Volume 16 Number 3 1988 Nucleic Acids Research

Mutational analysis of the tobacco mosaic virus 5'-leader for altered ability to enhance translation

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Received October 28, 1987; Accepted December 30, 1987

ABSTRACT

Mutational analysis of the 5'-untranslated leader sequence (Ω) of tobacco mosaic virus (TMV) was carried out to determine those sequences necessary for the translational enhancement associated with Ω. Five deletion mutants, a single base substitution, and a 25 base replacement mutant were tested for alterations in Ω's ability to enhance expression of β-glucuronidase (GUS) mRNA in tobacco mesophyll protoplasts and Escherichia coli or chloramphenicol acetyltransferase (CAT) mRNA in Xenopus laevis oocytes. Alteration of an eight base subsequence required for the binding of a second ribosome resulted in the loss of translational enhancement in X. laevis oocytes but not in protoplasts. Substantial increases in enhancement were observed for several of the mutants in E. coli.

INTRODUCTION

The 5'-untranslated leader (Ω) of tobacco mosaic virus will form disome initiation complexes with 80S wheat germ ribosomes in vitro in the presence of sparsomycin (1). In addition to the initiation codon (AUG), an AUU within Ω, 51 bases upstream to the initiator AUG, has been implicated in the binding of a 80S ribosome (2). The 51 base sequence separating the AUU from the AUG provides adequate spacing for two ribosomes to bind simultaneously without stearic hindrance. That initiation of translation can occur at the AUU as well as the AUG codon is supported by the production of two unique initiation dipeptides, Met-Thr and Met-Ala (3), which could arise from these initiation sites, respectively. In addition to TMV RNA, several other viral RNAs (4,5,6,7) have been shown to form disome or trisome initiation complexes. We have shown previously that translation of prokaryotic (8) or eukaryotic (9) mRNAs is greatly enhanced in vitro or in vivo when Ω is present at the 5' end. Recently we demonstrated that the ability of a RNA to form a disome structure is not consistantly associated with the ability to enhance translation. Although TMV RNA, brome mosaic virus (BMV) RNA3, Rous sarcoma virus (RSV) RNA, and turnip yellow mosaic virus (TYMV) RNA can all form disomes, only the leader sequences of the first three possess the ability to

enhance translation (10). For those leader sequences which do form disomes and enhance translation, it is not known whether the former phenomenon may be responsible for the latter.

We report here the effect that mutations, introduced throughout Ω (derived from the U1 strain of TMV) have on the translational enhancement of GUS or CAT mRNA expression in vivo.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and media

Escherichia coli strains HB101 and JM101 were obtained from F. Bolivar and J. Messing, respectively. The pSP64 derivative pJII101 has been described (8). The chloramphenicol acetyltransferase (CAT) reporter gene from Tn9 was obtained from T.J. Close. The β-glucuronidase gene (GUS) and its derivatives were obtained from R. Jefferson and M. Bevan. SP6 RNA-polymerase, human placental RNase inihibitor, DNA polymerase I (Klenow fragment), T4 DNA ligase and all restriction endonucleases were purchased from Boehringer (Mannhein), Pharmacia Ltd., or New England BioLabs. Purified CAT was bought from Pharmacia Ltd. SOC medium (11) was used to prepare competent E. coli cells, and L-broth (12) was used for all other cultures.

Plasmid DNA purification and manipulation

Preparative scale (13) and small scale (14) DNA isolations were as described. Standard DNA manipulations were performed as described (13).

Oligodeoxyribonucleotide synthesis

Oligodeoxyribonucleotides were synthesized by S. Gilmore and A.J. Northrop (Institute of Animal Physiology, Babraham, Cambridge) using a Biosearch 8600 4-channel DNA synthesizer and the β-cyanoethyl-phosphoramidite method (15). For each full-length dsDNA viral leader, one complete strand (the coding strand) was synthesized with a 5'-HindIII site and a 3'-Sal I site, for subsequent insertion into the transcription plasmid pJII1. A second complementary oligodeoxyribonucleotide (24-mer) was then annealed, and the dsDNA filled-in by polymerization with either DNA polymerase I (Klenow fragment) or reverse transcriptase.

trp promoter construct

The construction of the trp promoter expression vector, pJII168, has been described previously (10). The sequence of the region containing the promoter and transcriptional start site is:

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  -35                -10          ┌►
CTGTTGACAATTAATCATCGAACTAGTTAACTAGTACGAAGCTTGTCGACGGATCC
                                  HindIII SalI BamHI
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RNA synthesis

In vitro transcription of linearized plasmid DNAs was carried out using bacteriophage SP6 RNA polymerase as described (16). Capped transcripts were obtained by modifying the published reaction conditions to include 200μM GTP and 1.5mM G5'ppp5G (Pharmacia, Ltd). RNAs were quantitated either by trace-labelling with α-[^{32}P]-UTP or by formaldehyde-agarose gel electrophoresis as described (16).

Preparation and electroporation of tobacco mesophyll protoplasts

Mesophyll protoplasts were isolated from leaves of Nicotiana tabacum (cv. Xanthi) and stored in 0.7M mannitol (17). Electroporation of RNA into protoplasts and incubations were carried out as previously described (8).

Microinjection of Xenopus laevis oocytes

Preparation and microinjection of X. laevis oocytes was carried out as described previously (10).

GUS and CAT assays

Protoplast or E. coli extracts were prepared and assayed for GUS activity as described previously (10). Cultures containing trp promoter constructs were induced for 4 hr with 20 μg/ml indole acrylic acid before assaying. X. laevis oocytes extracts were prepared and assayed for CAT activity as described previously (10).

RESULTS

The rational behind the construction of Ω based mutants to test for alteration in translational enhancement

The Ω sequence is a multifunctional region involved in efficient uncoating of virions (18), enhanced expression of mRNAs (8), and probably involved as a replicase recognition site during viral replication. Inspection of this 68 base untranslated leader revealed the presence of three 8 base direct repeats, the 5' copy of which is separated from the other two by a 27 base sequence essentially composed of a repetitive CAA element (Fig. 1). Although the 5' untranslated leader sequences from four different strains of TMV vary in length they all contain roughly equivalent repeats and a poly(CAA) sequence (19,20). These conserved structural features of TMV leaders may reflect their role as information sources for the Ω-associated phenomena observed. In our mutational approach towards delineating those sequences necessary for translational enhancement, we constructed a series of Ω-based oligonucleotide sequences incorporating deletions of each structural feature (Fig. 1).

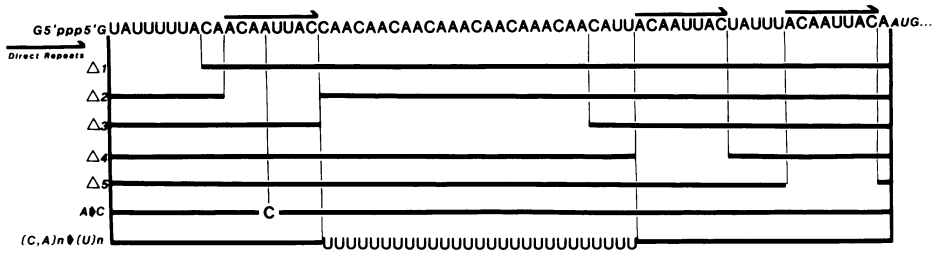

Fig. 1. Ω-derived mutants designed to test for alterations in translational enhancement. The 5' untranslated leader sequence of TMV is shown at the top. The AUG initiation codon of the first open reading frame (126 Kd protein) is shown at the right end. Ω is indicated by the sequence in large letters and the 8 base direct repeats by the arrows above. The sequence present in each mutant construct is designated by a solid line.

In addition, as the AUU contained within the 5' copy of the 8 base repeat has been implicated as the binding site for the second ribosome, an oligonucleotide incorporating a single base substitution of this sequence, from AUU to CUU was also synthesized. Lastly, if this AUU is involved in ribosome binding, sufficient spacing would be necessary between it and the other site of ribosomal binding, the AUG initiation codon for the first open reading frame (126 Kd protein) 51 bases downstream. As ribosomes are estimated to protect approximately 20 bases on either side of a binding site, the 51 bases between the AUU and AUG of the TMV leader could allow a disome structure to exist without steric hindrance between the ribosomes. The poly(CAA) sequence itself may not be important for translational enhancemnt but may function, in part, to provide the correct spacing between the AUU and AUG ribosomal binding sites. To test this hypothesis, the poly(CAA) region was replaced by an unrelated sequence (poly(U)) which maintained the equivalent spacing between the AUU and AUG codons.

For each Ω-based oligonucleotide synthesized, a Hind III and a Sal I site were incorporated at the 5' and 3' ends, respectively, and the double stranded form of the oligonucleotide introduced into the SP6 based derivative, pJII1. A Sal I ended fragment containing the appropriate reporter gene was then inserted into the Sal I site immediately downstream of each derivative to act as sensitive assay system for alterations in translational enhancement.

Mutational analysis of Ω in X. laevis oocytes

Due to the high levels of endogenous GUS activity in X. laevis oocytes, CAT was selected as the reporter mRNA. CAT mRNAs with the Ω-leader derivatives were transcribed in vitro using SP6 polymerase and the mRNAs

microinjected into oocytes. As observed previously (8,10), when Ω was present at the 5' end, translation of the CAT mRNA was stimulated, in this case 9.5 fold (Fig. 2). Deletions within the Ω sequence, regardless of position, resulted in a significant loss of enhancement ability (45% or more). $\Omega_{\Delta 2}$, the mutant in which the sequence for the second ribosome binding site has been deleted, was the most severely affected, having lost 71% of its enhancing ability in relation to the intact Ω sequence. The single base substitution mutant, $\Omega_{A\rightarrow C}$, in which the AUU implicated in the binding of the second ribosome was changed to CUU, lost 55% of its enhancing ability. Although the deletion of the poly(CAA) resulted in a reduction of enhancement by 46%, substitution of this same region with poly(U) ($\Omega_{C,A\rightarrow U}$) surprisingly did not alter the ability of this sequence to enhance translation from that seen for Ω itself.

Mutational analysis of Ω in tobacco mesophyll protoplasts

Ω has been shown to enhance the translation of both CAT and GUS mRNAs in electroporated tobacco mesophyll protoplasts (8,10). The GUS system has proven to be the more sensitive of the two reporter genes in these protoplasts. In addition, a derivative of the GUS gene containing a good initiation codon context (21) has been constructed (9) and, as a result, is several fold more sensitive as a reporter mRNA in tobacco protoplasts than GUS mRNA which contains the native initiation codon context (10). Both GUS constructs were used to test the Ω-derivatives in protoplasts. Capped mRNA of each leader-GUS construct was produced in vitro using SP6 polymerase and delivered to tobacco protoplasts by means of electroporation. The presence of Ω at the 5' end of "bad" and "good" initiation context GUS mRNAs stimulated translation of these mRNAs 18 and 46 fold, respectively (Table 1). The effect of the mutations on Ω's ability to enhance GUS mRNA translation resulted in the same pattern for both the "good" context and "bad" context" mRNAs. Deletions introduced throughout Ω did not significantly alter translational enhancement with the exception of $\Omega_{\Delta 3}$, which lost 39% and 48% of its activity with GUS mRNA of "bad" and "good" initiation context, respectively. Replacement of the poly(CAA) with poly(U) resulted in total loss of enhancing ability using "bad" context mRNA and an 87% reduction in activity with "good" context mRNA. The single base subtitution mutant, $\Omega_{A\rightarrow C}$, actually resulted in a small but reproducible increase (13% to 28%) in translational enhancement over that seen for Ω.

Mutational analysis of Ω in E. coli

Although there exists many differences between translational initiation between prokaryotes and eukaryotes, Ω stimulates translation in both, though

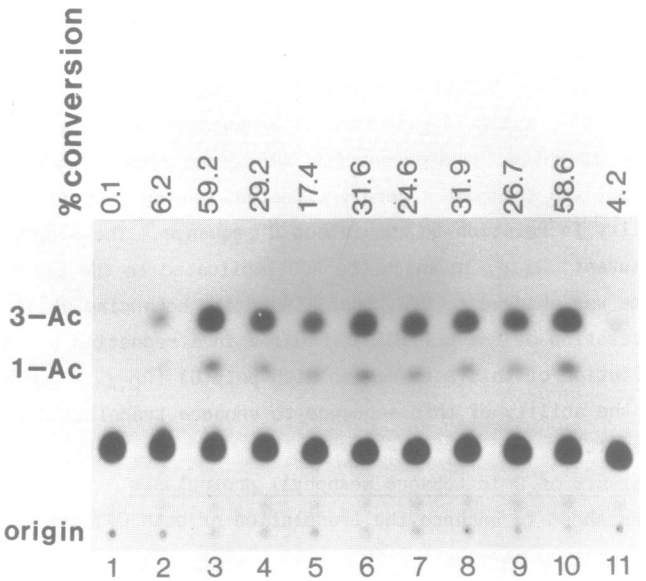

Fig. 2. The effect of mutations within the Ω sequence on the expression of CAT mRNA in microinjected X. laevis oocytes. Equivalent oocyte volumes (0.2 x cell) were assayed in each case. Conversion (%) of ^{14}C-chloramphenicol into its mono-acetylated forms is shown above each lane. Microinjected RNAs were: lane 1, no RNA (mock); lane 2, CAT mRNA; lane 3, Ω-CAT mRNA; lane 4, $\Omega_{\Delta 1}$-CAT mRNA; lane 5, $\Omega_{\Delta 2}$-CAT mRNA; lane 6, $\Omega_{\Delta 3}$-CAT mRNA; lane 7, $\Omega_{\Delta 4}$-CAT mRNA; lane 8, $\Omega_{\Delta 5}$-CAT mRNA; lane 9, $\Omega_{A \rightarrow C}$-CAT mRNA; lane 10, $\Omega_{C,A \rightarrow U}$-CAT mRNA; lane 11, 0.1 unit purified CAT enzyme added to an equivalent volume of extract as in lane 1. The dried tlc plate was autoradiographed at room temperature for 4 hours before excising and counting the relevent ^{14}C-labeled spots.

not necessarily by the same mechanism. Whether the Ω associated enhancement seen in E. coli is artifactual or not, the fact that Ω can stimulate prokaryotic translation is of value in identifying those factors which influence translation in prokaryotes.

A vector construct containing a derivative of the trp promoter by which the leader-GUS mRNAs can be produced in vivo has been described previously in detail (10) and briefly here (see Materials and Methods). The leader-GUS constructs were introduced just downstream of the transcriptional start site so that only four additional bases were present at the 5' end of the mRNA. Although neither form of the GUS gene described in this study possesses a Shine-Dalgarno region (22), the GUS derivative with the native initiation codon context, when introduced downstream of the trp promoter, is capable of

TABLE 1
The effect of mutations within Ω on translational enhancement of GUS mRNAs electroporated into tobacco protoplasts

mRNAs	Bad initiation codon context		Good initiation codon context	
	Specific activity (nmoles MUG hydrolysed/ min/μg protein)	Fold stimulation	Specific activity (nmoles MUG hydrolysed/ min/μg protein)	Fold stimulation
GUS	0.03	1	0.054	1
Ω-GUS	0.55	18	2.5	46
$\Omega_{\Delta 1}$-GUS	0.55	18	2.3	43
$\Omega_{\Delta 2}$-GUS	0.50	17	2.7	50
$\Omega_{\Delta 3}$-GUS	0.32	11	1.3	24
$\Omega_{\Delta 4}$-GUS	0.54	18	2.1	39
$\Omega_{\Delta 5}$-GUS	0.54	18	2.5	46
$\Omega_{A\rightarrow C}$-GUS	0.69	23	2.8	52
$\Omega_{A,C\rightarrow U}$-GUS	<0.01	<1	0.33	6

expressing a low level of GUS (10). In contrast, the GUS construct containing the altered initiation codon context ("good" context for eukaryotes) results in almost no detectable GUS activity (Table 2) and therefore was used to assay the Ω derivatives for translational enhancement in E. coli. As observed previously, Ω stimulated translation of GUS mRNA 8 fold (10). Of the five deletion mutants, three had little or no effect on Ω's enhancing ability. The replacement of the poly(CAA) region with poly(U) ($\Omega_{C,A\rightarrow U}$) resulted in only a small reduction in enhancement. Surprisingly though, two mutants, $\Omega_{\Delta 2}$ and $\Omega_{\Delta 3}$, at 73 and 75 fold enhancement, respectively, exhibited a nine fold increase in translation over that seen for Ω. In addition, $\Omega_{A\rightarrow C}$ possessed elevated enhancing potential as it was over six fold more active in stimulating translation than Ω itself.

DISCUSSION

Little is understood about the role that the 5' untranslated leader sequences play in the translation of mRNAs. Only recently has work begun to examine the effect of untranslated viral leader sequences on reporter mRNA expression (8,10,23). In this report, we concentrated on the leader sequence (Ω) from one virus, TMV, and described the effect that mutations, introduced within the Ω sequence, have on its ability to enhance translation. The

TABLE 2
The effect of mutations within Ω on translational enhancement of GUS mRNAs in E. coli

mRNAs	Specific activity (nmoles MUG hydrolyzed/ min/μg protein)	Fold stimulation
GUS	3.8	1
Ω-GUS	32.0	8
$\Omega_{\Delta 1}$-GUS	30.6	8
$\Omega_{\Delta 2}$-GUS	276	73
$\Omega_{\Delta 3}$-GUS	285	75
$\Omega_{\Delta 4}$-GUS	30.4	8
$\Omega_{\Delta 5}$-GUS	41.9	11
$\Omega_{A \rightarrow C}$-GUS	199	52
$\Omega_{A,C \rightarrow U}$-GUS	18.1	5

Ω sequence has been shown to bind two 80S ribosomes in presence of sparsomycin and the binding site for the second ribosome (in addition to the binding site at the AUG initiation codon) mapped to a region which includes an AUU codon. This AUU falls within an eight base direct repeat present in three copies in Ω, but only the copy nearest the 5' end has been implicated in ribosome binding. Moreover, this same repeat has been suggested by Yokoe et al. (24) to interact with the 3' end of wheat 18S rRNA. If the translational enhancement observed for Ω is associated with Ω's ability to form a disomic structure, then deletion of the region responsible for binding of the second ribome may result in the loss of translational enhancement. This is, in fact, what is observed in X. laevis oocytes. When the first direct repeat is deleted or the AUU contained within changed to a CUU, substantial lost of enhancing potential is seen. The poly(CAA) sequence itself seems to be nonessential for translational enhancement in oocytes as it can be replaced with an unrelated sequence without altering enhancement. But deletion of the poly(CAA) region does result in some loss of activity, suggesting that this region may play a role in the secondary structure of Ω or may serve to maintain the proper spacing between more essential elements of the leader.

The data from the tobacco protoplast system highlights the differences that exist between eukaryotic systems with respect to factors influencing translation efficiency. Ω-associated enhancement was much less sensitive to mutations introduced throughout the leader sequence. Although replacement of the poly(CAA) region with poly(U) resulted in a dramatic loss of enhancement (87%-100%), the deletion of this same poly(CAA) region resulted in a much less severe reduction (39%-48%). Instead of implicating this poly(CAA) region as the essential enhancing element, these data might suggest that the 27 U's which make up the poly(U) region are detrimental to the 40S scanning process or to 80S formation in protoplasts, although such a sequence poses no problem in X. laevis oocytes. Also intriguing is the effect of the single base substitution mutant, which in protoplasts actually increased enhancement potential by 13%-28%, but resulted in 55% loss of activity in oocytes. Such substantial differences in translational efficiency have also been observed for human interferon-β mRNA, assayed in wheat germ extracts and X. laevis oocytes. The 5', as well as the 3', untranslated regions of the mRNA have been identified as the responsible elements by which this difference arises (25).

That the deletion mutations throughout Ω do not result in a substantial loss of translational enhancement in protoplasts may be a consequence of the repetitive nature of Ω. Although the X. laevis results suggest the 5' 8 base repeat does play an important role in enhancing translation in oocytes, in the protoplast system, any of the direct repeats may suffice for this purpose. Deletion of any one repeat, therefore, would still leave two present in a leader construct. For the protoplast system, at least, it would be necessary to test a single copy of the 8 base repeat or the poly(CAA) sequence separately for translational enhancement potential. However, the data from the Ω-derivatives in protoplasts do not support the hypothesis that Ω's ability to form disomes is responsible for its enhancing ability.

In the E. coli system, the differences between factors influencing translation become even more marked. That Ω enhances translation in E. coli may be entirely fortuitous and may do so in a manner completely different from that in eukaryotes. The fact that eukaryotic 80S ribosomes are thought to be limited in their binding of a mRNA to the 5' end, whereas prokaryotic 70S ribosomes are not might suggest different mechanisms of interaction with Ω. Ω remained relatively insensitive to mutations in E. coli except for the region delineated by the first direct repeat and the poly(CAA) sequence. Those mutants which represented deletions or alterations of these regions

actually increased their ability to enhance translation 9 fold over that observed for Ω itself.

ACKNOWLEDGEMENTS

We are grateful to Peter Watkins and Clive Mason for excellent technical assistance. We thank Richard Jefferson and Michael Bevan for pRAJ235 and pRAJ275. This work was funded by Diatech Ltd., London. D.E.S. was supported by a CASE studentship from the Science and Engineering Research Council. These results form part of New British Patent Application No. 86 13481.

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