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Cap-Independent Enhancement of Translation by a Plant Potyvirus 5′ Nontranslated Region

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The RNA genome of tobacco etch virus (TEV), a plant potyvirus, functions as an mRNA for synthesis of a 346-kilodalton polyprotein that undergoes extensive proteolytic processing. The RNA lacks a normal 5′ cap structure at its terminus, which suggests that the mechanism of translational initiation differs from that of a normal cellular mRNA. We have identified a translation-enhancing activity associated with the 144-nucleotide, 5′ nontranslated region (NTR) of the TEV genome. When fused to a reporter gene encoding β-glucuronidase (GUS), the 5′ NTR results in an 8- to 21-fold enhancement over a synthetic 5′ NTR in a transient-expression assay in protoplasts. A similar effect was observed when the 5′ NTR-GUS fusions were expressed in transgenic plants. By using a cell-free translation system, the translation enhancement activity of the TEV 5′ NTR was shown to be cap independent, whereas translation of GUS mRNA containing an artificial 5′ NTR required the presence of a cap structure. Translation of GUS transcripts containing the TEV 5′ NTR was relatively insensitive to the cap analog m^7GTP, whereas translation of transcripts containing the artificial 5′ NTR was highly sensitive. The 144-nucleotide TEV 5′ NTR synthesized in vitro was shown to compete for factors that are required for protein synthesis in the cell-free translation reaction mix. Competition was not observed when a transcript representing the initial 81 nucleotides of the TEV 5′ NTR was tested. These results support the hypothesis that the TEV 5′ NTR promotes translation in a cap-independent manner that may involve the binding of proteins and/or ribosomes to internal sites within the NTR.

The plant potyviruses contain monopartite RNA genomes of approximately 10 kilobases (9). The complete nucleotide sequences of four potyviruses have been elucidated to date (1, 7, 28, 40). Each contains a genetically encoded polyadenylate tail at the 3′ end, a single functional open reading frame for translation of a large polyprotein, and (for at least two potyviruses) a protein termed VPg covalently linked to the 5′ terminus (9, 16, 42). These features also characterize the animal picornaviruses and plant comoviruses, both of which share significant amino acid sequence similarities with the potyviruses (8, 9, 11).

The absence of a conventional cap structure at the genome 5′ terminus presents an interesting situation with regard to translation. The eucaryotic 5′ cap structure is believed to perform at least two major functions. The cap serves both to stabilize the mRNA from exonucleolytic attack (12) and to direct the entry of 40S ribosome subunits via interaction with the cap-binding complex eIF-4F (39). It has not been determined whether the VPg of potyviruses remains linked to the RNA during translation or is removed. However, the 5′-terminal structure of polyribosome-bound poliovirus RNA is 5′ pUp (17, 35), indicating that the VPg is removed immediately before or after initiation of translation. These viruses, therefore, must use a translational initiation mechanism that bypasses the normal cap recognition step. Recent evidence suggests that initiation of translation of two picornaviruses, poliovirus and encephalomyocarditis virus, involves binding of ribosomes to sequences within the 5′ nontranslated sequence (20, 37).

Although the potyviruses and picornaviruses have a number of features in common, there exist important differences with regard to translation. The well-studied picornaviruses such as poliovirus shut off host cell translation by inducing proteolytic inactivation of p220, an eIF-4F component, shortly after initiation of infection (10). There is no evidence that potyviruses selectively inhibit host cell protein synthesis. The 5′ nontranslated regions (NTRs) of picornaviruses are extremely long, contain multiple AUG codons that do not signal initiation of translation, and often contain unusual structures such as long poly(C) tracts. The potyviruses possess 5′ nontranslated sequences that average approximately one-fourth the size of those associated with picornaviruses and contain no apparent unusual primary structures. The mechanisms associated with translational initiation may therefore be quite different.

We have begun an analysis of the properties of the tobacco etch potyvirus (TEV) 5′ NTR. This is an adenosine-plus-uridine-rich sequence of 144 nucleotides (nt) that contains no AUG codons (the coding sequence for the polyprotein starts at nt 145 [1]). The present study was initiated to test the hypothesis that the TEV 5′ NTR functions as a translational enhancer in a cap-independent fashion. We have analyzed in vivo and in vitro the effects of fusing the TEV 5′ NTR to a reporter gene. The results suggest that this sequence dramatically stimulates translation and that this effect is probably exerted via a cap-independent mechanism.

MATERIALS AND METHODS

Plasmid constructions. A number of plant and cell-free expression vectors were used in this study. pRT101 and pRT104 are plant transient-expression vectors that contain the 35S transcriptional promoter and terminator and a multiple cloning site (46). These vectors differ only in the sequence of the multiple cloning site. pRT104 contains an *Nco*I site embedded within the consensus ribosome initiation sequence ...ACCATGG..., and pRT101 lacks a translation start site.

pRTL was assembled by insertion of an *Eco*RI-*Bam*HI cDNA fragment representing TEV nt 12 to 205 into the multiple cloning site of pRT101; the TEV fragment contained

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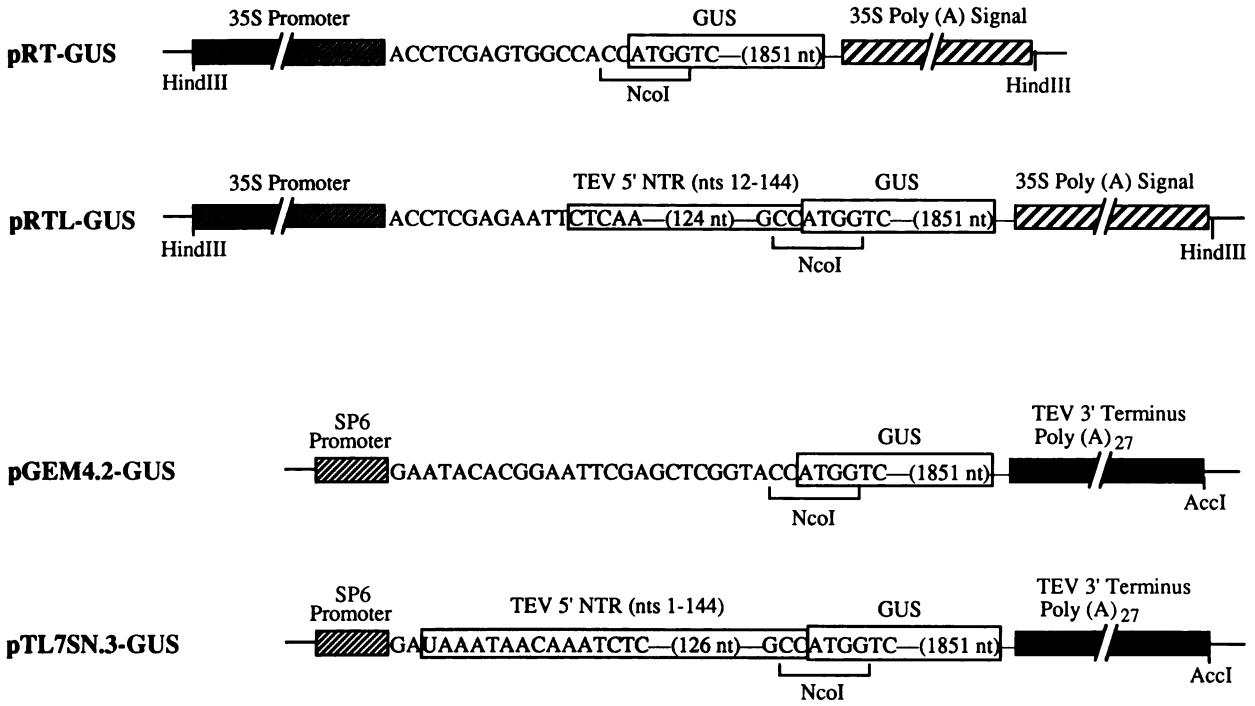

FIG. 1. Structures of relevant portions of recombinant plasmids assembled for this study. pRT-GUS and pRTL-GUS contain the 35S promoter and polyadenylation signal from cauliflower mosaic virus. The transcribed sequence is shown immediately after the 35S promoter box. The coding sequence for GUS is shown within the open box. The TEV 5′ NTR is shown within the shaded box in the pRTL-GUS schematic. pGEM4.2-GUS and pTL7SN.3-GUS contain a bacteriophage SP6 promoter for in vitro transcription and cDNA representing the 3′-terminal 154 nt of the TEV genome. The 3′-terminal fragment also contains a poly(A) region of 27 residues. A unique *Acc*I site immediately after the poly(A)$_{27}$ sequence permits linearization of these plasmids prior to transcription. The beginning of the transcribed sequence is shown adjacent to the SP6 promoter. Further details of these constructions are given in Materials and Methods.

most of the noncoding region (nt 12 to 144) and the coding sequence for the first 20 amino acid residues of the polyprotein (nt 145 to 205). The sequence of this fragment was identical to the wild-type sequence, with the exception of an adenosine-to-cytosine mutation at nt 144. This alteration, resulting in the sequence GCCATGG, was introduced by site-directed mutagenesis to form an *Nco*I site at the translational initiation codon (underlined).

pRT-GUS was constructed by insertion of the gene encoding β-glucuronidase (GUS), contained in an *Nco*I-*Eco*RI fragment (filled in with Klenow fragment) from pRAJ275 (23), between the *Nco*I and *Sma*I sites of pRT104 (Fig. 1). The resulting sequence context around the translational start site was ...ACCATGG.... pRTL-GUS was assembled by insertion of the GUS gene into pRTL (Fig. 1).

The 35S promoter, GUS-encoding sequence, and transcriptional terminator in pRT-GUS and pRTL-GUS were flanked by *Hin*dIII sites (Fig. 1). The entire GUS expression cassette from both plasmids was excised with *Hin*dIII and inserted into the unique *Hin*dIII site of the *Agrobacterium tumefaciens* binary vector pGA482 (2). This plasmid confers tetracycline resistance for selection in *Escherichia coli* and *A. tumefaciens*. The *Hin*dIII site into which the GUS cassette was inserted was situated adjacent to the gene encoding neomycin phosphotransferase II and between left and right border sequences from a Ti plasmid of *A. tumefaciens*. The recombinant plasmids, termed pGA-GUS (derived from the pRT-GUS cassette) and pGTL-GUS (from the pRTL-GUS cassette), were selected and screened in *E. coli* HB101 and then mobilized by triparental mating (6) into *A. tumefaciens* LBA4404 carrying a disarmed Ti plasmid.

The GUS coding sequence was inserted into two cell-free expression vectors, pTL7SN.3 and pGEM4.2, so as to permit positive-sense transcripts to be synthesized by using bacteriophage SP6 polymerase. Vector pTL7SN.3 possessed the following properties: (i) an intact TEV 5′ region consisting of nt 1 to 205, containing the engineered *Nco*I site encompassing the translational start codon; (ii) a polylinker region derived from pGEM4 (Promega Biotec) minus the *Sal*I/*Acc*I site which was destroyed by *Sal*I digestion, fill-in with Klenow fragment, and religation; and (iii) an intact TEV 3′ region consisting of nt 9341 to 9495-poly(A)$_{27}$, contained within a *Hin*dIII-*Hin*dIII fragment from pTL-8595 (5). The 3′ end of the poly(A) tail was flanked by *Sal*I/*Acc*I, *Pst*I, *Sph*I, and *Hin*dIII sites, each of which was contributed by the 3′ *Hin*dIII-*Hin*dIII fragment. The GUS coding sequence was removed from pRTL-GUS with *Nco*I and *Bam*HI and inserted into *Nco*I-*Bam*HI-digested pTL7SN.3 (Fig. 1). pGEM4.2 was nearly identical to pTL7SN.3, except that it lacked the TEV 5′ NTR sequence and therefore lacked a translational start codon. Hence, pRTL-GUS was cut with *Nco*I (containing the start codon for translation), filled in with Klenow fragment, and then cut with *Bam*HI to liberate the GUS coding sequence. This fragment was inserted into pGEM4.2 that had been cut with *Ava*I, treated with mung bean nuclease, then cut with *Bam*HI. The resulting recombinant, pGEM4.2-GUS, contained the GUS gene translational start site within the context ACCATGG (Fig. 1).

Protoplast preparation and electroporation. Protoplasts were isolated from surface-sterilized *Nicotiana tabacum* cv. Xanthi-nc grown under controlled greenhouse conditions (25°C). The undersurfaces of leaves (10 to 15 cm) were

abraded with carborundum and then floated overnight (usually 15 h) in enzyme solution (1.5% cellulase RS [Yakult Honsha, Tokyo, Japan], 0.1% Driselase [Sigma Chemical Co., St. Louis, Mo.], 0.5% Macerozyme R-10 [Yakult Honsha], K_3 macronutrients [24], 0.55 M mannitol [pH 5.9]). Protoplasts were isolated by flotation on 0.55 M sucrose (45 × *g* for 7 min) and then washed by two sedimentation cycles (100 × *g* for 4 min) in 0.55 M mannitol. Protoplasts were suspended in electroporation buffer (30 mM Tris hydrochloride [pH 9.0], 6 mM CaCl, 150 mM NaCl, 275 mM mannitol) to a concentration of 2×10^6 to 3×10^6/ml. The best yields were obtained from plants that were 50 to 60 days old.

Parameters for electroporation of plasmid DNA and synthetic RNA transcripts were similar to those defined as optimal by Taylor and Larkin (45). Supercoiled plasmid DNA or transcripts (10 μg) were mixed rapidly with 0.2 ml of ice-cold protoplasts and then subjected to five electric pulses of 1 ms each at the voltages indicated in the figure legends. The protoplasts were allowed to recover on ice for 10 min and then transferred to protoplast culture medium (1.2 ml) containing KP medium (32), 1 μg of benzylaminopurine per ml, 2 μg of naphthaleneacetic acid per ml, and 0.1 mg of cefotaxime (pH 5.9) per ml. The cultures were incubated at room temperature for 20 h under diffuse light.

In one experiment, polyethylene glycol-mediated transfection (34) was substituted for electroporation.

Production of transgenic plants. Transgenic *N. tabacum* cv. Havana 425 plants harboring integrated DNA from pGA482, pGA-GUS, and pGTL-GUS were produced by the leaf disk transformation protocol (18). Shoots were induced on MS medium (33) containing 1 μg of benzylaminopurine per ml, 0.01 μg of naphthaleneacetic acid per ml, 0.5 mg of carbenicillin per ml, 0.1 mg of kanamycin per ml, and 0.8% phytagar. Shoots were excised from the leaf piece after approximately 1 month and transferred to the same medium minus the growth hormones. Plants that regenerated roots were transplanted to boxes (Magenta Corp.) containing sterilized soil. Over the course of approximately 1 week, the lids were slowly removed to gradually decrease the humidity around the plants. The plants were finally transferred to pots and placed in a contained greenhouse.

In vitro transcription and translation. Plasmids pTL7SN.3-GUS and pGEM4.2-GUS were purified by cesium chloride-ethidium bromide gradient centrifugation (29). The DNAs were digested with *Acc*I, which recognized a unique site immediately downstream of the poly(A) tract. Transcription with SP6 polymerase was as described previously (5, 30), except that 20 μCi of [^{35}S]UTP was added. In addition, some reaction mixtures contained the cap precursor m^7GpppG or GpppG (0.4 mM) and reduced levels of GTP (40 μM). These conditions have been shown to promote the synthesis of capped transcripts (25). In some experiments, the competitor effects of the TEV 5′ NTR supplied in *trans* to translation reactions were tested. Transcripts were synthesized from pTL7SN.3 that had been digested with *Nco*I and mung bean nuclease, resulting in RNA representing nt 1 to 143 of the 5′ NTR. Additionally, competitor RNA representing nt 1 to 81 of the TEV 5′ NTR was produced by using *Dra*I-digested pTL7SN.3. The quantity of transcript synthesized in each reaction was measured by determining the ratio of incorporated to nonincorporated [^{35}S]UTP by established methods (29). Transcripts were precipitated twice with ethanol-ammonium acetate and suspended in deionized water.

Transcripts were translated in rabbit reticulocyte lysates essentially as described previously (5), except that [^{35}S] methionine was omitted. Translation reactions were terminated by the addition of 10 volumes of GUS lysis buffer (21). All mRNA transcripts were added to the translation reaction at a concentration of 15 nM.

GUS assays. GUS activity in protoplasts, plants, and cell-free translation reactions was measured as described previously (21). Protoplasts (4.0×10^5) were harvested by brief centrifugation and lysed completely (as judged by microscopic examination) in GUS lysis buffer (100 μl). GUS activity was tested by using 50-μl reactions (37°C for 30 min) containing 25-μl portions of 1:10, 1:100, or 1:1,000 dilutions of lysate. Conversion of 4-methylumbelliferyl glucuronide to methylumbelliferone (MU) was measured by using a fluorometer (TKO-100; Hoefer Scientific Instruments, San Francisco, Calif.). The molar concentration of MU that accumulated in the reactions was calculated from standard curves obtained with known concentrations of MU. One unit of GUS activity was defined as the amount required to produce 1 pmol of MU in 1 min at 37°C.

Extracts were prepared from transgenic plant leaves in GUS lysis buffer with the aid of a Kontes pestle and 1.5-ml microcentrifuge tube. The concentration of protein in each extract was measured by using a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard. The GUS assays were identical to those described above, except that the lysate dilutions were carried out to 1:10,000. Activity assays for in vitro-synthesized GUS were conducted by using 1:10 and 1:100 dilutions of lysate.

RESULTS

Activity of 5′ NTR-GUS fusions in protoplast transient-expression assays. To study the role of the TEV 5′ NTR in translational initiation, we have fused the TEV 5′ NTR, as well as an artificial NTR, to the coding sequence for a reporter gene encoding GUS (Fig. 1). The start codons in all constructs containing an artificial 5′ NTR were situated within the local sequence context, ACCATGG (initiator codon underlined), which has been shown to be the most effectual environment in which to initiate eucaryotic translation in the scanning model of Kozak (26). The initiator codon linked to the TEV 5′ NTR resided within the sequence GCCATGG. The chimeric genes were expressed transiently in protoplasts following electroporation with plasmid DNA or SP6 transcripts. To identify the optimal voltage to use during electroporation, we tested a series of voltage settings by using pRTL-GUS DNA. The highest GUS activity was generated in protoplasts subjected to 250 or 300 V (Fig. 2). Since the 250-V electroporation appeared to be less detrimental to cell viability (based on microscopic examination), this voltage was used in subsequent experiments.

The GUS activity produced after electroporation of protoplasts with pRT-GUS (without the TEV 5′ NTR) or pRTL-GUS (with the TEV 5′ NTR) is shown in Fig. 3A. GUS activity was detected in protoplasts electroporated with pRT-GUS or pRTL-GUS, but not with water. Electroporation with pRTL-GUS resulted in approximately eight-fold-higher GUS activity than that seen with pRT-GUS, suggesting that fusion of the TEV 5′ NTR substantially stimulated translation. However, the increased GUS activity could also be attributed to other indirect effects of the NTR on the 35S transcriptional promoter.

To verify that the enhanced GUS activity was the direct consequence of the TEV 5′ NTR at the level of translation, we synthesized capped or noncapped transcripts from pTL7SN.3-GUS or pGEM4.2-GUS and introduced them by

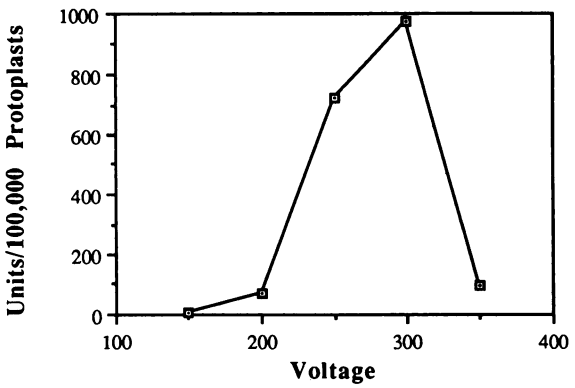

FIG. 2. Effect of voltage during electroporation of tobacco protoplasts with pRTL-GUS DNA. Equal numbers of protoplasts (4×10^5) were electroporated at 150, 200, 250, 300, or 350 V (five pulses of 1-ms duration each). Lysates were prepared after an incubation of 15 h and assayed for GUS activity.

electroporation into protoplasts. Transcripts derived from these plasmids carried a 3′ poly(A) tail (27 nt) that originated from the 3′ terminus of a TEV cDNA clone. GUS activities were determined and plotted in Fig. 3B. Transcripts from pGEM4.2-GUS lacking both an m^7GpppG cap and the TEV 5′ NTR failed to stimulate GUS activity significantly above that of the negative control (protoplasts electroporated in the presence of water). Addition of a 5′ cap structure to the artificial NTR-GUS transcript resulted in measurable GUS activity that was at least 10-fold higher than the activity detected with noncapped transcripts. However, the use of capped transcripts derived from pTL7SN.3-GUS (containing the TEV 5′ NTR) resulted in a 21-fold increase in GUS activity over that with capped transcripts lacking the TEV sequence.

GUS activity also was detected after electroporation of noncapped transcripts from pTL7SN.3-GUS. The level of expression was approximately 12-fold lower than that observed with capped transcripts, but was slightly higher than the level measured with capped transcripts lacking the TEV 5′ NTR (Fig. 3B). To test whether pTL7SN.3-GUS transcripts required a methylated cap for the enhancement activity, we synthesized transcripts that contained either m^7GpppG or GpppG as the cap structure at the 5′ terminus. Transcripts containing the nonmethylated cap were as active as those containing the methylated cap (Fig. 3C). These data suggest that the low level of GUS activity produced after transfection with the noncapped pTL7SN.3-GUS transcripts (Fig. 3B) was due to transcript instability within the cells rather than a requirement for a methylated cap.

Expression of 5′ NTR-GUS fusions in transgenic plants. To determine whether the translational enhancement activity associated with the TEV 5′ NTR functions in whole plants rather than just protoplasts and to explore the potential of this sequence to serve as a translational enhancer for foreign genes in transgenic plants, we integrated the NTR-GUS constructs into the tobacco genome by using *A. tumefaciens*-mediated transformation. The 35S transcriptional promoter-NTR-GUS-transcriptional terminator cassette from both pRT-GUS and pRTL-GUS was subcloned into the binary vector pGA482, forming pGA-GUS and pGTL-GUS, respectively. The GUS expression cassette was transferred and integrated into the tobacco genome by the leaf disk transformation procedure. Plants expressing the antibiotic marker neomycin phosphotransferase were selected by their ability to form roots in a medium containing kanamycin. A total of 15 pGA-GUS- and 20 pGTL-GUS-transformed plants formed roots under kanamycin selection, and 13 of each were found to express GUS activity. Of five plants transformed with pGA482 (vector alone without a GUS-encoding sequence), none were positive for GUS activity.

The levels of activity in each GUS-positive plant were quantified as shown in Fig. 4. The mean activity value (±

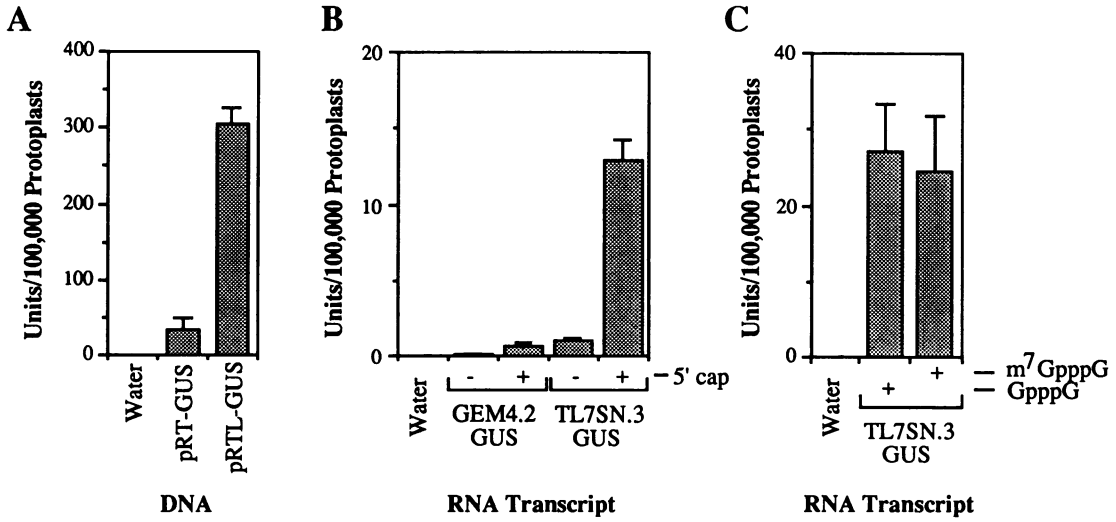

FIG. 3. Effect of the TEV 5′ NTR on the expression of GUS activity in protoplasts. Electroporation conditions were as described in the legend to Fig. 2 (with 250 V) and in Materials and Methods. (A) GUS activity measurements from protoplasts electroporated in the presence of water, pRT-GUS (with an artificial 5′ NTR), or pRTL-GUS (with the TEV 5′ NTR). Covalently closed supercoiled plasmid DNA (40 μg/ml) was used. (B) GUS activity measurements from protoplasts electroporated in the presence of water or in vitro-synthesized transcripts (10 μg/ml) from *Acc*I-linearized pGEM4.2-GUS or pTL7SN.3-GUS. Transcripts were synthesized with (+) or without (−) an m^7GpppG 5′ cap. (C) GUS activity measurements from protoplasts that were polyethylene glycol transfected with transcripts (10 μg/ml) from *Acc*I-linearized pTL7SN.3-GUS. Transcripts were synthesized with either an m^7GpppG or a nonmethylated GpppG 5′ cap.

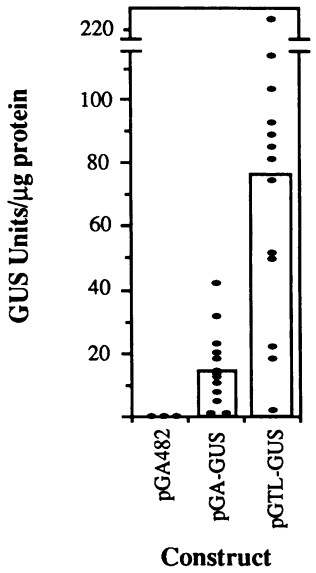

FIG. 4. Effect of the TEV 5′ NTR on expression of GUS activity in transgenic plants. Transgenic tobacco plants containing the GUS expression cassettes from pRT-GUS (pGA-GUS) or pRTL-GUS (pGTL-GUS) were generated by the leaf disk transformation procedure (18). Transgenic plants carrying vector sequences (pGA482) also were produced as controls. GUS measurements were performed on leaves of 13 independently transformed plants (represented by dots) carrying pGA-GUS or pGTL-GUS sequences. Three plants harboring pGA482 sequences also were assayed for GUS activity. The mean GUS values (in units per microgram of soluble protein) for each group are indicated by the bars.

standard error of the mean) for pGTL-GUS-transformed plants (77.7 ± 14 GUS units/μg of protein) was approximately fivefold higher than the mean value measured from pGA-GUS plants (16.7 ± 3.2). A wide range of activity values was observed among plants in each group, probably reflecting the influence of different chromosomal integration points. However, the activity values for individual plants were consistent between two sampling dates.

Expression of the 5′ NTR-GUS fusions in vitro. To further investigate the translational enhancement activity of the TEV 5′ NTR and to address the mechanism through which the enhancer functions, we have used a cell-free system (rabbit reticulocyte lysate) to translate SP6 transcripts containing the NTR-GUS fusions. Equimolar concentrations of capped and noncapped transcripts from pTL7SN.3-GUS and pGEM4.2-GUS were translated in vitro, and samples were withdrawn and analyzed for GUS activity at various time points. Translation of pGEM4.2-GUS transcripts (with the artificial 5′ NTR) was highly dependent on the presence of an m^7GpppG cap structure at the 5′ terminus (Fig. 5). In contrast, efficient translation of pTL7SN.3-GUS transcripts was relatively independent of the 5′ cap. (The difference in GUS activity induction between capped and noncapped pTL7SN.3-GUS transcripts in Fig. 5 was not reproducible; differences between these two transcripts from experiment to experiment were generally less than 10%.) Consistent with results of experiments with protoplasts and transgenic plants, fusion of the TEV 5′ NTR to the GUS-encoding sequence resulted in enhanced levels of GUS activity. Noncapped transcripts containing the TEV 5′ NTR were approximately 11-fold more active than noncapped transcripts containing the artificial NTR. The enhancement activity was less pronounced (fourfold) when capped transcripts of

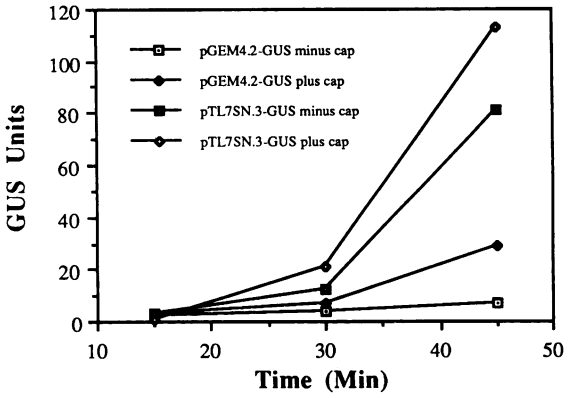

FIG. 5. Time course of GUS activity accumulation during cell-free translation of capped or noncapped transcripts derived from pGEM4.2-GUS (with artificial 5′ NTR) or pTL7SN.3-GUS (with TEV 5′ NTR). Translation reactions (30 μl) were programmed with in vitro-synthesized transcripts at a concentration of 15 nM. Samples were withdrawn after translation periods of 15, 30, and 45 min, and GUS activity measurements were taken. The GUS unit values are the average of two replicates from a typical experiment and are represented as total units per 30-μl translation reaction.

pTL7SN.3-GUS and pGEM4.2-GUS were compared. The translational enhancement activity of the TEV 5′ NTR also was observed by using a wheat germ-derived cell-free system (data not shown).

The translational enhancement activity of the TEV 5′ NTR could operate by increasing the mRNA stability. However, the relative translational enhancement caused by the TEV 5′ NTR at the 30-min time point was approximately equal to the enhancement detected at the 45-min time point, suggesting that the mRNAs were not decaying at substantially different rates. Additionally, incubation of ^{35}S-labeled mRNA transcripts in reticulocyte lysate, followed by withdrawal of samples at various time points and analysis of the RNAs by agarose gel electrophoresis, revealed no detectable differences between the stabilities of transcripts derived from pTL7SN.3-GUS and pGEM4.2-GUS (data not shown).

Results of the cell-free translation experiments described above suggested that efficient synthesis of protein from pTL7SN.3-GUS transcripts was cap independent. To further test this hypothesis, we conducted translation reactions in the presence of various concentrations of the cap analog m^7GTP. This nucleotide competitively interferes with association of the cap-binding complex with mRNA, thereby inhibiting cap-dependent initiation of translation. At a relatively low m^7GTP concentration (4.0 μM), translation of capped pGEM4.2-GUS transcripts was reduced 95%, to the level observed with noncapped transcripts from the same plasmid (Fig. 6). The same concentration of m^7GTP had little effect on translation of either capped or noncapped transcripts derived from pTL-7SN.3-GUS. This indicated that the inhibitory action of the cap analog was at the level of translation initiation rather than elongation. The addition of cap analog at 40 μM resulted in a 45% decrease in translation of capped pTL7SN.3-GUS transcripts relative to noncapped transcripts. This differential effect between capped and noncapped pTL7SN.3-GUS transcripts was reproducible, since it was seen in four separate experiments.

A possible explanation for the apparent cap-independent translation enhancement activity of the TEV 5′ NTR in vitro is that proteins required for initiation and/or ribosomes bind internally within the NTR. This model has been proposed as

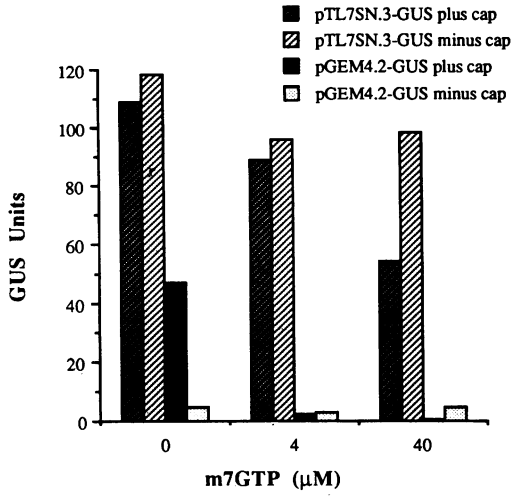

FIG. 6. Effect of the cap analog m^7GTP on cell-free translation of capped or noncapped transcripts derived from pGEM4.2-GUS (with artificial 5′ NTR) or pTL7SN.3-GUS (with TEV 5′ NTR). Translation reactions were conducted for 45 min in the presence of 0, 4.0, or 40 μM m^7GTP, after which GUS activity measurements were taken. The GUS unit values are the averages of two replicates from a typical experiment and are represented as total units per 30-μl translation reaction.

the mechanism whereby picornaviruses initiate translation (see Discussion). This hypothesis predicts that the TEV 5′ NTR (noncapped) will compete for factors required for translation initiation if supplied in excess over a functional mRNA. The TEV 5′ NTR (minus the start codon) was synthesized in vitro from pTL7SN.3 and added to cell-free translation reactions programmed with one of three mRNAs (capped and noncapped transcripts from pTL7SN.3-GUS and capped pGEM4.2-GUS transcripts). The competing NTR was supplied at 75, 150, or 300 nM in the translation reactions, representing 5-, 10-, and 20-fold molar excesses over NTR-GUS mRNA transcripts (15 nM), respectively. Addition of the TEV 5′ NTR to all three reactions inhibited translation in a dose-dependent manner (Fig. 7A). Translation was nearly completely abolished in the presence of a TEV 5′ NTR at 300 nM.

The possibilities that the inhibitory effect of using the TEV 5′ NTR in *trans* was due to the presence of DNA or to a nonspecific effect of excess RNA were tested. Translation reactions using noncapped pTL7SN.3-GUS transcripts were conducted in the presence of competitor RNAs (300 nM) synthesized from *Nco*I- or *Dra*I-linearized pTL7SN.3. *Dra*I cleaves pTL7SN.3 after nt 81 in cDNA representing the TEV 5′ NTR. In addition, reactions were carried out with *Dra*I-linearized pTL7SN.3 DNA (at 9 nM, or approximately 500 ng per reaction) as the competitor. This amount of DNA was present along with each competitor RNA tested, since no effort was made to remove DNA after transcription. Only transcripts representing the entire TEV 5′ NTR (synthesized from *Nco*I-linearized pTL7SN.3) functioned to inhibit translation (Fig. 7B). There was no significant difference between the level of translation measured from reactions with water, DNA, or *Dra*I-terminated transcripts as competitor.

DISCUSSION

We have presented evidence that the TEV 5′ NTR functions as an enhancer of translation when fused to the coding sequence of a reporter gene (GUS). The enhancement activ-

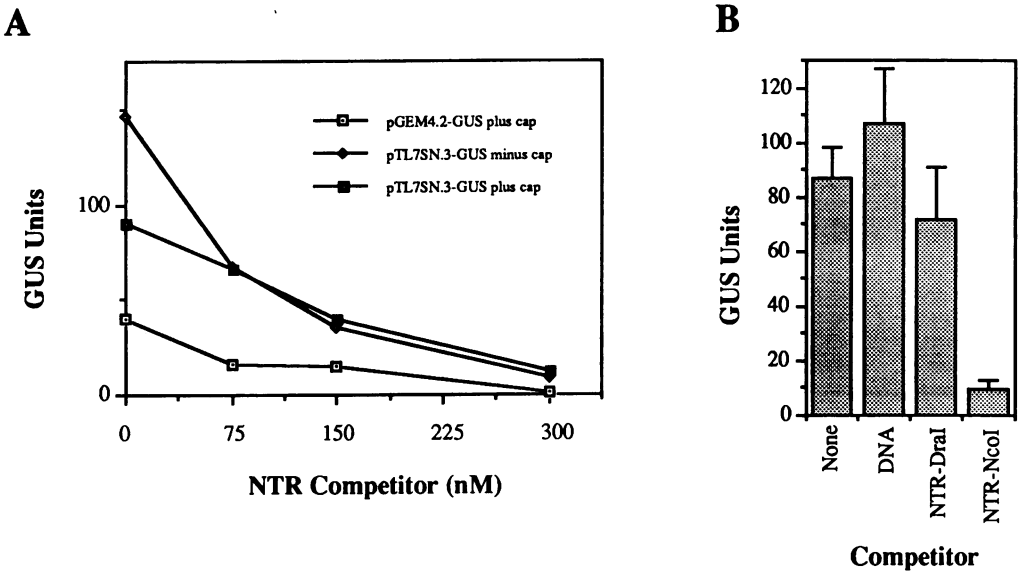

FIG. 7. *trans*-Inhibitory effect of the TEV 5′ NTR on translation of GUS mRNAs in vitro. (A) Translation reactions (30 μl for 45 min) were programmed with capped transcripts (15 nM) from pGEM4.2-GUS or capped or noncapped transcripts from pTL7SN.3-GUS. The reactions were supplied with 0, 75, 150, or 300 nM TEV 5′ NTR (nt 1 to 143) synthesized from pTL7SN.3. These concentrations were equivalent to 5-, 10-, and 20-fold molar excesses over the GUS mRNAs. The GUS unit values are the averages of two replicates from a typical experiment and are represented as total units per 30-μl translation reaction. (B) Translation reactions (30 μl for 45 min) were programmed with noncapped transcripts (15 nM) from pTL7SN.3-GUS. Reactions were conducted without any additional supplements (None) or in the presence of pTL7SN.3 DNA (9 nM) (DNA), transcripts (300 nM) from *Dra*I-linearized pTL7SN.3 (NTR-DraI), or transcripts (300 nM) from *Nco*I-mung bean nuclease-treated pTL7SN.3 (NTR-NcoI). Since the SP6 polymerase transcription reactions were not DNase treated, both competitor transcript preparations also contained DNA at a concentration of 9 nM. Transcripts prepared from *Dra*I- and *Nco*I-linearized pTL7SN.3 represent 5′ NTR segments between TEV nt 1 to 81 and 1 to 143, respectively. The GUS values are expressed as total units per 30-μl reaction.

ity was detected in transient-expression assays with protoplasts after electroporation with either DNA or RNA containing the NTR-GUS fusion, in transformed plants containing integrated copies of NTR-GUS expression cassettes, and in a cell-free translation system programmed with synthetic transcripts. Further analyses revealed that translation initiation on TEV 5′ NTR-containing mRNAs did not require a methylated cap structure. The cap-independent enhancement effect was seen even though the local sequence context of the start codon following the TEV 5′ NTR was less favorable, according to Kozak (26), than that found in the control constructs containing the artificial NTR.

A translation enhancement activity has also been found associated with the 5′ NTRs of a few other positive-strand RNA plant viruses, including alfalfa mosiac virus (AMV) and tobacco mosaic virus (TMV) (14, 23). This feature, however, is not characteristic of all positive-strand viral mRNAs, since fusion of the 5′ NTRs from turnip yellow mosaic or black beetle virus genomes to heterologous coding sequences failed to stimulate translation (13, 22). In contrast to TEV, AMV and TMV possess typical cap structures at the 5′ termini of their genomic RNAs. In vitro translation reconstitution experiments involving reporter gene fusions with the AMV or TMV 5′ NTRs suggest that different molecular mechanisms underlie the enhancer function for each viral sequence. A reduced requirement and/or higher affinity for initiation factors may boost translation initiation on mRNAs linked to the AMV 5′ NTR (4). There is also evidence that sequences within the mRNA coding region influence the interaction of initiation factors with the NTR (4). Interestingly, AMV RNA 4 is capable of translating in poliovirus-infected cell extracts (44), which contain a defective cap-binding complex. On the other hand, interaction with ribosomes appears to mediate the enhancer activity associated with the TMV 5′ NTR (15), although this association does not involve binding of ribosomes internally within the NTR (27, 43). The TEV 5′ NTR may use one or both mechanisms to stimulate translation.

The translation initiation pathway involving the TEV 5′ NTR should account for the following results. (i) Translation did not require a methylated 5′ cap structure. This makes intuitive sense, since TEV genomic RNA possesses a protein linked to the 5′ terminus (16), and, by analogy with the picornaviruses (17, 35), TEV mRNA in vivo probably is not capped. (ii) Translation in vitro was relatively resistant to the presence of a cap analog that inhibited initiation on an mRNA lacking the TEV NTR, suggesting that the cap-binding function of the cap-binding complex was dispensable for translation of mRNA containing the TEV NTR. (iii) In NTR competition experiments, transcripts representing nt 1 to 143 of the TEV 5′ NTR inhibited translation in vitro when supplied in *trans* with functional mRNAs. This inhibitory activity required sequences within the last 63 nt of the NTR, since equivalent molar concentrations of transcripts representing only nt 1 to 81 exhibited no inhibition.

These aggregate data can be fit into a model in which initiation of protein synthesis involves the binding of proteins and/or ribosomes to sequences within the TEV 5′ NTR. This model predicts that initiation of translation is independent of the 5′-terminal structure on the mRNA, since the mRNA recognition pathway would bypasses the RNA 5′ terminus. It predicts that the cap-binding function of the cap-binding complex is dispensable as the initial step in mRNA recognition and subsequent translation: hence, the relative insensitivity to cap analog inhibitor. A final prediction is that binding of putative factors can be competitively inhibited by excess TEV NTR molecules. The soluble factors that bind to the TEV 5′ NTR might include initiation factors, perhaps those normally associated with cap recognition and/or ATP-dependent unwinding of RNA in the mRNA NTRs. This NTR-eIF complex then would recruit ribosomes into the protein synthesis cycle.

The cap-independent, internal initiation model has been proposed previously for entry of ribosomes on picornaviral RNA. The 5′ NTRs from poliovirus and encephalomyocarditis virus are sufficient to direct internal entry of ribosomes on dicistronic mRNAs in vivo and in vitro (20, 37). Transfer of picornavirus 5′ NTRs to reporter genes permits translation of the chimeric mRNAs in poliovirus-infected cells and cell extracts (19, 36, 38, 48), which lack a functional component (p220) within eIF-4F and thus are unable to translate cellular or capped RNAs (10). Results of several studies suggest that sequences spanning a few hundred nucleotides within the picornavirus 5′ NTR confer cap-independent, internal initiation properties on mRNA (3, 20, 36, 41, 47) and that this may be mediated by a specific interaction with a soluble cellular protein(s) (31).

It should be pointed out that our data do not exclude the possibility that translation initiation mediated by the TEV 5′ NTR involves a traditional scanning mechanism in which the requirement for eIF-4F is decreased, perhaps owing to the absence of significant secondary structure within the NTR. This idea and the internal initiation model discussed above will be tested by construction and expression of dicistronic transcripts, as well as RNAs containing strong structure elements and open reading frames positioned within the TEV 5′ NTR.

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