The choice of alternative 5' splice sites in influenza virus M1 mRNA is regulated by the viral polymerase complex

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ABSTRACT  The influenza virus M1 mRNA has two alternative 5' splice sites: a distal 5' splice site producing mRNA3 that has the coding potential for 9 amino acids and a proximal 5' splice site producing M2 mRNA encoding the essential M2 ion-channel protein. Only mRNA3 was made in uninfected cells transfected with DNA expressing M1 mRNA. Similarly, using nuclear extracts from uninfected cells, in vitro splicing of M1 mRNA yielded only mRNA3. Only when the mRNA3 5' splice site was inactivated by mutation was M2 mRNA made in uninfected cells and in uninfected cell extracts. In influenza virus-infected cells, M2 mRNA was made, but only after a delay, suggesting that newly synthesized viral gene product(s) were needed to activate the M2 5' splice site. We present strong evidence that these gene products are the complex of the three polymerase proteins, the same complex that functions in the transcription and replication of the viral genome. Gel shift experiments showed that the viral polymerase complex bound to the 5' end of the viral M1 mRNA in a sequence-specific and cap-dependent manner. During in vitro splicing catalyzed by uninfected cell extracts, the binding of the viral polymerase complex blocked the mRNA3 5' splice site, resulting in the switch to the M2 mRNA 5' splice site and the production of M2 mRNA.

Alternative splicing of pre-mRNA plays an important role in regulating the expression of genes during development, differentiation, and virus infection (1, 2). Insight into alternative splicing mechanisms has come from the identification of proteins that regulate the choice of alternative splice sites. Some of these proteins apparently serve this specialized function only, e.g., the Drosophila melanogaster sex lethal (Sxl) protein controls the choice of 3' splice sites during the splicing of both Sxl and transformer (tra) pre-mRNAs (2–7). Other proteins function in constitutive as well as alternative splicing: the constitutive splicing factors, the SR proteins, also function in the selection of 5' splice sites in some pre-mRNAs (8–12). High concentrations of SR proteins favor the use of the proximal (downstream) 5' splice site.

The alternative splicing of the M1 mRNA of influenza A virus exhibits several distinctive features. There are two alternative 5' splice sites. The distal 5' splice site, which closely fits the consensus 5' splice site sequence, is used when the M1 gene is transcribed into uninfected cells (13–15). The resulting mRNA, termed mRNA3, has a reading frame for only a 9-amino acid peptide. The proximal 5' splice site, which does not fit the consensus as well, is not detectably used when the M1 gene is transcribed into uninfected cells (refs. 14 and 15 and the present study). However, this proximal 5' splice site is used in influenza virus-infected cells, generating the M2 mRNA encoding the essential M2 ion-channel protein (16, 17). How is the M2 mRNA 5' splice site activated in virus-infected cells? Here we demonstrate that the complex of the three viral polymerase proteins that function in processes unrelated to splicing, namely, the transcription and replication of the viral genome (18), are responsible for the activation of the M2 mRNA 5' splice site.

MATERIALS AND METHODS

Transfection and Influenza Virus Infection. Full-length wild-type and mutated M1 DNAs were inserted into pBC12 plasmids, which were transfected into 293 cells by using the calcium phosphate method (15, 19). In other experiments, 293 cells were infected with 20 plaque-forming units of influenza A (Udorn) virus per cell (20).

Detection of mRNA3 and M2 RNA in Transfected and Infected Cells. Transfected 293 cells were harvested 40 h after transfection, and influenza virus-infected cells were collected at the times indicated. RNA was extracted by the guanidinium isothiocyanate method (15, 19). Equivalent amounts of RNA were assayed by reverse transcriptase–PCR (RT–PCR) (21). The 5' primer was identical to positions 1–11 of the M1 viral sequences, and the 3' primer was complementary to nt 840–812 of the M1 sequences. The RT–PCR DNA products were resolved by electrophoresis on a 2% agarose gel for 1 h at 140 V.

In Vitro Splicing Assays. The M1 DNA containing a large deletion in the intron (see Fig. 2A) was inserted into pGEM1 via HindIII and BamHI sites. This DNA was linearized with Xho II (M1 DNA position 814) and was transcribed with SP6 RNA polymerase in the presence of [α-32P]UTP (22). A chimeric M1–simian virus 40 (SV40) early DNA containing M1 positions 1–71 and SV40 nt 4406–4617 was constructed by using PCR. This plasmid was linearized with BamHI for in vitro transcription. The transcripts contained m7GpppGm 5' ends and an additional 13 nt 5' to the M1 sequence. Splicing reactions and RNA extraction were carried out, and the RNA was analyzed by electrophoresis on 7% polyacrylamide/8 M urea gels (22, 23).

Preparation of the Influenza Virus Polymerase Complex. Monolayer HeLa cells were infected simultaneously with each of the recombinant vaccinia viruses that express the influenza virus PB1, PB2, or PA protein (24) at 3 plaque-forming units per cell. At 18 h after infection, nuclear extracts were prepared, and the polymerase complex was purified by sucrose gradient centrifugation (24–26).

RNA Binding Assays. The fragment of M1 DNA corresponding to positions 1–45 was transcribed by SP6 RNA polymerase in the presence of [α-32P]UTP and either m7GpppGm or GpppG. The indicated amount of the viral polymerase complex was incubated with the labeled transcript (10,000 cpm, 1 pmol) in an RNA binding buffer in a final volume of 20 μl for 30 min at 20°C (26). The RNA and RNA–protein complexes were resolved by electrophoresis on a 4% nondenaturing gel.

Abbreviations: RT–PCR, reverse transcriptase–PCR; SV40, simian virus 40.
RESULTS

Identification of the 5' Splice Sites of M1 mRNA Utilized in Uninfected and Influenza Virus-Infected Cells. M1 mRNA and its two alternatively spliced products, mRNA3 and M2 mRNA (15–17, 27), contain 10–13 host-coded nucleotides at their 5' ends as a result of capped RNA-primed initiation of viral RNA transcription (18, 28) (Fig. 1A). The mRNA3 5' splice site is 11 nt downstream from the beginning of the viral-encoded sequence, i.e., 21–24 nt from the 5' end of the mRNA. The M2 mRNA 5' splice site occurs 40 nt downstream from the mRNA3 5' splice site. The mRNA3 5' splice site more closely fits the consensus 5' splice site than the M2 mRNA 5' splice site, which has a cytidine rather than a guanosine at the 5' end of the 5'exon.

Most (14, 15), but not all (29), previous studies indicated that only the mRNA3 5' splice site, but not the M2 mRNA 5' splice site, is used in uninfected cells. To clarify this issue, a plasmid containing the M1 DNA sequence under the control of the cytomegalovirus promoter was transfected into 293 cells. The M1 mRNA transcribed from this sequence contained 13 nt 5' to the viral M1 sequence and would thus be similar to the M1 mRNA synthesized in influenza virus-infected cells. At 40 h after transfection, the total RNA was assayed for the presence of mRNA3 and M2 mRNA by using RT–PCR. The double-stranded DNAs derived from mRNA3 and M2 mRNA should be 111 and 151 nt long, respectively. After PCR the resulting double-stranded DNAs were resolved by agarose gel electrophoresis (Fig. 1B). The double-stranded DNA generated from the RNA from the transfected cells had the mobility expected of mRNA3 (lane 2), and sequence analysis verified that this was indeed the DNA amplified from mRNA3 (data not shown). No DNA corresponding to M2 mRNA was detected.

To determine whether the M2 5' splice site could be recognized in uninfected cells, the mRNA3 5' splice site in the M1 DNA plasmid was inactivated by mutating the guanosine at position +12 in the M1 sequence to a cytidine. The RT–PCR detected the production of M2 mRNA in cells transfected by this plasmid (Fig. 1B, lane 3). Thus, the cellular splicing machinery was capable of recognizing the M2 5' splice site, but only when the mRNA3 5' splice site was absent (15).

The time course of M1 mRNA splicing to form mRNA3 and M2 mRNA in infected cells was determined by using the RT–PCR assay (Fig. 1B, lanes 4–15). The first splicing product of M1 mRNA was detected at 1.0 h after infection (lane 5). Only mRNA3 and not M2 mRNA was produced between 1.0 h and 1.75 h after infection (lanes 5–8). The production of M2 mRNA was delayed until 2.0 h after infection (lane 9). At subsequent time points, both M2 mRNA and mRNA3 were present (lanes 10–15). The delay in the appearance of M2 mRNA suggested that some newly synthesized viral gene product was needed to activate the M2 5' splice site.

In Vitro Splicing of M1 mRNA. As a prerequisite for determining the mechanism by which the mRNA3 5' splice site is activated in infected cells, we established conditions under which the splicing of M1 mRNA occurred in vitro. By using nuclear extracts from uninfected HeLa cells, M1 mRNA splicing was achieved only when deletions were made in the middle of the intron and at the 3' end of the 5' exon, resulting in a 333-nt precursor (Fig. 2A). This M1 mRNA construct was spliced, albeit inefficiently, to produce mRNA3 (Fig. 2B, lane 2). The identity of this RNA was verified by DNA sequencing.

Fig. 1. Determination of the 5' splice sites of influenza viral M1 mRNA utilized in uninfected and virus-infected cells. (A) Diagram of the structures of M1 mRNA and its two alternatively spliced mRNA products, mRNA3 and M2 mRNA. The arrows indicate the positions and polarities of the two primers used for RT–PCR. (B) The spliced products of M1 mRNA in 293 cells transfected with either wild-type M1 DNA (lane 2) or M1 DNA with a mutated mRNA3 5' splice site (lane 3). Other 293 cells were infected with Udorn virus, and the RNA was isolated at the indicated time points (lanes 4–15). After RT–PCR of these RNA samples, the resulting double-stranded DNAs were separated by electrophoresis. Lane 1 contains a 100-bp DNA ladder. The upper band in lanes 2–15 is the DNA copy of M1 mRNA; the lower band is the 26-nt 5' primer.
after RT–PCR amplification (data not shown). No M2 mRNA was detected. The inefficient splicing was largely due to sequences in the 3′ half of the M1 DNA. When the 3′ half of the M1 mRNA construct was replaced by the corresponding region of an SV40 early pre-mRNA (Fig. 2A), splicing was stimulated >20-fold (Fig. 2C). Again, only the mRNA3 and not the M2 mRNA 5′ splice site was used, as was the case for the M1 mRNA construct itself. The uninfected cell nuclear extract was capable of using the M2 5′ splice site only when the mRNA3 5′ splice site in the M1 mRNA construct was inactivated by mutation (Fig. 2B, lane 4). These results are the same as those obtained in vivo.

Identification of the Viral Proteins that Cause the Activation of the M2 mRNA 5′ Splice Site. Recent experiments from others have shown that the complex of the three viral polymerase proteins (PB1, PB2, and PA) binds to the first 11 or 12 nt at the 5′ end of both virion RNA and its complementary RNA (26, 27), but it was not known whether this complex would bind to viral mRNA, which contains an additional 10–13 nt 5′ to the virus-encoded sequence. To test this possibility, we carried out gel shift experiments by using a partially purified viral polymerase complex and a 58-nt RNA corresponding to the 5′-terminal virus-encoded sequence of M1 mRNA plus an additional 13 nt 5′ to the M1 sequence (Fig. 3A). Increasing amounts of the viral polymerase complex bound increasing amounts of this M1-specific RNA (Fig. 3B, lanes 1–4). This binding was sequence specific: when the sequence of the first 6 M1-specific nucleotides was changed (mut6, Fig. 3A), the binding of the viral polymerase complex was reduced 10- to 20-fold (Fig. 3B, lanes 5–8). This binding was also dependent on the presence of a 5′-terminal methylated cap structure. The 58-nt M1-specific RNA containing a GpppG 5′ end bound to the viral polymerase complex ~10-fold less efficiently than the same RNA containing a m7GpppGm 5′ end (Fig. 3C).

To determine whether the binding of the viral polymerase complex to the 5′ end of M1 mRNA affects the choice of 5′ splice sites, increasing amounts of the viral polymerase complex were bound to the 333-nt wild-type M1 precursor (see Fig. 2A) in a short (15 min) preincubation, followed by a 2-h splicing reaction catalyzed by uninfected cell nuclear extracts (Fig. 4A). The binding of the viral polymerase complex caused a dramatic shift from the production of mRNA3 to the production of M2 mRNA by splicing (compare lanes 3–6 to lanes 1 and 2). The switch to the M2 5′ splice site was dependent on the preservation of the M1 mRNA binding site for the viral polymerase complex. This was established by using a 333-nt M1 precursor that contained the same 5′-terminal mutations that severely reduced polymerase binding (see mut6, Fig. 3). The mutated M1 mRNA was spliced to form mRNA3, but preincubation of this mRNA with increasing amounts of the viral polymerase complex did not cause a switch to the production of M2 mRNA (Fig. 4B).

**DISCUSSION**

Our results indicate that the choice of alternative 5′ splice sites in influenza virus M1 mRNA is regulated by the viral polymerase complex (Fig. 5). In uninfected cells, the distal mRNA3 5′ splice site is exclusively used, thereby generating mRNA3. This splice site continues to be used at early times of infection, until sufficient amounts of the polymerase complex are synthesized. These polymerase complexes bind to the 5′-terminal

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**Fig. 2.** *In vitro* splicing of M1 mRNA and of an M1–SV40 chimeric RNA. (A) Schematic representation of the M1 construct and the M1–SV40 chimeric construct. The boxes represent the exons. The nucleotide positions in the M1 and SV40 sequence are shown above and below the diagram, respectively. Each construct contained 13 nt (denoted by the line) 5′ to the influenza virus-specific M1 sequence, ss, Splice site. (B) Labeled RNA transcribed from the wild-type (wt) M1 construct (lanes 1 and 2) or the M1 construct containing a mutated mRNAs 5′ splice site (mutant) (lanes 3 and 4) was incubated with uninfected HeLa cell nuclear extract under splicing conditions for the indicated times at 30°C. The RNA was analyzed by electrophoresis on a 7% denaturing gel. Far left lane: 5′-32P-labeled HindIII digestion products of pBR322. (C) Labeled RNA transcribed from the M1–SV40 chimeric construct was incubated with uninfected HeLa cell nuclear extract under splicing conditions for the indicated times at 30°C. The RNA denoted as mRNA3 is actually the mRNA generated by using the mRNAs 5′ splice site and the SV40 3′ splice site.
M1-specific sequences in M1 mRNAs plus the 5' terminal cap structure, thereby blocking the mRNA3 5' splice site that is located at position 11 in the M1 virus-specific sequence. The cellular splicing machinery then switches to the downstream (proximal) M2 5' splice site. Consequently, after a delay of ~1 h, the production of M2 mRNA commences.

M2 mRNA encodes the M2 ion-channel protein that functions during the uncoating of the virus (16, 17). The M2 protein associated with the incoming virus particles permits ions to enter the virions, leading to a lowering of the pH (17, 30). This apparently dissociates the viral nucleocapsids from viral matrix proteins (30, 31). Newly synthesized M2 proteins do not participate in this uncoating but, rather, are needed for incorporation into progeny virus particles. The requirement for prior synthesis of viral polymerase complexes to activate the M2 5' splice site and hence the production of M2 mRNA ensures that the M2 protein is made only when it is needed. It may also be that the presence of increased amounts of the M2 protein at early times would actually be deleterious to early virus-specific events.

Fig. 3. Influenza viral polymerase complex bound to the 5' end of M1 mRNA in a sequence-specific and cap-dependent manner. (A) The structure of the 58-nt RNA used for the gel shift assay. The +11 position of the mRNA3 5' splice site is indicated. The sequence of the first 6 M1 virus-specific nucleotides in the wild-type (wt) and mut6 RNAs is shown. (B) Labeled wt (lanes 1–4) or mut6 (lanes 5–8) 58-nt RNA containing a m7GpppGm 5' end (10,000 cpm, 1 pmol of each RNA) was incubated with the indicated amounts of the purified viral polymerase complex (3P). The mixtures were subjected to electrophoresis on a nondenaturing 4% gel. (C) Labeled wt RNA containing either a m7GpppGm (lanes 1–4) or GpppG (lanes 5–8) 5' end was incubated with the indicated amounts of the purified viral polymerase complex.

Fig. 4. Influenza viral polymerase caused the splicing machinery to switch from the mRNA3 to the M2 mRNA 5' splice site in M1 mRNA. (A) Labeled wild-type M1 precursor (see Fig. 2A) (100,000 cpm, 1.6 pmol) was incubated with the indicated amounts of the viral polymerase complex (3P) at 20°C for 15 min. HeLa nuclear extract (25 μl) was added, and splicing reaction mixtures were incubated for 2 h at 30°C. RNA was analyzed by electrophoresis on 7% denaturing gels. (B) Labeled M1 precursor containing the mut6 mutations at its 5' end was substituted for the wild-type M1 precursor.

The primary role of the viral polymerase complex is the transcription and replication of the viral genome (18). During transcription this complex cleaves capped RNA fragments (10–13 nt) from host nuclear pre-mRNAs and mRNAs and then utilizes these fragments as primers to catalyze viral mRNA synthesis. The resulting viral mRNAs do not participate in viral RNA replication. For viral RNA replication, the polymerase complex acquires the ability to catalyze the unprimed copying of virion RNA into its complement and the subsequent unprimed copying of the complement into virion RNA. Recent experiments indicate that the polymerase complex containing the three polymerase proteins binds to the 5' end of the virus-specific RNA (virion RNA or its complement) that is to be copied or transcribed (26, 27). The binding site encompasses the first 11 or 12 5' terminal nucleotides. After binding to the 5' end of virion RNA the viral polymerase complex acquires the cap-binding activity but not the endonucleolytic activity required for transcription initiation (27). Here we show that the viral polymerase complex also binds to the 5' end of M1 mRNA. This occurs even though this RNA contains 13 nt 5' to the previously described polymerase binding site and even though this RNA does not serve as a template for virus-specific RNA synthesis. The binding to the 5' end of M1 mRNA is sequence-specific and is stimulated 10-fold by the presence of an m7GpppGm cap on the mRNA. This indicates that the viral polymerase complex acquires cap-binding activity when it binds to M1 mRNA, thereby enhancing the binding of the
to an alternative 3′ splice site, thereby resulting in a mRNA encoding the tra protein. This is similar to the situation when the alternative influenza viral M2 mRNA 5′ splice site is used after the binding of the viral polymerase complex to the constitutive mRNA5 5′ splice site. This alternative M2 mRNA 5′ splice site was used very efficiently when the mRNA5 5′ splice site was blocked by the viral polymerase complex, thereby generating significant amounts of M2 mRNA. This is likely to be of advantage to the virus. It will be important to determine how the splicing machinery utilizes the M2 mRNA 5′ splice site so efficiently under these conditions.

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