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The sequence between nucleotides 161 and 512 of cowpea mosaic virus M RNA is able to support internal initiation of translation *in vitro*

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Cowpea mosaic virus M RNA is translated *in vitro* as well as *in vivo* into two C-coterminal polyproteins of M_r 105K and 95K. Initiation of translation of the 95K protein gene occurs at an AUG codon at position 512 of M RNA, 351 nucleotides downstream of the initiation codon of the 105K protein gene at position 161. By employing an *in vitro* transcription and translation system it was determined that this 351 nucleotide sequence has the capacity to direct ribosomes to initiate translation at a downstream start codon. This effect is independent of the position of this sequence in an mRNA. Furthermore, evidence has been obtained that scanning ribosomes can bypass the AUG at position 161. Thus, both leaky scanning and internal entry are mechanisms for the initiation of translation of the 95K protein gene.

Introduction

Cowpea mosaic virus (CPMV) is a bipartite plant virus with a positive-sense ssRNA genome (for a recent review see Eggen & van Kammen, 1988). Both RNAs are covalently linked to a small protein (VPg) at the 5′ end, are polyadenylated and contain one large open reading frame (ORF). The RNAs are translated into large polyproteins which are cleaved by a virus-encoded protease into smaller products (Vos *et al.*, 1988). B RNA has a 5′ non-translated region (5′ NTR) of 206 nucleotides (nts) and M RNA a 5′ NTR of 160 nts (Fig. 1). The first 50 nts of both 5′ NTRs are very similar, but the remainder of the sequence is widely divergent. Whereas B RNA is translated into a single polyprotein of M_r 200K, M RNA is translated both *in vitro* and *in vivo* into two polyproteins of 105K and 95K that are C-coterminal (Pelham, 1979; Vos *et al.*, 1984; Rezelman *et al.*, 1989; Holness *et al.*, 1989). The 105K protein is encoded by a gene which initiates at the AUG at position 161 of M RNA (Vos *et al.*, 1984; Holness *et al.*, 1989), whereas the 95K protein gene initiates at the AUG at position 512 (Fig. 1; J. Verver, unpublished results). To determine how ribosomes initiate translation at this internal AUG codon we used full-length cDNAs of both CPMV RNAs to manipulate sequences involved in translation. Transcripts obtained after *in vitro* transcription of these mutant cDNAs with T7 RNA polymerase were translated in reticulocyte lysates and wheatgerm extracts. These experiments revealed that the sequence between nts 161 and 512 (161–512 sequence) of M RNA can effect internal initiation of translation at a downstream AUG codon independent of the position of this sequence in an mRNA.

Methods

Construction of plasmids. All constructs were derived from plasmids pTB1G and pTM1G, which contain full-length cDNA of CPMV B and M RNA respectively, from which infectious RNA can be obtained by transcription with T7 RNA polymerase (Eggen *et al.*, 1989). All DNA manipulations were essentially as described by Sambrook *et al.* (1989).

Plasmid pTMlac was created by inserting a 141 bp *Sau*3AI fragment from M13mp19 (from the *Bam*HI site in the multiple cloning region to the *Pvu*I site at nt 6409), containing *lacZ* sequences, into the *Bgl*II site (at nt 189 in the M cDNA; van Wezenbeek *et al.*, 1983) of pTM1G (see Fig. 2).

Plasmids pTMγBgl and pTMγHind contain cauliflower mosaic virus (CaMV) gene I sequences inserted into the *Bgl*II and *Hin*dIII sites (nt 482) of pTM1G. A 474 bp *Bam*HI–*Cla*I (filled-in) fragment of pAcAS4 (Vlak *et al.*, 1990) containing the gene I sequence (including the gene I start codon, but without the authentic stop codon) was inserted into *Bam*HI- and *Xba*I (filled-in)-digested plasmid Bluescriptks (pBSks) (Stratagene) to produce pBSγI. Plasmids pTMγBgl and pTMγHind were constructed by inserting 525 and 522 bp fragments, containing gene I sequences from pBSγI, into the *Bgl*II site (nt 189) and *Hin*dIII site (nt 482) of pTM1G.

Plasmid pTM58ΔH is a deletion mutant of pTM1G lacking the 210 bp *Hin*dIII fragment between positions 272 and 482.

The construction of mutants lacking the initiation codons, pTMΔAUG1 (lacking AUG 161 and containing a new *Sal*I site at nt 156 in the M cDNA), pTMΔAUG2 (lacking AUG 512 and containing a new *Eco*RI site at position 509 in the M cDNA) and pTMΔAUG1/2 (containing both sites), will be described in detail elsewhere.

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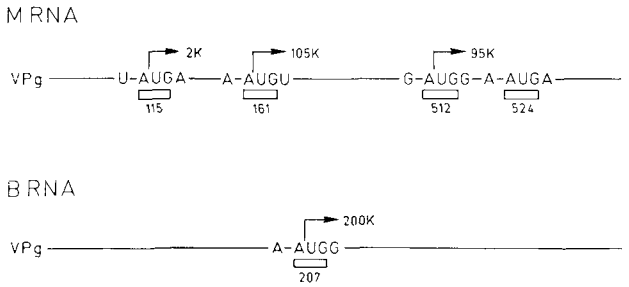

Fig. 1. Schematic representation of the positions of all the AUG codons present in the 5′ region of the CPMV RNAs. The nts at positions +4 and −3 around each AUG codon are shown, and the M_r of the polypeptide potentially initiated at each codon is indicated. The stop codon at the end of the 2K ORF of M RNA is at nt 175.

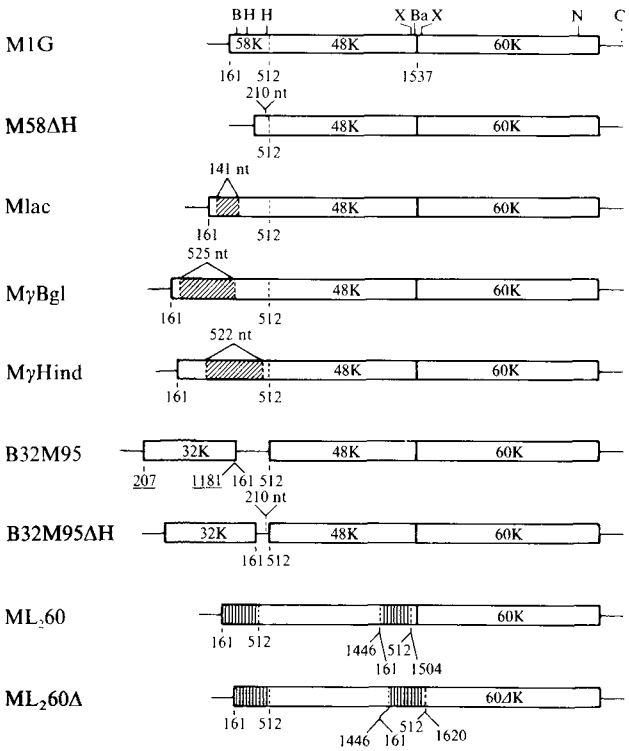

Fig. 2. Schematic representation of the constructs used in this study. The open bars represent the ORFs present in the transcripts. Translation from AUG 161 results in a 105K protein and from AUG 512 in a 95K protein. Processing of the 105K and 95K proteins at the glutamine/methionine site (at position 1537, indicated by a black line) results in 58K and 48K proteins, and the 60K capsid precursor protein. The positions of the restriction sites used for the construction of the mutants are shown above the wild-type transcript (B, *Bgl*II; H, *Hin*dIII; X, *Xho*I; Ba, *Bam*HI; N, *Nco*I; C, *Cla*I); relevant nt positions are indicated beneath the transcripts. The numbering is according to the positions of the nts in the full-length M RNA and B RNA (underlined). The sizes of the insertions (hatched) and deletions are indicated above the transcripts. The 161–512 segments present in $ML_2$60 and $ML_2$60Δ are indicated by vertical lines.

To construct pTMbL, which contains the 5′ NTR of B cDNA fused to the coding region of M cDNA, the 300 bp *Sal*I–*Eco*RI (filled-in) fragment of pTB1G and the 1340 bp *Sal*I (filled-in)–*Bam*HI fragment of pTMΔAUG1 were inserted into *Sal*I- and *Bam*HI-linearized M13mp18RF in a three point ligation. The 5′ NTR of B cDNA was linked to the M cDNA coding region by site-directed mutagenesis (Kunkel, 1985) using oligonucleotide 5′ CTTGACCCAA-CATGTTTTCTTTCACTGAAG. The 1590 bp *Sal*I–*Bam*HI fragment of the mutagenized phage M13 replicative form (RF) DNA was used to replace the 1550 bp *Sal*I–*Bam*HI fragment of pTM1G, producing pTMbL. To construct pTBmL, which contains the 5′ NTR of M cDNA fused to the coding region of B cDNA, the 200 bp *Bam*HI–*Sal*I fragment of pTMΔAUG1 and the 2350 bp *Sal*I–*Sst*I fragment of pTB1G were inserted into *Bam*HI- and *Sst*I-linearized M13mp18RF in a three point ligation. The 5′ NTR of M cDNA was positioned in front of the B cDNA coding sequence by site-directed mutagenesis (Kunkel, 1985) using oligonucleotide 5′ GCTTCGGCACCAGTA-CAATGGGTCTCCCAGAATATG. The 2300 bp *Sal*I–*Sst*I fragment of the mutagenized phage RF DNA was used to replace the 2350 bp *Sal*I–*Sst*I fragment of pTB1G, producing pTBmL.

Plasmids pTB32M95 and pTB32M95ΔH contain two ORFs separated by the 161–512 sequence of M cDNA. To construct these plasmids, additional restriction sites were created in pTB32S (which contains the 5′ NTR and 32K coding region of B RNA followed by a stop codon) (Vos *et al.*, 1988) after the stop codon of the 32K coding region. The small *Pvu*I–*Sst*I fragment of pT7-5 was inserted into pTB32S digested with the same enzymes, producing pTB32Sa. The 3·45 kb fragment from *Sal*I- and *Cla*I-digested pTMΔAUG1 was inserted into pTB32Sa digested with the same enzymes, producing pTB32M95 (Fig. 2). Plasmid pTB32M95ΔH was constructed by exchanging the 2·9 kb *Bgl*II–*Nco*I fragment of pTB32M95 with the similar fragment from pTM58ΔH.

To construct $pTML_2$60 and $pTML_2$60Δ, in which the 161–512 sequence of M cDNA has been inserted in frame in the middle of the coding region of M cDNA, the 357 bp *Sal*I–*Eco*RI fragment of pTMΔAUG1/2 was subcloned into pBSks linearized with the same enzymes to produce $pBSML_2$. A three point ligation of the 371 bp *Xho*I–*Sma*I fragment of $pBSML_2$ with the 2kb *Bam*HI (filled-in)–*Cla*I and the 3·8 kb *Xho*I–*Cla*I fragments of pTM1G produced $pTML_2$60. Plasmid $pTML_2$60Δ was constructed by ligating the 1·9 kb *Xho*I (filled-in)–*Cla*I fragment of pTM1G to the 4·2 kb *Eco*RI (filled-in)–*Cla*I fragment of $pTML_2$60.

In vitro *transcription and translation. In vitro* transcription reactions were performed as described previously (Vos *et al.*, 1988). The yield and integrity of the RNA was checked by agarose gel electrophoresis. *In vitro* translation was for 1 h at 30 °C in reticulocyte lysates in the presence of [^{35}S]methionine as described (Vos *et al.*, 1988), or for 1 h at 25 °C in wheatgerm extract containing 100 mM-potassium acetate and 3 mM-magnesium acetate, as described in the protocol supplied by the manufacturer (Promega). Care was taken that similar amounts of RNA were added to the translation mixes. The translation products were analysed in 7·5% SDS-polyacrylamide gels (Laemmli, 1970).

Results

The effect of the exchange of the 5′ NTR on translation in vitro

In vitro transcription of pTMbL and pTBmL produced M RNA in which the 5′ NTR (nt 1 to 160) was replaced by the 5′ NTR of B RNA (nt 1 to 206), and B RNA in

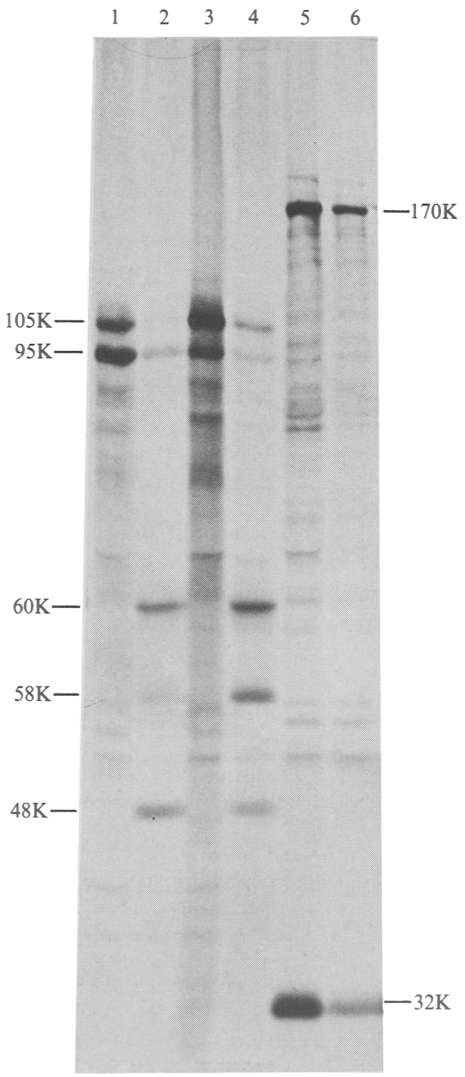

Fig. 3. Translations of the 5′ NTR exchange mutants in reticulocyte lysate. In some cases, there was an additional incubation for 3 h at 30 °C with 3 volumes of unlabelled translation products of CPMV RNA, which results in processing of the 105K and 95K protein to yield 58K and 48K proteins, and a 60K protein (lanes 2 and 4). The products were analysed in a 7·5% polyacrylamide gel. Lanes 1 and 2, M RNA; lanes 3 and 4, MbL RNA; lane 5, B RNA; lane 6, BmL RNA products.

which the 5′ NTR was replaced by that of M RNA. Both transcripts, MbL RNA and BmL RNA respectively, were translated in reticulocyte lysates to examine to what extent the synthesis of the two polyproteins encoded by M RNA was dependent on the sequence of the 5′ NTR. Fig. 3 shows that MbL RNA is still translated into 105K and 95K polyproteins, as is wild-type M RNA, but the ratio of the amount of 105K and 95K proteins produced changed from 1:2 for M RNA to 2:1 for MbL RNA (lanes 1 and 3). The amount of the N-terminal cleavage products (58K and 48K), obtained after processing of the 105K and 95K proteins with B RNA translation products, also reflects this change (Fig. 3, lanes 2 and 4). Translation of BmL RNA produced the same 200K polyprotein (which rapidly cleaved into 32K and 170K proteins) as obtained upon translation of B RNA, but BmL RNA appears to be a less efficient messenger as less protein was produced (Fig. 3, lanes 5 and 6). The results demonstrated that the ability of M RNA to produce a second polyprotein is independent of the 5′ NTR, and may well be located in the sequence between nts 161 and 512 of M RNA.

Evidence for leaky scanning of AUG 161 of M RNA

The AUG start codon at position 161 is in a suboptimal context for initiation of translation according to the rules of Kozak (1986) (Fig. 1). It is possible that the 95K protein is produced from the AUG codon at position 512 by leaky scanning, which is supported by the fact that the 161–512 sequence of M RNA does not contain an AUG triplet in any reading frame. To study this we inserted at nt 189 of M RNA part of the CaMV gene I sequence (525 nt) containing 10 AUG codons (MγBgl) and *lacZ* sequences (141 nt) without AUG codons (MLac) (see Fig. 2). In MγBgl the 5′-most AUG in the insert is the actual start site of gene I and this AUG is in the same reading frame as the AUG at position 161. When leaky scanning occurs at AUG 161, translation of MγBgl was expected to result in a 125K protein, by initiation at AUG 161, and in one or several faster migrating products as a result of initiation at the AUG(s) present in the gene I insert. Translation of transcripts in reticulocyte lysate revealed that insertion of a sequence without AUG codons (MLac) slightly increased translation initiation at AUG 161 and slightly decreased initiation at AUG 512 (Fig. 4, lane 3). Surprisingly, a small amount of protein migrating faster than the product initiated at AUG 161 was also produced. As no additional AUG codons were introduced into this mutant, translation of this protein could only have been initiated at a codon other than AUG (Peabody, 1989).

The gene I insertion reduced initiation at AUG 161 (producing a 125K protein), but several proteins migrating slightly faster were also produced (Fig. 4, lane 4). The largest of these proteins, which was probably initiated at the gene I start codon, was equivalent in amount to the 125K protein initiated at AUG 161.

When the gene I sequence was inserted at position 482 (MγHind), just in front of the start codon of the 95K protein, the 95K protein was not produced (Fig. 4, lane 5). On the other hand a 115K protein was synthesized which was probably initiated at the 5′-most AUG in the gene I insert. Initiation of translation at AUG 161 of MγHind resulted in a protein that migrated slightly more

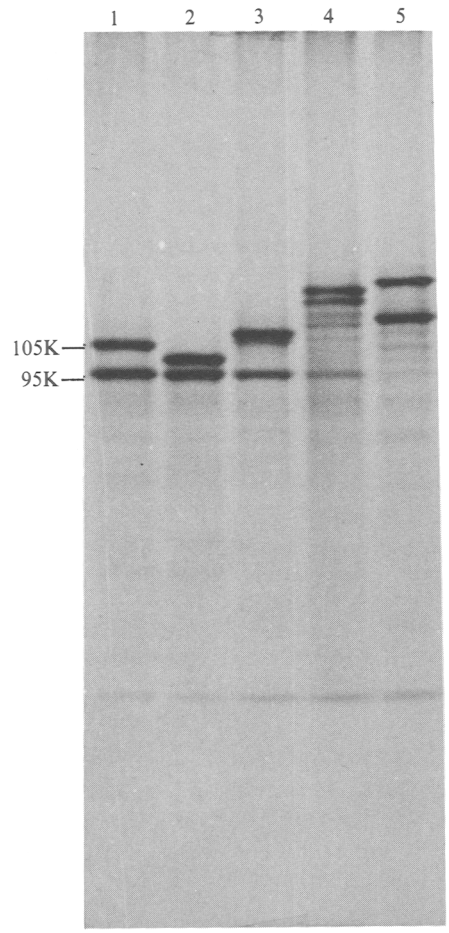

Fig. 4. Translation of the insertion mutants in reticulocyte lysate. The products were analysed in a 7·5% polyacrylamide gel. Lanes 1 to 5: M RNA, M58ΔH RNA, Mlac RNA, MγBgl RNA and MγHind RNA products.

slowly than the largest protein translated from MγBgl RNA, although both proteins contain very similar insertions. Presumably this effect was caused by the different positions of the inserts in the two polyproteins.

A deletion mutant that lacked 210 nt (272 to 482) in the 3′ region of the 161–512 sequence (M58ΔH) still produced two polyproteins (Fig. 4, lane 2), and the ratio of the polyproteins (a deleted 105K and 'normal' 95K) produced from this RNA was almost unaffected.

The result with MγBgl indicated that AUG 161 could be bypassed by initiation complexes, thus making it possible to start protein synthesis at a downstream AUG codon. However, even in the presence of multiple AUG codons between AUG 161 and AUG 512, considerable amounts of the 95K protein were still produced (Fig. 4, lane 4). The observation that MγHind no longer synthesizes the 95K protein showed that these AUG codons function as a 'trap' for all scanning ribosomes, because scanning is the only possible mechanism for

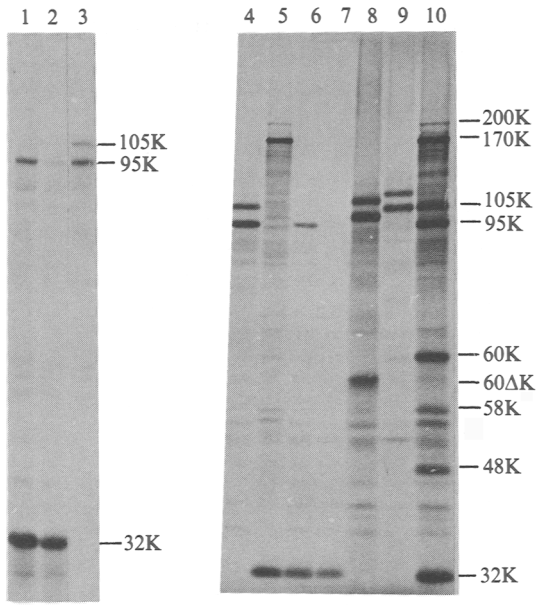

Fig. 5. Translation of the dicistronic messengers in wheatgerm extract (lanes 1 to 3) and reticulocyte lysate (lanes 4 to 10). The products were analysed in a 7·5% polyacrylamide gel. Lanes 1 to 10: B32M95 RNA, B32M95ΔH RNA, M RNA, M RNA, B RNA, B32M95 RNA, M32B95ΔH RNA, $ML_2$60Δ RNA, $ML_2$60 RNA and CPMV RNA products.

initiating translation at AUG 512 in this construct. Therefore, these results indicated that the 95K protein synthesized by MγBgl is not the result of initiation by ribosomes that have scanned past all these AUGs, but is more likely to be the result of internal entry of ribosomes onto a sequence downstream of the insert.

Evidence for internal entry of ribosomes on the 161–512 sequence of M RNA

The results of the experiments described above suggested that the internal binding of initiation complexes to the 161–512 sequence of M RNA may play a role in the production of the 95K protein. To test this hypothesis two types of constructs were created. B32M95 RNA is a dicistronic messenger containing the 5′ NTR and 32K coding region (with a stop codon at its 3′ end) of B RNA, followed by M RNA sequences from nt 161 to the 3′ end (without AUG 161, see Fig. 2). Translation of this transcript in reticulocyte lysate resulted in the synthesis of the 32K protein from the first cistron and the 95K protein from the second cistron (Fig. 5, lane 6). The amount of 95K produced was clearly less than that produced in a comparable translation of M RNA (lane 4). A 210 nt deletion in the 161–512 sequence fragment (B32M95ΔH) resulted in a large reduction in the amount of 95K protein produced by this type of messenger (lane 7). Translation of these transcripts in wheatgerm extract

gave essentially the same result. However, the amount of 95K protein produced in this system was approximately the same as that synthesized by M RNA (Fig. 5, lanes 1, 2 and 3).

In the second type of construct, the 161–512 sequence was duplicated in a position further downstream in M RNA without disturbing the reading frame and taking advantage of the fact that this sequence is also a coding region (Fig. 2; $ML_260\Delta$). It was anticipated that as a result of this duplication the transcript would be translated into three proteins. The new product would result from initiation of translation at an AUG codon immediately downstream of the inserted sequence. Translation of $ML_260\Delta$ RNA in reticulocyte lysate did result in the synthesis of three proteins, 110K, 105K (equivalent to a modified 105K and 95K protein) and 60ΔK (Fig. 5, lane 8). The 60ΔK protein probably originates from the AUG codon at position 1643 of M RNA, which is the first AUG downstream of the insertion. This codon is in frame with the large ORF of M RNA. Immunoprecipitation with an anti-VP23 serum confirmed that all three proteins contained VP23-related sequences (data not shown). Translation of ML_260 resulted in only two major products (Fig. 5, lane 9). Small amounts of a 60K protein, comigrating with the 60K capsid precursor, were detectable after immunoprecipitation with an anti-VP23 serum (data not shown). In ML_260 RNA, the AUG at position 1538 is the first AUG that is in frame with the large ORF, but the first two AUGs present downstream of the duplicated 161–512 sequence (at positions 1515 and 1521) are in other reading frames. Internal entry of ribosomes probably also occurs in this construct. Since the first downstream AUG (which has a favourable context) is not in the large reading frame, it initiates synthesis of a small product. The small amount of 60K protein detected could be due to leaky scanning of these first two AUGs. The insertions in ML_260 and $ML_260\Delta$ showed no effect on the production of the proteins initiated at AUG 161 and AUG 512. (In the experiment shown in Fig. 5 the amount of $ML_260\Delta$ RNA and CPMV RNA was greater than that of the RNA in the other lanes.) In wheatgerm extracts $ML_260\Delta$ RNA was translated into three proteins (data not shown), confirming the results in a different *in vitro* translation system.

Discussion

CPMV M RNA is translated both *in vitro* and *in vivo* into two C-coterminal polyproteins. Translation of the 105K protein gene is initiated at AUG 161, whereas that of the 95K protein gene is initiated at AUG 512. The experiments described in this paper reveal that both leaky scanning of the AUG codon at position 161 and internal entry of ribosomes onto the 161–512 sequence of M RNA are mechanisms for the synthesis of the smaller 95K protein.

The results with MγBgl, which contains an insert with several AUG codons derived from CaMV gene I at position 189, just behind AUG 161, show that AUG 161 can be bypassed by initiation complexes. The 161–512 sequence of M RNA does not contain AUG codons in any of the reading frames, so leaky scanning of AUG 161 of M RNA will initiate synthesis of the 95K protein. However, the results with the bicistronic messenger B32M95 and $ML_260\Delta$ clearly show that internal entry of ribosomes onto the 161–512 sequence in rabbit reticulocyte lysate can occur. It may be argued that the 95K protein produced by B32M95 RNA is the result of reinitiation of ribosomes that resume scanning after termination of translation of the 32K cistron, or is just the result of translation from degraded RNA. However, with the B32M95ΔH transcripts (which contain a 210 nt deletion in the 161–512 sequence) the amount of 95K protein is considerably reduced. Such a deletion should not have an effect on reinitiation of ribosomes because the length of the 'intercistronic region' (140 nt) is still sufficient for optimal reinitiation (Kozak, 1987) and there is no indication that the stability of B32M95ΔH RNA in the lysate would be much greater than that of B32M95. Furthermore, the 60ΔK protein translated from $ML_260\Delta$ RNA, which contains the 161–512 sequence duplicated in a position further downstream in M RNA, is certainly not the result of a reinitiation event and has to be the result of genuine internal entry of ribosomes onto the inserted 161–512 sequence. The observation that the translation of $ML_260\Delta$ RNA still results in synthesis of large amounts of the two large polyproteins shows that insertion of the 161–512 sequence does not make the RNA more susceptible to degradation and rules out the possibility that the 161–512 sequence is specifically cleaved in the translation assay. Therefore, aberrant translation initiation of degraded RNA, which has been used as an argument against several cases of internal initiation (Kozak, 1989), seems to be an unlikely explanation of our results. However, we cannot rule out the possibility that the small amount of 95K protein produced by B32M95ΔH is the result of initiation of translation of degraded RNA.

At the moment it is not possible to determine which mechanism is more important for synthesis of the 95K protein from wild-type M RNA because it is difficult to study one mechanism without interfering with the other. For instance, the 210 nt deletion in the 161–512 sequence in the dicistronic messenger B32M95ΔH greatly reduces the capacity of this sequence to direct internal entry of ribosomes. The same deletion in M58ΔH RNA shows

almost no effect on the amount of 95K produced from this RNA. Thus it seems likely that the 95K protein produced from M58ΔH RNA is due largely to leaky scanning of AUG 161. However we cannot rule out the possibility that sequences between nts 1 and 161 of M58ΔH RNA also contribute in some way to the internal binding of ribosomes.

That ribosomes can bypass AUG 161 may be explained by the suboptimal context of this start codon (Fig. 1; A at −3 and U at +4; Kozak, 1986). However, it also is possible that ribosomes bypass AUG 161 by initiating at AUG 115, terminating at nt 175 and then re-initiating (Fig. 1). We have obtained evidence that ribosomes can initiate at AUG 115 *in vitro* (P. Vos, M. Flipphi & L. Frenken, unpublished observations). However it is important to note that this situation is unique for CPMV and that M RNA sequences of other comoviruses do not contain this small ORF (Shanks *et al.*, 1986; G. Lomonossoff, personal communication). The presence of AUG 115 can also explain the change in the ratio of the amount of the 105K and 95K proteins produced from M RNA (1 : 2) and MbL RNA (2 : 1), and the difference in translation efficiency between B and BmL RNA (Fig. 3). We have not carried out experiments to confirm this possibility.

At the moment we do not know how initiation of translation takes place at the 5′ end of M RNA (nts 1 to 161). The mechanism has to be cap-independent because natural RNAs are covalently linked to VPg at their 5′ end. Furthermore, capping of the M RNA transcripts has no effect on the synthesis of the 105K and 95K proteins in rabbit reticulocyte lysate (data not shown). Experiments are in progress to determine which sequences are important for translation of the 105K protein. Preliminary data show that the 161–512 sequence has no role in this process (G. Kroon & J. Wellink, unpublished results). Furthermore it is of importance to determine whether internal initiation in the 161–512 sequence can also take place in cowpea cells. To test this we are currently developing a transient expression assay in cowpea protoplasts.

Nevertheless, we have demonstrated that the 161–512 sequence of CPMV M RNA is sufficient to allow internal entry of ribosomes *in vitro*, extending the growing list of viral mRNAs capable of supporting this mechanism (Herman, 1986; Hassin *et al.*, 1986; Pelletier & Sonnenberg, 1988; Jang *et al.*, 1988; Curran & Kolakofsky, 1989; Chang *et al.*, 1989) to the plant kingdom.

The 161–512 sequence of M RNA is very rich in U (37·2%) and relatively poor in A residues (21·9%) (total M RNA is 30·4% U and 29·2% A residues; van Wezenbeek *et al.*, 1983), and no extensive secondary structures have been predicted to occur (data not shown). Several stretches of RNA are probably in a single-stranded form, and it is possibly the absence of secondary structures that supports the internal initiation on CPMV M RNA (Sonenberg, 1991).

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