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


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) "ΔNPT-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 (IRESCr) promoting internal initiation of translation in vitro. Dicistronic IRESCP, containing chimeric mRNAs with the 5' terminal stem-loop structure preventing translation of the first gene (MP, ΔNPT, or CP), expressed the CP or GUS genes despite their 3' proximal localization. The capacity of crTMV IREScr 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 IRESCr. It was concluded that integrity of IRESCr 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.

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-Manifacier, 1993; Basso et al., 1994), cowpea mosaic virus (Thomas et al., 1991) and some cellular RNAs (Macejak and Samow, 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.
(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 (IRESC₃₉).

**MATERIALS AND METHODS**

**Viruses and RNA**

TMV UI and crTMV were isolated from systematically 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 Ncol 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 Smal-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 (Smal site) of crTMV RNA. Cloning of the HindIII/Sacl fragment from pG3-208 into pBluescript II SK+ resulted in formation of pTBSMPC₃₉PSmal (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 EcoRI/Sacl fragment of pG3A16 was ligated with EcoRI/Sacl-cut pTBSMPC₃₉PSmal to yield pTBSMPCp (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 Smal-cut pGEM-7z to give pG7S20. The Xhol/BglII fragment of pG7S20 was inserted into the Xhol/BglIII-cut pTBSMPCp to give pLMPCp (Fig. 2A). To construct pLMPΔCPSmal (Fig. 2D), the Xhol/BglII fragment of pG7S20 was ligated to Xhol/ BglIII-cut pTBSMPCp.

To obtain pΔMPecoCP (Fig. 2F) the EcoRI/Sacl (filled) fragment of pTBSMPCp was inserted into the EcoRI/Sacl-cut pBluescript II SK+. The plasmid pTBSMPCp was digested with Ncol and Xhol, filled with Klenow fragment, and religated, yielding pΔTBSMPCp (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 pH₂ΔNPTCP (Fig. 4C) contained T7 promoter, inverted tandem repeat to form a stable hairpin structure (ΔGᵣ = –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 pΔMPecoCP and contained no crTMV-derived sequence upstream from the initiation codon of the CP gene. Therefore the pH₂ΔNPTCP construct contained two genes, separated by polylinker-derived spacer. (ii) The construct pH₂ΔNPTIRESC₃₉CP (Fig. 4D) differs from previous constructs by the presence of IRES₃₉ right upstream of the CP gene in the intercistronic area. (iii) The construct pH₂ΔNPTΔ₃₉IRESC₃₉CP (Fig. 4E) differs from the pH₂ΔNPTIRESC₃₉CP by the deletion of the 5’-terminal 113-nt region of IRES₃₉. (iv) The construct pH₂ΔNPTΔ₃₉IRESC₃₉CP (Fig. 4F) contains the 5’-terminal part of IRES₃₉ 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-
pept (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 \( \Delta M P E c o C P \) plasmid as it was described in (i). (vi) The construct pHCPIRESCP-GUS (Fig. 7A) contained T7 promoter, inverted tandem repeat, CP gene of crTMV, IRES CP, and \( \beta \)-glucuronidase gene (GUS). To clone GUS gene right downstream of the Translation of the agarose gel-purified preparations of IRESCP NcoI site was introduced at the 3'-end of IRESCP, using corresponding oligonucleotides for PCR. (vii) In constructs pHCPUI 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 dyeoxy sequencing.

In vitro transcription and translation; Immunoprecipitation of the CP

The plasmids pLMPCP, pTBSMPCP, p\( \Delta T B S M P C P \), pLMPCPSmal, pTBSMPCPSmal, p\( \Delta M P E c o C P \), HCPIRESCP, HCPUI SP-GUS, HCP\( \alpha \), GUS, \( \alpha \) GUS, and UI SP-GUS were linearized by SacI; the plasmid pHUMP\( \beta \)NPT3, pH\( \beta \)NPTCP, pH\( \beta \)NPTA3\( \Delta \)IRESCP, CP, and pH\( \beta \)NPTA3\( \Delta \)IRESCP were linearized by HindIII; the plasmid pH\( \beta \)NPTIRESCP, CP was linearized by SphI 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 extracts as described by Pelletier and Sonenberg (1988) and in wheat germ extracts (WGE) according to the manufacturer's protocol in the presence of \( [35S] \)methionine for 60 min at 30°C. Radiolabeled translation products were analyzed by SDS–PAGE and localized by autoradiography on the dried gel. Immunoprecipitation of \( 35S \)-labeled CP, the product of in vitro translation, was carried out as described by Morozov et al. (1990).

Stability of dicistronic IRESCP containing RNA transcripts upon in vitro translation

Radioactive transcripts were obtained by standard procedure (see above) using \( [\alpha-32P] \)UTP instead of nonradioactive UTP. Radioactivity of transcripts was about 3 \( \times \) 10^6 cpm/\( \mu \)g RNA; 5 \( \mu \)g of each transcript was added to in vitro translation mixture (RRL) and incubated at 30°C for 60 min. Then RNA was isolated from the translation mixture with phenol, preincubated with ethanol, and solubilized in RNase-free water. Aliquots (radioactivity about 150 \( \times \) 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 detected 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 sqRNA 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 followed 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'–proximal 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 \( [35S] \)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 \( \Delta M P E c o C P \) (with the CP gene and 3’NTR deleted; Fig. 2D) and \( \Delta M P E c o C P \) 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 + IgpcrTMV)-immunoprecipita-
tion of translation product of ΔMPecoCP transcript with antibodies against crTMV CP; (crTMV + IgcptMv)-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-reinitia-
tion. To examine the translation mechanism of CP gene
further, we constructed a second type of dicistronic
script (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 (TBSMPΔCPSmal; Fig.
3B). This effect is likely due to the ability of TBS to pro-
duce 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 MPCR tran-
script 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) ne-
omycin phosphotransferase I gene (ΔNPTI) and the CP
gene of crTMV (Figs. 4C–F). The ΔNPTI ORF corre-
sponds 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" cTMV 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) ΔTBSMPCP transcript from which 57-nt 5’-terminal region of TBS was deleted; (D and E) LMPΔCP SmaI and TBSMPCPΔCP SmaI transcripts with the CP gene deleted; (F) LMPΔEcoCP 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 cTMV 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 αβΔNPT transcript (Fig. 4A), containing the 5’-nontranslated sequence (αβ) corresponding to the 83-nt 5’-leader of potato virus X (PVX) RNA upstream of the ΔNPTI ORF. The αβ-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 HβΔNPT (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 αβ, the translation enhancer, the ΔNPTI ORF was efficiently translated, directing synthesis of the 19-kDa protein as the major product (αβΔNPT in Fig. 5A). However, expression of the ΔNPTI ORF was completely blocked by an upstream hairpin-loop structure (H) (HβΔNPT in Figs. 5A and B).

Neither of the two cistrons could be translated from the dicistronic transcripts (HβΔNPTCP 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
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\textsubscript{CP}) was inserted as the intercistronic spacer in the chimeric dicistronic transcript \(\beta\Delta\text{NPTIRES}_{\text{CP}}\) (Fig. 4D), the CP gene was efficiently translated (Figs. 5A and 5B). These observations strongly suggest that the IRES\textsubscript{CP} of crTMV mediates internal initiation of the CP gene translation.

**FIG. 4.** Schematic representation of chimeric dicistronic "\(\Delta\text{NPTI-CP}\)" mRNA and its derivatives generated in vitro: (A) \(\alpha\beta\Delta\text{NPTI}\), the 3'-truncated \(\Delta\text{NPTI}\) gene with the 83-nt \(\alpha\beta\)-sequence of PVX RNA as the 5'-leader; (B) \(\text{H} \beta\Delta\text{NPTI}\), the \(\Delta\text{NPTI}\) gene with upstream sequence consisting of 80 nts forming a potentially stable hairpin (H) and the 42-nt \(\beta\)-sequence (\(\beta\)) of PVX RNA; (C) \(\text{H} \beta\Delta\text{NPTI CP}\), dicistronic transcript with 12 polylinker-derived (pd) nucleotides inserted between the \(\Delta\text{NPTI}\) and CP genes; (D) \(\text{H} \beta\Delta\text{NPTIRES}_{\text{CP}}\), in addition to the 12-nt pd insert, the 148-nt sequence located upstream of the CP gene of crTMV (IRES\textsubscript{CP}) was inserted; (E) \(\text{H} \beta\Delta\text{NPTI\textsubscript{5'}IRES}_{\text{CP}}\), only the 5'-proximal 35 nucleotides of the IRES\textsubscript{CP} were inserted upstream of the CP gene; (F) \(\text{H} \beta\Delta\text{NPTI\textsubscript{3'}IRES}_{\text{CP}}\), the 5'-proximal 113 nucleotides of the IRES\textsubscript{CP} were inserted upstream of the CP gene.
FIG. 5. Analysis of proteins directed in vitro by the "ΔNPTI-CP" dicistronic chimeric mRNAs. Autoradiogram of gradient PAGE–SDS gels containing [35S]methionine-labeled products directed by uncapped transcripts in RRL (A) and Krebs 2 extracts (B). Concentration of transcripts is 40 μg/ml. The positions of CP and ΔNPTI are marked. The designations above the panels were described in the legend to Fig. 4.

under conditions which abolish translation initiation of the first ORF.

The IRES<sub>CP</sub> sequence contains a purine-rich motif and can be folded into a simple secondary structure (Fig. 6). The functional role of the separate sequence elements as well as the minimum length of IRES<sub>CP</sub> essential for the CP gene expression is obscure. To study the role of different parts of the IRES<sub>CP</sub> in internal initiation two additional transcripts were tested. The first was the dicistronic HβΔNPTIΔ5′IRES<sub>CP</sub>CP transcript (Fig. 4E), which contained only the 35-nt 3′-terminal region of IRES<sub>CP</sub> as internal spacer between the ΔNPTI and CP genes. The second transcript was HβΔNPTIΔ3′IRES<sub>CP</sub>, which retained the 5′-terminal 113-nt region of IRES<sub>CP</sub> and the 3′-terminal 35 nucleotides were deleted (Fig. 4F). Figure 5 shows that deletion of either of the two regions of IRES<sub>CP</sub> resulted in complete blockage of translation of the second gene.

Translation of dicistronic "H-CP-IRES<sub>CP</sub>-GUS" chimeric RNA

The third type of dicistronic chimeric constructs contained the 5′-proximal CP gene of crTMV and the

FIG. 6. Proposed secondary structure of the 148-nt IRES<sub>CP</sub> containing region of crTMV RNA upstream of the CP gene AUG codon (boxed). The purine-rich tract of nucleotides is indicated; the direct repeat of nucleotides is marked by bold letters. An arrow points to the position resulting in Δ5′IRES<sub>CP</sub> and Δ3′IRES<sub>CP</sub> formation.
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<sub>Cp</sub>-containing sequence from crTMV RNA (HCPF<sub>Cp</sub>GUS in Fig. 7A); second was the 148-nt region (UI<sup>SP</sup>) upstream from start codon of the CP gene in TMV UI RNA (HCPUI<sup>SP</sup>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<sup>SP</sup>GUS) contained as 5'-nontranslated leaders the αβ-sequence and UI<sup>SP</sup>-sequence, respectively. Two dicistronic transcripts (CPIRES<sub>Cp</sub>GUS and CPUI<sup>SP</sup>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 U1SβGUS and αβGUS 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 HCPIRESCPUS transcripts containing the 148-nt IRESCP sequence as intercistronic spacer (Fig. 7B). It should be emphasized that neither of the two genes could be translated from the dicistronic transcript HCPIRESUSGUS in which the genes were separated by the 148-nt region preceding the AUG of the CP gene in TMV UI RNA (HCPIRESUSGUS in Fig. 7B). The same was true for dicistronic construct HCPαβGUS with the PVX αβ-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 HCPIRESCPUS but not from HCPIRESUSGUS 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. HCPIRESCPUS and CPIRESCPUS 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 LMP-CP and TBSMP-CP 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 IRESCP 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 IRESCP is instead an efficient cleavage site, particularly susceptible to nuclease in translation extracts. In order to show that the second cistron is not being translated from degraded IRESCP containing dicistronic RNAs the [32P]-labeled dicistronic transcripts (HCPIRESCPUS and HCPIRESUSGUS) 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 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 IRESCP) was inserted as the intercistronic spacer in chimeric dicistronic mRNA (Fig. 4D). It was found that IRESCP mediated initiation of the 3'-proximal CP gene translation under conditions which abolished the translation of the first cistron (see HβΔNPTIRESCP in Fig. 5). Although the boundaries of IRESCP have not been defined precisely, the element is contained within a 148-nt region upstream of CP gene. RNA secondary structure indicates that the IRESCP 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 IRESCP of the 3'-terminal 35-nt region (transcript HβΔNPTΔ3'IRESCP) completely blocks internal ribosome entry. At
INTERNAL ENTRY OF RIBOSOMES ON crTMV RNA

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/DDBJ Accession No. D38444), PMMV, pepper mild mottle virus (Alonso et al., 1991), Ob (Ikeda et al., 1993), TMV UI, common strain (Goelet et al., 1982), TMV U2 (tobacco mild green mosaic virus, TMGMV) (Solis and Garcia-Arenal, 1990), TMV L (tomato strain) (Ohno et al., 1984), ORSVM, 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.

In the same time, the 35-nt region retained after deletion of the 5'-terminal 116-nt part of IRESCP (transcript HbD5A) cannot promote the initiation of CP gene translation as well (Fig. 5). This indicates that integrity of IRESCP is important for internal translation of the crTMV CP gene.

The question arises as to whether the CP-coding sequences immediately adjacent to IRESCP are essential for internal initiation. Therefore, the third type of chimeric mRNA (H-CP-IRESCP-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 IRESCP of crTMV tobamovirus was efficient in mediating the 3'-proximal GUS gene expression.

In order to demonstrate that IRESCP-mediated translation is unusual for tobamoviruses, the equivalent dicistronic construct (HCPUI SPGUS 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 IRESCP-containing dicistronic
IRESCP-mediated translation was stimulated by blockage of the 5’-proximal gene (CP and GUS) was influenced by the 5’-proximal ORF translation. In the first type transcripts (TB5MPCP in Figs. 2 and 3; HCPRESCPGUS 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 (LMCP in Figs. 2 and 3; CPRESCPGUS in Figs. 7A and 7C). It was found that IRESCP-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 IRESCP, 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 IRESCP 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 (CPRESCPGUS) 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/DBB 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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