 SD as the top candidate within the segment (data not

shown). Of the two tools able to predict BP sites, HSF did not identify the site around A720

previously proposed to form a lariat structure (41) but instead identified the potential BP

around A713 with reasonable confidence, while SVM-BP identified both candidates with low

confidence but scored A713 more highly. SVM-BP also identified the same short 7 nucleotide

PPT adjacent to the potential BP at A720 as previously proposed (41) with moderate

confidence.

Since *in silico* analysis of segment 7 did not unambiguously identify which of the

mRNA2 splice signals might be responsible for downregulating splicing efficiency, we next

performed a systematic mutagenic analysis of each motif, starting from the 3'-end SA site. To

avoid confounding effects from non-synonymous mutations in the M1 gene or overlapping

segment-specific RNA packaging signals (54), we utilised a transfection-based minireplicon

system, using segment 7 as the reporter construct (35). To further mimic viral infection, we

also included segment 8, given the potential effects of NS1 on segment 7 mRNA splicing,

nuclear export and overall cellular translation (14, 35, 60-63).

Figure 2. The segment 7 SA has an optimal sequence. (A and E) Design of mutations (blue shades) to

change the nucleotide preceding or following the acceptor AG dinucleotide (orange). (B-D and F-H) HEK

293T cells were co-transfected (or mock transfected) with the viral polymerase and NP (3PNP) and NS

segment plasmids along with the indicated WT or mutant segment 7 constructs (pDUAL indicates an

empty vector control without segment 7) and harvested 48 h later. (B, F) Total RNA was extracted from

cells and segment 7 mRNAs detected using RT-PCR followed by agarose gel electrophoresis. (C, G) Cells

were lysed in Laemmli buffer and protein expression was analysed by SDS-PAGE and western blotting.

(D, H) Relative M2:M1 ratios were calculated by densitometry from three independent experiments.

Statistical annotations are the result of an ordinary One-way ANOVA with Dunnett's post-test relative to

WT. **** = $P<0.0001$; *** = $P<0.001$; ** = $P<0.01$; * = $P<0.05$; absence of significance symbols =

$P>0.05$. Error bars indicate the standard error of the mean.

PR8 segment 7 has an optimal SA sequence

The core sequence of the PR8 segment 7 SA is an exact match to cellular consensus.

However, to test for context-dependent effects, we examined the consequences of mutating

the nucleotides either side of the essential AG motif, as not all cellular SA sites meet the

extended consensus (Figure 1) (64, 65). Accordingly, two mutants were made in which the

SA AG motif was preceded by either A or G at position 737 (Figure 2A). Uracil (U) was not

tested as this would introduce a premature stop codon into the M1 gene. We then tested

segment 7 RNA synthesis and protein expression from the mutated segments using the

minireplicon system, measuring mRNA levels and relative M2:M1 ratios by RT-PCR and

western blotting respectively. The WT segment produced a mix of unspliced mRNA1 and

spliced mRNA2 (Figure 2B) as expected (35, 38), as well as appreciable quantities of both

M1 and M2 polypeptides (Figure 2C). However, the C737A and C737G mutations strongly

inhibited both mRNA splicing and M2 expression, reducing the latter to below the level of

detection when replicate experiments were quantified (Figure 2D). Therefore, 737C is a key

component of the segment 7 SA, consistent with its 100% conservation in segment 7

sequences (Figure 1) (66) and the likelihood of this nucleotide to cause under splicing in

cellular mRNA (65).

In contrast to nucleotide 737, single nucleotide polymorphisms are seen at segment 7

nucleotide 740 after the AG, albeit with a clear preference for G and A over C or U (Figure 1).

To test whether these variations affect segment 7 splicing, we made constructs in which the

WT 740G was replaced with A, U or C (Figure 2E) and monitored their splicing efficiency by

RT-PCR and western blotting as before (Figure 2F-H). WT segment 7 WT exhibited the

highest splicing efficiency by the qualitative RT-PCR assay (Figure 2F), while all 740

mutants had significantly lower splicing efficiency, as judged by quantitative western blots

(Figures 2G, H). However, the G740A variant resulted in higher mRNA splicing efficiency

higher than the other two mutants (Figure 2F-H). Thus, the G/A SNP at position 740 may

contribute to regulation of segment 7 splicing in some strains of IAV. Altogether, none of the

tested segment 7 mutations enhanced splicing efficiency, suggesting that IAV segment 7 SA is

inherently strong, and any substitution in the nucleotides either side of the SA AG

dinucleotide weaken splicing efficiency.

The segment 7 polypyrimidine tract is key to setting M1:M2 balance.

PPT sequences functions via recruiting spliceosomal U2AF, which then brings the U2

snRNP to the BP which is required for lariat formation (31, 32). The PPT is located upstream

of the SA (29) and its strength is determined by proximity and continuity: a long PPT in close

proximity to the SA is generally stronger than a short one or one further away from the SA

(67). The PPT of segment 7 has been predicted to be nucleotides 721-727 of the pre-mRNA

(underlined in Figure 1) (41), but this would be both short and relatively far from the SA.

Alternatively, a more conventionally sited PPT ending two nucleotides upstream of the SA at

position 735 would extend 5'-wards into the predicted PPT but is interrupted by 5 consecutive

purines centred at position 730. To experimentally test these options, we first mutated

pyrimidines within nucleotides 721 and 727 to either adenines or guanosines whilst avoiding

the creation of cryptic SA (AG) motifs (Figure 3A, mutants 721-24A and 724-27G). As

before, pre-mRNA splicing in the minireplicon system was measured using RT-PCR and

western blotting to detect M1 and M2 expression. Supporting the identification of the 721-

727 region as a PPT, mRNA2 splicing and M2 protein production were decreased when

pyrimidines were replaced by purines (Figure 3B lanes 3-4 and C lanes 4-5, Figure 3D for

quantification). However, the two sets of mutations affected splicing differentially. While

mRNA2 splicing was still detected for the 721-24A mutant, 724-27G almost totally abrogated

splicing and subsequent M2 expression.

Figure 3. Identification of the segment 7 PPT. (A). Sequences of segment 7 PPT mutants with altered

polypyrimidine tracts. Mutations are highlighted in blue shades and the segment 7 SA is highlighted in

orange. (B-J) The RNP components, NS segment and the indicated segment 7 or pDUAL empty vector

plasmids were co-transfected (or mock transfected) with the indicated segment 7 mutants in HEK 293T

cells. (B, E, H) Segment 7 mRNA was detected by RT-PCR followed by agarose gel electrophoresis. (C, F,

I) Protein expression was analysed by SDS-PAGE and western blotting. (D, G, J) Densitometric

quantification of the relative M2:M1 ratios was analysed by an ordinary One-way ANOVA test with

Dunnett's post-test relative to WT (N=3-5). **** = P<0.0001; *** = P<0.001; ** = P<0.01; * = P<0.05;

absence of significance symbols = P>0.05. Error bars indicate the standard error of the mean.

However, while mutations within nucleotides 721-727 confirmed that the segment 7

PPT extends to position 721 upstream of the SA, they did not necessarily define its full extent.

Therefore, we mutated regions downstream of the predicted PPT to either match (mutants

728-30U and 728-32U) or violate (U733A, U734G and U735G) the cellular PPT consensus.

RT-PCR results from these constructs showed that mRNA1 levels decreased and mRNA2

levels increased with the introduction of uridines (Figure 3E, lanes 3-4), while removing

uridines at positions 734 and 735 had the converse effect (Figure 3H, lanes 4 and 5),

consistent with uridine content close to the SA site modulating splicing. Supporting this, all

up and down mutations also caused significant alterations to the balance of M1 and M2

protein expression (Figures 3F, G, I and J; note that the y axis of G is reversed relative to

panels D and J). However, while reduced mRNA1 levels in the 728-32U mutant correlated

with a drastically lowered M1:M2 ratio, overall protein expression was low (Figure 3F, lane

5). This particular mutation introduced a 10 nucleotide long polyuridine stretch, which in

other contexts has been shown to induce viral polymerase slippage (68-70), potentially

providing an explanation for reduced levels of M1. In agreement, Sanger sequencing of PCR

products spanning the region showed a marked reduction in sequence fidelity at this region

(data not shown).

Overall, these data indicate that segment 7 has a bi-partite PPT located between nucleotides

721-735, consisting of strong 5' and weaker 3' elements, with the latter crucial to preventing

oversplicing of the primary transcript and lowered M1 expression.

Segment 7 has flexibly defined branch points

Splicing of cellular pre-mRNAs requires the formation of a lariat structure (29). The

BP is an essential *cis*-acting element that interacts with the SD site to initiate lariat formation.

In most cases, the BP is an adenine residue, as its 2'-OH group is necessary to attack the

phosphate backbone of the SD sequence (29). Previous bioinformatic analysis predicted that

nucleotide 720 might be the segment 7 BP (41). To test this, we mutated A720 to each of the

other three nucleotides (Figure 4A; BP2). If nucleotide 720 were the sole BP, these mutations

would be expected to destroy its function, block splicing and reduce M2 protein expression.

Unexpectedly, no reduction in M2 expression was detected for any of the three mutants,

which instead gave slightly increased (but not statistically significant) M2:M1 ratios relative

to WT segment 7 (Figure 4B and C). Thus, either nucleotide 720 is not the segment 7 BP or

an alternative BP can be utilised.

Figure 4. Mutational tests of putative BP2 at nucleotide 720. (A) Sequences of mutants with

substitutions at the putative BP. Mutated sites are highlighted in blue and the segment 7 SA is highlighted

in orange. (B) HEK 293T cells were co-transfected (or mock transfected) with the minireplicon

components, NS segment and the indicated segment 7 or pDUAL empty vector plasmids. Cells were lysed

and analysed by SDS-PAGE and western blotting as labelled. (C) Densitometric quantification of the

relative M2: M1 ratio was analysed by an ordinary One-way ANOVA test with Dunnett's post-test relative

to WT (N=3). Absence of significance symbols = P>0.05. Error bars indicate the standard error of the

mean.

As noted above (Figure1 and Table 1), another potential BP in segment 7 is predicted at

nucleotides 713, in addition to the one at position 720. To systematically test if these

adenosines can act redundantly as the segment 7 BP, we mutated them both to Gs, either as

single or double mutants (mutants A731G, A720G and A713G+A720G) (Figure 5A). As

before (Figure 4), mutation of A720 did not reduce mRNA2 or M2 expression. However,

decreases in mRNA2 levels and M2 expression were seen in the A713G and A713G+A720G

mutants (Figure 5B, lanes 3 and 5 and Figure 5C, lanes 4 and 6; quantification in 5D),

although without completely blocking splicing. This result suggests that while A713 is

important for splicing, it is not the only potential BP residue. Instead, the segment 7 BP could

be loosely defined (71), and nearby adenosines could be used in the absence of canonical BPs

(72). To test this, we mutated two additional adenosines at nucleotides 714 and 715 (A713-

5G). This mutant showed a more pronounced drop in mRNA2 and M2 levels, but low levels

of splicing still occurred (Figures 5B, lane 6; 5C lane 7, 5D). However, further addition of the

A720G mutation (A713-5G+A720G) led to barely detectable mRNA2 levels and

undetectable M2 expression (Figure 5B, lane 7, Figure 5C lane 8, Figure 5D). These results

support the hypothesis that segment 7 can use more than one BP adenosine, at positions 713-

715 and 720.

A previous study identified serine and arginine-rich splicing factor 5 (SRSF5) to be an

mRNA2 splicing factor that interacts with nucleotides 707-715 (73), overlapping our putative

BPs. To distinguish whether the absence of mRNA2/M2 in the A713-5G+A720G mutant was

caused by the loss of BP function or potential lack of SRSF5 binding, we further tested the

mutations in the context of mRNA4 splicing, which does not depend on SRSF5 (73).

However, given that WT PR8 only produces low levels of mRNA4, we altered the segment

splicing pattern to improve M42 expression. To achieve this, the mRNA4 SD was improved

through the introduction of a U148A and the mRNA2 SD was destroyed with a G52C change

(22). As expected, single mutation of U148A upregulated mRNA4 at the expense of mRNA2,

without abolishing its production (Figure 5E, lane 4), while G52C severely reduced mRNA2

levels (Figure 5E, lane 3). The combination of the two mutations reduced mRNA2 below the

level of detection (Figure 5E, lane 5). We then added the set of A713-5G-A720 potential BP

adenosine mutations on these two backgrounds. This caused a similar reduction in mRNA4

levels to that seen for mRNA2 (compare lane 6 with 7 and 8). Next, we used two anti-M2

antibodies with differing specificities to differentiate accumulation of the M2 and M42

proteins: the 14C2 antibody recognises residues 4 to 17 of the PR8 M2 protein but does not

bind M42 (22, 74), while a polyclonal antibody (PA5-32233) that was raised against amino

acids 11-97 of M2 recognises both M2 and M42 but is unable to distinguish them. M1 levels

were relatively consistent across all samples transfected with segment 7 plasmids, while

M2/M42 levels showed greater variation. The G52C mutant did not produce detectable

amounts of M2 and its M42 protein expression was also below the detection limit (Figure 5F,

lane 4). For the U148A mutant, 14C2 reactivity was weaker than the WT, whereas PA5-

32233 reactivity was comparable to WT, indicating that U148A produced both M2 and M42

(Figure 5F, lane 5). The double mutant G52C-U148A produced only M42, as evidenced by

reactivity with PA5-32233 but not with 14C2 (Figure 5F, lane 6). Consistent with the RT-PCR

data, once 713-715 and 720 adenosines were mutated, both M2 and M42 production were

dramatically reduced (Figure 5F, lanes 7-9; quantification in 5G). Given that SRSF5 does not

affect mRNA4 splicing and M42 expression (73), the much decreased mRNA4 and M42

production seen here suggests that adenosines 713-715 and 720 play a distinct role in

segment 7 splicing beyond serving as an SRSF5 binding motif. Overall, these data suggest

that segment 7 has redundancy in BP function to mediate mRNA2 and mRNA4 splicing.

Figure 5. Mutational identification of the segment 7 BP. (A) Mutational strategy for identifying BP

adenosines in segment 7. Adenosines that were predicted as potential BPs are shown in red on the WT

sequence, mutations are highlighted in purple, while the SA is highlighted in orange. The region of the

SRSF5 binding motif overlapping with BP mutations is underlined. (B-G) HEK 293T cells were co-

transfected (or mock transfected) with the minireplicon components, NS segment and the indicated

segment 7 or pDUAL empty vector plasmids, harvested at 48h and analysed by (B, E) RT-PCR and

agarose gel electrophoresis and (C, F) western blotting as labelled. (D, G) M2:M1 ratios were quantified

by densitometry of replicate experiments and normalised to the value of the WT construct. The relative M2:

M1 ratios were analysed by an ordinary One-way ANOVA test with Dunnett's post test relative to WT

451 (N=4-5). **** = P<0.0001; *** = P<0.001; ** = P<0.01; * = P<0.05; absence of significance symbols =

452 P>0.05. Error bars indicate the standard error of the mean.

The strength of the segment 7 SD is set by nucleotides C51 and C55

The SD motif of cellular pre-mRNAs is recognised by base-pairing between the U1

snRNP and 9 nucleotides either side of the mRNA splice site (Figure 6A) (28). The IAV

segment 7 mRNA2 SD sequences are highly conserved (except for nucleotide 55) and a close

match to cellular consensus. The two exceptions are nucleotides 51 and 55 (Figure 6B),

although the presence of a pseudo-uridine in U1 snRNA may allow some flexibility in base

pairing for the latter residue.

To systematically investigate how the mRNA2 SD sequence might regulate segment 7

splicing, we created a panel of all possible point mutations in the mRNA2 SD region and

analysed their behaviour in the minireplicon assay as before. RT-PCR analysis of segment 7

mRNAs indicated that altering the three nucleotides in the exon side of the motif had only

slight effects on splicing levels, although mutation C51G made splicing slightly stronger

(mRNA2:mRNA1 ratio 2.3 ± 0.3-fold (n=3) higher than WT) (Figure 6C, lanes 11). However,

most substitutions in the intron side of the SD motif influenced splicing (Figures 6D and E).

All variations of G52, U53, G56 and U57 inhibited mRNA2 splicing, mostly drastically

(Figure 6D, lanes 3-8 and Figure 6E, lanes 6-11). Defective recognition of the mRNA2 SD

was also associated with upregulation of mRNA4 levels, presumably due to competition

between these two SDs for U1 snRNP interaction (22). Conversely, C55, the only intronic

position with any variability in segment 7 (Figure 6B), was more tolerant of mutation.

Consistent with the IAV consensus, C55U showed a similar level of splicing to WT, while

mutating C to a purine (especially A) made splicing stronger (mRNA2:mRNA1 ratio 3.1 ±

0.1-fold higher than WT, n = 3). In addition, we used western blotting to measure viral

protein expression. Since some of the introduced point mutations are non-synonymous in

M1/M2 and amino acid substitutions in the M2 ectodomain interrupt the 14C2 anti-M2

antibody epitope, the polyclonal anti-M2 antibody (PA5-32233) was used. NP and NS1 levels

were similar between all samples, indicating equivalent levels of transfection (Figures 6F-H).

Consistent with the RNA analyses, point mutation C51G reduced M1 accumulation and gave

higher levels of M2, suggesting mRNA1 oversplicing (Figure 6F; compare lanes 4 and 13).

The C55A and C55G substitutions formed premature stop codons in M1 so predictably, no

M1 was detected in these two samples (Figure 6H, lanes 5 and 7). Even so, C55A showed

increased M2 levels, consistent with the increased mRNA2 splicing detected by RT-PCR.

Also consistent with the RT-PCR data, M2 antibody reactivity was absent (or very much

reduced; U57 series) from all mutants having mRNA2 splicing defects (Figure 6G, lanes 5-12

and 6H, lanes 8-13). However, even though defective mRNA2 SD sequences upregulated

mRNA4 SD recognition, M42 levels did not reach the detection limit in our experiments.

Thus, combining the RT-PCR and western blotting results, all point mutations of

nucleotides 52, 53, 54, 56 and 57 inhibited mRNA2 splicing. Only two mutations, C51G and

C55A, increased segment 7 splicing. Thus, the mRNA2 SD is strong, but not entirely

optimised to support mRNA2 SD recognition and splicing.

Figure 6. Mutational analysis of the mRNA2 SD sequence. (A) Cartoon diagram of base-pairing

between U1 snRNP and mRNA SD sequences. Ψ represents pseudouridine, an isomer of uridine found in

U1 snRNA. (B) Sequence logo indicating conservation of the segment 7 mRNA SD site, generated from

13,280 IAV segment 7 sequences. (C-H) The mRNA2 SD was systematically mutated and individual

constructs were tested by minireplicon system. HEK 293T cells were co-transfected (or mock transfected)

with viral polymerase and NP (3PNP), NS segment and the indicated segment 7 or pDUAL empty vector

plasmids. Lysates were harvested 48h post transfection and (C-E) segment 7 mRNA detected by RT-PCR

followed by agarose gel electrophoresis. (F-H). Viral protein expression levels were analysed using

western blotting.

Splicing regulation in viable viruses

So far, we analysed segment 7 mRNA splicing using a minireplicon system to avoid

any confounding effects of splice site mutations on the primary amino-acid sequence of M1

and segment-specific packaging signals. However, the kinetics and regulation of segment

splicing in the minireplicon system may not fully reflect that of the authentic virus. Therefore,

to test how the *cis*-acting splicing elements regulate mRNA production in the context of viral

infection, as well as to explore the evolutionary conflicts between the multiple functional

elements in segment 7, we attempted to rescue a panel of viruses containing mutations that

altered the balance of mRNA1 and mRNA2 production in the minireplicon system. For the

PPT, we redesigned the mutations to break homopolymeric adenosine runs with guanosines

(Figure 7A; 728C-30U, 728C+730U, 731-32Y and 728C+730-32Y), to avoid the potential for

transcriptional slippage by the viral polymerase (70).

Viruses were rescued by transfecting bidirectional reverse genetics plasmids into HEK

293T cells, amplified by one passage in MDCK cells, and plaque titred. WT PR8 grew to an

average titre of 6×10^8PFU/ml and formed large plaques (Figures 7B, C). No mutants with

alterations to the SD motif or the preceding nucleotide of the SA site could be successfully

rescued. Two of three mutants with altered first nucleotides downstream of the SA in the exon

grew to WT-like levels, albeit with small-plaque phenotypes, but G740C could also not be

rescued. Most PPT mutants rescued successfully with only 728-30U giving similar titres to

WT, albeit forming significantly smaller plaques. 728C+ 730-32Y could be rescued but

replicated to 10-fold lower titres than WT, while 734-36R was apparently non-viable. BP2

mutations at position 720 rescued readily and replicated to titres approaching that of WT

virus, with only moderate small plaque phenotypes. The BP1 mutation A713G caused a far

more severe growth defect. Overall, more than half the splice mutations gave viable viruses

with only moderate fitness penalties, indicating a degree of evolutionary flexibility in the 3'

splice site sequence motifs.

Most of the mutations in the splicing motif are non-synonymous in M1 protein, which

could potentially influence both splicing signals and M1 function. To assess the potential

effects of these mutations on M1 functions we mapped our mutations onto the output (75) of

a deep mutational scan of the PR8 M1 protein (Figure 7D). Some of the sites altered in our

study exhibited a high mutational tolerance, for example, D232 (A720U, A720C and A720G),

E235 (728-30U and 731-32Y), suggesting a lower likelihood of impact on M1 function. In

contrast, the lower diversity of amino acids recovered at positions 9 - 11 of M1 suggests

greater functional conservation and could be one of the reasons why the SD mutants tested

were lethal. This defect could be caused by impaired splicing, compromised M1 function, or

a combination of both.

Next, we investigated segment 7 mRNA splicing levels in cells infected with the set

of WT and mutant viruses that grew to sufficient titres to permit high multiplicity infection.

NP mRNA was analysed as an infection control, and this confirmed that all infections had

proceeded successfully (Figure 7E). In cells infected with WT PR8, a mixture of unspliced

mRNA1 and spliced mRNA2 was detected (Figure 7E, lane 2). Mutations at the nucleotide

immediately following the AG (especially G740U) resulted in increased mRNA1 levels and

reduced mRNA2 production, confirming that this position serves as an essential *cis*-acting

element for regulating splicing (Figure 7E, lanes 3 and 4). In contrast, mutant viruses

containing additional pyrimidines in the PPT enhanced splicing efficiency, thereby shifting

the mRNA1 and mRNA2 production ratio in viable viruses. The A720G BP mutant had

relatively lower levels of NP mRNA, suggesting impaired infectivity. However, mRNA2 was

still detectable in all the A720 mutant viruses. This result was consistent with the previous

observation in the non-infectious mini-replicon system, indicating that nucleotide 720 is

either not the BP or not the only BP for segment 7 splicing. Thus overall, the RT-PCR results

for segment 7 splicing were consistent with our previous minireplicon assay results.

Next, we analysed segment 7 protein expression from the mutant viruses by western

blotting. NP and tubulin levels were comparable in most samples, suggesting successful

infection and recovery of cellular protein. However, M2 levels were far more variable. The

G740A and G740U mutations flanking the AG acceptor dinucleotide gave very low and

undetectable amounts of M2, (Figure 7F, lanes 3 and 4 respectively). Quantification of

replicate experiments confirmed that the M2:M1 ratio of these two mutants was significantly

lower than that of WT, indicating M2 protein expression defects. Conversely, the 728-30U

and 731-32Y PPT up-mutations led to abundant synthesis of M2 proteins (Figure 7F, lanes 5-

6). The BP2 mutations at position 720 gave conflicting phenotypes. A720U and A720C

matched the minireplicon and viral mRNA results, producing abundant quantities of M2 and

a small (but not statistically significant) increase in M2:M1 ratios. However, in the context of

virus, A720G drastically underproduced M2, despite only a slight decrease in mRNA2 levels.

Nevertheless, M2 expression in all BP mutants confirm that A720 is not the sole segment 7

BP.

Overall, the data from viral infections were broadly consistent with our previous

minireplicon results and thus support the proposed mechanism by which *cis*-acting elements

in segment 7 are the primary regulator of mRNA splicing. They also reveal that the virus has

a surprising degree of tolerance for altered M1 to M2 ratios.

Figure 7. Virus phenotypes following mutation of *cis*-acting segment 7 splice elements. (A) Design of
a new panel of mutants with mutated polypyrimidine tracts. Mutations are highlighted in blue. The
canonical splice acceptor site is shown in orange and the positions of BP2 and PPT are labeled. (B) P1
stock titres of WT and mutant viruses. Data represent the mean of at least two independent rescues. (C)
Quantification of the plaque sizes of PR8 WT and mutant viruses. Data are the mean ± SEM of 5-20
plaques normalised to the average WT value from at least three independent experiments. (D) Logo plot
representing the amino acid preferences at each M1 residue site (75). Mutations tested here are labelled,
and amino acid substitutions are shown. Mutants that could not be rescued are shown in red; mutants
exhibiting titres less than 10% of WT are coloured in amber; mutants which grew to near WT levels
(sufficient to allow high multiplicity synchronous infections) are coloured in green. (E) The effects of
splice element mutations on viral mRNA splicing. MDCK cells were infected (or mock-infected) with the

indicated viruses at an MOI of 5. Total RNA was isolated at 6 h p.i and the indicated mRNAs were

detected by RT-PCR. (F) MDCK cells were infected (or mock-infected) with the viruses at an MOI of 5,

and lysated at 8 h p.i. The viral protein expressions were analysed by SDS-PAGE and western blotting. (G)

Quantification of the relative M2: M1 ratio was conducted using an ordinary One-way ANOVA test with

Dunnett's post test relative to WT (N=4-6). **** = $P<0.0001$; *** = $P<0.001$; ** = $P<0.01$; * = $P<0.05$;

absence of significance symbols = $P>0.05$. Error bars indicate the standard error of the mean. Panel D was

adapted with permission from Hom et al., Journal of Virology 2019;93:e00161-19 (CC BY 4.0

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Discussion

IAV uses alternative splicing of segment 7 to encode two proteins, M1 and M2, and

impaired production of either of these two proteins can reduce virus fitness (22, 76-79). The

regulation of segment 7 alternative splicing is complex, and previous studies have reported

various control mechanisms, involving *cis*-acting sequences in segment 7 and *trans*-acting

protein factors, both host and viral. For example, a polypyrimidine and polycytosine tract

downstream of the mRNA2 SD has been shown to influence splicing by binding to the NS1-

BP and hnRNP K proteins (34). Alternative splicing factor 1/splicing factor 2 (ASF/SF2)

which binds to the downstream purine-rich stretch of SA is also involved in mRNA2 splicing

as a *trans*-acting element (36). Potential viral *trans*-acting factors include the viral

polymerase binding to the cRNA 5'-promoter element downstream of the 5'-cap-snatched

leader sequence to block usage of the mRNA3 SD site, itself proposed to regulate usage of

the mRNA2 SD (37). NS1 also potentially modulates splicing through direct or indirect

interactions with mRNA1 and mRNA2 as well as by promoting nuclear export of unspliced

mRNA1 (35, 80-82). There may be elements of virus strain-specificity to some of these

mechanisms, given (for instance) the variable amounts of mRNA3 production and

disagreements over whether or not M2 is essential for virus replication (22, 78). Analyses of

the role of NS1 in controlling viral mRNA splicing have also given variable results (38).

Here, we describe how suboptimal splice signals in IAV segment 7 maintain the

desired ratio of unspliced and spliced transcripts and consequently balanced production of

M1 and M2 proteins (Figure 8). A strong SA and redundant BP adenosines promote efficient

mRNA2 splicing and M2 protein production. Negative sequence elements in the SD and PPT

prevent oversplicing to preserve sufficient essential viral protein M1 production from

unspliced mRNA.

Figure 8. Diagrammatic summary of how IAV segment 7 uses suboptimal splicing signals to maintain

the balance of mRNA1/M1 and mRNA2/M2 production. Segment 7 has a strong splice acceptor (SA),

redundant and strong branch points (BPs; potential lariat-forming adenosines indicated in bold) and a

medium-strong splice donor (SD) which allow the virus to produce sufficient M2 protein. A purine-rich

stretch in a medium strength polypyrimidine tract (PPT) prevents oversplicing, thereby preserving levels of

unspliced mRNA1 required for the synthesis of the essential M1 protein.

Thus, although multiple factors can regulate segment 7 mRNA splicing, we propose

that the four required *cis*-acting elements, SD, BP, PPT, SA are the primary determinants that

regulate viral mRNA splicing. This model is consistent with the high conservation of the

segment 7 splice motifs (Figure 1B) and would maintain a basal regulation of M1/M2

production across the frequent reassortment events seen in avian and other influenza A

viruses that could pair the segment with variants of the viral polymerase and/or NS1 with

altered splicing regulatory activity. Given the evolutionary conservation of the basal splicing

machinery across birds and mammals (83, 84), segment-autonomous regulation may also

help underpin a broad host range. Further "fine-tuning" of splicing ratios could then evolve

for the particular gene constellation in an individual virus lineage or host.

Achieving the correct balance of M1 and M2 exerts a selection pressure on the virus

to evolve suboptimal splicing signals. Further sequence constraints arise from maintaining

function of the translation products encoded by the mRNAs, as well as any other *cis*-acting

functions of the segment. All splice site elements considered in this study overlap with the

M1 open reading frame (ORF) and some of them overlap the M2 ORF. Consequently, most

mutations that alter splicing efficiency are non-synonymous in M1 and sometimes in M2. The

mRNA2 SD sequence is also involved in the segment-specific packaging signal (54).

Mutations in this region therefore might not only change splicing efficiency (27) but also

affect virus genome packaging, or even M1/M2 translation via codon bias effects. Consistent

with a severe constraint on the segment sequence, all our attempts to rescue three mRNA2

SD mutants failed. A previous study also reported failure to rescue mRNA2 SD mutants,

finding that virus could only be generated successfully when the mutations within mRNA2

SD motif (G52 and U53, M1 codons 9 and 10) were synonymous (16). Other mutants which

also failed to produce mRNA2 but which also altered codons 9 or 10 of M1 were non-viable.

Therefore, either M1 sequence and/or packaging signal sequences are critical in this region,

hampering modifications to the splice site.

In contrast to the relative mutational inflexibility of the SD sequence, the purine-rich

stretch (728-733 of segment 7) in PPT tract was much more tolerant of changes. When we

introduced pyrimidines, which are non-synonymous in M1, to this region, viable viruses with

only limited fitness defects were produced; WT-level titres but small-plaque phenotypes,

indicating mutational flexibility in this region. This is consistent with the output of the deep

mutational scan of PR8 M1 protein where M1 E235 and N236 sites exhibited a high

mutational tolerance for amino acid substitutions (75) and (Figure 7D). Furthermore, this

region does not overlap with any currently defined packaging signals in segment 7 (85, 86).

Thus, unlike the SD region, which plays multiple essential roles (packaging signal, splice site

and encoding M1 and M2) during the viral life cycle, the PPT region has "only" two defined

functions: M1 protein coding and acting as a PPT. The combination of relative functional

simplicity and high mutational tolerance suggests that the virus can more easily regulate

splicing by changing the PPT sequence during evolution. This might explain why the PPT is

the most important down-regulator of splicing as it allows more flexibility without severe

impacts on viral fitness.

Although the majority of mutations in this study were introduced into the M2 intron,

expression of M2 was directly affected by changes in splicing efficiency. In most situations,

M2 is considered essential for normal virus replication (22, 78). However, our data show that

the virus can tolerate a low M2 expression level and still replicate to high titres. The G740U

mutant exhibited a severe splicing defect and failed to produce detectable amounts of

mRNA2/M2 during high multiplicity synchronous infection. Despite this, it only exhibited

modest growth defects: a slightly reduced titre compared to WT PR8 and a small-plaque

phenotype. These data suggest that the PR8 virus only needs a very low level of M2 when

replicating in tissue cultured cells. This finding aligns with previous studies (16, 22, 54, 78,

79), showing a variety of IAV strains can undergo multiple cycles of replication despite

lacking M2 ion channel activity or overall M2 expression. However, the degree of replication

attenuation seen varies by over 1000-fold, depending on strain and mutational strategy,

raising the possibility that the requirement for M2 may vary across different strains.

To avoid confounding effects from mutations affecting M1 or M2 protein function

and/or packaging signals, we based most of our study around a non-infectious minireplicon

system, which may not completely recapitulate the temporal aspects of virus infection (15, 76,

87). Nevertheless, when we compared our results from minireplicon and viable virus

infection, the majority of mutants behaved similarly. The exception was the A720G mutation,

which showed different splicing patterns under the two conditions. In the minireplicon

system, A720G produced slightly more mRNA2 and an elevated (though not statistically

significant) M2: M1 ratio (Figures 4B and 5B). In contrast, during single-cycle infection,

A720G produced seemingly normal amounts of mRNA2 but had a significantly reduced

M2:M1 ratio (Figure 7E). Potentially, this could reflect cell type differences, as the

minireplicon system used HEK 293T cells while infection was carried out in MDCK cells (13,

15, 76). Further experiments are required to test this hypothesis.

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