Splicing of influenza virus matrix protein mRNA expressed from a simian virus 40 recombinant

Juan Valcárcel,1,2† Purificación Fortes1,2 and Juan Ortín1,2*

1 Centro Nacional de Biotecnología (CSIC) and 2 Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Influenza virus RNA segment 7 encodes two proteins, M1 and M2, depending on the optional removal of an intron from its primary transcript. To investigate the mechanism of this regulated splicing, an influenza virus segment 7 cDNA was cloned under the control of simian 40 virus (SV40) early promoter and poly(A) signals in an SV40 recombinant virus (SVM), and expressed in COS-1 cells. Expression of both M1 and M2 proteins was detected in SVM-infected cells, suggesting (i) the appropriate splicing events to generate M2 mRNA occur in these cells and (ii) significant amounts of unspliced M1 mRNA are transported to the cytoplasm. Analysis of the relative proportion of M2 mRNA to mRNA3 indicated that the use of the alternative 5' splice sites is reversed in SVM-infected cells compared with those infected with influenza virus. In addition, a different intranuclear distribution of segment 7 transcripts was found in each type of infected cell. We speculate that these differences in splicing efficiency and splice site choice might be related to different subnuclear localizations of segment 7 transcripts synthesized by the different transcriptional machineries.

Introduction

The influenza A virus genome consists of eight ssRNA segments of negative polarity, encoding at least 10 polypeptides. Each of the six largest RNA segments encode a single polypeptide, but the two smallest ones each contain the information for two different proteins (for a review, see Lamb, 1989). The segment 8 collinear transcript encodes the NS1 protein, whereas the NS2 protein is expressed from a spliced mRNA which has lost a single intron (Inglis et al., 1979; Lamb & Choppin, 1979; Lamb & Lai, 1980). In the case of RNA segment 7, three mRNA species are found in infected cells: (i) a collinear mRNA (M1 mRNA) encoding the M1 protein, (ii) a spliced mRNA (M2 mRNA) resulting from the removal of a single intron which encodes the M2 protein and (iii) an alternative spliced mRNA (mRNA3) that differs from M2 mRNA by the use of a different 5' donor site and has very little coding capacity (Inglis & Brown, 1981; Lamb et al., 1981).

The M1 protein is an abundant, evolutionary conserved component of the virion (Ito et al., 1991; Ortín et al., 1983), that interacts with the ribonucleoprotein complex, (RNP) repressing its transcription activity (Ye et al., 1989; Zvonarjev & Ghendon, 1980), and with the membrane (Ye et al., 1987). At early times in the infection, the M1 protein localizes to the cell nucleus (Bucher et al., 1989; our unpublished results), where it may start virion morphogenesis mediating RNP transport to the cellular membrane (Martin & Helenius, 1991). M2 protein is an integral membrane protein, with its amino-terminal tail exposed extracellularly (Lamb et al., 1985; Zebedee et al., 1985). It is abundant in the infected cell, but a minor component in the virion (Zebedee & Lamb, 1988). Antibodies specific for the extracellular tail reduce viral infectivity and the viral resistant mutants show changes in the M1 cistron, suggesting a possible M1–M2 protein interaction during morphogenesis (Zebedee & Lamb, 1988, 1989). The location of mutations to amantadine resistance in the M2 cistron (Hay et al., 1985) has led to a proposal that M2 functions as a proton pump (Sugrue et al., 1990). Direct demonstration of an ion channel activity of the M2 protein was provided by Pinto et al. (1992) by measuring the membrane currents obtained in frog oocytes expressing wild-type or amantadine-resistant M2 proteins. The biological activity of such a pump would allow the dissociation of the M1–NP interaction by acidification of the infecting particle (Bukrinskaya et al., 1982; Martin & Helenius, 1991), and would avoid a premature change of the haemagglutinin molecule to the acidic conformation during its exocytic progress to the cellular membrane (Sugrue et al., 1990; Helenius, 1992; Skehel, 1992).

† Present address: Program in Molecular Medicine, University of Massachusetts Medical Center, 373 Plantation Street, Worcester, Massachusetts 01605, U.S.A.
Fig. 1. Construction of SVM. Segment 7 cDNA was synthesized onto the linearized and dT-tailed pBSV9 plasmid as described (Portela et al., 1985a). The pBR322-derived sequences in the resulting pBSVa982 recombinant were substituted by the SV40 structural protein genes derived from plasmid pSLtsl (Portela et al., 1985b).

Since both NS1 and NS2 proteins on one hand, and M1 and M2 proteins on the other, are required for viral infectivity, the splicing of the corresponding collinear mRNAs is not constitutive, but is regulated to allow a steady-state accumulation of cytoplasmic processed mRNAs at a few percent of total segment-specific mRNA. For segment 8, evidence has been presented that suggests that both cis and trans elements mediate this regulation (Agris et al., 1989; Alonso-Caplen et al., 1992; Nemeroff et al., 1992; Plotch & Krug, 1986; Smith & Inglis, 1985). On the other hand, the level of M2 mRNA has been shown to increase during the productive infection, probably due to an increase in the splicing efficiency (Valcárcel et al., 1991).

In this report, we have analysed the splicing of M1 mRNA when expressed from an SV40 recombinant as an RNA polymerase II transcript. Our results indicate that the M1 mRNA synthesized from a DNA recombinant clone associates efficiently with the nuclear matrix, whereas the influenza virus transcript does not, and it is spliced to both M2 mRNA and mRNA3. The efficiency of splicing to produce M2 mRNA is higher in SV40 recombinant virus-infected cells, whereas the opposite is true for mRNA3. A significant fraction of the unspliced M1 mRNA is transported efficiently to the cytoplasm of recombinant virus-infected cells.

Methods

Viruses and cells. The MDCK cell line was obtained from the ATCC at passage 53 and used within the following 20 passages. The COS-1 cells (Gluzman, 1981) were obtained from Y. Gluzman (Cold Spring Harbor Laboratory, New York, U.S.A.). The influenza virus A/Victoria/3/75 strain was cloned by two successive plaque isolations and used throughout this study.

Construction of recombinant plasmids and viruses. Influenza virus segment 7 cDNA was cloned into the pBSV9 vector by oriented cDNA synthesis, as described (Portela et al., 1985a). In brief, influenza virus mRNA synthesized in vitro (del Rio et al., 1985) was used as a template for reverse transcription, using a BglI-cut, dT-tailed pBSV9 vector as primer. Second-strand synthesis was primed with the common influenza virus terminal oligodeoxynucleotide. Finally, the 5'-terminal sequence of the eDNA was directly fused to the filled-in HindIII site of the vector, generating a unique NheI site in plasmid pSVa982 (Fig. 1). To construct an SV40 recombinant virus, the large BglI–EcoRI fragment of pSVa982 plasmid was ligated to the BglI–EcoRI fragment that contains the structural protein genes of SV40 DNA, obtained from the pSLtsl plasmid (Portela et al., 1985b). Upon transfection into COS-1 cells, SVM was obtained. It contains the segment 7 cDNA under transcriptional control of the SV40 early promoter and termination signals (Fig. 1). For riboprobe synthesis, the NheI–BglII fragment (positions 1 to 141 in segment 7 cDNA sequence) and PvuII–BglII fragment (positions 270 in the SV40 DNA sequence to 141 in the
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Poly(A)⁺ RNA was isolated by two rounds of oligo(dT)-cellulose chromatography (Aviv & Leder, 1972), as described previously (Valcärèl et al., 1991).

RNA analyses. The initiation and termination sites of transcription were identified by S1 nuclease mapping as described (Berk & Sharp, 1977; Weaver & Weissman, 1979). The probes were isolated from recombinant pSVa982 and consisted of fragment BgII–PvuII (probe B), which contains the 5' terminus of segment 7 cDNA and the SV40 early promoter, and fragment ClaI–HpaI (probe C), which includes the 3' terminus and SV40 poly(A) signals (Fig. 2). Probe B was 5'-labelled with [γ-³²P]ATP and polynucleotide kinase and probe C was 3'-labelled with [α-³²P]CTP by filling in with Klenow polymerase, using standard procedures (Sambrook et al., 1989).

Amplification of DNA was performed as described (Kawasaki, 1990), except that primer concentration was reduced to 8 nM. Oligonucleotide CR (TTCCTTAGAAGGAAGCCCT; positions 911 to 894 in the segment 7 sequence) was employed to prime first-strand cDNA synthesis on poly(A)⁺ cytoplasmic RNA. To amplify M1 cDNA specifically, oligonucleotide m1 (CCAGTGTGGTGTTAAAG; positions 699 to 716 in the segment 7 sequence) was used. Oligonucleotides m2 (GAGGTCGAAAC-GCCT) and m3 (AGCAAG-AGCAG-GCCT), corresponding to the splicing junctions of M2 mRNA and mRNA3 respectively, were employed for specific amplification of their corresponding cDNAs.

The ribonuclease protection assay was carried out as follows. Labelled riboprobes were synthesized on plasmids pG7NB or p7GPB linearized with restriction with HindIII or Stul, respectively (Melton et al., 1984) and purified by denaturing gel electrophoresis. Approximately 1 fmol of segment 7-specific RNA, as determined by previous dot blot hybridizations, was mixed with a 10-fold excess of probe in a buffer containing 80% formamide, 0.5 M NaCl, 5 mM-EDTA, 40 mM-PIPES pH 6.4. RNAs were denatured for 5 min at 85 °C and hybridized for approx. 60 h at 50 °C. Under these conditions, hybridization equilibrium was obtained, as determined in kinetic experiments (data not shown). Samples were diluted into 0.3 M NaCl, 5 mM-EDTA, 10 mM-Tris–HCl pH 7.5 and digested for 1 h at 37 °C with 20 μg/ml of Rnase A. The products were analysed by electrophoresis on sequencing gels and autoradiography. Controls to ensure that a riboprobe excess was used and to check the linearity of film exposure were included in each experiment. Protected bands were quantified by microdensitometry.

For dot blot hybridizations, RNAs were denatured by heating at 65 °C in 0.5 M-formaldehyde, diluted in 0.5 M-formaldehyde–10 × SSC and applied to nylon filters (Thomas, 1983). Hybridization conditions were as described (Sambrook et al., 1989) and the probes were as follows. As a cross-reactive probe, the oligonucleotide CR (TTCCGTAGAAGGAAGCCCT; positions 911 to 894) was used. The probes specific for M1 and M2 mRNAs, and mRNA3 were oligonucleotides M1 (TCATCTTTTAGACCAGCACTGG; positions 720 to 699), M2 (TTCTGATAGGC-GTTTCGACCTC; positions 750 to 740 and 11 to 1), and M3 (TTCTGATAAGGGCTTTTGTGCT; positions 750 to 740 and 11 to 1), respectively. Oligonucleotides were end-labelled with [γ-³²P]ATP and polynucleotide kinase, as described (Sambrook et al., 1989).

Protein analyses. Infected or mock-infected COS-1 cells were starved for 90 min in cysteine- and methionine-free DMEM and labelled for 2 h with a mixture of [³⁵S]Cys and [³⁵S]Met (300 μCi/ml) in the same medium. The pulse was started at 4 h post-infection (p.i.) or at 72 h p.i. in influenza virus- or SVM-infected cells, respectively. Total cell extracts were prepared in 50 mM-Tris–HCl, 150 mM-NaCl, 2 mM-MgCl₂, 2 mM-DTT, 0.5% NP40, 1 mM-PMSF, 0.1 mM-TLCK, 0.1 mM-TPCK, pH 8.0. Immunoprecipitations were carried out as described (Harlow & Lane, 1988), using M1-specific monoclonal antibodies (MAbs) m2 and m3 (Sánchez-Fauquier et al., 1987).
Results and Discussion

Structure of segment 7 transcripts in SVM-infected cells

The splicing efficiency of the segment 7 collinear, M1-specific transcript is regulated in influenza virus-infected cells to allow a limited accumulation of processed M2-specific mRNA (Valcárcel et al., 1991). To study the possible factors involved in this regulation, the cDNA of segment 7 RNA was cloned into a SV40 expression vector. The strategy used involved the oriented synthesis of cDNA onto a linearized pBSV9 vector (Portela et al., 1985a). Second-strand synthesis was primed with an influenza virus-specific oligonucleotide and the recombinant DNA was circularized by blunt-end ligation (Fig 1). This approach led to the fusion of cDNA downstream from the SV40 transcription initiation site and to the generation of a unique NheI recognition sequence at the junction site. To increase the efficiency of DNA transfer to COS-1 cells, the pBR322-derived sequences in the pSVa982 plasmid were substituted by the region encoding the SV40 structural proteins, generating SVM DNA (Fig. 1). This recombinant DNA was amplified and packaged within SV40 pseudovirions by transfection into COS-1 cells.

The transcription initiation and termination sites used in the expression of segment 7 RNA in SVM-infected cells were identified by S1 nuclease mapping. The probes used and the sizes of the fragments protected are shown in Fig. 2. With probe B, the expected protection bands
would be 136 nucleotides (nt) long for influenza virus-infected cell mRNA and approximately 207 or 237 nt for SVM-infected cell mRNA, depending on the use of early-early or early-late major initiation sites in SV40 DNA (Das & Salzman, 1985). Experimentally, protected bands of approximately 130 and 220 nt plus 250 nt were obtained for mRNAs isolated from influenza virus and SVM-infected cells, respectively, in reasonable agreement with those expected. The protected fragment of 420 nt obtained with probe C and mRNA isolated from SVM-infected cell mRNA, and approximately 207 or 237 nt early-early or early-late major initiation sites in SV40 for SVM-infected cell mRNA, depending on the use of the probe.

In a previous report (Lamb & Lai, 1982), the segment 7 cDNA was expressed under the control of the SV40 late transcription signals. Under those circumstances, mRNA3 was the only spliced transcript found, with no apparent production of M2 mRNA. The generation of spliced M2 mRNA by SVM was studied by reverse transcriptase-dependent DNA amplification mediated by specific oligonucleotides (Fig. 3a): oligonucleotide CR, corresponding to the second exon (positions 911 to 894) was used as a primer for reverse transcription on infected cell mRNA and as common leftward primer for DNA amplifications. The rightward primers were specific for each species of mRNA and included oligonucleotide m1, corresponding to the intron (positions 699 to 716; M1-specific), oligonucleotide m2, corresponding to the splice junction (positions 41 to 51 and 740 to 743; M2-specific) and oligonucleotide m3 (positions 1 to 11 and 740 to 743; mRNA3-specific). The specificity of the spliced oligonucleotides as primers for M2 mRNA and mRNA3 was checked by amplification on plasmid pSVa982. As shown in Fig. 3(b), no amplification bands were detected for this template when the spliced primers were used, whereas a band of approximately 213 nt was amplified with the M1-specific primer. As expected, a band of approximately 184 nt was obtained when the spliced primers were used for amplification of influenza virus-infected cell mRNA. The amplification of a band of the same size as the mRNA obtained from SVM-infected cells indicated the production of M2 mRNA and mRNA3 by splicing of M1 mRNA transcribed from the recombinant. The discrepancy between these results and those reported previously (Lamb & Lai, 1982) could be attributed to the differences in the SV40-specific sequences present in the 5' untranslated region of each mRNA. Those found in the late recombinant mRNA are much longer and obviously different from the ones present in the early recombinant mRNA and may interfere with the 5' splice site selection.

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Fig. 4. Synthesis and stability of M1 protein in SVM-infected cells. Cultures of COS-1 cells were infected with influenza virus (FLU) or with SVM. Infected cells were pulse-labelled with [35S]methionine (P) or pulse-labelled and chased with unlabelled methionine for 1, 3 or 9 h. Total cell extracts were immunoprecipitated with M1-specific MAbs (Sánchez-Fauquier et al., 1987) (αM1) or with control MAbs (αC). The numbers to the left indicate the position and the size of M1 protein. The synthesis of functional M1 and M2 mRNAs was checked further by the analysis of the corresponding coded proteins. Extracts from influenza virus- or SVM-infected cells were either pulse-labelled or pulse-labelled and chased for 1, 3 or 9 h. Immunoprecipitation with a mixture of M1 protein-specific MAbs and analysis by PAGE showed that a protein band of approximately 25K Mr was apparent in the extracts from influenza virus- and SVM-infected cells (Fig. 4). This protein band was not present when a control MAAb was used for the immunoprecipitation. Furthermore, the pulse–chase experiments indicated that M1 protein was metabolically stable (Fig. 4).

When a similar approach was applied to the analysis of M2 protein expression using the 14C2 MAAb (Zebedee & Lamb, 1988), no specific precipitation was observed in extracts of either influenza virus- or SVM-infected cells, although an M2-specific band could be immunoprecipitated when extracts of cells infected with the PR8 strain of influenza virus were used (data not shown). This negative result could be due to an amino acid sequence change in the epitope [Asn (13) to Ser] in the VIC strain as compared to PR8 virus (unpublished results), which may lower the affinity of the 14C2 MAAb for this particular M2 protein (Zebedee & Lamb, 1988). In contrast, an interaction of 14C2 MAAb with VIC virus M2
Fig. 5. Accumulation of M2 protein in SVM-infected cells. Cultures of COS-1 cells were mock-infected (MOCK), or infected with influenza virus (FLU) or with SVM for 6 and 72 h, respectively. After fixation, the preparations were incubated with 14C2 (MOCK, FLU and SVM) or control MAb (CTRL MAB) and subsequently treated with rabbit anti-mouse and rhodamine-tagged goat anti-rabbit antibodies. Photographs were taken in a Zeiss Axiophot fluorescence microscope using identical exposure time.

protein could be detected by indirect immunofluorescence. Specific staining of influenza virus- or SVM-infected cells was observed, but such staining was absent from mock-infected cells or when a control MAb was used (Fig. 5). It could be argued that the reactivity seen in SVM-infected cells reflects an artefactual recognition of the M1 protein. However no immunofluorescence signal could be detected in such preparations with M1-specific MAbs and the M1 protein was not immunoprecipitated by the 14C2 MAb from SVM-infected cell extracts (data not shown).

Modulation of splicing efficiency and alternative splicing site choice in SVM-infected and influenza virus-infected cells

To measure the efficiency of M1 mRNA splicing, the proportion of M1 and M2 mRNAs present in the cytoplasm and nucleus of COS-1 cells infected with SVM was determined by the quantitative RNase protection assay described in Methods. The results are presented in Fig. 6. Protection bands of 155 and 136 nt (M1-specific) and 71 and 52 nt (M2-specific) were observed for mRNAs isolated from SVM-infected and influenza virus-infected cells, respectively. However, no protection band of 30 nt (mRNA3-specific) was detected, suggesting a low level of accumulation of such a transcript. Likewise, no 11 nt protection band was detected in influenza virus-infected cell mRNA, presumably owing to the instability of such a short dsRNA (however, see below). The steady-state molar ratio of M2 to M1 mRNAs ranged between 0.4 and 1.0 in different experiments when cytoplasmic RNAs of SVM-infected cells were analysed, compared to approx. 0.03 for influenza virus-infected cells at 3 h p.i. (Valcárcel et al., 1991) or to approx. 0.15 for the particular preparation shown in Fig. 6, obtained at 5 h p.i. Similar results were obtained when nuclear RNAs were used (Fig. 6), indicating that the splicing of M1 mRNA, and not the relative nucleocytoplasmic transport of M1 mRNA and M2 mRNA, is altered in SVM-infected cells, as compared to influenza virus-infected cells. These results indicate that the inhibition of splicing of M1 mRNA in influenza virus-infected cells is partially overcome when segment 7 is expressed by RNA
polymerase II, independent of any other viral genes. However, the splicing was not complete and the unspliced M1 mRNA was transported efficiently to the cytoplasm, unlike what happens to most of the cellular pre-mRNAs.

To investigate further the relative ratio of unspliced to spliced mRNA species derived from segment 7, dot blot hybridizations were carried out using specific oligonucleotides (M1, M2 and M3) and a common oligonucleotide (CR), as probes. The specificity of the probes, as tested by hybridization with M1-, M2- and M3-specific amplified DNA (see Fig. 3), was appropriate for the quantification of the various mRNAs present in the samples (Fig. 7). The results obtained indicate that the relative proportion of the spliced transcripts present in SVM-infected cells was dramatically different from that in influenza virus-infected cells. M2 mRNA was predominant in SVM-infected cells (74% of total spliced products), whereas mRNA3 was the most abundant spliced transcript in influenza virus-infected cells (81% of total spliced products). This is remarkable, since the first exon of mRNA3 has only 11 virus-specific nucleotides plus approx. 15 nt of heterogeneous cellular primer, usually too short a length for efficient splicing to occur.

Correlation between changes in the splicing efficiency and in splice site choice and subnuclear distribution of segment 7 transcripts

Pre-mRNA splicing is a nuclear event probably coupled to transcription and polyadenylation (Beyer & Osheim, 1988; Niwa & Berget, 1991), at a time assumed to be controlled by factors distributed all over the nucleoplasm. This view has been challenged by results from a variety of technical approaches suggesting that subnuclear RNPs (snRNPs) and splicing factors are non-uniformly distributed in the nucleus. Thus, snRNP Sm antigens appear in a speckled pattern of 20 to 50 bright dots (Spector, 1990), which is also shared by splicing factor SC-35 (Fu & Maniatis, 1990; Spector et al., 1991). snRNAs appear particularly concentrated in five to 10 particularly bright foci (Carmo-Fonseca et al., 1991a, b), now identified as coiled bodies (Carmo-Fonseca et al., 1992), a pattern that is shared by the splicing factor U2AF (Zamore & Green, 1991). Although the functional significance of these structures is not yet clear, the emerging picture is that discrete nuclear regions contain different concentrations of splicing factors and snRNPs, allowing the possibility that spliceosome assembly or splicing itself could be compartmentalized. Interestingly, c-fos nascent transcripts have been found associated with speckles (Huang & Spector, 1991) and highly localized nuclear tracks have been described for specific mRNAs (Lawrence et al., 1989). In this regard, SV40 transcripts have been shown to be tightly bound to the nuclear matrix (Ben et al., 1981), a subnuclear structure that has been proposed to be the site for pre-mRNA processing (Zeitlin et al., 1987).

We analysed the nucleocytoplasmic distribution as well as the intranuclear localization of segment 7 transcripts in SVM- and influenza virus-infected cells. As shown in Fig. 8, the relative nuclear accumulation of
Fig. 7. Quantification of segment 7 transcripts in influenza virus- and SVM-infected cells by hybridization with specific oligonucleotide probes. Samples of M1-, M2- and M3-specific DNAs amplified by PCR (see Fig. 3), as well as poly(A)+ RNAs isolated from mock-infected cells (CTRL) or cells infected with SVM recombinant virus (SVM) or influenza virus (FLU), were fixed to nylon filters and hybridized to oligonucleotide probes M1, M2 or M3, as described in Methods. After quantification, the probes were removed from the filters which were then rehybridized with oligonucleotide CR. The relative concentration of the different mRNA species was calculated from the intensities of the spots, once the relative efficiencies of hybridization of the probes were determined from the signals obtained for the DNA samples using the specific versus cross-hybridizing (CR) probes.

Fig. 8. Subcellular distribution of segment 7 transcripts in influenza virus- and SVM-infected cells. Cultures of COS-1 cells were infected with either influenza virus (FLU) or SVM and fractionated into cytoplasmic, nuclear or subnuclear fractions as described in Methods. Poly(A)+ RNA was obtained from each fraction and cell-equivalent aliquots were applied to nylon filters and hybridized with riboprobe 7NB.

segment 7 transcripts in SVM-infected cells is higher than in influenza virus-infected cells, suggesting a longer nuclear residence time for the SVM-specific transcripts. More interestingly, the subnuclear distribution of segment 7 transcripts is completely different: transcripts generated in SVM-infected cells were found predominantly associated with the nuclear matrix fraction, consistent with previous data for SV40 mRNA localization (Ben et al., 1981). In contrast, influenza virus transcripts were essentially excluded from this compartment. As the nuclear matrix has been functionally implicated in splicing we speculate that the differences in splicing efficiencies of segment 7 transcripts derived from SV40 could be due to their tight association with this structure in vivo. A corollary of these observations would be that influenza virus transcription machinery does not associate with the subnuclear structures with which RNA polymerase II associates and that this might be the basis for the regulation of splicing of influenza virus segment 7 transcripts.

Concluding remarks

The splicing of M1 mRNA transcribed from an SV40 recombinant virus as an RNA polymerase II transcript differs substantially from the process observed in
influenza virus-infected cells. The chimeric transcript associates with the nuclear matrix and is spliced in a different way than the influenza virus transcript, albeit not completely. The rapid transport to the cytoplasm of the latter might be mediated by a specific transport signal or by a particular intranuclear localization of the viral transcription machinery, which would avoid the entrance of the viral transcripts into the normal splicing pathway. Finally, the 5' splice selection appears to be a complex process, not exclusively dependent on the similarity to the consensus sequence, since M2 mRNA is the minor spliced product in influenza virus-infected cells, is also not detected when transcription takes place from the late SV40 promoter (Lamb & Lai, 1982), but is the major spliced product in the SVM-infected cell.

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