

A Tobamovirus Genome That Contains an Internal Ribosome Entry Site Functional *in Vitro*

P. A. Ivanov, O. V. Karpova, M. V. Skulachev, O. L. Tomashevskaya, N. P. Rodionova,
Yu. L. Dorokhov, and J. G. Atabekov¹

Department of Virology and A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia

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Most eukaryotic mRNAs are translated by a "scanning ribosome" mechanism. We have found that unlike the type member of the genus *Tobamovirus*, translation of the 3'-proximal coat protein (CP) gene of a crucifer infecting tobamovirus (crTMV) (Dorokhov *et al.*, 1993; 1994) occurred *in vitro* by an internal ribosome entry mechanism. Three types of synthetic dicistronic RNA transcripts were constructed and translated *in vitro*: (i) "MP-CP-3'NTR" transcripts contained movement protein (MP) gene, CP gene and the 3'-nontranslated region of crTMV RNA. These constructs were structurally equivalent to dicistronic subgenomic RNAs produced by tobamoviruses *in vivo*. (ii) " Δ NPT-CP" transcripts contained partially truncated neomycin phosphotransferase I gene and CP gene. (iii) "CP-GUS" transcripts contained the first CP gene and the gene of *Escherichia coli* β -glucuronidase (GUS) at the 3'-proximal position. The results indicated that the 148-nt region upstream of the CP gene of crTMV RNA contained an internal ribosome entry site (IRES_{CP}) promoting internal initiation of translation *in vitro*. Dicistronic IRES_{CP}, containing chimeric mRNAs with the 5'-terminal stem-loop structure preventing translation of the first gene (MP, Δ NPT, or CP), expressed the CP or GUS genes despite their 3'-proximal localization. The capacity of crTMV IRES_{CP} for mediating internal translation distinguishes this CP tobamovirus from the well-known-type member of the genus, TMV UI. The equivalent 148-nt sequence from TMV RNA was incapable of mediating internal translation. Two mutants were used to study structural elements of IRES_{CP}. It was concluded that integrity of IRES_{CP} was essential for internal initiation. The crTMV provides a new example of internal initiation of translation, which is markedly distinct from IRESs shown for picornaviruses and other viral and eukaryotic mRNAs. © 1997 Academic Press

INTRODUCTION

According to the ribosome scanning model, traditional for most eukaryotic mRNAs, the 40S ribosomal subunit binds to the 5' cap and moves along the nontranslated 5'-sequence until it reaches an AUG codon (Kozak, 1986, 1989). Although, for the majority of eukaryotic mRNAs, only the first ORF is translationally active, there are different mechanisms by which mRNA may function polycistronically (Kozak, 1986). If the first AUG has unfavorable sequence context, 40S subunits may bypass it and initiate at downstream AUG codons (leaky scanning mechanism). Termination-reinitiation has also been suggested to explain the initiation of translation of functionally dicistronic eukaryotic mRNAs (Kozak, 1989). Another mechanism for discontinuous ribosome migration ("shunting") on mRNA has been recently proposed for cauliflower mosaic virus 35S RNA (Fütterer *et al.*, 1993).

In contrast to the majority of eukaryotic mRNAs, the initiation of translation of a variety of viral and cellular RNAs takes place by an alternative mechanism of internal ribosome entry. A picornaviral 5'-nontranslated region (5'NTR) contains a so-called internal ribosome entry site (IRES) or a ribosome landing pad (Jang *et al.*, 1988;

Pelletier and Sonenberg, 1988; Jackson *et al.*, 1990), which is folded into a complex secondary structure and contains a pyrimidine-rich tract followed by an AUG codon (reviewed by Agol, 1991; Wimmer *et al.*, 1993; Sonenberg and Pelletier, 1989; Belsham and Sonenberg, 1996). Internal ribosome entry has also been reported for other viral RNAs, such as hepatitis C virus (Tsukijama-Kohara *et al.*, 1992; Wang *et al.*, 1993; Reynolds *et al.*, 1995; Rijnbrand *et al.*, 1995), murine leukemia virus (Berlioz and Darlix, 1995), Moloney murine leukemia virus (Mo-MULV) (Vagner *et al.*, 1995b), Harvey murine sarcoma virus (Berlioz *et al.*, 1995), tricistronic subgenomic RNA of infectious bronchitis virus (Liu and Inglis, 1992; Le *et al.*, 1994), some potyviral RNAs (Levis and Astier-Manificier, 1993; Basso *et al.*, 1994), cowpea mosaic virus (Thomas *et al.*, 1991) and some cellular RNAs (Macejak and Sarnow, 1991; Jackson, 1991; Oh *et al.*, 1992; Vittorioso *et al.*, 1994; Vagner *et al.*, 1995a; Gan and Rhoads, 1996).

The genome of tobamoviruses (TMV UI is the type member) contains four large open reading frames (ORFs). *In vitro* translation experiments have shown that the two components of the replicase (the 130-kDa and its readthrough 183-kDa proteins) are translated directly from the genomic RNA (Pelham and Jackson, 1976). The other two proteins (30-kDa movement proteins, MP and CP) are translated from two individual subgenomic RNAs

¹ To whom correspondence and reprint requests should be addressed. Fax: (095) 9380601; E-mail: Atabekov@virus.genebee.msu.u.

(sgRNAs). The structurally dicistronic I₂ sgRNA is translated to give the 30-kDa MP, while its 3'-terminal CP gene is silent and a monocistronic sgRNA codes the CP (for review, see Palukaitis and Zaitlin, 1986).

Recently we have isolated from *Oleracia officinalis* L. plants and sequenced the genome (6312 nucleotides) of a new tobamovirus, crTMV (Dorokhov *et al.*, 1993, 1994). A peculiar feature of crTMV is its ability to infect systemically the members of *Cruciferae* family. The crTMV RNA contains four ORFs encoding the proteins of 122 kDa (ORF1), 178 kDa (ORF2), the readthrough product of 122-kDa, 30-kDa MP (ORF3), and 17-kDa CP (ORF4). Unlike other tobamoviruses, the coding regions of the MP and CP genes of crTMV overlap for 25 codons; i.e., 5' of the CP coding region are sequences encoding MP (Dorokhov *et al.*, 1994). In this study we have shown that, unlike the RNA of typical tobamoviruses, translation of the 3'-proximal CP gene of crTMV RNA occurs *in vitro* by a mechanism of internal ribosome entry, which is mediated by a specific sequence element (IRES_{CP}).

MATERIALS AND METHODS

Viruses and RNA

TMV UI and crTMV were isolated from systemically infected *Nicotiana tabacum* L. cv. Samsun plants as described previously (Dorokhov *et al.*, 1994).

Northern blot analysis

The general procedure of Sambrook *et al.* (1989) was used for Northern blot hybridizations. The filters were probed with ³²P-labeled cDNA prepared with a random deoxyhexamers cDNA labeling kit (Boehringer) on the isolated cloned DNA fragment specific for crTMV CP gene as described in the manufacturer's protocol. RNA analyzed was extracted from crTMV-infected *N. tabacum* leaves according to Vaerword *et al.* (1989). RNA transcripts corresponding to 3'-terminal 1400- and 700-nt fragments of crTMV RNA used as a size controls were obtained by *in vitro* transcription of cDNA constructs (Dorokhov *et al.*, 1994).

Plasmid constructs

A cDNA clone p208 was obtained in RT-PCR using genomic crTMV RNA with the 5'-oligonucleotide primer corresponding to nucleotides 4877–4900 of crTMV RNA and containing an *Nco*I site, and the 3' oligonucleotide primer, complementary to nucleotides 5788–5808 of crTMV RNA. This cDNA fragment was given blunt ends with T4-DNA polymerase and ligated to *Sma*I-cut pGEM-3z (Promega) to give pG3-208. The T7 RNA-polymerase transcript from plasmid pG3-208 contains a region corresponding to nucleotides 4877–5808 (*Sma*I site) of crTMV RNA. Cloning of the *Hind*III/*Sac*I fragment from pG3-208 into pBluescript II SK+ resulted in formation of

pTBSMPΔCPS_{*Sma*I} (Fig. 2E). The RNA transcript from this plasmid contains a 98-nt-long 5'-nontranslated leader which can be folded into a putative hairpin-loop structure presented in Fig. 2G and referred to as translation blocking sequence (TBS).

A cDNA synthesis kit (Promega), based on the method of Gubler and Hoffman (1983), was used to prepare the double-stranded oligo(dT)-primed cDNA from 3'-polyadenylated crTMV RNA and to obtain clone pG3A16. This clone corresponded to nucleotides 5431–6312 of crTMV RNA. The *Eco*RI/*Sac*I fragment of pG3A16 was ligated with *Eco*RI/*Sac*I-cut pTBSMPΔCPS_{*Sma*I} to yield pTBSMP_{CP} (Fig. 2B). A cDNA synthesis system (Promega) was used to prepare the double-stranded blunt-ended cDNA from genomic crTMV RNA using specific primers corresponding to nucleotides 5020–5040 and 4627–4647 of genomic crTMV RNA. This cDNA corresponded to nucleotides 4647–5040 of TMV RNA and contained a 5'-terminal 230-nt sequence (L) upstream from the 30-kDa MP gene and the 5'-terminal coding part of the MP gene. It was inserted into the *Sma*I-cut pGEM-7z to give pG7S20. The *Xho*I/*Bgl*II fragment of pG7S20 was inserted into the *Xho*I/*Bgl*II-cut pTBSMP_{CP} to give pLMP_{CP} (Fig. 2A). To construct pLMPΔCPS_{*Sma*I} (Fig. 2D), the *Xho*I/*Bgl*II fragment of pG7S20 was ligated to *Xho*I/*Bgl*II-cut pTBSMPΔCPS_{*Sma*I}.

To obtain pΔMPE_{*Eco*CP} (Fig. 2F) the *Eco*RI/*Sac*I (filled) fragment of pTBSMP_{CP} was inserted into the *Eco*RI/*Sma*I-cut pBluescript II SK+. The plasmid pTBSMP_{CP} was digested with *Nco*I and *Xho*I, filled with Klenow fragment, and religated, yielding pΔTBSMP_{CP} (Fig. 2C), which contained a polylinker-derived 5'-nontranslated sequence (41-nt) upstream of the 30-kDa MP gene.

The following constructs were obtained using standard gene engineering techniques and pGEM-3z and pBluescript plasmids. (i) The construct pHβΔNPT_{CP} (Fig. 4C) contained T7 promoter, inverted tandem repeat to form a stable hairpin structure (ΔG = –90 kcal/mol) in mRNA, the β-sequence of potato virus X (PVX) genomic RNA (Tomashevskaya *et al.*, 1993), the 5'-terminal part of the neomycin phosphotransferase I gene (ΔNPTI), and the CP gene of crTMV, which was inserted as a PCR product from the plasmid pΔMPE_{*Eco*CP} and contained no crTMV-derived sequence upstream from the initiation codon of the CP gene. Therefore the pHβΔNPT_{CP} construct contained two genes, separated by polylinker-derived spacer. (ii) The construct pHβΔNPTIRES_{CP}CP (Fig. 4D) differs from previous constructs by the presence of IRES_{CP} right upstream of the CP gene in the intercistronic area. (iii) The construct pHβΔNPTΔ5'IRES_{CP}CP (Fig. 4E) differs from the pHβΔNPTIRES_{CP}CP by the deletion of the 5'-terminal 113-nt region of IRES_{CP}. (iv) The construct pHβΔNPTΔ3'IRES_{CP}CP (Fig. 4F) contains the 5'-terminal part of IRES_{CP} and lacks the 3'-terminal 35-nt region of the IRES between ΔNPT and CP genes. (v) The construct pHCP contained T7 promoter, inverted tandem re-

peat (hairpin structure H in Fig. 7), and the CP gene of crTMV, which was inserted into pBluescript SK II + plasmid as a PCR product obtained from p Δ MP Eco CP plasmid as it was described in (i). (vi) The construct pHCP $IRES_{CP}$ -GUS (Fig. 7A) contained T7 promoter, inverted tandem repeat, CP gene of crTMV, $IRES_{CP}$, and β -glucuronidase gene (GUS). To clone GUS gene right downstream of the $IRES_{CP}$ Nco I site was introduced at the 3'-end of $IRES_{CP}$, using corresponding oligonucleotides for PCR. (vii) In construct pHCP UI^{SP} -GUS (Fig. 7A) the 148-nt region preceding the AUG of the CP gene in TMV UI RNA was cloned as intercistronic spacer (UI^{SP}) between the CP and GUS genes. (viii) The construct UI^{SP} -GUS (Fig. 7A) contained T7 promoter, the same 148-nt sequence UI^{SP} from TMV UI and GUS gene, cloned downstream of the UI^{SP} sequence. Important regions of plasmids were analyzed by dideoxy sequencing.

In vitro transcription and translation; Immunoprecipitation of the CP

The plasmids pLMPCP, pTBSMPCP, p Δ TBSMPCP, pLMP Δ CP Sma I, pTBSMPCP Δ CP Sma I, p Δ MP Eco CP, HCP $IRES_{CP}$ -GUS, HCP UI^{SP} -GUS, HCP $\alpha\beta$ -GUS, $\alpha\beta$ -GUS, and UI^{SP} -GUS were linearized by *Sac*I; the pLC, pH β Δ NPT3, pH β Δ NPTCP, pH β Δ NPT Δ 5' $IRES_{CP}$ CP, and pH β Δ NPT Δ 3' $IRES_{CP}$ CP were linearized by *Hind*III; the plasmid pH β Δ NPT $IRES_{CP}$ CP was linearized by *Sph*I and filled in by T4 DNA polymerase. The recombinant plasmids were transcribed *in vitro* as described earlier (Tomashevskaya *et al.*, 1993) and agarose gel electrophoresis of RNA transcripts confirmed that they were intact. The RNA concentration was quantified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and spectrophotometry.

Synthetic mRNA transcripts were translated in RRL as described earlier (Morozov *et al.*, 1990; Smirnyagina *et al.*, 1991) in Krebs-2 ascite cell-free extract as described by Pelletier and Sonenberg (1988) and in wheat germ extracts (WGE) according to the manufacturer's (Promega) protocol in the presence of [35 S]methionine for 60 min at 30°. Radiolabeled translation products were analyzed by SDS-PAGE and localized by autoradiography on the dried gel. Immunoprecipitation of 35 S-labeled CP, the product of *in vitro* translation, was carried out as described by Morozov *et al.* (1990).

Stability of dicistronic $IRES_{CP}$ containing RNA transcripts upon *in vitro* translation

Radioactive transcripts were obtained by standard procedure (see above) using [α - 32 P]UTP instead of nonradioactive UTP. Radioactivity of transcripts was about 3×10^6 cpm/ μ g RNA; 5 μ g of each transcript was added to *in vitro* translation mixture (RRL) and incubated at 30° for 60 min. Then RNA was isolated from the translation mixture with phenol, preincubated with ethanol, and solu-

bilized in RNase-free water. Aliquots (radioactivity about 150×10^3 cpm) of each sample were electrophoresed in a thin layer (4 mm) of 1.5% agarose and labeled transcripts were detected by autoradiography.

RESULTS

Translation of the agarose gel-purified preparations of crTMV genomic RNA in WGE (Fig. 1A) and RRL (not shown) resulted in synthesis of a product which corresponded in size to CP and was specifically immunoprecipitated by antibodies raised against crTMV CP. No CP was directed by TMV UI RNA taken as negative control (Fig. 1A). These results suggested that the crTMV CP gene expression may occur by an internal ribosome entry mechanism. Alternatively, a monocistronic CP sgRNA could be synthesized to produce the CP in parallel with the internal translation of the CP gene from genomic and/or I_2 sgRNA. In a separate series of Northern blot analyses of RNAs from crTMV-infected tobacco leaves, a traditional pair of subgenomic-size RNAs closely following I_2 sgRNA and CP sgRNA bands, respectively, was revealed (Fig. 1B). Consequently, our assumption that the CP gene in genomic crTMV RNA was accessible to the ribosomes was hampered by the presence of the CP sgRNA *in vivo*. To confirm unambiguously that initiation of the crTMV CP gene translation may proceed through an internal ribosome entry we constructed a series of dicistronic RNAs to show that a putative IRES positioned between the coding sequences allows initiation from the 3'-proximal gene.

Translation of dicistronic "MP-CP-3'NTR" crTMV RNA

In the first series of experiments two types of synthetic dicistronic transcripts that contained the MP gene, CP gene, and the 3'-NTR (Fig. 2) were translated in RRL (Fig. 3) and Krebs-2 ascite cell extracts (data not presented). The transcripts of the first type (LMPCP) represented the 3'-proximal 1665-nt fragment of crTMV RNA and contained the 230-nt 5' nontranslated leader sequence (L) derived from the replicase gene of crTMV RNA (Fig. 2A). Translation of structurally dicistronic LMPCP transcript *in vitro* yielded two major products of 30 and 17 kDa (Fig. 3). The latter product was identified as the crTMV CP by immunoprecipitation of [35 S]methionine-labeled translation products with antibodies against crTMV (data not presented). Additional evidence for the identification of CP and 30-kDa MP in cell-free systems was provided by examining the translation products of the monocistronic transcript LMP Δ CP Sma I (with the CP gene and 3'NTR deleted; Fig. 2D) and Δ MP Eco CP transcript (with deleted 30-kDa gene; Fig. 2F). These transcripts produced single proteins, the 30-kDa MP or CP, respectively (Fig. 3B). Our results indicate that the LMPCP transcripts are functionally dicistronic in RRL translation system (LMPCP in Fig. 3), although it does not allow us to conclude unambigu-

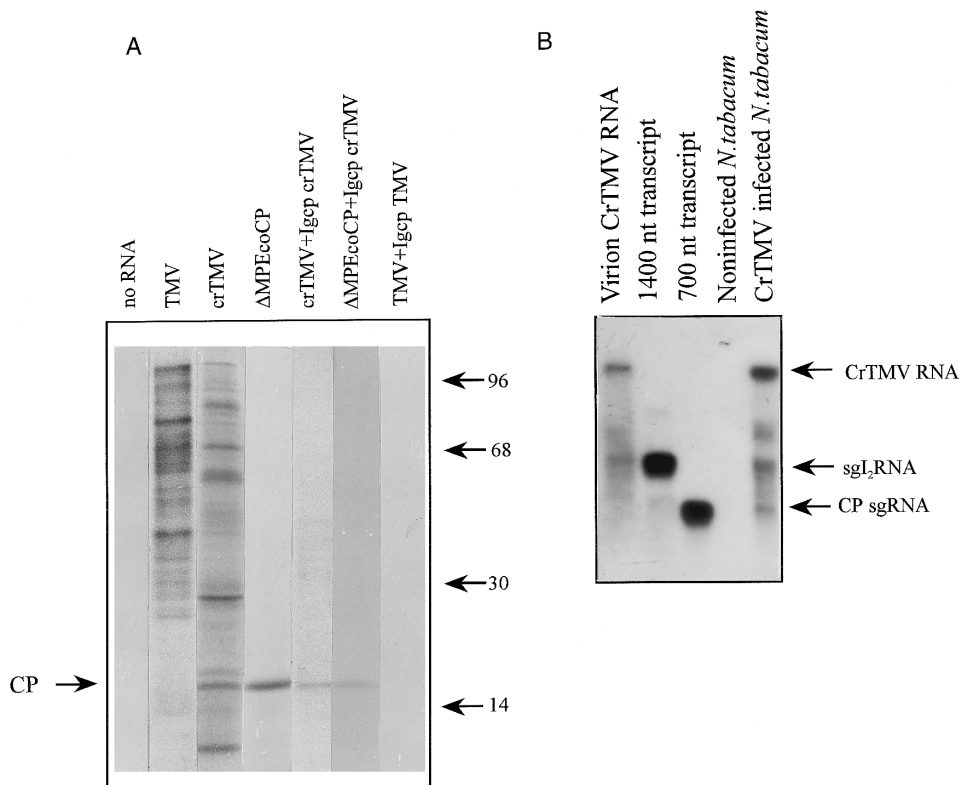


FIG. 1. (A) Production of the coat protein in wheat germ extracts directed by crTMV genomic RNA. Autoradiogram of gradient 8–20% polyacrylamide–SDS gel of [³⁵S]methionine products. Concentration of RNA is 40 μg/ml. Lanes from left to right: no RNA added; the products directed by TMV UI genomic RNA; by crTMV genomic RNA; by ΔMPEcoCP-monocistronic RNA transcript carrying the crTMV CP gene (Fig. 2F); (crTMV + IgCPCrTMV)-immunoprecipitation of translation products of crTMV genomic RNA with antibodies against crTMV CP; (ΔMPEcoCP + IgcpCrTMV)-immunoprecipitation of translation product of ΔMPEcoCP transcript with antibodies against crTMV CP; (TMV + IgcpCrTMV)-immunoprecipitation of translation product of TMV UI RNA with antibodies against TMV UI CP (negative control). The positions of CP and of marker proteins (in kDa) are indicated. (B) Northern blot analysis of crTMV-specific RNAs accumulated in crTMV-infected *N. tabacum*: virion crTMV RNA; RNA transcripts comprising 3'-terminal 1400- and 700-nt fragments of crTMV RNA, respectively; RNA extracted from noninfected and crTMV-infected tobacco 3 days postinoculation. Positions of subgenomic I₂ RNA, CP-coding RNA, and genomic crTMV RNA are marked.

ously if expression of the second CP gene is due to internal initiation, leaky scanning, or termination-reinitiation. To examine the translation mechanism of CP gene further, we constructed a second type of dicistronic transcript (TBSMPCP, Fig. 2B), which contained a polylinker-derived 98-nt sequence upstream from the MP gene. This 5'-leader is referred to as the translation blocking sequence (TBS), since the presence of TBS upstream of the 30-kDa MP ORF abolished translation of the MP gene from monocistronic transcript (TBSMPΔCPS_{mal}; Fig. 3B). This effect is likely due to the ability of TBS to produce a potentially stable hairpin loop structure at the 5' terminus of the chimeric mRNA (Fig. 2G).

Figure 3 shows that the TBS abolished translation of the first 30-kDa MP gene within dicistronic (TBSMPCP) transcripts, whereas the CP gene was still expressed. This implies that expression of the 3'-proximal CP gene from synthetic dicistronic transcript is mediated by IRES localized upstream of the CP gene. This conclusion is substantiated by the results of translation of the ΔTBSMPCP transcript (Fig. 3A) from which the most part

(57 5'-terminal nucleotides) of TBS was deleted (Fig. 2C). Deletion of the 5' TBS restored the dicistronic character of mRNA (Fig. 3A).

It should be noted that the efficiency of the 3'-proximal CP gene translation from dicistronic mRNA depended significantly on the possibility of the 5'-proximal MP gene translation. Expression of the internally translated CP gene from functionally monocistronic TBS MPCP transcript was higher than that of functionally dicistronic LMPCP RNA (Figs. 3A and 3B). This result could be due to the competition of the CP gene with the MP gene for the ribosomes or/and a translation initiation factor(s) in cell-free translation system.

Translation of dicistronic "ΔNPTI-CP" chimeric RNA

We next constructed several dicistronic chimeric T7 RNA transcripts containing modified (3'-truncated) neomycin phosphotransferase I gene (ΔNPTI) and the CP gene of crTMV (Figs. 4C–F). The ΔNPTI ORF corresponds to the N-terminal 19-kDa fragment of neomycin phosphotransferase I. Two different types of construct

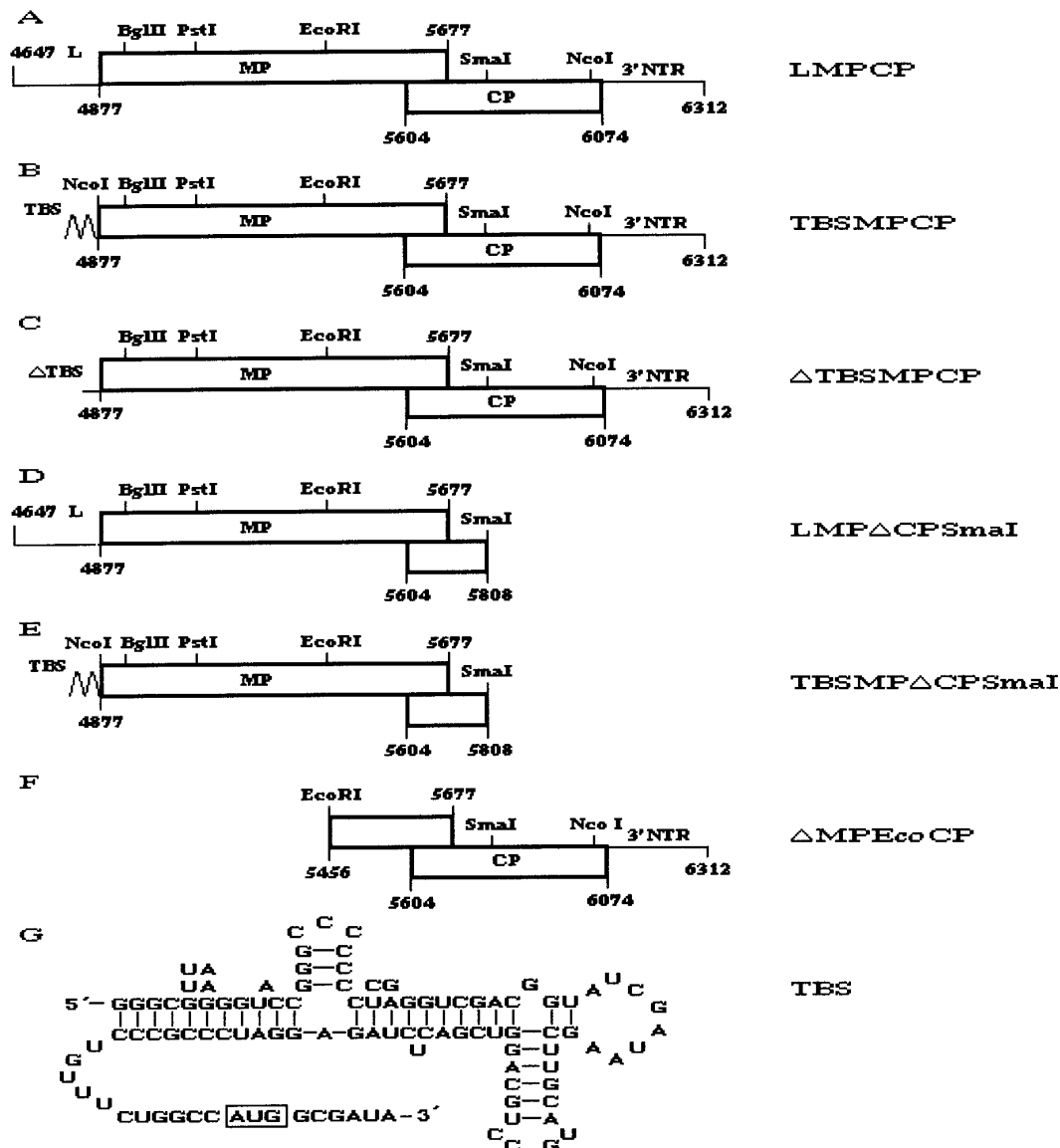


FIG. 2. Schematic representation of the dicistronic "MP-CP-3'NTR" crTMV RNA transcript and its derivatives generated *in vitro*: (A) Wild-type (LMPCP) transcript with 230-nt 5'-leader; (B) transcript TBSMPCP with the 5'-terminal 98-nt translation blocking sequence (TBS); (C) Δ TBSMPCP transcript from which 57-nt 5'-terminal region of TBS was deleted; (D and E) LMP Δ CPSmaI and TBSMP Δ CPSmaI transcripts with the CP gene deleted; (F) Δ MPEcoCP transcript from which the 5'-region of the 30-kDa MP gene was deleted. There are no AUG codons downstream of the *EcoRI* site within the 30-kDa ORF. Numbers indicate the corresponding nucleotides of crTMV RNA sequence (Dorokhov *et al.*, 1994). Boxes represent the ORFs, which are drawn to scale. The first nucleotide of each relevant start and stop codons is indicated. Abbreviations of transcripts correspond to appropriate cDNA clones (e.g., LMPCP transcript corresponds to pLMPCP cDNA). (G) Predicted secondary structure of the 5' terminal translation blocking sequence (TBS).

were used: (i) the monocistronic $\alpha\beta\Delta$ NPT transcript (Fig. 4A), containing the 5'-nontranslated sequence ($\alpha\beta$) corresponding to the 83-nt 5'-leader of potato virus X (PVX) RNA upstream of the Δ NPTI ORF. The $\alpha\beta$ -leader has been shown to strongly enhance the expression of adjacent genes in chimeric mRNAs (Smirnyagina *et al.*, 1991); however, the β -sequence (42 nt) was dispensable for translation (Tomashevskaya *et al.*, 1993). (ii) Monocistronic H β Δ NPT (Fig. 4B) and the various dicistronic transcripts contained a potentially stable (-90 kcal/mol) hairpin structure (H) inserted upstream of the 42-nt β -element immediately at their 5'-termini.

Under control of $\alpha\beta$, the translation enhancer, the Δ NPTI ORF was efficiently translated, directing synthesis of the 19-kDa protein as the major product ($\alpha\beta\Delta$ NPT in Fig. 5A). However, expression of the Δ NPTI ORF was completely blocked by an upstream hairpin-loop structure (H) (H β Δ NPT in Figs. 5A and B).

Neither of the two cistrons could be translated from the dicistronic transcripts (H β Δ NPTCP in Fig. 5A) in which the ORFs were separated only by a short (12-nt) polylinker-derived intercistronic spacer and the 5'-terminus was blocked by the H-sequence (Fig. 4C). Consequently, no internal initiation of the 3'-proximal CP

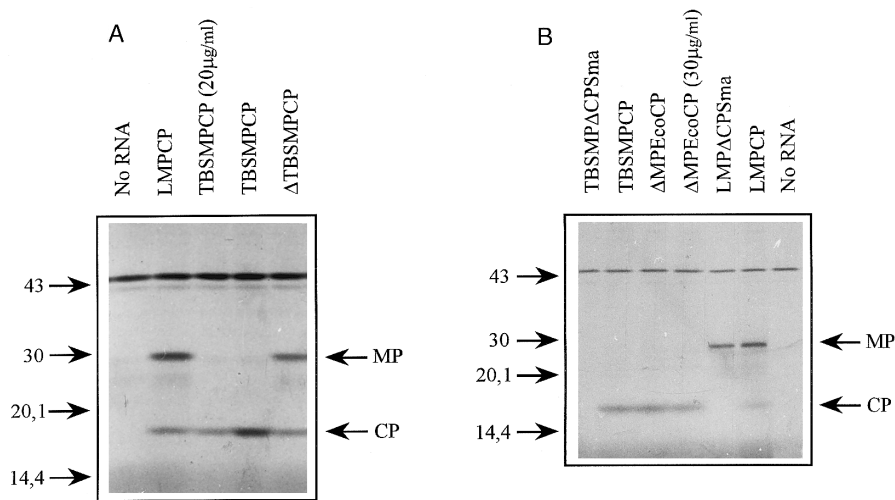


FIG. 3. Analysis of proteins directed *in vitro* by the "MP-CP-3'NTR" crTMV transcripts. Autoradiogram of gradient 8–20% polyacrylamide–SDS gels containing [³⁵S]methionine-labeled products directed by uncapped transcripts in RRL. Concentration of transcripts is 40 µg/ml unless otherwise indicated. The positions of CP and MP are marked and the positions of marker proteins (in kDa) are indicated. The designations above the panels are described in Fig. 2.

gene translation occurred in dicistronic transcript of this type. On the other hand, when the 148-nt region preceding the AUG codon of the CP gene of crTMV (IRES_{CP}) was inserted as the intercistronic spacer in the chimeric

dicistronic transcript βΔNPTIRES_{CP}CP (Fig. 4D), the CP gene was efficiently translated (Figs. 5A and 5B). These observations strongly suggest that the IRES_{CP} of crTMV mediates internal initiation of the CP gene translation

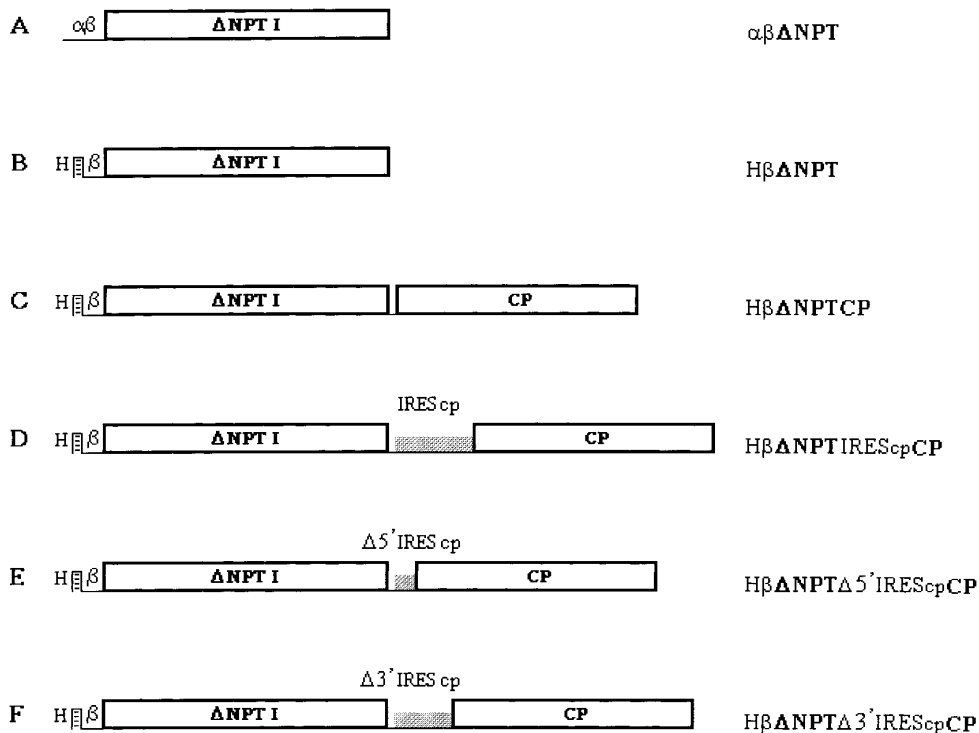


FIG. 4. Schematic representation of chimeric dicistronic "ΔNPTI-CP" mRNA and its derivatives generated *in vitro*: (A) αβΔNPT, the 3'-truncated ΔNPTI gene with the 83-nt αβ-sequence of PVX RNA as the 5'-leader; (B) HβΔNPT, the ΔNPTI gene with upstream sequence consisting of 80 nts forming a potentially stable hairpin (H) and the 42-nt β-sequence (β) of PVX RNA; (C) HβΔNPTCP, dicistronic transcript with 12 polylinker-derived (pd) nucleotides inserted between the ΔNPTI and CP genes; (D) HβΔNPTIRES_{CP}CP, in addition to the 12-nt pd insert, the 148-nt sequence located upstream of the CP gene of crTMV (IRES_{CP}) was inserted; (E) HβΔNPTΔ5' IRES_{CP}CP, only the 3'-proximal 35 nucleotides of the IRES_{CP} were inserted upstream of the CP gene; (F) HβΔNPTΔ3' IRES_{CP}CP, the 5'-proximal 113 nucleotides of the IRES_{CP} were inserted upstream of the CP gene.

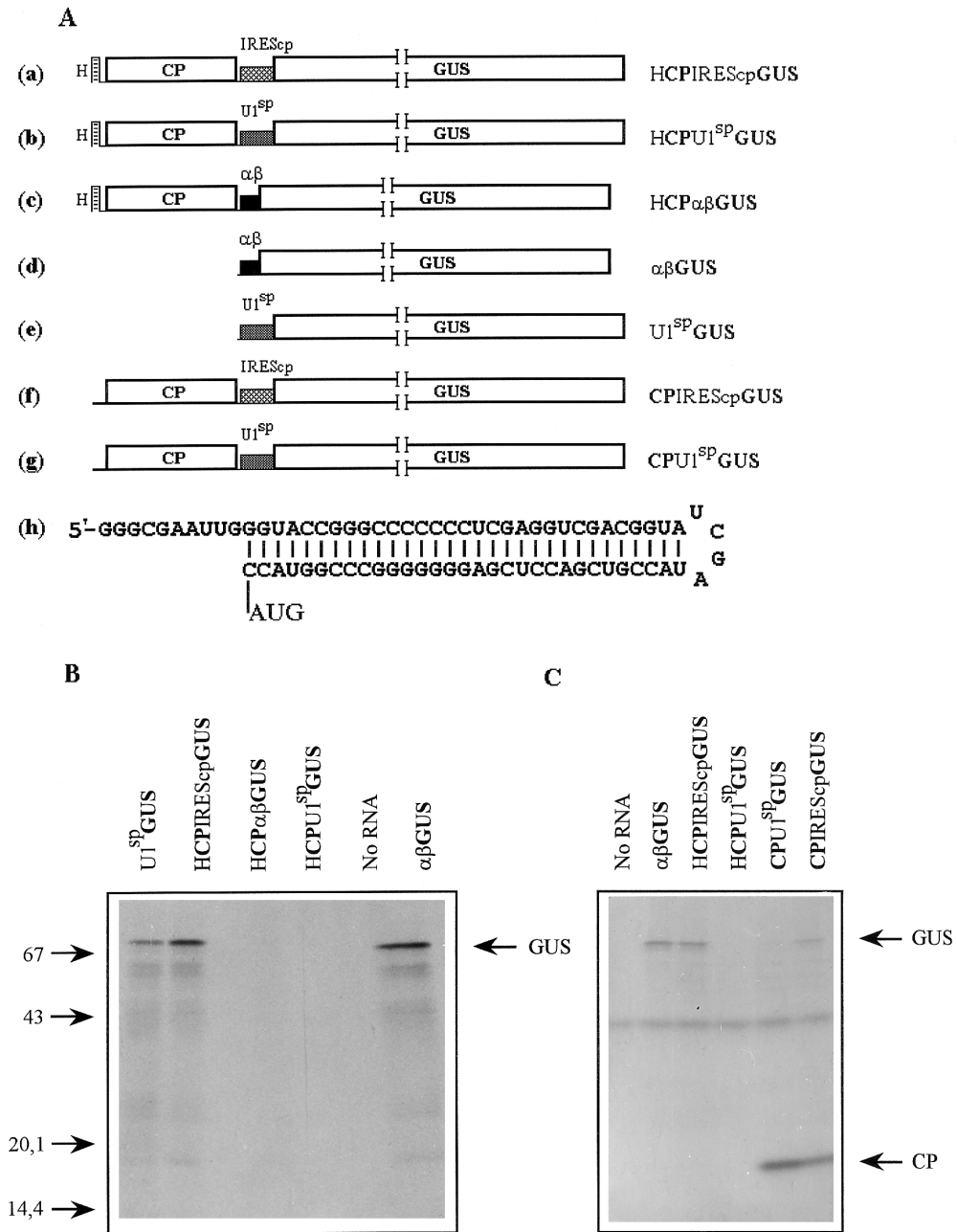


FIG. 7. (A) Schematic representation of the dicistronic "H-CP-IRES_{CP}GUS" transcript and its derivatives: HCPIRES_{CP}GUS, the 5'-proximal CP gene with upstream sequence forming a potentially stable hairpin (H) and GUS gene are separated by the IRES_{CP} (a); H-CP-UI^{SP}-GUS, the 148-nt region upstream of TMV UI CP gene (UI^{SP}) is inserted as the intercistronic spacer (b); HCPαβ-GUS, the αβ leader of PVX RNA was inserted as the intercistronic spacer (c); αβGUS, the GUS gene controlled by the 5'-αβ-translational enhancer (d); UI^{SP}GUS, the GUS gene carrying the 5'-terminal UI^{SP} sequence (e); CPIRES_{CP}GUS (f); and CPU^{SP}GUS (g); bicistronic transcripts corresponding to those in a and b, respectively, but lacking hairpin H at their 5'-termini; predicted secondary structure of the 5'-terminal stem-loop H upstream from the start codon of CP gene. Analysis of proteins directed in WGE (B) and RRL (C) by chimeric RNAs. The designations above the panels are described in A.

3'-proximal GUS gene (Fig. 7A). Three dicistronic constructs (a, b, and c in Fig. 7A) contained a potentially stable hairpin structure (H) upstream of the CP gene aimed at inhibiting this gene translation. Three different sequences were used as intercistronic spacers in these constructs. First was the 148-nt IRES_{CP}-containing sequence from crTMV RNA (HCP IRES_{CP}GUS in Fig. 7A); second was the 148-nt region (UI^{SP}) upstream

from start codon of the CP gene in TMV UI RNA (HCPU^{SP}GUS in Fig. 7A), and third was the 83-nt αβ-sequence from PVX RNA (HCPαβGUS in Fig. 7A). Two control monocistronic transcripts (αβGUS and UI^{SP}GUS) contained as 5'-nontranslated leaders the αβ-sequence and UI^{SP}-sequence, respectively. Two dicistronic transcripts (CPIRES_{CP}GUS and CPU^{SP}GUS) differed from those in Fig. 7A, a and b, in that they lacked

the 5'-terminal H sequence; i.e., their 5'-proximal CP gene was functional.

Figure 7B shows that the monocistronic transcripts UI^{SP}GUS and $\alpha\beta$ GUS were translated in WGE producing GUS. Expression of the 5'-proximal CP gene was completely blocked by the H-structure (Fig. 7B), whereas the 3'-proximal GUS gene was translated from dicistronic HCPIRES_{CP}GUS transcripts containing the 148-nt IRES_{CP} sequence as intercistronic spacer (Fig. 7B). It should be emphasized that neither of the two genes could be translated from the dicistronic transcript HCPUI^{SP}GUS in which the genes were separated by the 148-nt region preceding the AUG of the CP gene in TMV UI RNA (HCPUI^{SP}GUS in Fig. 7B). The same was true for dicistronic construct HCP $\alpha\beta$ GUS with the PVX $\alpha\beta$ -sequence as intercistronic spacer. Analogous results were obtained when the transcripts were translated in RRL (Fig. 7C): the 3'-proximal GUS gene was translated from HCPIRES_{CP}GUS but not from HCPUI^{SP}GUS dicistronic transcripts, which contained the 5'-terminal H sequence blocking the CP gene expression. On the other hand, translation of the second GUS gene from dicistronic transcript was reduced significantly when the first CP gene was functional (cf. HCPIRES_{CP}GUS and CPIRES_{CP}GUS in Fig. 7C). This observation could be due to the competition of two genes upon translation as it was proposed above in the case of the MP and CP genes translation from dicistronic LMPCP and TBSMPCP transcripts (Figs. 3A and 3B). These results show that in contrast to the type member of the genus *Tobamovirus* (TMV UI), the region upstream of the CP gene in crTMV RNA mediates an internal initiation of translation *in vitro*.

Stability of dicistronic IRES_{CP} containing RNA transcripts in *in vitro* translation system

The data presented above suggested that the 148-nt region upstream of the CP gene of crTMV RNA contained an IRES. However, an alternative model would be that the sequence thought to be an IRES_{CP} is instead an efficient cleavage site, particularly susceptible to nucleases in translation extracts. In order to show that the second cistron is not being translated from degraded IRES_{CP} containing dicistronic RNAs the ³²P-labeled dicistronic transcripts (HCPIRES_{CP}GUS and HCPUI^{SP}GUS) were incubated in translation system. The RNAs were then extracted with phenol, and RNA integrity was determined by gel electrophoresis and autoradiography. Figure 8 shows that no significant changes in electrophoretic mobility or integrity were observed after incubation.

DISCUSSION

It has long been known that only the 5'-proximal gene of tobamovirus genomic RNA can be directly translated by ribosomes. A dicistronic uncapped sgRNA called I₂ directs translation of only MP, while a second, capped

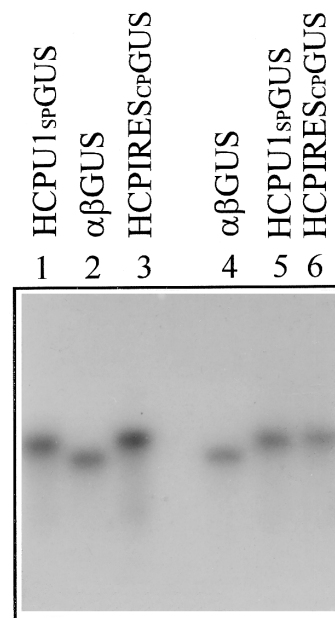


FIG. 8. Stability of dicistronic RNA transcripts upon *in vitro* translation. Autoradiogram of agarose gel containing ³²P-labeled transcripts incubated in RRL for 60 min at 30° (lanes from 1 to 3) and not incubated controls (lanes from 4 to 6). The designations above the panels are described in the legend to Fig. 7A.

monocistronic sgRNA directs synthesis of the CP (reviewed by Palukaitis and Zaitlin, 1986). Unexpectedly, our experiments have shown that, unlike TMV UI RNA, genomic RNA of crTMV tobamovirus directs synthesis of CP *in vitro* (Fig. 1A).

Dicistronic uncapped T7 RNA transcripts of the "MP-CP-3'NTR" series (Fig. 2) were synthesized which were analogous in genes organization to tobamovirus I₂ sgRNAs. It was found that the 3'-proximal CP gene could be translated from RNAs of this type even when the first gene (30K MP) translation was abolished (see TBSMPCP in Figs. 2 and 3). These results implied that an internal ribosome entry mechanism promoted translation initiation of CP gene in crTMV RNA *in vitro*.

The 148-nt region preceding CP gene of crTMV (designated as IRES_{CP}) was inserted as the intercistronic spacer in chimeric dicistronic mRNA (Fig. 4D). It was found that IRES_{CP} mediated initiation of the 3'-proximal CP gene translation under conditions which abolished the translation of the first cistron (see H β Δ NPTIRES_{CP}CP in Fig. 5). Although the boundaries of IRES_{CP} have not been defined precisely, the element is contained within a 148-nt region upstream of CP gene. RNA secondary structure indicates that the IRES_{CP} sequence may contain two stem-loop structures (Fig. 6). It remains to be investigated whether the purine-rich tract (Fig. 6) and/or the stem-loop structures are essential for internal initiation of translation. Our results show that deletion from IRES_{CP} of the 3'-terminal 35-nt region (transcript H β Δ NPT Δ 3'IR-ES_{CP}CP) completely blocks internal ribosome entry. At

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5537 ... atttcgcaat caatctaaga aaggaagtaa taagtatgta ggtaagagaa PMMV
5606 ... tataggagtt tccaaacctg tcggtttaga aagaaataaa gtaaggagtg Ob
5564 ... cgcgaaaggg aaaaatagta gtaatgatcg gtcagtgccc aacaagaact TMV
5555 ... aagagggtccg aaaaataata ataatttagg taaggggctg tcaggcgcaa L
5515 ... ggttccgaa aacaaaaaag aaatggtagg taataatggt aataataaga TMGMV
5580 ... taatagtcgt aataaatata attatgaaa tggtgatagt gatgttgga ORSV
5612 ... aagttcctat ttcagcggct gtaatggcaa gagatcccag ttataggtct CGMMV
-851 ... gtgccgagta cgatgtgaaa aggagtgtta aaaggacaaa atctgaaaac SHMV

5456 acg aattcgtcga ttcggttgca gcatttaag cggttgacaa ctttaaaaga CrTMV
5455 acg aattcgtcga ttcggttgca gcatttaag cggttgacaa tttcaggaag TVCV
5447 ... aattcgtcga ttcggttgca gcattcaagg cggttgacac tttcagaaag TMV-Cg
..... *X***** *****R*** *****R**N* *****X R**R*NN*NN consensus

atgataataa ggggttgaat aaggaagga agctgtttga taaggtaga attgggcaga PMMV
tagttagaaa aggggttagg agtgatagta gtttaggtgt gactgatag agtcaggacg Ob
atagaaatgt taaggatttt ggaggaatga gtttataaaa gaataattta atcgatgatg TMV
ggcctaacc aaaaagtttt gatgaagttg aaaaagagtt tgataattg attgaagatg L
aaataataa cagtgtgaag aagggtttta aaattgagga aattgaggat aatgtaagt TMGMV
ctagtgtgt agatgatatt gtggttgga atggtgttag tgatattcgt attgatgatg ORSV
aggtcgagc ctgctgggtg cggtattcta aacctccaaa tcggaggttg CGMMV
actccgggaa aaagaagggt gaatgttgat agtgtgagtt tgggattag aaagggaaag SHMV

aggaaaaaga aggttgaaga aaaggggtgta g.taag.ta agtataagta cagaccggag CrTMV
aggaaaaaga aggttgaaga aagggatgta g.taag.ta agtataaata tagaccggag TVCV
aaaaagaaa ggattggagg aaaggatgta aataataata agtttagata tagaccggag TMV-Cg
*N**N**N* N*N**N**N **N***** N.**X.** **X**NN** R****X*** consensus

actcggagtc atcggacgcc gagtcttctt cgtttact. ATG 5687 PMMV
gtagctcaag cgagatatca tccgattcgt ttatttaact. ATG 5756 Ob
attcggagtc tactgtcgcc gaatcggatt cgttttaa. ATG 5714 TMV
aagccgagac gtcggtgctg gattctgatt cgtattaa. ATG 5705 L
atgacgagtc tctcgcgca tcgagtagt tttaatca. ATG 5668 TMGMV
attgtgagtc atttgacgca caatctgaat cgtattga. ATG 5730 ORSV
gactctgctt ctgaagagtc cagttctggt tctttgaa. ATG 5762 CGMMV
agtgtgtctg ctaaaaacga agacacagag tctgtatt. ATG -701 SHMV

aagtagcggc gtcctgattc gtttaatttg aaagaagaaa. ATG 5606 CrTMV
aaatagcggc gtcctgattc gtttaattta aaagaagaaa. ATG 5605 TVCV
agatagcggc gtcaggattc gtttaattat aaagaagaaa. ATG 5597 TMV-Cg
*NN***** ***XX***** ***X***XX *****.*** consensus

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FIG. 9. Nucleotide sequence comparison of 150-nt region upstream of different tobamovirus CP genes: crTMV (Dorokhov *et al.*, 1994), TVCV (Lartey *et al.*, 1995), TMV-Cg (EMBL/Genbank/DBJ Accession No. D38444), PMMV, pepper mild mottle virus (Alonso *et al.*, 1991), Ob (Ikeda *et al.*, 1993), TMV UI, common strain (Goellet *et al.*, 1982), TMV U2 (tobacco mild green mosaic virus, TMGMV) (Solis and Garcia-Arenal, 1990), TMV L (tomato strain) (Ohno *et al.*, 1984), ORSV, odontoglossum ringspot virus (Isomura *et al.*, 1990, 1991), CGMMV (cucumber green mottle mosaic virus) (Ugaki *et al.*, 1991), SHMV, sunn-hemp mosaic virus or cowpea strain of TMV (Meshi *et al.*, 1982). Bold letters indicate common nucleotides in crucifer infecting tobamoviruses. Lower line is consensus sequence of crucifer-infecting tobamoviruses. The nucleotide positions are numbered from their distance from the 5'-terminus of tobamovirus genomic RNA besides SHMV RNA which was numbered from the 3'-terminus. The start AUG codon of CP gene is denoted by capital bold letters.

the same time, the 35-nt region retained after deletion of the 5'-terminal 116-nt part of IRES_{CP} (transcript H β Δ NPT Δ 5'IRES_{CP}CP) cannot promote the initiation of CP gene translation as well (Fig. 5). This indicates that integrity of IRES_{CP} is important for internal translation of the crTMV CP gene.

The question arises as to whether the CP-coding sequences immediately adjacent to IRES_{CP} are essential for internal initiation. Therefore, the third type of chimeric mRNA (H-CP-IRES_{CP}-GUS in Fig. 7A) containing the 3'-proximal foreign GUS gene was translated in WGE (Fig.

7B) and RRL (Fig. 7C). It was found that IRES_{CP} of crTMV tobamovirus was efficient in mediating the 3'-proximal GUS gene expression.

In order to demonstrate that IRES_{CP}-mediated translation is unusual for tobamoviruses, the equivalent dicistronic construct (HCPUI^{SP}GUS in Fig. 7A) was made containing the 148-nt region upstream of TMV UI CP gene as the intercistronic spacer. Figures 7B and 7C show that TMV UI-derived sequence was incapable of mediating internal ribosome entry. It is important that the second ORF was translated from IRES_{CP}-containing dicistronic

RNA-transcripts that retained their integrity during incubation in translation extract (Fig. 8).

Two types of dicistronic IRES_{CP}-containing transcripts were used in order to examine whether the IRES_{CP}-mediated translation of the 3'-proximal gene (CP and GUS) was influenced by the 5'-proximal ORF translation. In the first type transcripts (TBSMPCP in Figs. 2 and 3; HCPIRES_{CP}GUS in Figs. 7A and 7C) translation of the 5'-proximal ORF was abolished by potentially stable hairpin structures inserted immediately at their 5'-termini. By contrast, the 5'-proximal ORF was translationally active in the second type of transcripts (LMPCP in Figs. 2 and 3; CPIRES_{CP}GUS in Figs. 7A and 7C). It was found that IRES_{CP}-mediated translation was stimulated by blockage of the 5'-ORF expression (Figs. 3A, 3B, and 7C), suggesting that it was due to the competition between the genes for ribosomes or/and some factor(s) which are limited in cell-free translation system.

To our knowledge, this study is the first to describe a tobamovirus genome that contains an internal ribosome entry site. Our results indicate that the structure of IRES_{CP}, which is relatively short and simple (Fig. 6), is markedly distinct from IRESes of picornaviruses and other eukaryotic mRNAs described so far. An exception is provided by the 126-nt long IRES of Mo-MULV RNA (Vagner *et al.*, 1995b). One of the few features of picornavirus RNAs shared by all picornaviruses is the presence of an oligopyrimidine motif located some 25-nt upstream of the 3'-end of the IRES. It has been reported by Kaminski *et al.* (1994) that the oligopyrimidine tract is not the most critical functional element of the IRES and could be regarded as the 5'-proximal part of the unstructured spacer at the 3'-end of the IRES. Contrary to picornaviral IRESs the IRES_{CP} of crTMV RNA contains a purine-rich tract upstream of AUG codon (Fig. 6). The functional significance (if any) of this motif is obscure.

Evidently, our conclusion that the CP gene of crTMV can be internally translated *in vitro* does not exclude that a traditional mechanism of the CP gene expression via monocistronic sgRNA operates *in vivo* concurrently. Moreover, two canonical RNA species of subgenomic size revealed in crTMV-infected tobacco by Northern blot hybridization experiments corresponded in size to I₂ sgRNA and CP sgRNA, respectively (Fig. 1B). It is possible that both mechanisms contribute to crTMV CP gene expression, i.e., that monocistronic CP sgRNA translation occurs in parallel with internal initiation of CP gene translation from the genomic and/or I₂ sgRNA. Our recent experiments on microprojectile bombardment of tobacco leaves with dicistronic 35S promoter-based cDNA show that the 3'-proximal GUS gene can be expressed *in vivo* from dicistronic (CPIRES_{CP}GUS) construct as visualized by *in situ* GUS staining (unpublished).

The complete nucleotide sequence of three crucifer-infecting tobamoviruses have been reported including crTMV (Dorokhov *et al.*, 1994), turnip vein-clearing virus,

TVCV (Lartey *et al.*, 1995), and TMV-Cg (EMBL/Genbank/DBJ Accession No. D38444). The nucleotide sequence comparison of 150-nt region upstream of CP genes shows that this region is highly conservative between crucifer-infecting tobamoviruses and is strongly different from other tobamoviruses (Fig. 9). In crTMV, TMV-Cg, and TVCV genomes the MP and CP genes overlap by 25 codons; i.e., the 5'-proximal region of the CP gene are sequences encoding MP. This raises the question as to whether different crucifer-pathogenic tobamoviruses express the CP gene by the internal ribosome entry mechanism.

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