A comparison of eukaryotic viral 5′-leader sequences as enhancers of mRNA expression in vivo

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ABSTRACT

The 5′-untranslated leader sequences of several plant RNA viruses, and a portion of the 5′-leader of an animal retrovirus, were tested for their ability to enhance expression of contiguous open reading frames for chloramphenicol acetyltransferase (CAT) or β-glucuronidase (GUS) in tobacco mesophyll protoplasts, Escherichia coli and oocytes of Xenopus laevis. Translation of capped or uncapped transcripts was substantially enhanced in almost all systems by the leader sequence of either the U1 or SPS strain of TMV. All leader sequences, except that of TYMV, stimulated expression of 5′-capped GUS mRNA with the native prokaryotic initiation codon context, in electroporated protoplasts. Only the TMV leaders enhanced translation of uncapped GUS mRNAs in protoplasts and increased expression of uncapped CAT mRNA in microinjected X. laevis oocytes. In oocytes, the TYMV leader sequence was inhibitory.

In transformed E. coli, the TMV-U1 leader enhanced expression of both the native and eukaryotic context forms of GUS mRNA about 7.5-fold, despite the absence of a Shine-Dalgarno region in any of the transcripts. The absolute levels of GUS activity were all about 6-fold higher with mRNAs containing the native initiation codon context. In E. coli, the leaders of AlMV RNA4 and TYMV were moderately stimulatory whereas those of BMV RNA3, RSV and the SPS strain of TMV enhanced GUS expression by only 2- to 3-fold.

INTRODUCTION

Cis-acting features which influence the selection and translation of eukaryotic mRNAs are poorly understood. Surveys of sequences upstream from the AUG start codon have failed to identify a universal consensus sequence which might act as the eukaryotic equivalent of the prokaryotic Shine-Dalgarno region, a region essential for the expression of prokaryotic mRNAs in E. coli (1). Secondary structures within the 5′-untranslated leaders of some eukaryotic mRNAs have been claimed to promote
(2,3) or inhibit (4) translation initiation. In prokaryotic mRNAs, selection of start codons may also be influenced, in part, by low surrounding secondary structure (5). In the relaxed scanning model (6), 40S ribosomal subunits bind at the 5'-end of an mRNA and scan until the first AUG in the optimal context (5'-ACCAUGG-3') is reached, at which point translation begins. Beyond this, little is known about the longer-range effects of specific sequences on expression of eukaryotic mRNA.

We have shown that translation of prokaryotic (7) and eukaryotic (8) mRNAs is greatly enhanced by a contiguous derivative of the 68-nucleotide, 5'-leader sequence of tobacco mosaic virus (TMV), Ul strain (called Omega (Ω); 9,10). The stimulatory effect of this Ω-like sequence (referred to as Ul-Ω) has been observed in vitro and in vivo, in both eukaryotic and prokaryotic translation systems. Tyc and co-workers (11) identified a second 80S ribosome binding site, centred on residues 14-16 (AUU) within Ul-Ω (or Ω'), which was upstream of, and in frame with, the predicted ribosome binding site at the first AUG codon (residues 68-70 in Ul-Ω). The latter initiates synthesis of the 126,000 dalton (126Kd) protein encoded by TMV RNA. In Ul-Ω (and Ω'-Ul), 51 nucleotides separate the AUU and AUG sequences which, in the presence of an inhibitor of elongation (sparsomycin), permit two ribosomes to bind simultaneously without steric hindrance. Initiation of translation of genomic TMV RNA under these conditions has been claimed to result in two unique dipeptides, Met-Thr and Met-Ala, (12) which may arise by illegitimate or legitimate initiation at the AUU and AUG sites, respectively. Yokoe and coworkers (13) demonstrated RNA-RNA hybridization between the 5'-region of Ul-Ω, containing the AUU sequence, and the 3'-terminus of wheat germ 18S rRNA, again supporting the possibility of disome formation. In addition to TMV, several other viral RNA leader sequences have been shown to form disome (or even trisome) structures (2, 14-16). The 36-nucleotide leader of A1MV RNA4 binds only one ribosome (17), nevertheless it will stimulate expression of contiguous foreign gene transcripts in vitro (18).

We wished to determine whether translational enhancement was a general feature of 5'-untranslated viral leader sequences and
if the ability of a viral leader to form disomes could be correlated with its ability to enhance translation of a contiguous open reading frame. For this purpose, synthetic oligonucleotide sequences derived from the 5'-leaders of TMV (Ul strain; disome), TMV (SPS strain; disome), turnip yellow mosaic virus (TYMV; disome), alfalfa mosaic virus (AlMV) RNA 4 (monosome), brome mosaic virus (BMV) RNA 3 (disome), and the animal retrovirus, Rous sarcoma virus (RSV; disome) were analyzed for their relative abilities to stimulate expression of convenient reporter gene transcripts 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 derivatives pJIII1, pJIll101, pJI12, pJI1102 have been described (7). The chloramphenicol acetyltransferase (CAT) reporter gene from Tn9 was obtained from T.J. Close (CSIRO, Canberra, Australia). The p-glucuronidase gene (GUS) and its derivatives were obtained from R. Jefferson and M. Bevan (Plant Breeding Institute, Maris Lane, Trumpington, Cambridge). SP6 RNA-polymerase, human placental RNase inhibitor, DNA polymerase I (Klenow fragment), T4 DNA ligase and all restriction endonucleases were purchased from Boehringer (Mannheim), Pharmacia Ltd., or New England BioLabs. Purified CAT was bought from Pharmacia Ltd. SOC medium (19) was used to prepare competent E. coli cells, and L-broth (20) was used for all other cultures.

Plasmid DNA purification and manipulation

Preparative scale (21) and small scale (22) DNA isolations were as described. Standard DNA manipulations were performed essentially as described (21).

Oligodeoxyribonucleotide synthesis

Oligodeoxyribonucleotides were synthesized by S. Gilmore and A.J. Northrop (Institute of Animal Physiology, Babraham, Cambridge) using a Bioschere 8600 4-channel DNA synthesizer and the β-cyanoethyl-phosphoramidite method (23). For each full-length dsDNA viral leader, one complete strand (the coding strand) was synthesized with a 5'-HindIII site (+1 base) and a
3'- SalI site (+ 1 base), for subsequent insertion into the transcription plasmid pJIII. A second complementary oligodeoxyribo nucleotide (24-mer) was then annealed, and the dsDNA filled-in by polymerization with either DNA polymerase I (Klenow fragment) or reverse transcriptase.

Construction of trp promoter plasmid pJIII68 for E. coli transformation

A 90 base pair (bp) HindIII/BamHI fragment containing the tryptophan (trp) promoter (P-L Biochemicals, Inc.) was introduced into the HindIII/BamHI sites of pJIIl (7), from which a 400bp BamHI fragment containing the TMV origin-of-assembly sequence had been removed. The HindIII site upstream from the trp promoter was removed by digestion with HindIII, filling-in with DNA polymerase I (Klenow fragment), followed by re-ligation. A HindIII site was then introduced at the 3'-end of the promoter by replacing the 25bp HpaI/SalI fragment with a synthetic 17bp HpaI/SalI fragment, containing a HindIII site positioned at the transcription start site.

RNA synthesis

In vitro transcription of linearized plasmid DNAs was carried out using bacteriophage SP6 RNA polymerase (24). 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-[32P]-rUTP or by formaldehyde-agarose gel electrophoresis as described (24).

Preparation and electroporation of tobacco mesophyll protoplasts

Mesophyll protoplasts were isolated from leaves of Nicotiana tabacum (cv. Xanthi) and stored in 0.7M mannitol (25). Electroporation of RNA into protoplasts and incubations were carried out as previously described (7).

After incubation, electroporated protoplasts were sedimented, resuspended and broken by ten passages through a 26-gauge needle in 400μl of 0.25M Tris-HCl, pH 7.4, containing 10mM dithiothreitol (DTT). Extracts were microcentrifuged at 10,000xg for 10 min at 4°C.

Microinjection of Xenopus laevis oocytes

X. laevis were purchased from Xenopus Ltd., South Nuffield,
Two ng of each synthetic uncapped SP6 mRNA were injected into the cytoplasm of stage 6 oocytes in batches of 25 using standard procedures (26). Oocytes were incubated for 21 hours in Modified Barth's Saline, then washed briefly in distilled water. Extracts from Xenopus oocytes were prepared by resuspending each sample in 0.25M Tris-HCl, pH 7.4, 10mM DTT (20μl/oocyte), followed by sonication for 10 sec. Insoluble material was removed by microcentrifugation for 15 min and fractions of the supernatant representing equivalent numbers of oocytes were assayed for CAT activity.

**CAT assay**

The protein concentration of each supernatant from X. laevis oocytes or tobacco protoplasts was determined by the method of Bradford (27). The CAT assay was essentially as described (28), but used 0.25M Tris-HCl, pH 7.4, containing 10mM DTT and 30mM acetyl-CoA. Quantitation of the thin-layer chromatograph was achieved by cutting out the area corresponding to each 14C-labelled spot and counting in a toluene-based scintillant containing 4% (w/v) PPO and 0.005% (w/v) POPOP.

**GUS assay**

GUS activity was measured spectrophotometrically or fluorimetrically in 0.5ml assay buffer containing 50mM sodium phosphate, pH 7.0, 10mM 2-mercaptoethanol, 0.1% (v/v) Triton X-100 and either 1mM β-nitrophenyl-β-D-glucuronide (PNPG; for E. coli extracts) or 0.5mM 4-methyl-umbelliferyl-β-D-glucuronide (MUG; for tobacco protoplast extracts). Assays were carried out at 37°C and were terminated by addition of 0.4ml 2.5M 2-amino-2-methyl-1,3-propanediol for E. coli extracts, or 0.5ml 0.2M Na2CO3 for protoplast extracts. β-Nitrophenol absorbance was measured at 415nm using a Pye Unicam SP1800 Spectrophotometer. Fluorescence was measured by excitation at 365nm and emission at 455nm in a Perkin-Elmer 204 Fluorescence Spectrophotometer.

**In situ localization of GUS activity in SDS-polyacrylamide gels**

Samples of protoplast extracts containing equivalent amounts of protein were incubated with an equal volume of gel loading buffer (29) at room temperature for 15 min, followed by SDS-polyacrylamide gel electrophoresis (29) in a 12.5% (w/v) gel at 50V for 16 hours. The gel was rinsed 4 times in 100ml assay...
buffer (without the glucuronide substrate) for a total of 2 hours, incubated on ice in assay buffer containing 0.5mM MUG for 30 min, and transferred to a glass plate at 37°C for 30 min. The gel was then sprayed with 0.2M Na2CO3 and photographed under long-wavelength ultraviolet light using a Wratten 2E filter.

RESULTS

Quantitation of the effect of J'-U1 and initiation codon context on expression of GUS mRNAs in tobacco protoplasts.

A derivative of the TMV leader, J'-U1 (Fig. 1), has been shown to enhance translation of CAT mRNA in tobacco mesophyll protoplasts, and other eukaryotic and prokaryotic systems (7). To quantitate the effect of J'-U1 in protoplasts more precisely, we used the GUS reporter gene (30). A Sall-ended fragment containing the GUS gene from pRAJ235 (30) was introduced into the Sall site of the pSP64-derived vectors pJIHO1 and pJI11 (7), resulting in pJI120 and pJI119, with or without a 5'-proximal J'-U1 sequence, respectively. The native Sall GUS fragment had 19 nucleotides upstream of the AUG start codon (Fig. 1). The context of this AUG codon (5'- CCCUUAUGU-3') was, according to Kozak (6), inefficient for eukaryotic translation (hereafter referred to as "bad context" GUS). To determine whether the effect of J'-U1 on mRNA expression was influenced by the context of the initiation codon, a Sall fragment of a derivative of the GUS gene with an initiation codon context (5'-CGACCAUGG-3') close to the consensus sequence for optimal eukaryotic translation initiation was constructed (in pRAJ275; (8)). This derivative (hereafter referred to as "good context" GUS) had only 7 nucleotides upstream of the AUG (Fig. 1). This Sall fragment was introduced into pJI1101 and pJI1 as for "bad context" GUS, resulting in pJI140 and pJI139, with or without a 5'-proximal J'-U1 sequence, respectively. 5'-Capped or uncapped mRNAs were synthesized in vitro by SP6 RNA polymerase on BglII-linearized pJI1119, pJI1120, pJI1139, pJI1140 templates. Eight micrograms of each transcript were electroporated into tobacco mesophyll protoplasts and incubated for 20 hours at 25°C. Assaying protoplast extracts by GUS-activity gel (Fig. 2) revealed that "good context" GUS mRNAs (tracks 6-9) were expressed more efficiently.
Fig. 1. DNA constructs representing the 5′-untranslated viral leaders tested for translational enhancement. The sequence of the untranslated portion of each viral RNA up to position +4 (6) of the first open reading frame is shown. Each initiation codon (AUG) is underlined. The region of each leader sequence used in the construction of the corresponding oligodeoxyribonucleotide is marked above the RNA sequence by the bold line (the uppermost being \( \text{N}^-\text{U}^l \)). Terminal restriction sites for HindIII and SalI were present in each DNA construct. Additional nucleotides present between the SalI site and the start codon (underlined) of the CAT or GUS reporter gene cassettes are shown below as RNA sequences.

than "bad context" GUS mRNAs (tracks 2-5). In addition, the presence of \( \text{N}^-\text{U}^l \) on both the "good" and "bad context" GUS mRNAs enhanced expression considerably, whether the mRNAs were capped or not. Accurate fluorimetric quantitation of the kinetics of GUS activity (Fig. 3) and hence of GUS mRNA expression (Table 1), revealed that the levels of expression of uncapped "good" or "bad context" GUS mRNAs were below the limit of detection and only became detectable when the transcripts were capped and/or when \( \text{N}^-\text{U}^l \) was present. In all cases, the presence of \( \text{N}^-\text{U}^l \)
Fig. 2. β-glucuronidase activity-gel of extracts from electro-
porated tobacco mesophyll protoplasts. Extract volumes repre-
senting equivalent amounts of protein were loaded onto each
track. Both "bad" and "good context" GUS mRNAs were used to
quantitate the effect of 5'-Ul or a 5'-cap on expression of the
enzyme. Electroporated RNAs were: track 1, no RNA (mock); tracks
2-5, "bad context" mRNAs and tracks 6-9, "good context" mRNAs.
Tracks 2 and 6, GUS mRNA; tracks 3 and 7, 5'-Ul-GUS mRNA; tracks
4 and 8, 5'-capped-GUS mRNA; tracks 5 and 9, 5'-capped-5'-Ul-GUS
mRNA.

enhanced expression markedly, stimulating the "bad context" GUS
mRNA approximately 20-fold. Stimulation of "good context" GUS
mRNA by 5'-Ul was even greater, showing an 80-fold increase with
the capped form of the transcript (Table 1).

Other viral leader sequences as translational enhancers in
tobacco protoplasts.

To determine whether the phenomenon of translational enhan-
cement is associated with all viral RNA leader sequences or only
with those leaders which form disome structures, HindIII and
Sall-linked oligonucleotides were synthesized which incorpora-
ted the 5'-leader sequences of: TMV (SPS strain), TYMV, AlMV
RNA4, BMV RNA3, and part of RSV RNA (Fig.1). Due to constraints
of synthesis only the 5'-112 residues of the 380-nucleotide RSV
Fig. 3. Kinetic analysis of β-glucuronidase activity in extracts from electroporated tobacco mesophyll protoplasts. Graphical display of the rate of appearance of the reaction product (4-methyl-umbelliferone, 4-MU). Extract volumes representing equivalent amounts of protein were added to each assay. ▲, "bad" or "good context" GUS mRNAs; △, 5'-capped "bad" or "good context" GUS mRNAs; ■, 5'-U1-"bad context" GUS mRNA; □, 5'-U1-"good context" GUS mRNA; ●, 5'-capped-5'-U1-"bad context" GUS mRNA; ○, 5'-capped-5'-U1-"good context" GUS mRNA.

leader (3) were synthesized. This includes the region (residues 9-53) of the native RSV leader shown to act as the binding site for a second 80S ribosome (16). For cloning purposes, these oligonucleotides were manipulated in an identical fashion to 5'-U1 (7). A family of SP6-transcripts in which each leader was located upstream of the "bad context" GUS gene were electroporated into tobacco protoplasts. Only the 5'-U1 and, to a lesser extent, the 5'-SPS leaders proved stimulatory for uncapped transcripts (Table 2). However, when the transcripts were capped, stimulation was observed with the leaders of A1MV RNA4, BMV RNA3
TABLE 1
Translational enhancement by \( \nu^-U_1 \) on GUS mRNAs electroporated into tobacco protoplasts

<table>
<thead>
<tr>
<th>SP6-RNAs</th>
<th>Initiation codon context</th>
<th>Specific activity (nmoles MUG hydrolysed/ min/µg protein)</th>
<th>Fold-stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncapped</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUS</td>
<td>bad</td>
<td>&lt;0.01</td>
<td>1</td>
</tr>
<tr>
<td>( \nu^-U_1)-GUS</td>
<td>bad</td>
<td>0.18</td>
<td>&gt;18</td>
</tr>
<tr>
<td>GUS</td>
<td>good</td>
<td>&lt;0.01</td>
<td>1</td>
</tr>
<tr>
<td>( \nu^-U_1)-GUS</td>
<td>good</td>
<td>0.35</td>
<td>&gt;35</td>
</tr>
<tr>
<td>5'-Capped</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUS</td>
<td>bad</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td>( \nu^-U_1)-GUS</td>
<td>bad</td>
<td>0.61</td>
<td>20</td>
</tr>
<tr>
<td>GUS</td>
<td>good</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>( \nu^-U_1)-GUS</td>
<td>good</td>
<td>3.2</td>
<td>80</td>
</tr>
</tbody>
</table>

and "RSV" as well as \( \nu^-U_1 \) and \( \nu^-SPS \). Only with the TYMV leader did the level of GUS activity remain below the limit of detection.

Other viral leader sequences as translational enhancers in X. laevis oocytes

We have shown (7) that Xenopus oocytes, microinjected with capped or uncapped CAT mRNAs, gave approximately 3- to 4-fold more CAT activity when the \( \nu^-U_1 \) leader sequence was present. In common with most (or all) animal cells, Xenopus oocytes contain high levels of endogenous GUS activity. It was therefore not feasible to assay the different viral leader sequences using GUS mRNA as the reporter. Consequently, various pSP64-based leader constructs, each containing the CAT gene, were transcribed and the uncapped mRNAs microinjected into oocytes. In this experiment, the presence of \( \nu^-U_1 \) gave a 7.5-fold enhancement of CAT activity (Fig. 4). This probably reflects the better quality oocytes than were used previously (7). The \( \nu^-SPS \)
### TABLE 2
Translational enhancement by various viral leaders on "bad context" GUS mRNAs electroporated into tobacco protoplasts

<table>
<thead>
<tr>
<th>SP6-RNAs</th>
<th>Specific activity (nmol MUG hydrolysed/min/µg protein)</th>
<th>Fold-stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uncapped</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUS</td>
<td>&lt; 0.01</td>
<td>1</td>
</tr>
<tr>
<td>Jl'-U1-GUS</td>
<td>0.25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Jl'-SPS-GUS</td>
<td>0.15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>TYMV-GUS</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>AlMV RNA4-GUS</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>BMV RNA3-GUS</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>&quot;RSV&quot;-GUS</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td><strong>5'-Capped</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUS</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td>Jl'-U1-GUS</td>
<td>0.54</td>
<td>18</td>
</tr>
<tr>
<td>Jl'-SPS-GUS</td>
<td>0.43</td>
<td>14</td>
</tr>
<tr>
<td>TYMV-GUS</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>AlMV RNA4-GUS</td>
<td>0.23</td>
<td>8</td>
</tr>
<tr>
<td>BMV RNA3-GUS</td>
<td>0.23</td>
<td>8</td>
</tr>
<tr>
<td>&quot;RSV&quot;-GUS</td>
<td>0.23</td>
<td>8</td>
</tr>
</tbody>
</table>

sequence gave a similar (6-fold) level of enhancement. The BMV RNA3, "RSV", and AlMV RNA4 leaders were not stimulatory in this system. The TYMV leader sequence appeared to reduce expression of CAT mRNA.

**Enhancement by viral leader sequences in prokaryotic cells**

In previous work (7), Jl'-U1 was shown to be stimulatory in vitro in an E. coli translation system. The reporter gene sequences used encoded CAT or neomycin phosphotransferase (NPTII). In both cases, the transcripts contained the natural prokaryotic
Fig. 4. The effect of various viral leader sequences on expression of CAT mRNAs microinjected into X. laevis oocytes. Oocyte extract volumes (equivalent to 0.25 x cell) were assayed in each case. Conversion (%) of 14C-chloramphenicol into its mono-acetylated form, is shown above each track. Microinjected RNAs were: track 1, no RNA (mock); track 2, CAT mRNA; track 3, A'-Ul-CAT mRNA; track 4, A'-SPS-CAT mRNA; track 5, TYMV-CAT mRNA; track 6, AlMV RNA4-CAT mRNA; track 7, BMV RNA3-CAT mRNA; track 8, "RSV"-CAT mRNA; track 9, 0.1 unit purified CAT enzyme added to an equivalent volume of extract as in track 1. The dried tlc plate was autoradiographed at room temperature for 4 hours before excising and counting the relevant 14C-labelled spots.

Shine-Dalgarno (S-D) ribosome-binding site. The S-D sequence was located between the 3'-end of A'-Ul and the start of the open reading frames for CAT or NPTII. The S-D region is considered to be the most critical feature of a prokaryotic mRNA, signalling the attachment of a 30S ribosomal subunit to initiate translation at a downstream start codon. Nevertheless, with A'-Ul positioned upstream from the natural S-D region of CAT or NPTII, there was a significant enhancement of translation in vitro in
Fig. 5. Trp-promoter construct used to assay the effect of viral leader sequences on expression of various GUS gene transcripts in situ, in E. coli. -35, -10, and operator regions of the promoter are designated above the sequence. Restriction sites are underlined below. The arrow indicates the site of transcription initiation. GUS gene cassettes were introduced at the SalI site of pJII168 after the various HindIII/ SalI leader cartridges (Fig. 1) had first been inserted.

E. coli. Recently (8), we have shown that Jl-U1 also stimulates translation of eukaryotic mRNAs, which contain no S-D-like sequence, in vitro in an E. coli cell-free system.

To complement these observations, we examined the effect of Jl'-U1 on the in vivo expression of a prokaryotic mRNA which lacked a S-D region. A derivative of the tryptophan (trp) promoter was constructed (Fig. 5) in the plasmid pJII168. Although the HindIII site altered the native sequence of the trp operator region slightly, this derivative retained the regulation associated with the wild-type trp promoter (data not shown). The position of the HindIII site resulted in addition of only 4 nucleotides upstream of each leader construct, in contrast to the 12-additional nucleotides present in our in vitro SP6 transcripts. The SalI-ended "bad context" GUS gene fragment has the native (E. coli) context of the AUG codon and 13-nucleotides upstream from the AUG (Fig. 1). In the native GUS gene, the S-D region began just upstream of this 13-nucleotide leader, but this has now been replaced by a sequence containing the SalI site. When the "bad context" GUS gene was introduced downstream of the trp promoter and transformed HB101 cells were induced and assayed, a low but measurable level of GUS activity was detected (Table 3). This is in agreement with the previous observation (31) that the presence of a complete S-D sequence can be advantageous but is not essential for gene expression. Insertion of Jl'-U1 between the trp promoter and the GUS sequence resulted in a 7-fold increase.
TABLE 3
Translational enhancement by various viral leaders on GUS mRNAs in E. coli transformed with a recombinant trp promoter plasmid

<table>
<thead>
<tr>
<th>Leader-GUS construct</th>
<th>Initiation codon context</th>
<th>Specific activity (nmoles PNPG converted/min/µg protein)</th>
<th>Fold-stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUS</td>
<td>bad</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>J'-U1-GUS</td>
<td>bad</td>
<td>162</td>
<td>7</td>
</tr>
<tr>
<td>GUS</td>
<td>good</td>
<td>3.8</td>
<td>1</td>
</tr>
<tr>
<td>J'-U1-GUS</td>
<td>good</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>J'-SPS-GUS</td>
<td>good</td>
<td>7.4</td>
<td>2</td>
</tr>
<tr>
<td>TYMV-GUS</td>
<td>good</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>ALMV RNA4-GUS</td>
<td>good</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>BMV RNA3-GUS</td>
<td>good</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>&quot;RSV&quot;-GUS</td>
<td>good</td>
<td>6.4</td>
<td>2</td>
</tr>
</tbody>
</table>

in GUS activity, a level in good agreement with that observed previously for prokaryotic transcripts which contained a S-D region (7,8). This observation contradicts the view that, in all cases, E. coli transcripts must have a S-D region for efficient expression. The "good context" GUS mRNA has had the initiation codon context dramatically altered from that of the native gene, and it lacks all the native GUS leader sequence - now replaced with the SalI site and one C-residue (Fig. 1). The trp promoter construct containing this "good context" GUS resulted in extremely low, but detectable, levels of GUS activity. Even in this severely altered context, addition of the J'-U1 leader produced an 8-fold stimulation in expression of GUS mRNA.

As described above, some residual sequences of the natural GUS mRNA leader were present in the "bad context" GUS construct. Because these might provide some cryptic S-D function, the "good context" GUS construct was chosen as the most sensitive reporter to assay for the effect of the other viral RNA leaders on pro-
karyotic translation in vivo. The A1MV RNA4 and TYMV leaders produced a 6- and 5-fold stimulation, respectively (Table 3). The BMV RNA3 and "RSV" leaders provided only slight enhancement, 3- and 2-fold, respectively. Surprisingly, the J'-SPS leader sequence was much less stimulatory than J'-U1 in E. coli, causing only a 2-fold enhancement.

DISCUSSION

Work carried out by Kozak (6) showed that the initiation codon context of eukaryotic mRNAs has an important role in determining the selection of a particular start site and the level of mRNA expression. Our results from protoplasts, using two variants of GUS mRNA with either a "good" or "bad" initiation codon context, support these earlier findings (6).

The endogenous level of GUS activity in tobacco mesophyll protoplasts is extremely low. Thus we were able to quantitate accurately the stimulatory effect of J'-U1 and the other viral leaders on expression of GUS mRNA. Whether using "good" or "bad context" GUS mRNA, the presence of J'-U1 at the 5'-end resulted in a substantial enhancement of expression (approximately 20-fold; Table 1). When capped mRNAs were used (Table 1), the final level of enhancement by J'-U1 with "bad context" GUS mRNA, was greater than 60-fold and, with "good context" GUS mRNA, greater than 320-fold over that seen with the respective GUS mRNAs lacking both a cap and an J'-U1 sequence. Similar levels of enhancement were observed with J'-SPS (Table 2). In contrast, none of the other viral leader sequences were stimulatory with uncapped GUS mRNAs (Table 2). However, with capped GUS mRNAs, the leader sequences of A1MV RNA4, BMV RNA3, and "RSV" gave a 8-fold enhancement (Table 2). Only the TYMV leader failed to enhance, irrespective of whether the GUS mRNA was capped or not. It is of interest to note that the TYMV leader has been shown to form disomes (14), suggesting that the ability of a leader sequence to form disomes does not correlate with its ability to enhance translation. Alternatively, the 12 additional 5'-nucleotides added by our SP6 vector construct (7), may have selectively destroyed the ability of the TYMV leader to enhance translation. However, it appears that even in the absence of
these additional 5'-nucleotides, the TYMV leader sequence fails to stimulate translation (L. Gehrke, personal communication). In vivo, it may be that the TYMV leader is extremely host-dependent in its enhancing ability. Therefore even protoplasts made from tobacco mesophyll cells do not provide the proper machinery for the TYMV sequence. Certainly the ability of a viral leader sequence to enhance translation is not strictly dependent on its capacity to bind more than one ribosome, as shown by data (above and (18)) with the leader of A1MV RNA4 (a monosome former).

Translational enhancement of CAT mRNA by N'-U1 in microinjected oocytes was shown previously (7). In this report, enhancement was also observed with the related N'-SPS sequence. In contrast, leader sequences from A1MV RNA4, BMV RNA3 and "RSV" failed to enhance translation of CAT mRNA in oocytes. The TYMV leader construct reduced CAT mRNA expression by 80%.

The enhancing effect of N'-U1 in E. coli cells may be due to some fortuitous interaction with the prokaryotic translation machinery. However, as N'-U1 is devoid of G-residues, it cannot provide a sequence similar to that described by Shine and Dalgarno; (5'-AGGAGGU-3'; (1)) and shown to be present in, and required for efficient expression of, nearly all E. coli mRNAs studied to date. Of the other viral leaders, only those from TYMV and A1MV RNA4 displayed any significant enhancement of GUS activity in transformed E. coli cells.

In this survey of viral RNA leader sequences, only one, TYMV, consistently failed to enhance expression in the plant protoplast system. All the leader sequences were derived from positive-sense RNA viruses which must express their genetic information immediately and efficiently within the infected plant or animal cell to avoid the risk of degradation by host RNAses. Furthermore, they must compete effectively with the endogenous cellular mRNAs.

Sequence comparisons between the leaders tested here show no significant homologies other than a high A,U-content, a common feature of viral leader sequences. It is tempting to speculate that the viral leader sequence may circumvent the need for some rate-limiting initiation factor(s), or that it acts as an enhancing element for the association of ribosomes or initiation
factor(s). A precedent for the former possibility exists in the translational regulation of the prokaryotic IF3 gene (32). The sequence immediately surrounding the IF3 start codon allows 30S ribosomal subunits to bind, and translation to begin, without a requirement for IF3, an initiation factor normally essential for the initiation process. The findings of Yokoe and co-workers (13) suggest, at least for the two TMV leaders tested, that a eukaryotic equivalent of the S-D region exists to interact with the 3′-end of 18S rRNA. The lack of homology between the various viral leader sequences may indicate that no one strategy is followed by all, but that there may be several ways to achieve enhancement.

The high A,U-content of these leaders might suggest a low index of secondary structure, which would present fewer obstacles to scanning (4,6) by eukaryotic ribosomes. However, the weak but stable secondary structure potential of the BMV RNA3 leader (2), and the potential of the complete RSV leader to form extensive secondary structures (3), have been used to explain how the 5′-cap and the initiation codon are juxtaposed to facilitate ribosome binding and translation initiation.

The affinity of these sequences for translation initiation factors or other mRNA-binding proteins remains to be tested. Clearly substantial additional work is required to elucidate the mechanism(s) whereby these viral leader sequences can enhance expression of contiguous coding regions in such diverse translation systems.

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