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Characterization of an internal ribosome entry site within mRNA 5 of murine hepatitis virus

M. Jendrach, V. Thiel, and **S. Siddell**

Institute of Virology, University of Würzburg, Würzburg, Germany

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Summary. The unique region of mRNA 5 of murine hepatitis virus contains two open reading frames, ORF 5a and ORF 5b. The downstream ORF 5b encodes the envelope (E) protein, an integral membrane protein of the virus. We have shown previously that the expression of ORF 5b is mediated by the internal entry of ribosomes. In the experiments reported here, we have used the in vitro translation of synthetic mRNAs to identify the region of mRNA 5 that mediates internal ribosome entry. Our results show that the 5′ border of the MHV mRNA 5 IRES element is located between nucleotides 227 and 244 in ORF 5a, while the 3′ border is located between nucleotides 140 and 172 in ORF 5b. The MHV mRNA 5 IRES element, therefore, contains not more than 280 nucleotides and encompasses the ORF 5b initiation codon. As evidenced by electrophoretic mobility shift assays, the IRES element of mRNA 5 interacts specifically with protein factors present in an L-cell lysate.

Introduction

Murine hepatitis virus (MHV) belongs to the family of *Coronaviridae*. It has a positive-strand genomic RNA of approximately 31300 nucleotides [4, 24, 27]. In MHV-infected cells, viral gene expression is mediated by a characteristic 3′ co-terminal set of subgenomic mRNAs. These mRNAs are structurally polycistonic (with the exception of the smallest) but only the unique region of each mRNA, i.e. the region that is not present in the next smallest mRNA, is translated [25, 35]. Normally, the unique region of coronavirus mRNAs contain only one functional open reading frame (ORF). For MHV, however, three mRNAs, mRNA 1, mRNA 5 and mRNA 7, are exceptions [4, 6, 9, 36].

The unique region of the MHV mRNA 5 contains two ORFs, ORF 5a and ORF 5b. The ORF 5a encodes a polypeptide of approximately 110 amino acids but this gene product has only been detected by in vitro translation of synthetic mRNA [6, 38]. Also, it appears to be dispensible, at least for the replication of MHV in tissue

culture cells [41]. The second ORF, ORF 5b, encodes the envelope (E) protein of the virus [42]. The E protein is comprised of approximately 85 amino acids and it is a relatively minor component of the MHV virion. Nevertheless, this protein appears to play an important role in coronavirus assembly and morphogenesis [5, 40].

We have shown previously, by the in vitro translations of synthetic mRNAs, that the initiation of MHV (strain JHM) ORF 5b translation is mediated by the internal entry of ribosomes [38]. Protein initiation by internal ribosome entry is best studied amongst the picornaviruses but there are several examples of viral and cellular mRNAs that initiate translation by internal ribosome entry (for a review see [33]). The region of the mRNA that is required for the internal ribosome entry and translational initiation has been termed the internal ribosome entry site (IRES) [18]. In the experiments reported here, we have investigated the translation of mRNA 5 of MHV (strain A59). We demonstrate that the translation of MHV A59 ORF 5b is initiated by internal ribosome entry and show that the ribosome entry site encompasses nucleotides spanning ORF 5a and ORF 5b and, thus, includes the initiation codon of ORF 5b. Furthermore, as envidenced by electrophoretic mobility shift assays, the MHV A59 mRNA 5 IRES element interacts specifically with one or more L-cell proteins.

Materials and methods

Construction of plasmids

Sac (−) cells were infected with MHV-A59 at an m.o.i. of 5 and the poly(A)-containing RNA was isolated after 16 h using Dynabeads Oligo $(dT)_{25}$ [39]. The unique region of mRNA 5 was then amplified by RT-PCR. Reverse transcription was done with primer OLM 12 (see Table 1) which is located at the 3′ end of ORF 5b and includes a *Bam*HI site. PCR amplification was done using the reverse transcriptase reaction product as template DNA, an upstream primer OLM 13, which includes an *Eco*RI site, and the downstream primer OLM 12. The PCR product was digested with *Eco*RI and *Bam*HI and cloned in plasmid pGEM1

Table 1. Sequence, position and polarity of primers used in this study

Name	Sequence	Position	(nucleotide/sense)		Used for
OLM 12	5′-CGG GAT CCG GAT TAG ATA TCA TCC-3′	ORF 5b	235–249	−	RT-PCR, PCR
OLM 13	5′-GCG AAT TCC TCA TCT TAA TTC TGG TCG T-3′	NTR	1–20	+	RT-PCR
OLM 16	5′-TAC GGC ACA ACT GTC CAA	ORF 5a	244–262	+	PCR
OLM 21	5′-AAG AAG CTG TTG ATT TTA TTC-3′	ORF 5a	284–305	+	PCR
OLM 26/2	5′-TTA CTA GCT TCA CGG CCT-3′	ORF 5a	227–245	+	PCR
OLM 35	5′-CGG GAT CCT TAT AAA CCG CAA AGT TGA ATA CA-3′	ORF 5b	118–139	−	PCR
OLM 36	5′-CGG GAT CCT TAA ATA GAA GGG GAC AGC A-3′	ORF 5b	152–170	−	PCR

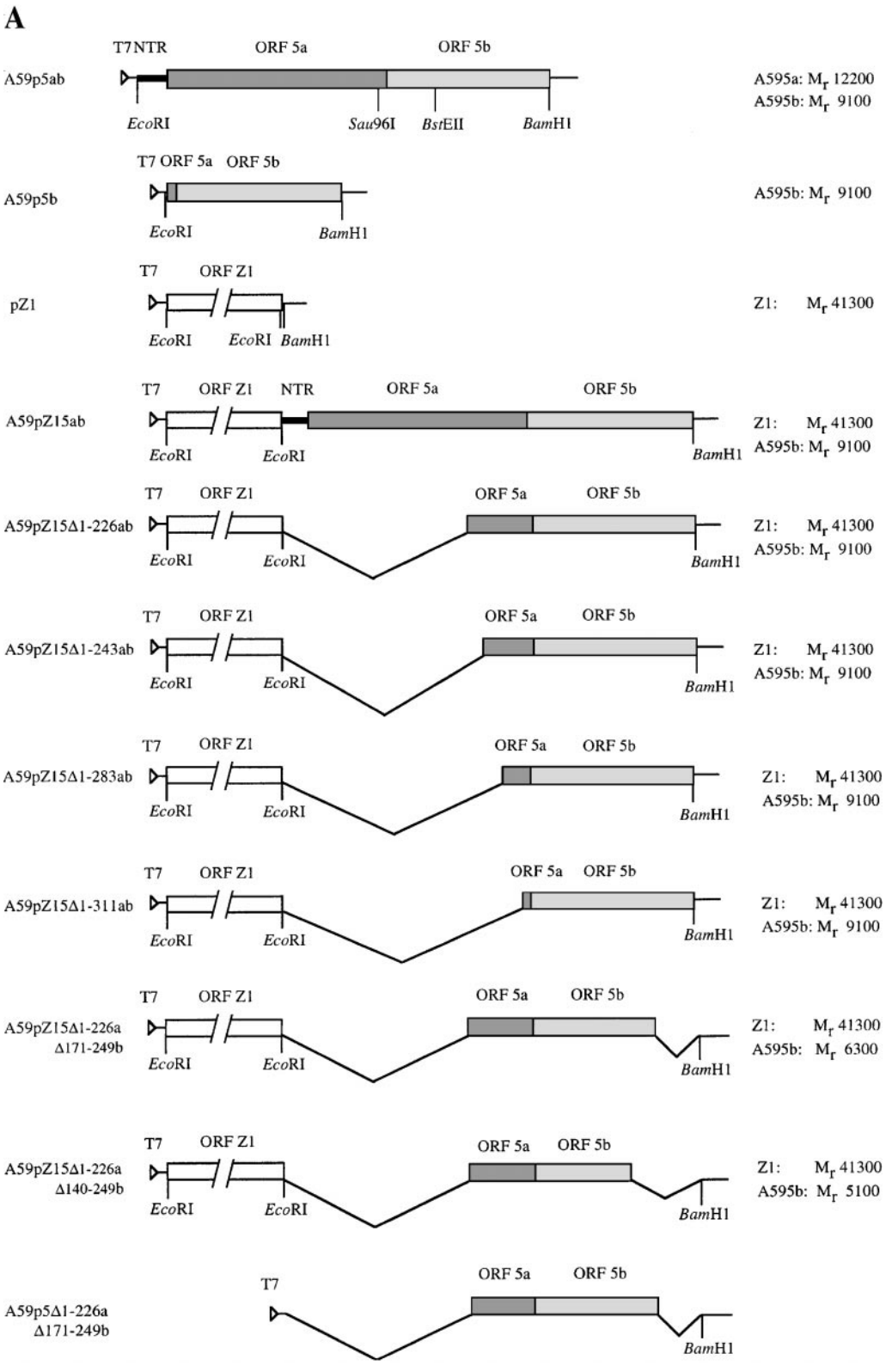

Fig. 1 (*continued*)

B

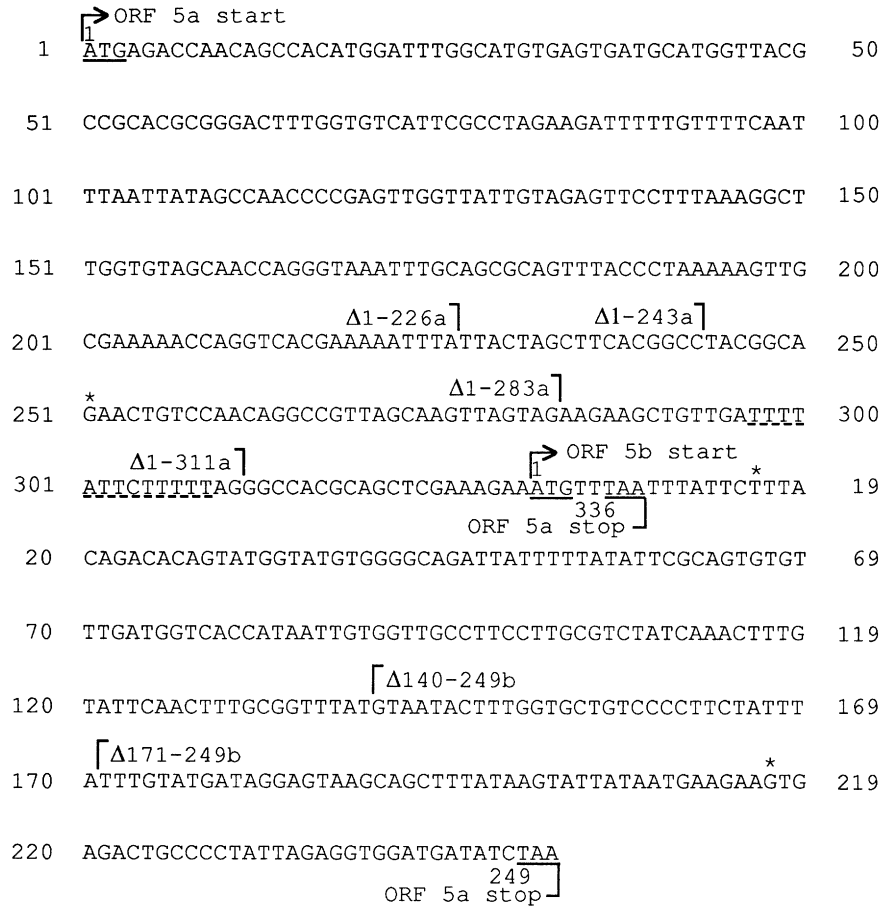

Fig. 1. Structure of the plasmids used in this study. **A** The relative positions of the T7 promotor (▷), ORF Z11 (□), ORF 5a (■), ORF 5b (▒) and relevant restriction enzyme sites are shown. The calculated sizes of the predicted in vitro translation products are indicated. **B** The sequence of the MHV A59 ORFs 5a and 5b are given and the nucleotides that differ from the published sequence [6] are marked by asterisks. The positions of the ORF 5a and 5b deletions are indicated. The initiation and termination codons are underlined and the putative pyrimidine tract (nucleotides 297–309) is highlighted

DNA that had been digested with the same enzymes. The resulting plasmid A59p5ab contains the non-translated region (NTR), ORF 5a and ORF 5b of the MHV A59 mRNA 5 (see Fig. 1).

The plasmid A59p5ab was digested with *Eco*RI and *Bam*HI and the resulting 635 bp fragment was digested with *Sau*96I and treated with the Klenow fragment of DNA polymerase I. The resulting 269 bp fragment was cloned in pGEM1 DNA that had been linearized with *Sma*I. The plasmid A59p5b contains 25 nucleotides of ORF 5a and the complete ORF 5b of MHV A59 mRNA 5 behind a T7 promotor.

The plasmid pZ1 contains a truncated β-galactosidase gene (1128 nucleotides) behind a T7 promotor in vector pGEM1. At the 3′ end of ORF Z1 are three stop codons in three different reading frames. To obtain this construct, plasmid pZ5ab[10] [38] was digested with *Sac*I, treated with the Klenow fragment of DNA polymerase I and the resulting 1140 bp

fragment was cloned in vector pGEM1 that had been digested with *Eco*RI and treated with the Klenow fragment of DNA polymerase I.

To construct the tricistronic plasmid A59pZ15ab, A59p5ab was digested with *Eco*RI, treated with the Klenow fragment of 6DNA polymerase I and digested with *Bam*HI. The resulting 635 bp fragment was cloned in the plasmid pZ1 after it had been linearized with *Sma*I and *Bam*HI.

To identify the region of the MHV A59 mRNA 5 that mediates internal ribosome entry, four plasmids with deletions in ORF 5a were constructed. To obtain the plasmid A59pZ15Δ1-311ab, the construct pZ1 was digested with *Eco*RI and the 1140 bp fragment was cloned in plasmid A59p5b DNA which had been linearized with *Eco*RI. The plasmid A59pZ15Δ1-311ab contains 25 nucleotides of ORF 5a and the complete ORF 5b. To obtain the plasmids A59pZ15Δ1-226ab, A59pZ15Δ1-243ab and A59pZ15Δ1-283ab, the upstream primers OLM 26/2, OLM 16 and OLM 261, respectively, were used in PCR together with the downstream primer OLM 12 and A59p5ab DNA as template. The resulting PCR products contain the complete ORF 5b and different deletions in ORF 5a. After amplification, the DNA was treated with the Klenow fragment of DNA polymerase I and alkaline phosphatase, digested with *Bam*HI and cloned in plasmid pZ1 DNA that had been linearized with *Sma*I and *Bam*HI. The plasmid A59pZ15Δ1-226ab contains 110 nucleotides of ORF 5a, the plasmid A59pZ15Δ1-243ab contains 93 nucleotides of ORF 5a and the plasmid pZ15Δ1-283ab contains 53 nucleotides of ORF 5a.

For the same purpose, two plasmids with deletions in both ORF 5a and ORF 5b were also constructed. To produce the plasmids A59pZ15Δ1-226aΔ171-249b and A59pZ15Δ1-226aΔ140-249b, the downstream primers OLM 36 and OLM 35, respectively, were used in PCR with the upstream primer OLM 26/2 and A59p5ab DNA as template. After amplification, the DNA was treated with the Klenow fragment of DNA polymerase I and alkaline phosphatase, digested with *Bam*HI and cloned in plasmid pZ1 DNA that had been linearized with *Sma*I and *Bam*HI. The plasmid A59pZ15Δ1-226aΔ171-249b contains 110 nucleotides of ORF 5a and 170 nucleotides of ORF 5b. The Plasmid A59pZ15Δ1-226aΔ140-249b contains 110 nucleotides of ORF 5a and 139 nucleotides of ORF 5b. All of the plasmid constructs described above are illustrated schematically in Fig. 1a and the positions of the deletions made in ORFs 5a and 5b are shown in Fig. 1b. The nucleotide sequences of all plasmid constructs were verified by dideoxynucleotide chain-termination sequencing.

In order to produce a polynucleotide substrate for mobility shift assays, the plasmid A59pZ15Δ1-226aΔ171-249b was digested with *Eco*RI and the 3413 bp fragment was isolated and religated. This resulted in the construct A59p5Δ1-226aΔ171-249b which could be linearized with *Bam*HI and transcribed in vitro using T7 RNA polymerase to produce a 289 nt RNA that essentially comprised ORF 5a nucleotides 227–336 and ORF 5b nucleotides 1–170.

In vitro transcription

Plasmid DNAs were linearized with *Bam*HI and transcripts were made with T7 RNA polymerase as described by Herold et al. [13]. The synthetic cap structure $m^7G(5')ppp(5')G$ (Pharmacia) was included in the transcription reaction when the synthetic RNA was used for in vitro translations [7]. The transcripts used in mobility shift assays were synthesized in presence of 10 μCi of [alpha-^{32}P]UTP. The radiolabeled mRNA was precipitated with ethanol and washed twice with 70% ethanol. The homogeneity of all synthetic transcripts was evaluated by agarose gel electrophoresis.

In vitro translation

Synthetic RNAs were translated in a L-cell lysate. Preparation of the L-cell lysate from $L_{929}S$ cells (ECACC 85011425) was done as described by Siddell [35], except that the run-

off translation was carried out at 34 °C in the presence of 5 mM methionine. The L-cell ribosomal wash factors were prepared from $L_{929}S$ cells as described previously [34]. All translations were done with 2.5 pmol (0.1 μg to 2 μg of synthetic RNA) in the L-cell lysate as described [38], except that treatment of the lysate with micrococcus nuclease was omitted. The translation products were analysed on discontinuous 17% SDS-polyacrylamide gels [21] and the radioactivity incorporated into the translation products was quantified using a PhosphorImager (Model 400E; Molecular Dynamics).

Electrophoretic mobility shift assays

Gel shift assays were done essentially as described by Haller and Semler [12]. Briefly, L-cell lysate (5 to 10 μl at 2 μg/μl) was preincubated in binding buffer (5 mM HEPES pH 7.5, 50 mM potassium acetate, 2.4 mM magnesium acetate, 0.1 mM EDTA, 0.01 mM DTT, 1mM ATP and 0.4 mM GTP) at 30 °C for 15 min. For competition experiments, calf liver tRNA (10 μg) or the unlabeled synthetic RNA (3 μg) transcribed from A59p5Δ1-226aΔ171-249b DNA was present. Then ^{32}P-labeled A59p5Δ1-226aΔ171-249b synthetic RNA (1×10^4 cpm) was added and the icubation continued at 30 °C for 15 min before glycerol was added to a final concentration of 10%. The free and bound polynucleotides were separated on a non-denaturing, Tris-Borate-EDTA-6% polyacrylamide gel.

Results

We have shown previously that the expression of the MHV JHM E protein i.e., the ORF 5b gene product, is mediated by the internal entry of ribosomes on mRNA 5 [38]. To confirm that the same mechanism is also used by the closely related murine hepatitis virus, strain A59, we first of all constructed the tricistronic plasmid A59pZ15ab. This plasmid contains a truncated β-galactosidase gene (ORF Z1) in front of the MHV A59 ORFs 5a and 5b. After the in vitro translation of synthetic RNA derived from A59pZ15ab, three major translation products could be observed (Fig. 2, lane 4). One of these, with an M_r of approximately 26000, is present in the water control and probably represents the translation of endogenous mRNA. The other two, with apparent molecular masses of approximately 45000 and 9000, can be identified as products of ORF Z1 and ORF 5b respectively. The product of M_r 45000 has the same mobility as the translation product of RNA derived from the construct pZ1, i.e., the truncated β-galactosidase protein (lane 4). The product of M_r 9000 has the same mobility as the translation product of RNA derived from the construct A59p5b, i.e., the MHV A59 E protein (lane 3). The possibility that the 9000 product is translated from the MHV A59 ORF 5a is excluded by the experiments described below. These results show clearly that the translation of the MHV-A59 ORF 5b protein is initiated by internal ribosome entry.

By analogy to the situation in picornaviruses, it seemed likely that, at least, part of the MHV A59 IRES element would precede and be adjacent to the ORF 5b initiation codon, i.e., in ORF 5a. To test this idea, we made four derivates of A59pZ15ab, each with a progressively larger deletion in ORF 5a. The in vitro translation products of synthetic RNAs derived from these plasmids, A59pZ15Δ1-226ab, A59pZ15Δ1-243ab, A59pZ15Δ1-283ab and A59pZ15Δ1-311ab are shown in Fig. 2 (lane 6 to 9). First, the deletion of more than two-thirds of ORF 5a,

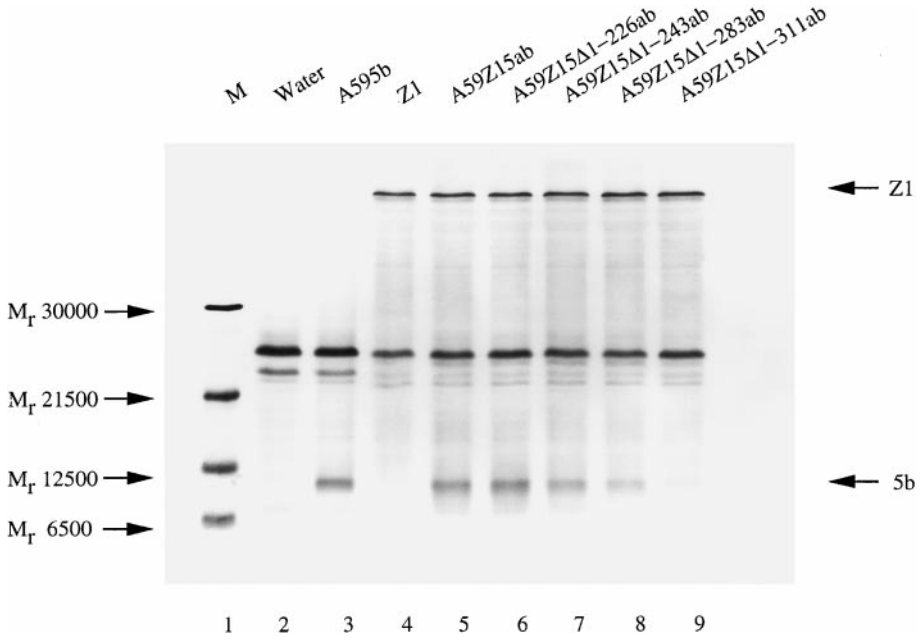

Fig. 2. In vitro translation of synthetic RNAs with deletions in ORF 5a. After translation in the L-cell lysate, the products of synthetic RNAs derived form A59p5b (*3*), pZ1 (*4*), A59pZ15ab (*5*), A59pZ15Δ1-226ab (*6*), A59pZ15Δ1-243ab (*7*), A59pZ15Δ1-283ab (*8*) and A59pZ15Δ1-311ab (*9*) were separated in a 17% SDS-polyacrylamide gel and analysed by autoradiography. Molecular mass markers (M) are shown in *1*

i.e., nucleotides 1 to 226 (A59pZ15Δ1-226ab) had no effect on the expression of the ORF 5b product (lane 6). However, the deletion of an additional 17 nucleotides (A59pZ15Δ1-243ab) resulted in a significant decrease in the amount of ORF 5b protein translated (lane 7). PhoshorImager quantitation of the radioactivity incorporated in the ORF 5b protein shown in lanes 6 and 7, indicated that this decrease was approximately 28%. Further deletions in ORF 5a (A59pZ15Δ1-283ab and A59pZ15Δ1-311ab) reduced the expression of the ORF 5b product even more significantly (lanes 8 and 9). In this case, the reductions were measured to be 45% and 89%, respectively. Second, it should be noted that the product of ORF Z1 is translated from all constructs in similar amounts. Thus, these data indicate that, in functional terms, the 5′ boundary of the MHV A59 mRNA 5 IRES lies between nucleotides 227 and 243 in ORF 5a.

Next we investigated where the 3′ boundary of the MHV A59 mRNA 5 IRES element is located. In the course of earlier experiments, we had noted that the insertion of additional nucleotides towards the 5′ end of the MHV ORF 5b (in the context of either bicistronic or tricistronic constructs) had a detrimental effect on the translation of the ORF 5b protein (Thiel, unpublished). Also, it is now well established that the IRES element of the hepatitis C virus extends over the initiation codon and into the coding region of the virus genome [30]. Thus, we decided to examine the translational activity of synthetic RNAs derived from constructs with additional deletions in the ORF 5b region.

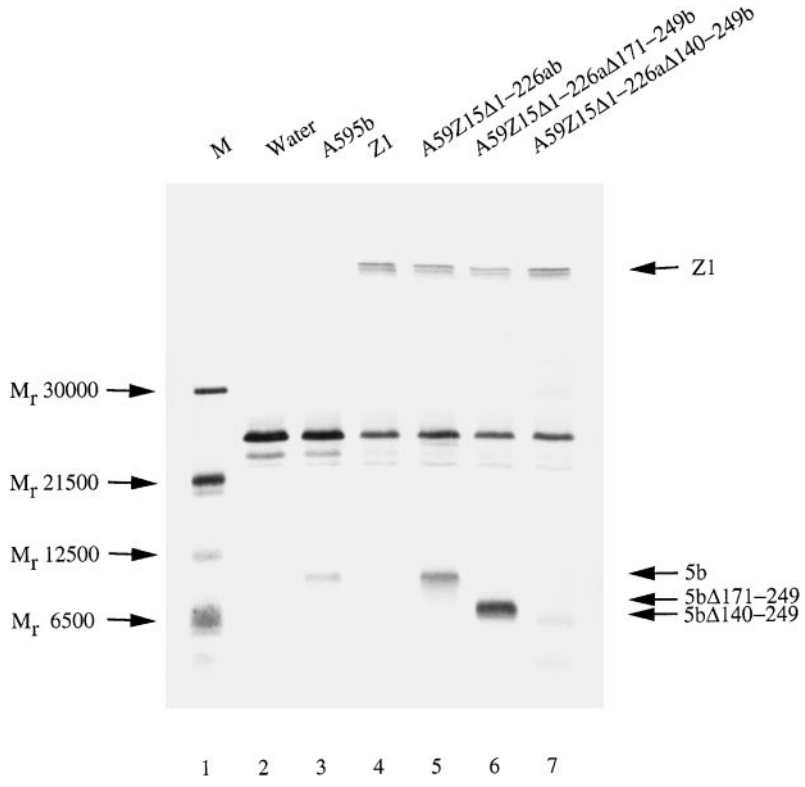

Fig. 3. In vitro translation of synthetic RNAs with deletions in ORF 5a and ORF 5b. After translation in the L-cell lysate, the products of synthetic RNAs derived form A59p5b (*3*), pZ1 (*4*), A59pZ15Δ1-226ab (*5*), A59pZ15Δ1-226aΔ171-249b (*6*) and A59pZ15Δ1-226aΔ140-249b (*7*) were analysed by electrophoresis in a 17% SDS-polyacrylamide gel, followed by autoradiography. Molecular mass markers (*M*) are shown in *1*

Synthetic RNAs, derived from constructs with deletions in both ORF 5a and ORF 5b, A59pZ15Δ1-226aΔ171-249b and A59pZ15Δ1-226aΔ140-249b, were examined by in vitro translation. The results are shown in Fig. 3. First, RNA derived from A59pZ15Δ1-226aΔ171-249b synthesized more ORF 5b translation product, which is truncated by 27 amino acids compared to the authentic E protein, than the equimolar amount of RNA derived from the construct A59Z5Δ1-226ab (lanes 5 and 6). The increase was measured to be 48%. Second, RNA derived from A59pZ15Δ1-226aΔ140-249b synthesized significantly less ORF 5b translation product, which is truncated by 37 amino acids compared to the authentic E protein, than RNA derived from the construct A59Z5Δ1-226ab (lanes 5 and 7). In this case, the reduction was measured to be 78%. It should be noted that the truncated β-galactosidase protein is expressed in equal amounts from RNA derived from all three constructs and that the number of methionines in the three different ORF 5b translation products is the same. These results suggest that the 3′ boundary of the MHV A59 mRNA 5 IRES element lies between nucleotides 140 and 171 in ORF 5b.

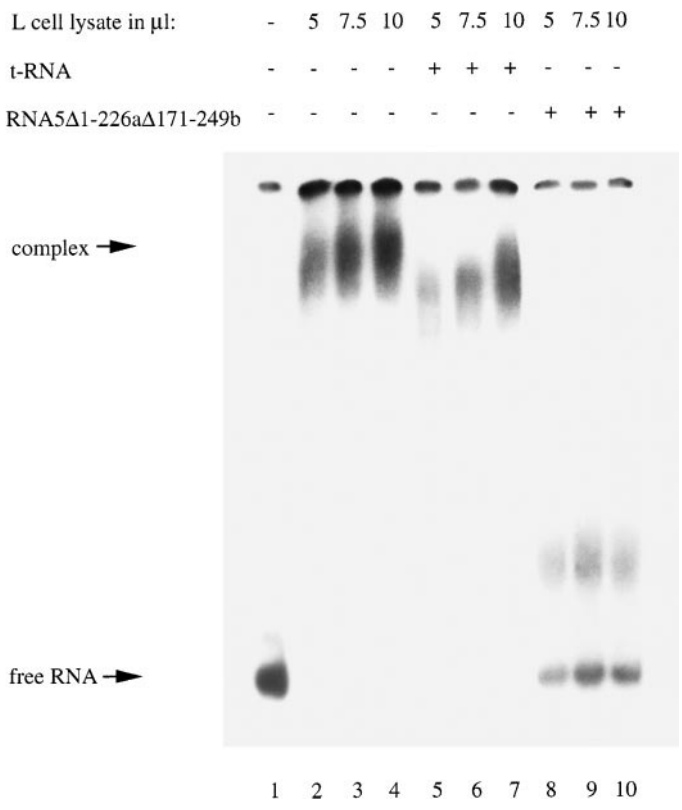

Fig. 4. Electrophoretic mobility shift assays showing the binding of L cell proteins to RNA derived from A59p5D1-226aD171-249b. The radiolabeled RNA was incubated without the addition of lysate (*1*), with 5, 7.5 and 10 µl of L cell lysate (µg/µl) (*2–4*), with 5, 7.5 and 10 µl of L cell lysate (2 µg/µl)and 10 µg tRNA (*5–7*), with 5, 7.5 and 10 µl of L cell lysate (2 µg/µl) and 3 µg of unlabeled RNA derived from A59p5D1-226aD171-249b (*8–10*). Protein complexes and free RNA were separated in a non-denaturing 6% polyacrylamide gel

After determining of the boundaries of the MHV A59 mRNA 5 IRES element, we were interested in detecting possible interactions between the IRES element and cellular proteins. To do this, we did electrophoretic mobility shift assays. Based upon the results described above, we constructed the plasmid A59p5Δ1-226aΔ171-249b, which contains the region of the MHV A59 mRNA 5 that is essential for internal ribosome entry and ORF 5b initiation. This plasmid was transcribed in vitro in the presence of [alpha- ^{32}P]UTP and the ^{32}P-labeled RNA obtained was incubated with different amounts of L cell lysate. The free and bound RNAs were then resolved on a non-denaturating 6% polyacrylamide gel. The results are shown in Fig. 4. By adding increasing amounts of L cell lysate to the binding reaction, an RNA-protein complex can be observed (Fig. 4, lanes 2 to 4) which is not present when the L cell lysate was omitted (lane 1). To examine the specificity of the complex formation, nonspecific and specific competitor RNA was used. The presence of tRNA (10 µg) in the binding reaction did not inhibit the formation of the RNA-protein complex (lanes 5 to 7). The presence of

homologous unlabeled RNA, i.e., the transcript of A59p5Δ1-226aΔ171-249b, in a 30 molar excess, prevented complex formation completely (lanes 8 to 10). These results indicate that the nucleotides extending from position 227 in ORF 5a to position 170 in ORF 5b are able to specifically bind proteins present in the L cell extract.

Discussion

The data presented here lead us to conclude that the translation of ORF 5b of the MHV A59 mRNA 5 is mediated by the internal entry of ribosomes, as we have already shown for the closely related MHV JHM [38]. Moreover, they indicate that a stretch of not more than 280 nucleotides, spanning a region from nucleotide 227 in ORF 5a to nucleotide 170 in ORF 5b is able to mediate this process. Finally, we have shown that these 280 nucleotides are able to specifically bind one or more proteins present in an L cell lysate.

It has been suggested by Jackson and colleagues that "all examples of internal initiation must exhibit some common features both at the level of the cis-acting RNA and the trans-acting factors" [16]. If this is true, then perhaps the MHV IRES element, which is relatively short compared to the IRES elements of other positive strand RNA viruses [8, 17, 26, 30], may be a suitable system in which to identify and analyse some of these features. Thus, for example, in relation to cis-acting elements, an oligopyrimidine tract, adjacent and upstream of the initiating codon, seems to be a conserved feature. Hence, it may be relevant that 22 nucleotides upstream of the MHV ORF 5b initiation codon, a stretch of 13 nucleotides, UUUUAUUCUUUUU (see Fig. 1b), is positioned. Our preliminary mutation analysis indicates that these nucleotides cannot be altered without a negative effect upon the initiation of ORF 5b translation (Jendrach, unpublished) but further experiments are required to link this putative element to, for example, the binding of a specific protein. Also, now that we have delineated the MHV mRNA 5 IRES element to approximately 280 nucleotides, it should be possible to predict potential secondary structures, or the presence of conserved structural motifs in loop regions (which may be indicative of tertiary structures) and to make comparisons with the postulated common RNA structural motifs involved in the internal initiation of translation [22, 23, 32].

The MHV mRNA 5 IRES also appears to be unusual in that it encompasses a large proportion of the coding region of ORF 5b. This situation is not unique; it has been shown that the IRES element of hepatitis C virus encompasses up to 30 nucleotides of the HCV coding region and that the HCV initiation codon is involved in a stem-loop structure [14, 15, 29, 30]. In the context of the MHV mRNA 5, it is clearly intriguing to imagine how ORF 5b translational initiation and ORF 5b translational elongation can occur simultaneously. Perhaps they do not and this may, at least in part, explain the low levels of E protein synthesized in the virus-infected cell [42].

In relation to trans-acting factors, we have demonstrated by electrophoretic mobility shift assays that the IRES of MHV mRNA 5 binds one or more proteins

present in the L cell lysate and the specificity of the interaction was shown by competition assays. The obvious next step will be to identify these proteins by UV-crosslinking assays. The ribosome entry sites of picornaviruses and hepatitis C virus are known to bind several proteins, including La autoantigen, pyrimidine tract-binding protein, p25 and the PCBP1 and PCBP2 proteins (for a review see [1–3, 10, 11, 19]). How these proteins mediate the internal initiation event is not known in detail but it seems likely that, at least in some situations, they facilitate the entry of the 40S ribosome subunit at a specific site and restrict initiation to a translation initiation window [28, 31]. Obviously, it would not be surprising to find that some of these proteins are also involved in the function of the MHV mRNA 5 IRES element.

Finally, we would like to draw attention to what, in our opinion, is a significant difference between the MHV mRNA 5 IRES element and, for example, the IRES elements of picornaviruses. In the course of infection, picornaviruses specifically shut off the cap-dependent translation of cellular mRNAs [37]. Therefore, of neccessity, they have evolved a mechanism that allows for the cap-independent translation of their own mRNA; namely internal ribosome entry and translation initiation. Evidently, this cannot have been the "evolutionary" explanation of the MHV mRNA 5 IRES element. All of the MHV mRNAs have an identical leader sequence of approximately 88 nucleotides which is capped at the 5′ end. Although it has not been experimentally proven, it is thought that, with the exception of mRNA 5, translation is initiated on these mRNAs in a cap-dependent manner according to the conventional ribosomal scanning model of Kozak [20]. To say the least, it is puzzling that, in the case of mRNA 5, the expression of an essential structural protein appears to take place from a functionally bicistronic mRNA using a different mechanism of translational initiation. A better understanding of the replication strategy and biology of MHV will, perhaps, help to resolve this apparent paradox.

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Authors' address: Dr. S. Siddell, Institute of Virology, Versbacher Str. 7, D-97078 Würzburg, Germany.

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