Internal ribosome entry in the coding region of murine hepatitis virus mRNA 5

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The unique region of murine hepatitis virus (MHV) mRNA 5 has two open reading frames, ORF 5a and ORF 5b, that encode small proteins of unknown function. In the experiments described here, we have used the in vitro translation of synthetic mRNAs to examine the expression of these ORFs. Our results show that a synthetic mRNA containing both ORFs is functionally bicistronic. More importantly, the expression of ORF 5b, but not ORF 5a, is maintained in a tricistronic mRNA containing an additional 5'-proximal ORF. Thus, in the context of the MHV mRNA 5 unique region, the initiation of protein synthesis on ORF 5b can occur independently of ribosomes that enter from the 5' end of the mRNA. We conclude that the translation of ORF 5b is mediated by the internal entry of ribosomes.

Introduction

Mouse hepatitis virus (MHV), a member of the Coronaviridae, has a positive-strand RNA genome of about 31300 nucleotides (Pachuk et al., 1989; Lee et al., 1991; Bonilla et al., 1994). In the infected cell, the expression of viral proteins is mediated by the genomic-length RNA, also known as mRNA 1, together with seven subgenomic mRNAs. These mRNAs form a 3'-co-terminal nested set and they contain a common leader sequence of about 70 nucleotides at their 5' end (Lai et al., 1983). Only the 5' unique region of each mRNA, i.e. the region that is absent from the next smallest mRNA, is translationally active (Leibowitz et al., 1982; Siddell, 1983).

Most MHV mRNAs contain only a single open reading frame (ORF) in their 5' unique region. These mRNAs appear to be functionally monocistronic. However, mRNA 1 and the subgenomic mRNA 5 are exceptions (Skinner et al., 1984; Budzilowicz & Weiss, 1987; Pachuk et al., 1989). The unique region of MHV mRNA 1 contains two large ORFs that constitute the coronavirus RNA polymerase locus. Expression of viral proteins from this locus involves both ribosomal frameshifting and autoproteolytic processing (Baker et al., 1989; Bredenbeek et al., 1990). MHV mRNA 5 also contains two large ORFs in its unique region, designated ORF 5a and ORF 5b. In vitro translation studies suggest that this mRNA is functionally bicistronic and the ORF 5b gene product has been detected in MHV (strain A59)-infected cells (Leibowitz et al., 1988). On the basis of sequence similarities, it is likely that the MHV ORF 5b gene product is equivalent to the small membrane (sM) proteins of infectious bronchitis virus (IBV) and transmissible gastroenteritis virus particles (Liu & Inglis, 1991; Godet et al., 1992).

Two mechanisms can be proposed for the translation of the MHV ORF 5b. The leaky scanning model, as proposed by Kozak (1989), is one possibility. In this case, the initiation of ORF 5b translation would be mediated by ribosomes that fail to recognize the ORF 5a initiation codon and scan to the next available AUG triplet, which is the ORF 5b initiation codon. An alternative model is a cap-independent mechanism involving ribosome entry at an internal position on the MHV mRNA 5. An analogous mechanism has been described for a variety of picornavirus RNAs and hepatitis C virus RNA (Jackson et al., 1990; Brown et al., 1992). If this model is correct, the initiation of MHV ORF 5b translation should occur independently of ribosomes that bind to the 5' cap structure of the mRNA.

In the experiments reported here, we have analysed the in vitro translation of synthetic mRNAs that contain the unique region of MHV mRNA 5 (minus the leader RNA and some 5' non-translated sequences) preceded by an ORF derived from the β-galactosidase gene of Escherichia coli. The results show that the β-galactosidase ORF is an effective barrier to the movement of ribosomes from the 5' end of the mRNA but, nevertheless, ORF 5b is efficiently translated. Our conclusion is that translation of ORF 5b is mediated by the internal entry of ribosomes.
To enlarge ORF 5b, a cDNA fragment of pHCV-N (J. Ziebuhr, unpublished results), which contains the N gene of the human coronavirus (HCV) strain 229E, was cloned into the ORF 5b coding region. The 718 bp EcoRI fragment of pHCV-N was treated with the Klenow fragment of DNA polymerase I and ligated to BamHI linkers. The BamHI restriction sites of p5b, p5ab and pZ5ab were eliminated by linearization, treatment with the Klenow fragment and religation. The plasmids were then linearized with BstEII, treated with Klenow fragment and alkaline phosphatase and ligated to BamHI linkers. The plasmids and the cDNA fragment were then digested with BamHI and ligated together to produce the constructs p5bN, p5abN and pZ5abN. The enlarged ORF 5b, designated ORF 5b°, contains 27 codons from the 5' end of ORF 5b, 241 codons from the HCV N protein gene, 63 codons from the 3' end of the ORF 5b and a total of six artefactual codons.

To increase the methionine content of the ORF 5b product, eight AUG codons were inserted into the ORF 5b coding region. First, in vivo recombinant–PCR mutagenesis (Yao et al., 1992) was used to generate a Bpu1102I restriction site 10 nucleotides upstream of the ORF 5b termination codon in p5b, p5ab and pZ5ab. Oligonucleotides OLV 9 (5' CAGCAATAAACCACCCAGAAG 3'), OLV 10 (5' CCGGCTG-GCTGGTTATTGCTG 3'), OLV 28 (5' TATAGGCTTAGATTA-TGAGTATGACCACCTACGGGA 3') and OLV 29 (5' ATATGCT-AAGGTTATATTATCTCCACCCCC 3') were used for this purpose. The constructs p5bN, p5abN and pZ5abN were then generated by exchanging the Bpu1102I–BamHI fragment of the derived plasmids with a synthetic double-stranded DNA fragment comprised of the annealed oligonucleotides OLV 30 (5' TTAGCAGAAGTGGT-ATG),ACATTATGACTAGGGA 3') and OLV 31 (5' GATCCCC-ACTAGTCTAAATG(3ATG)AAGCCCTATACG 3').

The nucleotide sequences of all the plasmids described above were confirmed by dideoxynucleotide chain-termination sequencing. Fig. 1 summarizes the structure of these plasmids and indicates the size of potential translation products encoded in mRNAs derived from them. For in vitro transcription, plasmid DNAs were linearized with restriction enzymes as shown in Fig. 1 and RNA was synthesized with T7 RNA polymerase in the presence of the synthetic cap structure m7G(5')ppp(5')G (Pharmacia) as described previously (Contreras et al., 1982; Melton et al., 1984).

Preparation of L cell ribosomal wash factors. L929S cells (1 x 10⁶; European Collection of Animal Cell Cultures) were used to prepare ribosomal wash factors as described by Schreier & Staehelin (1973). The crude initiation factors (IF) fraction, adjusted to 300 A₂₆₀/ml, was used without further purification.

Preparation of an L cell lysate and in vitro translation. The L cell lysate was prepared from L929S cells as previously described (Siddell, 1983). Prior to translation, the lysate was treated with micrococcal nuclease. A 340 lal incubation mixture containing 20 mM-Hepes pH 7.0, 85 mM-KCl, 1.2 mM-MgCl₂, 2 mM-dithiothreitol, 1 mM-ATP, 0.2 mM-GTP, 50 mM-spermine, 250 mM-spermidine, 12 mM-creatine phosphate, 0.1 mg creatine kinase, 0.2 mM of each amino acid except methionine, 1 mM-CaCl₂, 5.5 units of micrococcal nuclease (11000 units/mg), 20 lal of L cell ribosomal wash factors and 200 lal of the L cell lysate was incubated at 20°C for 10 min. After adding EGTA pH 7.0 to a final concentration of 2 mM, the mixture was incubated on ice for 5 min.

Translation reactions consisted of 20 lal of the nucleate-treated L cell lysate supplemented with 3 lal of [³⁵S]methionine (15 mCi/ml), 1 lal calf liver tRNA (10 mg/ml) and either 1 lal of water, 1 lal of water

**Methods**

Construction of recombinant plasmids and in vitro RNA synthesis. To construct recombinant plasmids corresponding to the unique region of MHV mRNA 5, the cDNA insert of pJMS1010 (Ebner et al., 1988) was isolated by excision with PstI, digested with DdeI and, after treatment with T4 DNA polymerase, a 630 bp fragment was cloned into Smal-linearized pGEM1. In the resulting construct, p5ab, the initiation codon of ORF 5a lies 37 bp downstream of the cloning site. For the construction of a plasmid corresponding to ORF 5b alone, pJMS1010 DNA was digested with PstI and the isolated cDNA insert was further digested with TauI and RsaI. The fragment containing the coding region of ORF 5b was treated with T4 DNA polymerase and cloned into Smal-linearized pGEM1 to produce the construct p5b.

In order to place an ORF upstream of ORF 5a, PCR was carried out with oligonucleotides OLV 25 (5'CACAGAGGTTCACAAATGCG-CATGATTCGAAAT 3') OLV 26 (5'GGGATCCGGTTCATGTTGATATCCTG 3') and Smal-linearized pROS DNA (Ellinger et al., 1989). The PCR product contains a SalI restriction site upstream of the Kozak consensus sequence ACCATGGG, which is in frame with a fragment of the β-galactosidase gene from nucleotides 6 to 1125, a stop codon that terminates this ORF and a second SalI restriction site. After digestion with SalI this fragment was cloned into SalI-linearized pGEM1 and p5ab. The resulting constructs were designated pZ and pZ5ab, respectively.

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containing 2.5 pmol (0.4 to 1.8 μg) of synthetic mRNA or 1 μl of water containing 0.5 μg of polyadenylated RNA from MHV-infected cells. Incubations were carried out at 34°C for 2 h and 15 μl aliquots of the translation reaction were electrophoresed on discontinuous, 17% SDS-polyacrylamide gels as described by Laemmli (1970). The radioactivity incorporated into the translation products was determined using a PhosphorImager (model 400E; Molecular Dynamics). Cytoplasmic poly(A) RNA from MHV-infected cells was prepared as previously described (Siddell, 1983).

**Results**

**Identification of MHV mRNA 5 translation products**

In order to identify the translation products of the MHV ORFs 5a and 5b, mRNAs were synthesized from BstEII-linearized p5ab (mRNA 5a) and BamHI-linearized p5b (mRNA 5b). *In vitro* translation of mRNA 5a directed the synthesis of a polypeptide with an apparent Mr of 12000 (Fig. 2a, b and c, lane 3). The predicted Mr of the ORF 5a product (107 amino acids) is 12500. The *in vitro* translation of mRNA 5b directed the synthesis of a polypeptide with an apparent Mr of 14000 (Fig. 2a, lane 4). The predicted Mr of the ORF 5b product (88 amino acids) is 10200.

The discrepancy between the expected and observed electrophoretic mobility of the ORF 5b product was surprising. We therefore performed two further experiments to confirm it’s identity. The *in vitro* translation of mRNA derived from BamHI-linearized p5b<sup>1°</sup> (mRNA 5b<sup>1°</sup>) directed the synthesis of a polypeptide with an apparent Mr of 11000 (Fig. 2b, lane 4). The predicted Mr of the ORF 5b<sup>1°</sup> product (99 amino acids) is 11500.

Thus, despite the incorporation of 11 additional amino acids in the ORF 5b translation product, its electrophoretic mobility had increased. In the translation products directed by mRNA 5b<sup>1°</sup>, we also observed a protein of 33000 apparent Mr, (indicated as X in Fig. 2b, lane 4). We assume that this is an aggregated form of the ORF 5b<sup>1°</sup> product.

Secondly, the identity of the ORF 5b product was also confirmed by the translation of mRNA derived from the HindIII-linearized plasmid p5b<sup>N</sup>. The *in vitro* translation of mRNA 5b<sup>N</sup> (Fig. 2c, lane 4) directed the synthesis of a polypeptide with an apparent Mr of 36000. The predicted Mr of the ORF 5b<sup>N</sup> product (337 amino acids) is 37200.

**In vitro translation of the bicistronic mRNA 5ab**

The *in vitro* translation of mRNA derived from BamHI-linearized p5ab (mRNA 5ab) directed the synthesis of both the ORF 5a and ORF 5b products (Fig. 2a, lane 5). This result is consistent with the idea that the MHV mRNA 5 is functionally bicistronic (Skinner et al., 1984; Budzilowicz & Weiss, 1987; Leibowitz et al., 1988). In the experiment shown, the ratio of ORF 5a to ORF 5b products translated from mRNA 5ab was about 8:1, showing that, at least in this system, ORF 5a is more efficiently translated than ORF 5b.

To strengthen the conclusion that the mRNA 5ab is functionally bicistronic we carried out two further experiments. Firstly, we translated mRNA derived from BamHI-linearized p5ab<sup>1°</sup> (mRNA 5ab<sup>1°</sup>). In this case, the detection of the ORF 5b<sup>1°</sup> product should be
enhanced by the incorporation of additional radioactivity and, indeed, this result can be clearly seen in Fig. 2(b), lane 5. Secondly, we translated mRNA derived from HindIII-linearized p5abS (mRNA 5abS). In this experiment, the ORF 5b product with an apparent Mr of 36000 was also clearly detected (Fig. 2c, lane 5).

To test whether ORF 5b can be expressed independently of ribosomes that enter from the 5' end of the mRNA, we translated mRNA derived from BamHI-linearized pZ5ab (the tricistronic mRNA Z5ab). The result is shown in Fig. 2(a), lane 6. As expected the upstream ORF Z was expressed, resulting in the synthesis of a polypeptide with an apparent Mr of 51000. The predicted Mr of the ORF Z product (376 amino acids) is 41500. Importantly, no ORF 5a product was detected in the translation reaction. This indicates that very few, if any, ribosomes scan through the upstream ORF Z and initiate the synthesis of an ORF 5a polypeptide. In contrast, the ORF 5b product was readily detected. The amount of ORF 5b product synthesized from the tricistronic mRNA Z5ab was similar to the amount of ORF 5b product expressed from an equimolar concentration of the bicistronic mRNA 5ab (compare Fig. 2a lanes 5 and 6).

To rule out the possibility that a polypeptide of 14000 apparent Mr can be synthesized by the aberrant translation of ORF Z (for example, premature termination or internal initiation), we translated mRNA derived from BamHI-linearized pZ (mRNA Z). As expected this mRNA directed the synthesis of the ORF Z gene product and no polypeptide with an apparent Mr of 14000 was detected (Fig. 2a, b and c, lane 7).

To strengthen the conclusion that ORF 5b, but not ORF 5a, is translated from a tricistronic mRNA containing an additional upstream ORF, we carried out two further experiments. Firstly, we translated mRNA derived from BamHI-linearized pZ5ab (mRNA Z5ab). The result is shown in Fig. 2(b), lane 6. In this translation reaction, the ORF Z product and the ORF 5b product were easily identified. Again, using equimolar concentrations of mRNA, approximately equal amounts of ORF 5b products were expressed from the bicistronic and tricistronic mRNAs, mRNA 5ab (mRNA Z5ab) and mRNA Z5ab (compare Fig. 2b lanes 5 and 6). The ORF 5a product was not expressed from the tricistronic mRNA Z5ab. Secondly, we translated mRNA derived from HindIII-linearized pZ5ab (mRNA Z5ab). The result is shown in Fig. 2(c), lane 6. Again, the ORF Z product and the ORF 5b product were easily identified but a translation product for ORF 5a was not detected.

**Discussion**

The results presented in this paper lead us to the conclusion that, in the context of the MHV mRNA 5 unique region, the initiation of protein synthesis on ORF 5b can occur independently of ribosomes that enter from the 5' end of the mRNA. This has been shown by the translation of the tricistronic mRNAs, Z5ab, Z5ab10 and Z5ab30, where the 5'-proximal and 5'-distal ORFs, ORF Z and ORF 5b, are translated, whilst the internal ORF, ORF 5a, is translationally inactive. Clearly, the upstream ORF Z, provides an effective barrier to scanning ribosomes but does not prevent the initiation of ORF 5b translation.

Our conclusions are based upon the translation of synthetic mRNAs in vitro. As always, it can be argued that the results are due to peculiarities of the in vitro system or, for example, the degradation of mRNA during the translation reaction. However, we have taken care to use a translation system derived from a murine cell line and, at least in the case of the tricistronic mRNAs Z5ab, Z5ab10 and Z5ab30, the mRNA degradation interpretation would require a ribonuclease activity that specifically renders the ORF 5b initiation codon accessible to scanning ribosomes.

Furthermore, our results are not the first evidence for the internal entry of ribosomes on a coronavirus mRNA. Liu & Inglis (1992), using an approach very similar to that described here, have concluded that the tricistronic mRNA 3 of IBV encodes three proteins, 3a, 3b and 3c, and that the translation of the most distal ORF 3c is mediated by a cap-independent mechanism involving internal initiation. Taken together, these data strongly suggest that the translation of the coronavirus sM proteins, i.e. the ORF 3c product of IBV and the ORF 5b product of MHV (Cavanagh et al., 1994), involves the internal entry of ribosomes on a polycistronic mRNA. Clearly, in vivo studies using reporter gene constructs and the naturally occurring mRNAs will be required to strengthen this conclusion.

The initiation of protein synthesis by internal ribosome entry has been most extensively studied by picornavirus RNAs (for a recent review see Meerovitch & Sonenberg, 1993). In this case, ribosome entry is mediated by a region in the 5' untranslated region of the genomic RNA, the so-called ‘internal ribosome entry site’ (IRES) or ‘ribosome landing pad’ (RLP). The function of the IRES/RLP is dependent upon conserved primary sequence, for example a polypyrimidine tract (Pestova et al., 1991), as well as base-pairing interactions that give rise to extensive RNA structures able to bind trans-acting factors and, directly or indirectly, components of the translational machinery (Jung & Wimmer, 1990; Meerovitch et al., 1993).
Recently, Le et al. (1992, 1993) have proposed a general model of conserved tertiary structural elements in picornavirus IRES/RLPs. This model includes RNA stem-loops that can be modelled into compact superstructures involving pseudoknots together with possible base-pairing interactions between the picornavirus IRES/RLP and 18S rRNA. Most interestingly, they have recognized similar structural elements in the unique region of the IBV mRNA 3 (Le et al., 1994) and suggest that these features may be the hallmark of elements that mediate the internal initiation of cap-independent translation.

An obvious question is whether or not the same features can be identified in the unique regions of the MHV mRNA 5. This question can be approached theoretically with software capable of predicting tertiary RNA structures and experimentally using chemical and enzymatic probing in combination with covariant nucleotide mutation analysis. We are currently using both approaches. Furthermore, it will be of interest to examine interactions between the putative MHV mRNA 5 IRES/RLP element and cellular proteins. The in vitro translation system we have used is prepared from a murine cell line and it may be possible to search for proteins that specifically interact with the MHV mRNA 5 unique region directly in this system.

In the long term, we are interested in the biological relevance of internal ribosome entry on the MHV mRNA 5. The MHV ORF 5b product is believed to be an essential structural protein of the virus, but its functional role(s) in the replication cycle is still unknown. Why, in contrast to all other MHV subgenomic mRNAs, does the unique region of mRNA 5 encode two proteins? And why is the initiation of ORF 5b translation mediated by a complex mechanism such as internal ribosome entry? Also, if the putative IRES/RLP element of mRNA 5 encompasses ORF 5a, it is difficult to envisage translation of both proteins from the same mRNA, at least at the same time. If there are two forms of the MHV mRNA 5 in the infected cell, then how are they distinguished? The answers to these and other questions must await further experiments.

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References


Lebowitz, J. L., Perlman, S., Weinstock, G., DeVries, J., R.


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