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Diversity of coding strategies in influenza viruses

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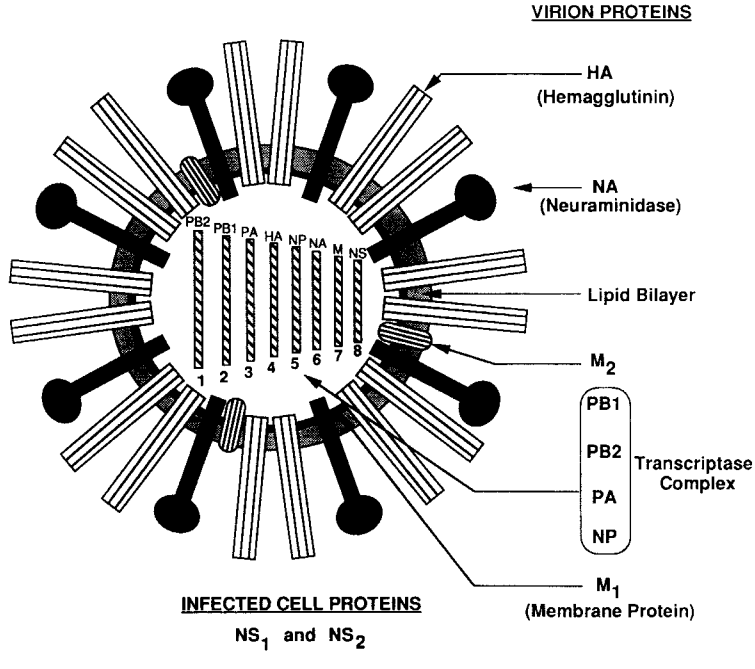
Influenza viruses have exploited a variety of strategies to increase their genome coding capacities. These include unspliced, spliced, alternatively spliced and bicistronic mRNAs, translation from overlapping reading frames and a coupled stop–start translation of tandem cistrons.

In recent years a remarkable catalog of mechanisms has been identified by which eukaryotic cells expand their genome coding capacity beyond the linear blocks of nucleotide sequence originally thought to encode individual proteins. These coding strategies not only create diversity by increasing the number of proteins encoded but also provide a means by which to regulate the expression of these proteins. Nowhere is this more apparent than with eukaryotic viruses. In most cases they have provided the prototypic example of molecular mechanisms that serve to maximize coding potentials. The compactness of the viral genome may be an important factor contributing to the success of the virus as a cellular parasite. The viruses with RNA genomes have used many different forms of gene expression, and the influenza viruses provide some illuminating examples of genome diversity. Although the functions of some of the influenza virus gene products described below are not yet known, the variety of mechanisms used for their synthesis provides a paradigm of successful exploitation of a genome.

The influenza A, B and C viruses comprise a family of related enveloped viruses with a segmented single-stranded RNA genome that has been called 'negative stranded' because the viral mRNAs are transcribed from the viral RNA segments (vRNAs) by a virus-encoded RNA-dependent RNA transcriptase. The complete

FIG 1



A schematic diagram of the structure of the influenza A virus particle. Three types of integral membrane protein – hemagglutinin (HA), neuraminidase (NA) and small amounts of M_2 – are inserted through the lipid bilayer of the viral membrane. The virion membrane protein M_1 is thought to underlie the lipid membrane. Within the envelope are the eight segments of single-stranded genome RNA contained in the form of helical ribonucleoproteins (RNP). The nucleocapsid protein (NP) is associated with approximately every 20 nucleotides of the RNA. Associated with the RNPs are small amounts of the transcriptase complex, consisting of proteins PB1, PB2 and PA. The coding assignments of the eight RNA segments are also illustrated. RNA segments 7 and 8 each code for more than one protein (M_1 and M_2, and NS_1 and NS_2, respectively). NS_1 and NS_2 are found only in infected cells and are not thought to be structural components of the virus. Influenza B virus does not encode an M_2 integral membrane protein, but the NB glycoprotein encoded by RNA segment 6 (which also encodes NA) is of very similar structure to M_2, and it seems possible it will perform a similar function in cells and virions. Influenza C virus contains seven RNA segments, lacking an RNA segment for NA. However, the HA also has a neuraminate-O-acetylesterase activity (reviewed in Ref. 1). An equivalent of M_2 or NB has yet to be identified in influenza C viruses.

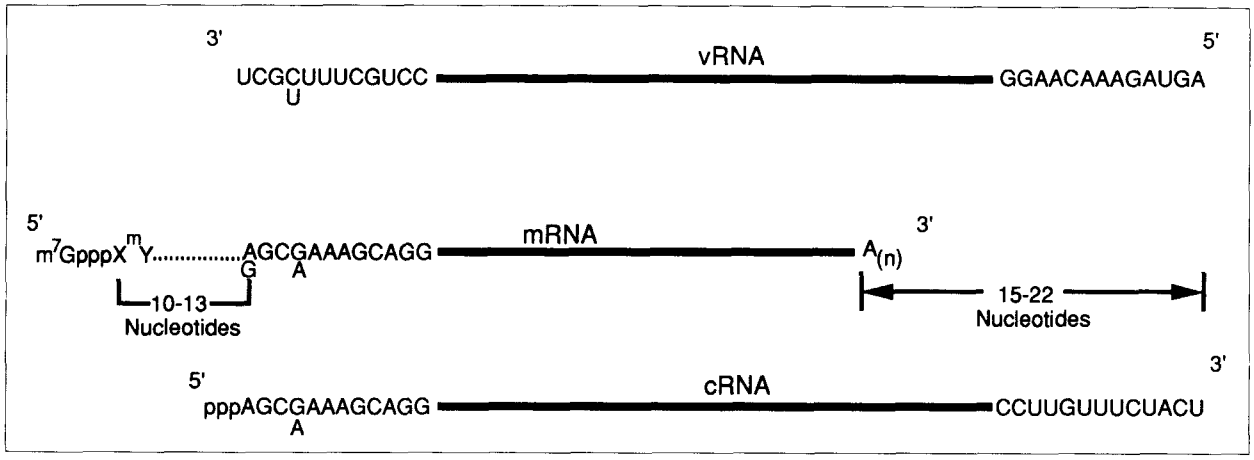


FIG 2

Schematic diagram to illustrate the differences between influenza virus virion RNA (vRNA) segments, mRNAs and full-length cRNA or template RNA. The conserved 12 nucleotides at the 3'-end and 13 nucleotides at the 5'-end of each of the influenza A virus vRNA segments are indicated. Similar conserved sequences are found at the 3'- and 5'-ends of the influenza B and C virus RNA segments. See text for details of the 5'- and 3'-terminal structure of the mRNA species.

nucleotide sequence of the eight RNA segments of influenza A and B viruses and the seven RNA segments of the influenza C viruses has been obtained: the PR/8/34 strain of influenza A virus contains 13 588 nucleotides (reviewed in Refs 1, 2). A schematic diagram of the structure of influenza A virus is shown in Fig. 1. The virion lipid envelope, which is derived from the host cell plasma membrane, contains three integral membrane proteins: hemagglutinin, neuraminidase and small amounts of M_2. The viral membrane protein (M_1) is thought to underlie the lipid bilayer. The nucleocapsid protein is the major structural protein that interacts with the vRNA segments to form the ribonucleoprotein particles (RNPs). Associated with each RNP core are small amounts of a complex consisting of the three transcriptase-associated polypeptides PB1, PB2 and PA.

The first 12 nucleotides at the 3'-end, and the first 13 nucleotides at the 5'-end of each vRNA segment are conserved in all eight RNA segments (see Fig. 2); recent studies using RNAs of altered sequence, and reconstitution of the transcriptase activity, indicate a promoter-like role for these sequences in transcriptase recognition[3]. In the nucleus of infected cells, transcription of the vRNAs into mRNAs by the virus-encoded RNA transcriptase requires cooperation with ongoing transcription by cellular RNA polymerase II. The synthesis of influenza virus mRNAs is an unusual process, as its initiation requires m^7GpppXm-containing capped primers that are generated from a subset of host cell RNAs by an influenza virus-encoded cap-dependent endonuclease. The primers average 10–13 nucleotides in length and in many cases contain the sequence 5'-GCA-3' immediately adjacent to the cleavage site, suggesting that the RNA endonuclease has some nucleotide sequence specificity (reviewed in Ref. 4). Viral transcription is initiated by the addition of a G residue complementary to the penultimate C residue on the vRNAs, and transcription elongates until it reaches the poly(A) addition site located 15–22 nucleotides short of the 5'-end of the vRNA. Like most cellular polymerase II transcripts, the influenza virus mRNAs are methylated on internal A residues, but the role of methylation is not known[4].

In addition to stealing caps, influenza virus mRNAs make use of another aspect of host cell nuclear function, namely the splicing machinery. As detailed below, the splicing of influenza virus mRNA transcripts provides the only known example of splicing of RNA that is not transcribed from DNA by RNA polymerase II. Since mRNAs synthesized by the viral RNA transcriptase are incomplete transcripts of the vRNA and contain host cell sequences at their 5'-end and poly(A) at their 3'-end, replication of influenza virus vRNA requires the synthesis of full-length copies of the vRNA segments, known as cRNA or template RNAs (reviewed in Ref. 4). The template RNAs are faithful copies of the vRNA, they have no 5' cap structure or 3' poly(A) tail, and they serve as the template for genome replication. The differences between the vRNA, mRNA and template RNA species are shown in Fig. 2.

Spliced mRNAs and overlapping reading frames

Genetic and biochemical evidence had shown that vRNA segment 8 (890 nucleotides), the smallest of the influenza virus RNA segments, encoded two discrete proteins, NS_1 (26 kDa) and NS_2 (14 kDa), and that NS_2 is translated from a small mRNA (~350 nucleotides)[5,6]. Nucleotide sequencing indicated that the NS_1 mRNA is unspliced, directly encoding the NS_1 protein (237 amino acids) whereas the NS_2 pre-mRNA contains a 473 nucleotide intron. NS_1 and NS_2 share the same AUG codon for initiation of protein synthesis and nine subsequent amino acids before the intron, and then translation of the body of the NS_2 mRNA continues in the +1 reading frame, which overlaps the NS_1 frame by 70 amino acids[7]. Figure 3A shows the arrangement of the NS_1 and NS_2 mRNAs and their open reading frames (ORFs). An analogous arrangement of unspliced and spliced mRNA transcripts is found to encode the NS_1 and NS_2 proteins derived from RNA segment 8 of influenza B virus and RNA segment 7 of influenza C viruses, respectively[8,9].

Analysis of the RNA transcripts derived from RNA segment 7 in cells infected with influenza A virus indicated that in addition to the unspliced transcript encoding the M_1 protein there are two alternatively spliced RNAs, M_2 and $mRNA_3$, that involve the use of different 5' splice sites[10,11] (Fig. 3B). The M_2 mRNA, which encodes the M_2 protein[10,12], contains a 51 nucleotide virus-specific leader sequence, a 689 nucleotide intron and a 271 nucleotide [excluding poly(A) tail] body region. The leader sequence of the M_2 mRNA contains the AUG initiation codon and codons for eight subsequent residues which are shared with the M_1 protein; the M_2 mRNA body region encodes 88 residues in the +1 reading frame and overlaps the M_1 protein by 14 amino acids. The alternatively spliced $mRNA_3$ has a 5' leader sequence of 11 virus-specific nucleotides, and shares the same 3' splice site as the M_2 mRNA. If this mRNA is translated it would yield a nine residue peptide identical to the carboxy-terminal region of the M_1 protein[10] but to date the peptide has not been identified in infected cells. An analogous situation does not occur with influenza B virus RNA segment 7, but this RNA segment does contain a second ORF and this is described in detail below.

In influenza C virus, another variation on the splicing theme has been found for the M protein-encoding RNA segment 6 (influenza C virus has just seven RNA segments as it lacks a separate RNA segment encoding a neuraminidase). The primary transcript mRNA contains an ORF encoding 374 amino acids and this RNA is spliced such that a translational termination codon is introduced after 242 residues of the ORF[13]. The available evidence suggests that the spliced mRNA encodes the M protein[13] (see Fig. 4). At present a protein product translated from the unspliced transcript mRNA has not been identified, which is surprising considering the abundance of the primary transcript RNA.

The 5' and 3' splice junctions of the influenza virus RNAs fit reasonably closely with the consensus sequences found at both sides of the exon–intron boundaries of eukaryotic mRNAs. Evidence that the cellular splicing machinery is used to generate the influenza virus spliced mRNAs comes from the findings that the

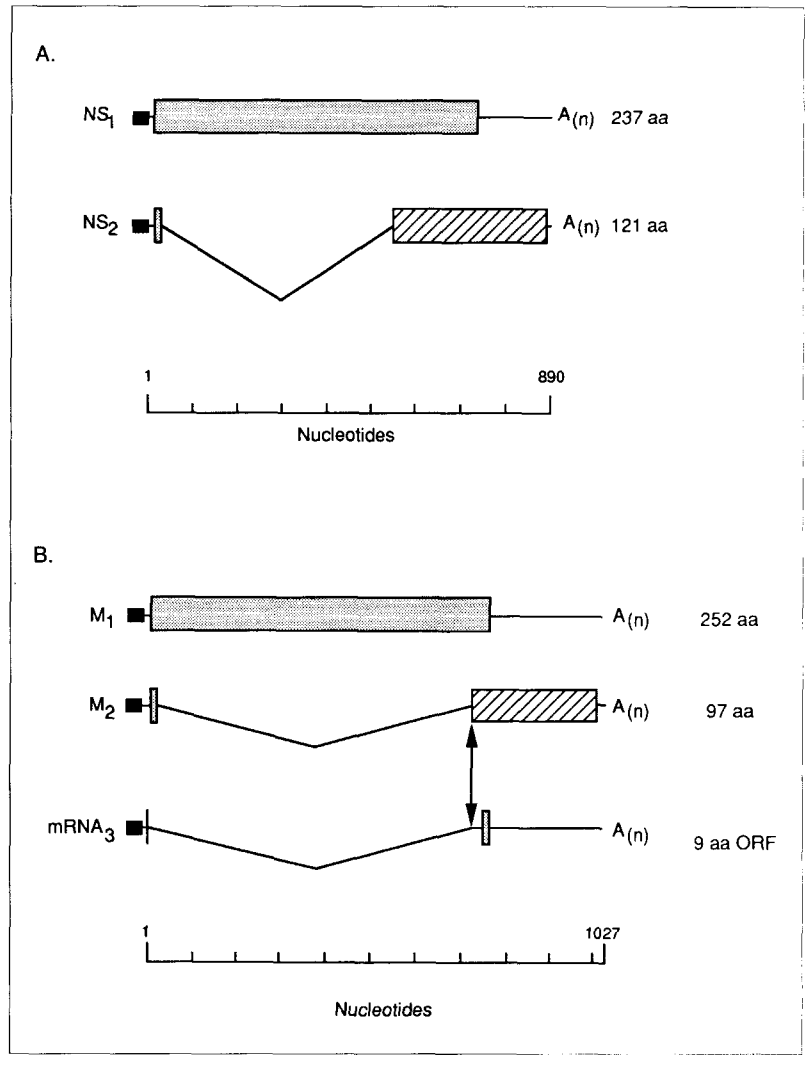


FIG 3

Model for the arrangement of (A) NS_1 and NS_2 mRNAs and their coding regions and (B) M_1, M_2 mRNAs and $mRNA_3$ and their coding regions. Thin lines at the 5' and 3' termini of the mRNAs represent untranslated regions. Shaded or hatched areas represent coding regions in 0 or +1 reading frames, respectively. The introns in the mRNAs are shown by the V-shaped lines, filled rectangles at 5'-ends of mRNAs represent heterogeneous nucleotides derived from cellular RNAs that are covalently linked to viral sequences. No evidence has yet been obtained that $mRNA_3$ is translated *in vivo*. Adapted from Refs 7, 10.

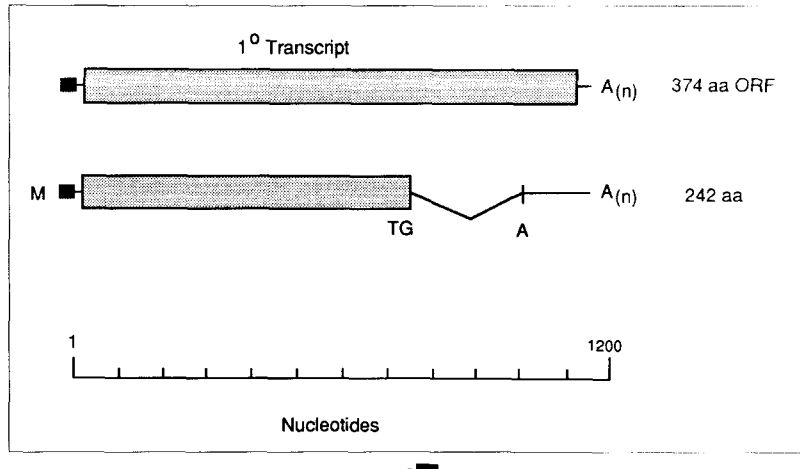


FIG 4

Schematic representation of the influenza C virus mRNAs derived from RNA segment 6. Key to symbols as in Fig. 3. Drawn from data provided in Ref. 13.

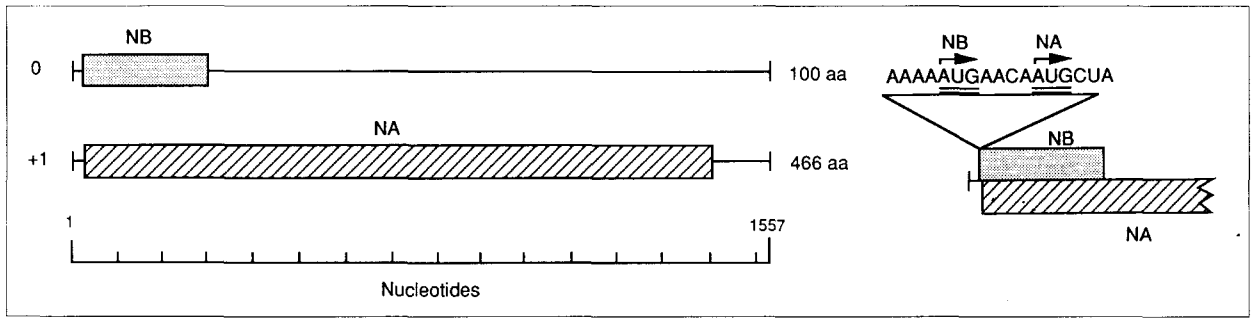


FIG 5

Schematic representation of ORFs in influenza B virus RNA segment 6 to show overlapping reading frames of NB and neuraminidase (NA). Nucleotide sequence surrounding the two AUG initiation codons, in mRNA sense, is shown to the right. Adapted from Ref. 1.

splice junctions are used when the cDNAs are expressed using DNA vectors[14,15]. In most cases of splicing of eukaryotic mRNAs, only the spliced mRNA products are detected in the cytoplasm. In influenza virus, and also in retroviruses, both spliced and unspliced RNAs code for proteins and thus both spliced and unspliced RNAs are transported from the nucleus to the cytoplasm.

For influenza virus, the extent of splicing is controlled, with a steady-state amount of about 10% spliced mRNA to unspliced mRNA (reviewed in Ref. 3). Some evidence suggests that this regulation is mediated by a *trans* mechanism requiring the action of a viral protein[16]. Other evidence from studies of the splicing of NS_1 mRNA *in vitro* indicates that the regulation is via a *cis*-acting mechanism, but the nucleotide sequences involved in the control system have yet to be identified[17,18]. In addition, the extent of splicing of the NS_1 mRNA can be regulated by the efficiency of nucleocytoplasmic transport of unspliced NS_1 mRNA[19]. In human immunodeficiency virus type 1, the *rev* gene product acts through a structured target RNA sequence located near the *env* gene to activate the transport of unspliced RNA from the nucleus[20]. It is possible either that the influenza virus NS_1 protein regulates the transport of unspliced NS_1 mRNA from the nucleus, analogous to *rev*, or that NS_1 acts as a *trans*-dominant negative regulator of NS_2 spliced mRNA transport to the cytoplasm (R.M. Krug, pers. commun.).

It is interesting to speculate how the overlapping genes might have evolved. The length of the NS_1 protein varies between 124 and 237 residues in different strains of influenza A virus, and analysis of the nucleotide sequence in the region of overlap between NS_1 and NS_2 indicates that the amino acid sequence of NS_2 is conserved at the expense of NS_1 (reviewed in Ref. 1). These observations support the earlier suggestion, made by Winter and colleagues[21], that perhaps NS_1 and NS_2 were originally collinear in the vRNA but not overlapping: readthrough of a terminator at the end of NS_1 then allowed the NS_1 protein to become longer and use more of the reading frame.

Bicistronic mRNAs

At the translational level, diversity of polypeptide production can arise by the selection of alternative initiation sites for translation on bicistronic or polycistronic mRNAs. The coding potential of the influenza B virus genome is expanded, since its RNA segment 6 gives rise to a functional bicistronic mRNA containing two initiating AUG codons that are separated by four nucleotides, so two discrete proteins are expressed from different reading frames: the 100 amino acid NB glycoprotein and the 466 amino acid neuraminidase glycoprotein[22–24]. The NB ORF overlaps the neuraminidase reading frame by 292 nucleotides (see Fig. 5).

The initiation of protein synthesis from eukaryotic mRNAs usually occurs at the 5' proximal AUG codon, although other AUG codons can be used for translational initiation in some cellular and many viral mRNAs. Most examples of translational initiation can be accommodated by the 'modified scanning hypothesis' of eukaryotic translation[25] in which 40S ribosomal subunits enter the 5'-end of the mRNA and 'scan' the nucleotide sequence, examining each AUG codon for its potential use as an initiator. From examination of the NB/neuraminidase mRNA nucleotide sequence in conjunction with the rules of the modified scanning hypothesis[25], it would be expected that only NB and not neuraminidase should be synthesized in infected cells. However, NB and neuraminidase accumulate in a 0.6:1 ratio, suggesting that approximately 60% of ribosome preinitiation complexes scanning the NB/neuraminidase mRNA do not initiate protein synthesis at the first AUG codon but continue scanning until reaching the second AUG codon four nucleotides downstream[24]. The importance of the nucleotide sequences surrounding the two AUG codons was investigated by altering many of the residues flanking the initiation codons. Most of the changes had very little effect on the ratio of NB/neuraminidase that accumulated. However, deletion of 40 of the 46 influenza virus-specific 5'-untranslated region nucleotides decreased neuraminidase synthesis tenfold, suggesting that an unrecognized feature of the entire region, such as a specific secondary structure, also has a major role in the initiation of protein synthesis at the second AUG codon[24].

Coupled translation of tandem cistrons

The nucleotide sequence of influenza B virus RNA segment 7 indicated that, in addition to the gene encoding the 248 amino acid M_1 protein, there is a second ORF in the +2 frame that has a coding capacity of 195 amino acids and is designated BM2ORF (reviewed in Ref. 1) (see Fig. 6). To search for a polypeptide product derived from BM2ORF, an antiserum to

an engineered β-galactosidase–BM2ORF fusion protein was produced and used to identify a polypeptide, designated BM2 protein (12 kDa), that is synthesized in cells infected with influenza B virus[26]. A mutational analysis of the cloned DNA was performed and the altered DNAs expressed in eukaryotic cells, in an attempt to reveal the mechanism by which the BM2 protein is synthesized. The data indicate that the BM2 protein initiation codon overlaps with the termination codon of the M_1 protein in a translational stop–start pentanucleotide UAAUG, and that the expression of the BM2 protein requires termination of M_1 synthesis adjacent to the 5'-end of the BM2 coding region (Fig. 6).

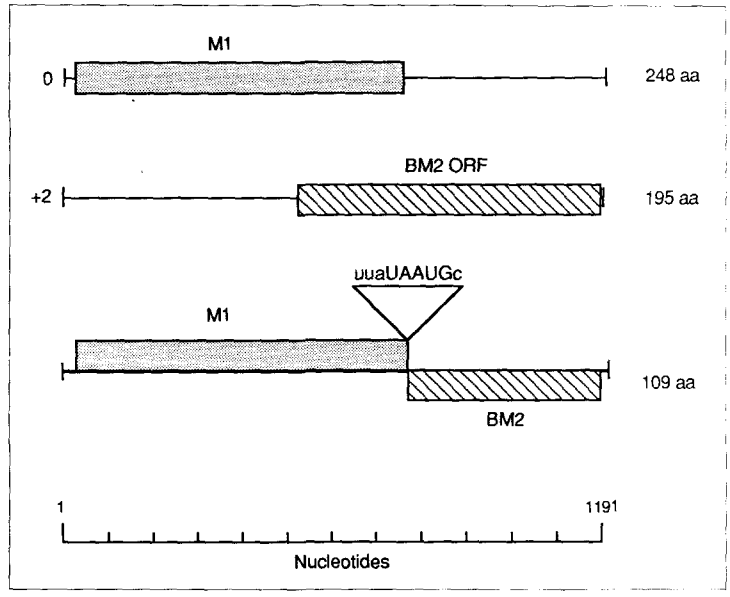


FIG 6

Schematic representation of ORFs in influenza B virus RNA segment 7 is shown in the first two lines and the third line shows the extent of the ORFs used to translate the M_1 and BM2 proteins. The stop–start pentanucleotide is illustrated. Adapted from Ref. 26.

The simple notion that initiation of BM2 synthesis is due solely to migration of the ribosome scanning from the 5'-end of the mRNA past 21 AUG codons in all three reading frames is unlikely, given that BM2 synthesis did not occur when the M_1 and BM2 reading frames were fused[26]. The results for translation of this construct also make it unlikely that the ribosome enters internally in the mRNA. Internal entry of ribosomes has been found to occur with picornaviruses, where initiation of protein synthesis occurs at the seventh AUG codon from the 5'-end of the viral RNA. A large body of data indicates that *cis*-acting RNA sequences adjacent to the initiator are involved in the 'ribosome landing pad' or 'internal ribosome entry site' and that specific cellular factors associate with these sequences (reviewed in Ref. 27). A related but seemingly different form of internal initiation of protein synthesis also appears to occur with the polymerase (reverse transcriptase) gene of the hepatitis B virus, since it is located 3' to an overlapping gene (C) yet mutations affecting translation of C have only minimal effects on expression of polymerase[28].

Reinitiation of translation at downstream AUG codons has been found to occur with some other naturally occurring and artificially constructed bicistronic mRNAs, the latter mostly using preproinsulin as a model system. In these cases, as with the BM2 protein, initiation of protein synthesis at the downstream AUG codon depends on translational termination of the polypeptide encoded by the upstream ORF. In almost all of these natural or artificially created mRNAs, the upstream ORF is small and has been characterized as a 'mini-cistron'[25]. In a study on the effect of intercistronic length on the efficiency of reinitiation at downstream AUG initiation codons, using altered preproinsulin mRNAs, it was found that when the terminator/initiator was in the nucleotide sequence UAAUG, reinitiation was inefficient, but when the intercistronic distance was lengthened the efficiency of reinitiation was greatly increased[25]. It has been suggested that initiation factors may be released stochastically during chain elongation, and if these factors are needed for reinitiation, then the ability to reinitiate would be inversely proportional to the size of the upstream ORF[25]. The comparatively large size of the influenza B virus M_1 ORF (248 amino acids) may in part explain the relative inefficiency of reinitiation (~25 mol % of M_1). In addition, the influenza B virus BM2 AUG initiation codon is not in the most favored nucleotide context for initiation of protein synthesis[25] (a C is present at the +4 position) and this may affect the efficiency of reinitiation. The major difference between the analysis of the naturally occurring influenza B virus M_1/BM2 bicistronic mRNA[26] and the data obtained using artificial RNAs[25] is that when the intercistronic distance was increased, the synthesis of BM2 could not be detected, whereas in the artificial constructions, translation from the second ORF was found to be increased. These data suggest that for BM2 synthesis to occur, the termination of translation and the reinitiation event have to be closely coupled.

In some respects, the finding that the BM2 protein initiation codon overlaps the M_1 protein termination codon is similar to the situation found for some coordinately regulated bacterial genes (e.g. the *trp* operon), where the termination codon of one gene overlaps the initiation codon for a downstream gene (reviewed in Ref. 29). When the intercistronic distance is expanded experimentally in prokaryotic genes, the efficiency of translation of the second ORF is greatly diminished, except in cases where an internal Shine–Dalgarno sequence precedes the second ORF. In these latter cases, it has been suggested that the ribosome translating the first ORF could expose a masked ribosome entry site by melting RNA secondary structure, thereby allowing recognition of the downstream initiation site by ribosomes[30]. However, for the vast majority of eukaryotic mRNAs, ribosomes enter only at the 5'-end of the mRNA. The frequent presence of internal ribosome entry sequences in prokaryotic but not eukaryotic mRNAs may account for one major difference between the M_1/BM2 situation and prokaryotic coupled stop–start systems.

in that prokaryotic reinitiation events are often highly efficient.

Although they make use of a wide range of coding strategies, influenza viruses have not been demonstrated to use some other known mechanisms to access 'hidden' reading frames. Among those employed by other viruses is the use of non-AUG initiation codons (ACG or AUA) for protein synthesis by hepatitis B virus, adeno-associated virus and Sendai virus, a paramyxovirus (reviewed in Ref. 31). In addition, frameshifting in the −1 frame occurs with most retroviruses to switch from the *gag* to *pol* reading frames, and also in coronaviruses to express their RNA replicase protein[32,33]. Suppression of termination codons occurs with Sindbis virus to synthesize its *ns*4 protein[34]. Finally, a form of RNA editing is used by paramyxoviruses (another group of negative strand RNA viruses) to add nontemplated G nucleotides at a specific site in an mRNA. The mechanism for this process is probably similar to the addition of poly(A), involving 'stuttering' of the RNA transcriptase. The consequence of the addition of extra nucleotides to some of the mRNA transcripts is to generate two (and sometimes three) different mRNAs encoding fusion proteins with different carboxy-terminal domains (Refs 35, 36 and references therein; reviewed in Ref. 31). Whether more proteins will be found to be encoded by the influenza virus genome by mechanisms such as non-AUG initiators, ribosomal frameshifting, suppression of termination or RNA editing is of considerable interest and remains to be determined.

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