The mechanism of translation of cowpea mosaic virus middle component RNA: no evidence for internal initiation from experiments in an animal cell transient expression system

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The possibility that internal initiation of translation is responsible for the synthesis of the middle component (M) RNA-encoded 95K protein of cowpea mosaic virus (CPMV) has been investigated by constructing plasmids in which the entire sequence of CPMV M RNA was cloned downstream of a chloramphenicol acetyltransferase gene. Expression of these plasmids in an animal cell expression system revealed that no synthesis of the proteins encoded by the downstream CPMV open reading frame takes place from RNA derived from these constructs under conditions where the internal ribosome entry site of foot-and-mouth disease virus is functional. The results indicate that internal initiation is not responsible for the synthesis of the 95K protein in this system.

The genome of cowpea mosaic virus (CPMV), the type member of the comovirus group of plant viruses, consists of two molecules of single-stranded RNA of positive (messenger-sense) polarity. The two molecules, termed middle (M) and bottom (B) component RNA, consist of 3481 and 5889 nucleotides, respectively (van Wezenbeek et al., 1983; Lomonossoff & Shanks, 1983). In a number of important aspects, the genomic RNAs of CPMV resemble those of picornaviruses. For example, both RNAs of CPMV are polyadenylated and have a small protein (VPg) covalently linked to their 5′ termini, and both direct the synthesis of large, precursor polyproteins, which are subsequently cleaved by a virus-encoded protease.

The translation strategy of B RNA can readily be interpreted in terms of the ‘scanning’ model of translation (Kozak, 1978, 1981). Initiation of translation has been shown to take place at the 5′ proximal AUG codon (Wellink et al., 1986) which is in an optimal context (Kozak, 1986a; Lütcke et al., 1987) for it to serve as an efficient initiator. By contrast, the translation strategy adopted by M RNA is more complicated. Translation of M RNA both in vitro and in vivo gives rise to two carboxy terminal proteins known as the 105K and 95K proteins (Davies et al., 1977; Pelham, 1979; Holness et al., 1989; Rezelman et al., 1989), which have been shown to arise through the use of alternative initiation codons (Vos et al., 1984; Holness et al., 1989). The AUG codons used to initiate translation of the two proteins have been identified (Holness et al., 1989) and are at positions 161 (105K protein) and 512 (95K protein) in the M RNA sequence; initiation of translation of the 95K protein can however, in certain circumstances, occur at the AUG at position 524 (Holness et al., 1989). Consideration of the ‘contexts’ of the AUG codons at positions 161 and 512 led to the suggestion (Holness et al., 1989) that the downstream initiation event is a result of ‘leaky scanning’ (Kozak, 1986b) in which a proportion of ribosomes scanning the RNA fail to initiate at AUG at position 161 because of its poor context and proceed to scan until they reach AUG at position 512. However, the recent demonstration of internal initiation in picornaviruses (for a review, see Jackson et al., 1990) raises the possibility that initiation of 95K protein synthesis at position 512 might be the result of the direct binding of ribosomes to an internal site on M RNA somewhere between residues 161 and 512. A suggestion that this does, in fact, occur has been made recently on the basis of in vitro translation data (Wellink et al., 1990; Thomas et al., 1991). Furthermore, recent studies on the mechanism of translation of the RNA of the polyvirus tobacco etch virus (TEV) have shown that initiation occurs in a cap-independent manner (Carrington & Freed, 1990). Since cap-independent initiation is a feature associated with internal initiation, these results provide some support to the notion that internal initiation may occur in plant as well as in animal viruses.

To determine whether internal initiation is either
wholly or partly responsible for the synthesis of CPMV 95K protein, we have investigated the translation of various artificial bicistronic mRNAs containing the entire sequence of CPMV M RNA using an in vivo transient expression system. The results demonstrate that an internal initiation mechanism, such as that found for picornaviruses, is not responsible for the synthesis of a detectable amount of the CPMV 95K protein, at least in the system used.

To construct a full-length cDNA copy of CPMV M RNA suitable for the work below, full-length double-stranded cDNA copies of M RNA were synthesized as described previously (Holness et al., 1989). The double-stranded cDNA was cleaved at its unique BamHI site (position 1504) and the 5' terminal 1.5 kb fragment ligated into BamHI/HincII-digested M13mp19 replicative form DNA. Clones containing the 5' terminal portion of M RNA were identified and one of them, M13-5'13, was selected for further manipulation. Double-stranded replicative form DNA from M13-5'13 and M13-3'4, a clone which contains the 3' terminal 2.0 kb of CPMV M RNA (Holness et al., 1989), was digested with PstI and BamHI, and the CPMV-specific fragments were isolated. The purified fragments were mixed, ligated into the PstI site of the dual promoter plasmid pSPT18 (Pharmacia) and transformed into Escherichia coli JM101. One recombinant, pSPM2, was identified by restriction enzyme analysis as having an apparently full-length copy of M RNA in such an orientation that transcription from the T7 promoter would give positivense RNA. Subsequent analysis revealed that pSPM2 had a single base deletion at position 2419 of the M RNA sequence which was corrected as previously described (Holness et al., 1989). The final construct was designated pSPM203 (Fig. 1). Transcription in vitro of SphI-linearized pSPM203 with T7 RNA polymerase resulted in the synthesis of RNA identical in size with natural virion M RNA. Translation of the transcripts in messenger-dependent rabbit reticulocyte lysates resulted in the synthesis of two proteins identical in size to the 105K and 95K proteins synthesized when the lysate was programmed with M RNA isolated from CPMV virus particles (results not shown).

To construct bicistronic transcription plasmids containing CPMV M RNA sequences, the SalI chloramphenicol acetyltransferase (CAT) cartridge (Close & Rodriguez, 1982) was obtained by digesting plasmid pJII-102 (Sleat et al., 1987) with SalI and isolating the 779 bp fragment. The SalI CAT cartridge was ligated into the unique SalI site of pSPM203 which lies between the T7 promoter and the start of the CPMV M RNA-specific region (see Fig. 1). Two recombinants, pCAT203-5 and pCAT203-8, which had the CAT gene inserted in opposite orientations, were identified by restriction enzyme analysis. As controls, two clones, pSPTCAT-8 and pSPTCAT-10, which had the SalI CAT cartridge alone cloned in opposite directions in the SalI site of the transcription vector pSPT18 were also constructed. The clone p203ΔBgII, in which the AUG at position 161 is deleted, was produced by digesting pSPM203 with BamHI and BgII, isolating the two

![Fig. 1. Representation of the pSPT18-derived plasmids used to investigate the translation of CPMV M RNA. Only the relevant portion of each plasmid is shown. The black box represents the T7 promoter, and the open reading frames encoding CAT and the CPMV-specific proteins are shown as open and shaded boxes, respectively. The arrow in the CAT gene indicates its orientation with respect to the T7 promoter. The positions of the AUG codons used to initiate the CPMV 105K and 95K proteins are indicated, as is the position of the UAA terminator for these proteins.](image-url)
largest fragments and re-ligating them (Fig. 1). The construction and properties of the T7 plasmids pKSMRClaCAT and pKSMRHCAT have been described previously (Belsham & Brangwyn, 1990). They are both bicistronic constructs containing an upstream fowl plague (avian influenza) virus matrix protein gene and a downstream CAT gene. The two genes are separated by either nucleotides 369 to 803 (pKSMRClaCAT) or nucleotides 369 to 742 (pKSMRHCAT) from the 5' non-translated region of foot-and-mouth disease virus (FMDV) RNA.

To investigate whether internal initiation occurs on CPMV M RNA, we used the eukaryotic transient expression system developed by Fuerst et al. (1986). BSC40 cells were infected with the recombinant vaccinia virus vTF7-3 (which expresses T7 RNA polymerase) and 5 μg of each of the various plasmids was introduced using lipofectin (BRL) as described previously (Belsham et al., 1990; Belsham & Brangwyn, 1990). The cells were labelled with [35S]methionine, extracts were prepared and immunoprecipitated with either anti-CPMV serum or anti-influenza virus matrix protein serum as appropriate, and analysed by SDS-PAGE. Immunoprecipitates from cells transfected with plasmid pSPM203 contain significant amounts of two high Mr (> 100K) proteins and lesser amounts of a smaller protein (approx. 97K) not seen in control extracts (Fig. 2a). The identity of these products was established by comparison with the results obtained with pSPMABgl in which synthesis of the largest protein is abolished (Fig. 2a). This is consistent with the largest protein being initiated at AUG at position 161 (i.e. being the 105K protein). The other major protein with an apparent Mr of > 100K is almost certainly the 95K protein. The identity of the protein of lower abundance with an apparent Mr of 97K has not been established but it is conceivable that it is the result of initiation of translation at position 524.

None of the CPMV-specific proteins is detectable in extracts from cells transfected with either pCAT203-5 or pCAT203-8 (Fig. 2a). Both these constructs contain a CAT gene inserted between the T7 promoter and a full-length cDNA copy of CPMV M RNA and differ only in the orientation of the CAT gene (Fig. 1). Most importantly they both contain the whole of the 5' non-coding region of CPMV M RNA between the CAT gene and the CPMV coding region. If picornavirus-like internal initiation was a significant mechanism for the synthesis of the 95K protein, one would expect to see detectable levels of it in extracts of cells transfected with either pCAT203-5 or pCAT203-8 but this is clearly not the case. We estimate that our methodology would easily have detected a level of synthesis of the CPMV protein 5% of that seen in the case of pSPM203. From these results, we conclude that internal initiation, if it occurs at all, is not a major mechanism for the synthesis of the CPMV 95K protein, at least in the transient expression system which we have used for these studies. It is also clear from Fig. 2 that the orientation of the upstream CAT gene has no effect on whether or not CPMV-specific proteins are synthesized.

Apart from the obvious conclusion from the above results that internal initiation does not occur on CPMV M RNA, there are two possible trivial reasons as to why no synthesis of CPMV-specific proteins occurred in response to transfection of cells with pCAT203-5 and pCAT203-8. The first is that no mRNA may have been synthesized, and the second is that the system used may have been incapable of detecting internal initiation. To eliminate the first possibility, extracts from cells transfected with the various constructs were assayed for CAT activity as described previously (Belsham & Brangwyn, 1990). Extracts of cells transfected with pSPTCAT-8 and pCAT203-8 which both contain CAT genes in the correct orientation immediately downstream of the T7 promoter (Fig. 1) contain high levels of CAT activity (Fig. 3a). In addition, large amounts of [35S]-labelled CAT...
Fig. 3. Assay of CAT gene expression in extracts of vTF7-3-infected BSC40 cells transfected with either the constructs used to investigate the translation of CPMV M RNA (a) or constructs containing portions of the FMDV 5' untranslated region (b). The control lane in (a) consists of unreacted 14C-labelled chloramphenicol (Cm). The acetylated forms in order of increasing mobility are 1-acetylchloramphenicol (1-AcCm), 3-acetylchloramphenicol (3-AcCm) and 1,3-diacetylchloramphenicol (1,3-DiAcCm). The origin (O) is also marked.

could be detected when extracts of cells transfected with pSPTCAT-8 or pCAT203-8 were immunoprecipitated with anti-CAT serum (data not shown). These observations demonstrate that mRNA is synthesized in both cases and that the failure of p203CAT-8 to synthesize CPMV-specific proteins is not due to the absence of mRNA. Not surprisingly, extracts of cells transfected with pSPTCAT-10 and p203CAT-5, which contain the CAT gene in the antisense orientation, or extracts of cells transfected with pSPM203 and p203ABgl, which do not contain the CAT gene, did not contain detectable CAT activity (Fig. 3a).

To confirm that in the assays used to investigate the translation of CPMV M RNA it was possible to detect internal initiation, cells were transfected with either pKSMRClaCAT or pKSMRHCAT. Synthesis of the upstream influenza virus matrix protein could be detected in extracts from cells transfected with either of the constructs (Fig. 2b). Nucleotides 369 to 803 from FMDV RNA have previously been shown to be sufficient to direct internal initiation on bicistronic constructs (Belsham & Brangwyn, 1990) and it was therefore anticipated that extracts from cells transfected with pKSMRClaCAT would contain detectable levels of CAT activity. This proved to be the case (Fig. 3b). Indeed, the amount of CAT activity synthesized from the bicistronic construct, pKSMRClaCAT, was very similar to the amount synthesized in cells transfected with pSPTCAT-8 or pCAT203-8 where the CAT gene is 5' proximal (Fig. 3b). This clearly demonstrates the efficiency of the FMDV internal ribosome entry site (IRES) at promoting internal initiation. Indeed, CAT synthesis driven by the FMDV IRES could easily be detected by immunoprecipitation of 35S-labelled cell extracts using anti-CAT serum (data not shown). That the presence of an intact FMDV IRES is required for the expression of the downstream CAT gene in this system is demonstrated by the lack of detectable CAT activity in extracts of cells transfected with pKSMRHCAT which contains only a portion of the IRES (Belsham & Brangwyn, 1990) (Fig. 3b). These results confirm that the system chosen to investigate the possibility of internal initiation on CPMV M RNA is capable of demonstrating the phenomenon. Furthermore, it is clear that if CPMV M RNA contained a sequence upstream of position 512 which promoted internal initiation with anything like the efficiency of the FMDV IRES, synthesis of the 95K protein would have been detected by immunoprecipitation of extracts of cells transfected with pCAT203-5 or pCAT203-8.

The results described above demonstrate that internal initiation of the type which occurs in picornaviruses does not occur to a significant extent on CPMV M RNA, at least in the system used here. The system was selected for a number of reasons: it is an in vivo rather than an in vitro system, it had previously been shown to be capable of demonstrating highly efficient internal initiation in FMDV (Belsham & Brangwyn, 1990) and it is not prone to give the false positives for internal initiation (see the results with pKSMRClaCAT and pKSMRHCAT) often associated with in vitro translation systems such as rabbit reticulocyte lysate (Dasso & Jackson, 1989; Kozak, 1989). The only defect in the system from the point of view of investigating the translation of CPMV is that it is heterologous, being an animal rather than a plant system. The same criticism, of course, applies to translation studies conducted in rabbit reticulocyte lysate (e.g. Thomas et al., 1991). The fact that correct synthesis of the 105K and 95K was observed within cells transfected with the monocistronic CPMV constructs pSPM203 and p203ABgl argues that the system chosen is a suitable one for studying the translation strategy of CPMV. From the results presented here, it therefore seems reasonable to conclude that internal initiation is not responsible for the synthesis of significant amounts, if any, of the CPMV 95K protein. From our experiments, however, we cannot rule out the possibility that internal initiation is responsible for the synthesis of minor, undetected
amounts of the 95K protein but it is certainly not the major mechanism for its synthesis.

The mechanism for the synthesis of most, if not all, of the 95K protein has not been unambiguously determined. However, it seems probable that it involves the ‘leaky scanning’ mechanism discussed earlier. An alternative ‘re-initiation’ mechanism (see Kozak, 1989) for the synthesis of the 95K protein is also conceivable. This would involve initiation at the 5’ proximal AUG in the CPMV M RNA sequence (position 115), termination at an in-phase UGA (position 175) and re-initiation at AUG at position 512. However, this mechanism would be specific to CPMV M RNA and could not operate with the M RNAs of two other comoviruses, red clover mottle virus (RCMV) or bean pod mottle virus (BPMV), which do not have an out of phase AUG upstream of the long open reading frame (Shanks et al., 1986; MacFarlane et al., 1991).

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References


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