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TRANSLATION OF THE MHV sM PROTEIN IS MEDIATED BY THE INTERNAL ENTRY OF RIBOSOMES ON mRNA 5

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INTRODUCTION

Mouse hepatitis virus (MHV) has a positive strand RNA genome of about 31 kilobases (1). In the infected cell, viral gene expression is mediated by translation from both genomic RNA and subgenomic mRNAs. These mRNAs form a 3′ co-terminal set and they contain a common 5'leader sequence (2). Only the region of each mRNA absent from the next smallest mRNA, the so-called unique region, is thought to be translationally active (3,4). Most coronavirus mRNAs contain a single open reading frame (ORF) in their unique region and appear to be functionally mono-cistronic. One exception is the MHV mRNA 5 which contains two ORFs in its unique region, designated as ORF 5a and ORF 5b. Studies on the in vitro translation of synthetic mRNAs suggest that the MHV mRNA 5 is funtionally bicistronic (5). The ORF 5b gene product has been detected in MHV infected cells and virus particles and is equivalent to the small membrane (sM) proteins of infectious bronchitis virus (IBV) and transmissible gastroenteritis virus (TGEV)(6,7,8,9). Two mechanisms can be proposed for the expression of the MHV ORF 5b product. One possibility is based upon the leaky scanning model, as proposed by Kozak (10). In this case, the expression of ORF 5b would be mediated by ribosomes that scan from the 5′ end of the mRNA, but fail to recognise the ORF 5a initiation codon. An alternative model is a cap-independent mechanism involving ribosome entry at an internal position on the MHV mRNA 5. Such a mechanism has been described for a variety of picornavirus RNAs and hepatitis C virus RNA (11,12). In the experiments reported here, we have analysed the in vitro translation products of synthetic mRNAs that contain the unique region of MHV mRNA 5, preceded by an ORF derived from the ß-galactosidase gene. The results show that the ß-galactosidase ORF prevents the movement of ribosomes from the 5′ end of the mRNA but ORF 5b is, nevertheless, translated. We conclude that translation of the sM protein is mediated by an internal ribosome entry mechanism.

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METHODS

Cloning Recombinant Plasmids and in Vitro RNA Synthesis

To construct recombinant plasmids corresponding to the unique region of MHV mRNA 5, a 630 bp *Dde*I fragment of pJMS1010 (13) was blunt end cloned into *Sma*I linearised pGEM1. In the resulting construct, p5ab, the initiation codon of ORF 5a is 37 bp downstream of the cloning site. For the construction of a plasmid corresponding to ORF 5b alone, a *Taq*I - *Rsa*I fragment of pJMS1010 DNA, containing the coding region of ORF 5b, was blunt end cloned into *Sma*I linearised pGEM1 to produce the construct p5b. In order to place an ORF upstream of ORF 5a, a PCR-product containing an ORF comprised of the ß-galactosidase gene from nucleotide 6 to nucleotide 1125, was cloned into pGEM1 and p5ab. The resulting constructs are designated as pZ and pZ5ab, respectively. To increase the methionine content of the ORF 5b product, 8 AUG codons were engineered into the ORF 5b coding region, 10 nucleotides upstream of the ORF 5b termination codons in p5b, p5ab and pZab. The resulting constructs are designated as $p5b^{10}$, $p5ab^{10}$ and $pZ5ab^{10}$. The nucleotide sequences of all the plasmids described above were confirmed by chain-termination sequencing. Figure 1 shows the structure of these plasmids. For in vitro transcription, plasmid DNAs were linearised with restriction enzymes (as shown in Figure 1) and RNA was synthesised with T7 RNA polymerase in the presence of the cap structure m7G(5′)ppp(5′)G as described previously (14).

In Vitro Translation in an L Cell Lysate

The L cell lysate was prepared from L929S cells as previously described (4). The lysate was treated with micrococcal nuclease and 2.5 pmols (0.4 to 1.8 μg) of synthetic mRNA was added to each translation reaction. Aliquots of the translation mixture (15 μl) were electrophoresed on 17% SDS-polyacrylamide gels as described by Laemmli (15). Cytoplasmatic, polyadenylated RNA from MHV-infected cells was prepared as previously described (4).

RESULTS

In Vitro Translation of MHV mRNA 5 Derived Constructs

In order to identify the translation products of the MHV ORFs 5a and 5b, mRNAs were synthesised from *Bst*EII-linearised p5ab (mRNA 5a) and *Bam*HI-linearised p5b (mRNA 5b). In vitro translation of mRNA 5a directs the synthesis of a polypeptide of 12 kDa (figure 2a lane 3) and mRNA 5b directs the synthesis of a polypeptide of 14 kDa (figure 2a, lane 4). The mRNA $5b^{10}$ translation product has an apparent size of 11 kDa (Figure 2b, lane 4). The in vitro translation of a structurally bicistronic mRNA derived from *Bam*HI-linearised p5ab, i.e. mRNA 5ab, directs the synthesis of both the ORF 5a and ORF 5b products (Figure 2a, lane 5). This result is consistent with the idea that the MHV mRNA 5 is functionally bicistronic (16,5,6). To strengthen this conclusion, we translated mRNA derived from *Bam*HI-linearised $p5ab^{10}$, i.e. mRNA $5ab^{10}$. In this case, the detection of the ORF $5b^{10}$ product should be enhanced by the incorporation of additional radioactivity and, indeed, this result is clearly seen in Figure 2b, lane 5.

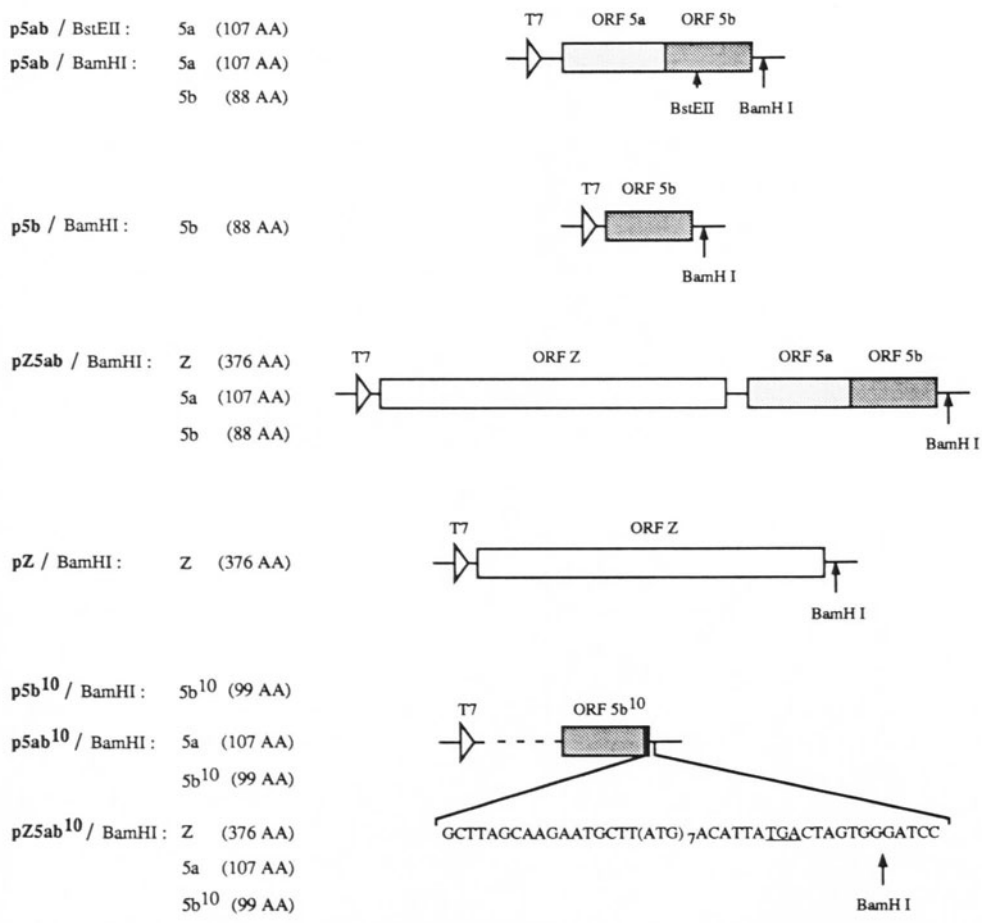

Figure 1. Structure of transcription plasmids. The transcription plasmids used in this study are illustrated. The position of the T7 promoter is shown and ORFs are indicated as boxes. The positions of relevant restriction enzyme recognition sites and the size of potential translation products are also indicated. The broken line represents sequences upsteam of ORF 5b in the plasmids $p5b^{10}$, $p5ab^{10}$ and $pz5ab^{10}$

In Vitro Translation of ORF 5b but Not ORF 5a from a Tricistronic mRNA Containing an Additional Upstream ORF

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 *Bam*HI-linearised pZ5ab, i.e. the tricistronic mRNA Z5ab. The result is shown in Figure 2a, lane 6. As expected, the upstream ORF Z is expressed, resulting in the synthesis of a polypeptide of 51 kDa. Importantly, no ORF 5a product can be 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 is readily detected. The amount of ORF 5b product synthesised from the tricistronic mRNA Z5ab is similar to the amount of ORF 5b product expressed from an equimolar concentration of the bicistronic mRNA 5ab (compare Figure 2a, lanes 5 and 6). To rule out the possibility that a polypeptide of 14 kDa can be synthesised by the aberrant translation of ORF Z (for example, premature termination

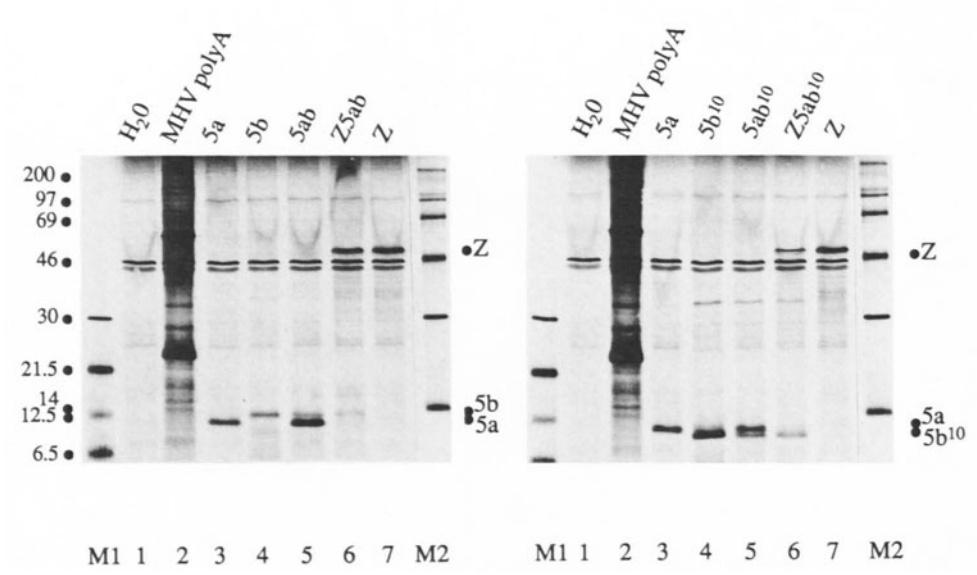

Figure 2. In vitro translation of mRNAs derived from transcription plasmids. The translation products of cell free protein synthesis were electrophoresed in 17% SDS polyacrylamide gels and detected by autoradiography

or internal initiation), we translated mRNA derived from *Bam*HI-linearised pZ, i.e. mRNA Z. As expected, this mRNA directs the synthesis of the ORF Z gene product and no 14 kDa polypeptide can be detected (Figure 2a, 2b, 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 a further experiment. We translated mRNA derived from *Bam*HI-linearised pZ5ab^{10}, i.e. mRNA Z5ab^{10}. The result is shown in Figure 2b, lane 6. In this translation reaction, the ORF Z product and the ORF 5b^{10} product are easily identified. Again, using equimolar concentrations of mRNA, approximately equal amounts of ORF 5b^{10} products are translated from the bicistronic and tricistronic mRNAs, mRNA5ab^{10} and mRNA Z5ab^{10} (compare Figure 2b, lanes 5 and 6). The ORF 5a product is not expressed from the tricistronic mRNA Z5ab^{10}.

DISCUSSION

The results presented in this study show that, in the context of the MHV mRNA 5 unique region, the initiation of ORF 5b protein synthesis occurs independently of ribosomes that enter from the 5′ end of the mRNA. This has been shown by the translation of the tricistronic mRNAs, Z5ab and Z5ab^{10}, where the 5′ proximal and 5′ distal ORFs, ORF Z and ORF 5b/b^{10}, are translated, whilst the internal ORF, ORF 5a, is not. Clearly, the upstream ORF Z, provides an effective barrier to scanning ribosomes but does not prevent the initiation of ORF 5b translation. Liu and Inglis (17) 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, involves the internal entry of ribosomes on a polycistronic mRNA. The initiation of protein synthesis by internal ribosome entry has been most extensively studied in picornavirus RNAs (18). In this case, ribosome entry is

mediated by the so-called "internal ribosome entry site" (IRES) or "ribosome landing pad" (RLP) An obvious question is whether or not similar structures can be identified in the unique region of the MHV mRNA 5 Furthermore, it will be of interest to examine interactions between the putative MHV mRNA 5 IRES/RLP element and cellular proteins In the long term, the biological relevance of internal ribosome entry on the MHV mRNA 5 has to be explained The MHV ORF 5b product is thought to be an essential structural protein of the virus, however, 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? The answers to these and other questions must await further experiments

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