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A translational enhancer derived from tobacco mosaic virus is functionally equivalent to a Shine–Dalgarno sequence

(translational control/ribosomal binding site/Gram-negative bacteria)

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ABSTRACT **When present at the 5′ end of mRNAs, the untranslated leader sequence (Ω) of tobacco mosaic virus RNA significantly enhances translation in eukaryotes and prokaryotes. We have tested a deletion derivative of the Ω sequence, $\Omega_{\Delta 3}$, for its enhancing ability on gene constructs in which the ribosomal binding site was either present or deleted, in several Gram-negative bacterial species including *Escherichia coli*, *Agrobacterium tumefaciens*, *Xanthomonas campestris* pv. *vitians*, *Erwinia amylovora*, and *Salmonella typhimurium*. *In vivo* production of chloramphenicol acetyltransferase from a gene construct lacking its native ribosomal binding site was enhanced 40- to 120-fold by the presence of $\Omega_{\Delta 3}$. Similar levels of enhancement (30- to 240-fold) were observed when the gene encoding β-glucuronidase was tested. With a chloramphenicol acetyltransferase construct containing a ribosomal binding site, enhancement was markedly less, between 1- and 3.8-fold. $\Omega_{\Delta 3}$ appeared to enhance translation independent of its position upstream of the AUG codon used for initiation.**

With only a few exceptions (1–3), the vast majority of *Escherichia coli* mRNAs possess a Shine–Dalgarno (SD) sequence (4), which is located 5–8 bases upstream of the initiation codon AUG or GUG (5) and which is essential for translational initiation (6). The sequence 5′-AGGAGG-3′, which is complementary to the 5′-CCUCCU-3′ sequence present at the 3′ end of *E. coli* 16S rRNA, is the sequence most often found in bacterial leader sequences (7), although other sequences close to the 16S rRNA 3′ terminus have been proposed to function in an analogous fashion (8). The removal or displacement, even by a few bases, of a SD sequence can have a significant effect on the translational efficiency of an mRNA (7). Although most of the interest in sequence requirements affecting translation has focused on the region extending from the SD site to just downstream of the initiation codon, sequences upstream of the SD region can play a role in modulating translational efficiency (9, 10).

The 5′ untranslated leader of tobacco mosaic virus RNA, which consists of a 68-base sequence (Ω), has been shown to be a general enhancer of eukaryotic and prokaryotic translation *in vitro* (11) and *in vivo* (12). Although genes that do not contain a SD region can continue to be expressed when Ω is present (13), the mechanism by which Ω brings this about is not well understood. The lack of any sequence similarities between a SD sequence and Ω prevents Ω from interacting with the 16S rRNA 3′ end in the way that a SD sequence can. This does not preclude, however, the possibility that Ω is functionally equivalent to a SD region. To test this hypothesis, an Ω-derived sequence was introduced upstream to reporter gene constructs in which the native SD region was either present or had been deleted. These plasmid constructs were then introduced into a variety of Gram-negative bacteria where the reporter genes could be expressed under *in vivo* conditions and assayed in order to determine the effect of a SD region on Ω-associated enhancement.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* HB101 *hsdS20* (r^-m^-), *recA13, proA2, leuB, thi, ara-14, xyl-5, mtl-1, galK2, lacY1, rpsL20, supE44* and *Agrobacterium tumefaciens* LBA4301 Rif^r, UV^s, Rec$^-$, pTiAch5$^-$ were obtained from R. Rodriguez (University of California, Davis) and R. Schilperoort (State University, Leiden), respectively. *Agrobacterium rhizogenes* 3D12R Rif^r, mannopine^c, Vir$^+$; *Erwinia amylovora* 1D32R Rif^r; *Salmonella typhimurium* 21D34 Rif^r; and *Xanthomonas campestris* pv. *vitians* 7D51R Rif^r were from this laboratory. *Rhizobium meliloti* U45 Rif^r was obtained from A. Downie (CSIRO, Canberra), and the plasmid-mobilizing *E. coli* strain S17-1 was obtained from A. Puhler (14). Plasmid pUCD1591 was a gift from P. Rogowsky (our laboratory); pUCD615 (15) from J. Shaw; and pSP64 (16) was purchased from Promega. The chloramphenicol acetyltransferase (CAT) and β-glucuronidase (GUS) genes, each flanked by *Sal* I linkers, have been described (17, 18).

Transformation and Conjugation. Transformation of *E. coli* strains was carried out as described (19). For conjugation, plasmid constructs were introduced into the mobilizing strain S17-1 and 100 μl of an overnight culture was mixed with an equal volume of the recipient strain, plated on medium 523 (20), and incubated at 30°C for 24 hr. For selection of transconjugants, the mixture was streaked for single colonies on medium 523 containing rifamycin (50 μg/ml) and kanamycin (50 μg/ml), except *S. typhimurium* transconjugants, which were isolated on SS medium (Difco) supplemented with kanamycin (50 μg/ml).

GUS and CAT Assays. GUS activity was assayed (21) in 0.5 ml of buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 0.1% (vol/vol) Triton X-100, and 1 mM *p*-nitrophenyl β-D-glucuronide (Sigma). The assay was carried out at 37°C, terminated by the addition of 0.4 ml of 2.5 M 2-amino-2-methyl-1,3-propanediol, and quantitated spectrophotometrically at 415 nm. The assay for CAT activity has been described (17). Protein levels were determined by the method of Bradford (22).

Construction of pUCD2009 and pUCD2010. Oligodeoxynucleotides representing a slightly altered tryptophan (*trp*) promoter and its complementary strand were synthesized to include a *Hin*dIII site at the site of transcriptional initiation, followed by *Sal* I and *Bam*HI sites. In order to introduce the *trp* promoter fragment into the *Hin*dIII/*Bam*HI sites of pSP64 (modified so that the *Sma* I site was converted to a *Bgl*

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Abbreviations: SD, Shine–Dalgarno; CAT, chloramphenicol acetyltransferase; GUS, β-glucuronidase.

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II site) and maintain the uniqueness of the *Hin*dIII site at the transcriptional start site, the 5′ end of the *trp* fragment was synthesized so that it possessed the appropriate "sticky end" but did not result in a regenerated *Hin*dIII site. The Ω-derived sequence, $\Omega_{\Delta 3}$, was introduced into the *Hin*dIII/*Sal* I sites of the pSP64–*trp* promoter construct (pUCD2007), resulting in pUCD2008. A *Sal* I-ended GUS gene fragment was then introduced into the *Sal* I site of pUCD2007 and pUCD2008.

The pSa-based vectors were constructed by first digesting the pUCD2007(−SD)GUS and pUCD2008(−SD)GUS constructs with *Eco*RI, filling in with DNA polymerase (Klenow fragment), and then digesting with *Xba* I. *Eco*RI cuts immediately upstream of the *trp* promoter and *Xba* I cuts just downstream of the GUS gene. The pSa-derived vector pUCD1591 [a derivative of pUCD615 (15)] was digested with *Sal* I, the sticky ends were filled in with DNA polymerase (Klenow fragment), and the linearized, blunt-ended vector DNA was digested with *Xba* I. Each of the *Eco*RI (made blunt)/*Xba* I *trp*–$\Omega_{\Delta 3}$–GUS fragments was introduced into the *Sal* I (made blunt)/*Xba* I sites of pUCD1591. The GUS gene was then removed from each construct by digestion with *Sal* I and the constructs were recircularized with phage T4 DNA ligase, resulting in pUCD2009 (no $\Omega_{\Delta 3}$ present) and pUCD2010 ($\Omega_{\Delta 3}$ present) (Fig. 1).

A *Sal* I-ended CAT gene including its native SD sequence (*) (17) was introduced into the *Sal* I site of pUCD2009 and pUCD2010. It contains 34 base pairs (bp) of DNA upstream to the initiation codon ATG (underlined).

```
 (Sal I)                   ********
5'-GTCGACGAGCTTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACT...(CAT gene)-3'
                                    MetGluLysLysIleThr...(CAT protein)
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The CAT gene construct in which the SD region had been deleted (the protein product from which contains an additional 4 amino acid residues at the N terminus) was introduced into pUCD2009 and pUCD2010. It contains 9 bp of DNA upstream to the initiator ATG.

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 (Sal I)
5'-GTCGACCCGATGCGACCTGCAGGGGAGAAAAAAATCACT...(CAT gene)-3'
            MetArgProAlaGlyGluLysLysIleThr...(CAT protein)
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A *Sal* I-ended GUS gene was also introduced into both pUCD2009 and pUCD2010. It does not contain a SD sequence (21) in the 19 bp upstream of the ATG.

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 (Sal I)
5'-GTCGACCGGTCAGTCCCTTATGTTACGTCCTG...(GUS gene)-3'
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As a consequence of the introduction of a *Hin*dIII site at the transcriptional start site, minor sequence changes were made in the region involved in the binding of the *trp* repressor, and as a result, it was necessary to test this promoter for the ability to be induced. Induction was observed in *E. coli* and *S. typhimurium* in the presence of 3-indoleacrylic acid. Only a low level of induction was detected in the other bacterial species used in this study. No significant induction was observed when 3-indoleacetic acid was used as the inducing compound.

RESULTS

$\Omega_{\Delta 3}$ Overcomes the Requirement for a SD Region in a Variety of Gram-Negative Bacteria. We wished to examine the effect of the Ω-derived sequence $\Omega_{\Delta 3}$ on translation of a gene with or without its native initiator region, a major determinant of which is the SD region. We selected the CAT gene for this purpose because its mRNA is very efficiently translated *in vitro*. In fact, the CAT gene contains two closely spaced sequences that are possible SD regions, one of which has a region of 8 bases complementary to the 16S rRNA 3′ terminus. In addition, the initiator AUG is flanked on both sides by purine-rich sequences, allowing the initiator region to remain free of secondary structure, the lack of which is thought to be important for efficient translational initiation (23). Moreover, the codon immediately downstream from the initiator AUG may also be important in the efficiency of translation (24).

In order to measure fully the enhancing effect of $\Omega_{\Delta 3}$ on a CAT gene construct, those features which make up the initiator region—i.e., the SD sequence, the flanking purine-rich regions, and the codon immediately downstream from the initiator AUG—were deleted or changed to an unrelated sequence. A *Sal* I site was then introduced 3 bp upstream to the ATG, and this CAT construct was introduced into the broad-host-range vectors pUCD2009 and pUCD2010, which contain the *trp* promoter and are identical to each other except for the presence of $\Omega_{\Delta 3}$ in pUCD2010 (Fig. 1). We then introduced and tested pUCD2009(−SD)CAT and pUCD2010-(−SD)CAT in a variety of Gram-negative bacteria and determined whether $\Omega_{\Delta 3}$ might replace the requirement for the SD sequence in these species.

As might be expected for a gene in which the SD region had been removed, there was no detectable CAT activity from pUCD2009(−SD)CAT in *E. coli* or in the other bacterial species tested (Table 1). The presence of $\Omega_{\Delta 3}$ in pUCD2010-(−SD)CAT resulted in a >120-fold increase in the level of expression in *E. coli*. In the two other enteric species tested, *S. typhimurium* and *Er. amylovora*, increases in CAT activity of >140- and >40-fold, respectively, were observed when $\Omega_{\Delta 3}$ was present. pUCD2010(−SD)CAT resulted in CAT activity that was >63-, >42-, and >71-fold greater than that observed for pUCD2009(−SD)CAT in *A. tumefaciens*, *A. rhizogenes*, and *R. meliloti*, respectively. No CAT activity was detectable from either construct when tested in *X. campestris* pv. *vitians*, suggesting that either the *trp* promoter does not function well in this strain or that there is required a ribosomal binding site of a type for which $\Omega_{\Delta 3}$ cannot substitute.

When a GUS gene construct from which the SD region had been deleted, but in which the sequence downstream from the initiator AUG had been left unchanged, was introduced into pUCD2009, a low level of GUS activity (9.4 units; Table 2) was detected in *E. coli*. However, when $\Omega_{\Delta 3}$ was present at the 5′ end, expression increased 243-fold to 2284 units of activity. In *S. typhimurium*, pUCD2009(−SD)GUS resulted in only 44 units of GUS activity. Again, the presence of $\Omega_{\Delta 3}$

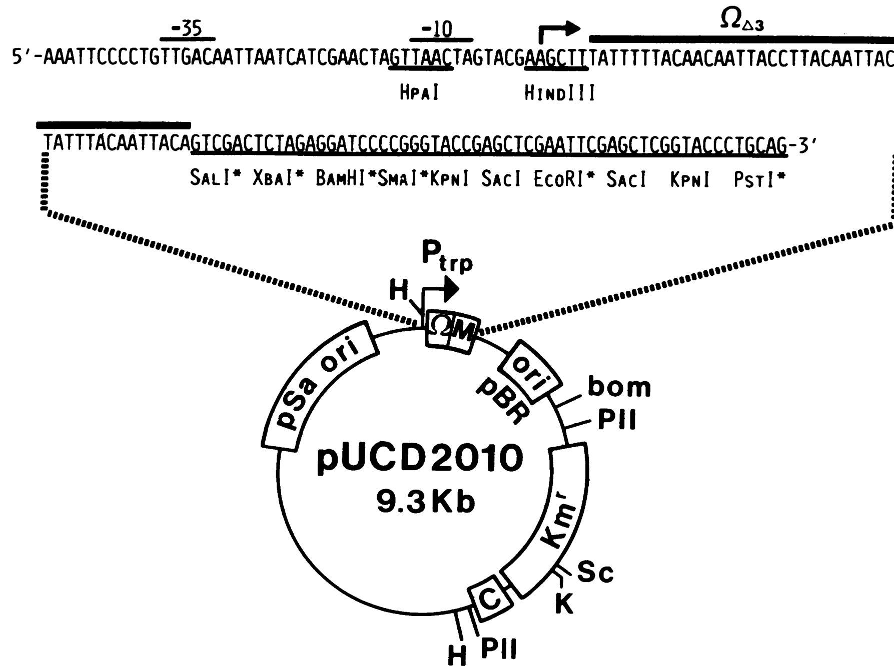

FIG. 1. The vector, pUCD2010, used to produce $\Omega_{\Delta3}$-containing mRNAs *in vivo*. The DNA sequence of the region containing the *trp* promoter, $\Omega_{\Delta3}$, and the multiple cloning site is shown above the vector. The −35 and −10 elements of the *trp* promoter are indicated and the transcriptional start site is indicated by the arrow. The $\Omega_{\Delta3}$ sequence is delineated by the line above the sequence. Unique restriction sites are indicated by asterisks. P_{trp}, *trp* promoter; Ω, $\Omega_{\Delta3}$ sequence; M, multiple cloning site; bom, basis of mobility; C, *cos* site; ori, origin of replication; Km^r, kanamycin-resistance gene; kb, kilobase pairs. PII, *Pvu* II; Sc, *Sac* I; K, *Kpn* I; H, *Hin*dIII. pUCD2009 is identical to pUCD2010 except for the absence of the $\Omega_{\Delta3}$ sequence.

produced a large increase in GUS activity (to 5597 units, a 127-fold increase). GUS activity resulting from pUCD2009-(−SD)GUS in *Er. amylovora* was also quite low (7 units), and $\Omega_{\Delta3}$ markedly elevated GUS activity to 393 units (a 57-fold increase). Similarly, for *A. tumefaciens, A. rhizogenes*, and *R. meliloti*, $\Omega_{\Delta3}$ enhanced expression >94-, 34-, and 103-fold, respectively. In *X. campestris* pv. *vitians*, as with the CAT constructs, no GUS activity was detected from either construct. These data demonstrate that $\Omega_{\Delta3}$ can overcome the requirement for a SD region in different Gram-negative bacteria, resulting in increased product formation.

Effect of $\Omega_{\Delta3}$ on the Expression of Genes with a SD Sequence. The rate of translation of an mRNA is largely determined by three factors: the rates of initiation, translocation, and termination. Of the three, initiation is the rate-limiting step. The presence of a SD region, its sequence composition, and its position relative to the initiator AUG can greatly enhance the rate of initiation relative to that of translocation (25–27). To a lesser extent, the codon immediately following the initiator AUG can also effect the efficiency of mRNA selection and initiation (23, 24). The impact of increases in the rate of initiation on the rate of translation will only be observed up to the point where the rate of initiation equals that of translocation and/or termination. Therefore, we hypothesized that $\Omega_{\Delta3}$-associated enhancement for mRNAs possessing a relatively high rate of initiation (those with a SD sequence) would be less than those observed for mRNAs possessing a low rate of initiation (those without a SD sequence). To test this idea, we introduced CAT, including its native SD sequence, into pUCD2009 and pUCD2010.

In *E. coli*, pUCD2009(+SD)CAT resulted in 11,030 units of CAT activity, while the presence of $\Omega_{\Delta3}$ [pUCD2010(+SD)-CAT] had virtually no effect on expression, resulting in 12,570 units of activity (Table 1). In *S. typhimurium* and *Er. amylovora*, the level of activity from pUCD2009(+SD)CAT was also high and no significant enhancement was observed with $\Omega_{\Delta3}$ (1.3- and 0.7-fold, respectively). In *X. campestris* pv. *vitians*, the pUCD2009(+SD)CAT construct resulted in a very low level of CAT activity (216 units), suggesting that the *trp* promoter is not well expressed in this species. The presence of $\Omega_{\Delta3}$ caused a 3.6-fold increase in the level of CAT expression (782 units), significantly higher than the level of enhancement observed in *E. coli*. pUCD2010(+SD)CAT, when present in *A. tumefaciens, A. rhizogenes*, and *R. meliloti*, resulted in $\Omega_{\Delta3}$-associated enhancements of 2.7-,

Table 1. Effect of $\Omega_{\Delta3}$ on CAT gene expression in the presence or absence of a SD sequence

Bacterial strain	CAT activity, nmol/(min·mg) pUCD2009-(−SD)CAT	CAT activity, nmol/(min·mg) pUCD2010-(−SD)CAT	Fold increase	CAT activity, nmol/(min·mg) pUCD2009-(+SD)CAT	CAT activity, nmol/(min·mg) pUCD2010-(+SD)CAT	Fold increase
E. coli HB101	<15	1888	>126	11,030	12,570	1.1
S. typhimurium 21D34	<15	2167	>144	31,273	40,691	1.3
Er. amylovora 1D32R	<15	634	>42	15,897	11,213	0.7
A. tumefaciens LBA4301	<15	941	>63	4,011	10,821	2.7
A. rhizogenes 3D14R	<15	631	>42	2,600	9,982	3.8
R. meliloti 1U45	<15	1068	>71	3,584	8,279	2.3
X. campestris pv. *vitians*	<15	<15	—	216	782	3.6

The $\Omega_{\Delta3}$ sequence is present in pUCD2010 constructs and absent in pUCD2009 constructs.

Table 2. Expression of a SD-deficient GUS gene with or without $\Omega_{\Delta 3}$ in Gram-negative bacteria

Bacterial strain	GUS activity, nmol/(min·mg)		Fold increase
	pUCD2009-(−SD)GUS	pUCD2010-(−SD)GUS	
E. coli HB101	9.4	2284	243
S. typhimurium 21D34	44.0	5597	127
Er. amylovora 1D32R	7.0	393	57
A. tumefaciens LBA4301	<1.0	94	>94
A. rhizogenes 3D14R	17.0	581	34
R. meliloti 1U45	5.3	544	103
X. campestris pv. *vitians*	<1.0	<1.0	—

See Table 1 legend.

3.8-, and 2.3-fold, respectively. These data show that the combination of SD and $\Omega_{\Delta 3}$ sequences does not appreciably enhance translational activity beyond that observed for a SD sequence alone.

DISCUSSION

We have demonstrated that $\Omega_{\Delta 3}$ can replace the requirement for a SD sequence in a variety of Gram-negative bacteria. $\Omega_{\Delta 3}$ does not significantly stimulate the translation of an mRNA that already contains a SD region, suggesting that $\Omega_{\Delta 3}$ might be functionally equivalent to a SD region. However, since SD sequences are G-rich and $\Omega_{\Delta 3}$ is devoid of guanine residues, $\Omega_{\Delta 3}$ most probably does not interact with the sequence, 5'-CCUCCU-3', close to the 16S rRNA 3' terminus in the manner that the SD region does. Moreover, there are significant functional differences between SD-associated enhancement and Ω-associated enhancement. Whereas a SD sequence must be positioned 3–8 bases upstream from the initiator AUG to function properly, Ω-derived sequences enhance translation whether they are present immediately upstream of the initiator AUG [pUCD2010(−SD)CAT], 19 bases upstream [pUCD2010(−SD)GUS], 35 bases upstream (11), or 110 bases upstream (D.R.G., unpublished results). In *E. coli*, the enhancement conferred by one copy of Ω can be more than doubled when two copies are present in a tandem arrangement (28).

It is interesting that the levels of CAT expression resulting from the presence of $\Omega_{\Delta 3}$ in the pUCD2010(−SD)CAT construct are lower than those observed for the wild-type CAT gene construct possessing its own SD sequence. It may be that $\Omega_{\Delta 3}$ is not as efficient in interacting with the translational machinery as the native CAT SD sequence. It is equally possible that the sequence alterations downstream of the CAT gene initiator AUG, which caused 4 amino acids to be added to the N terminus of the protein, may have affected its enzymatic activity.

What then may be the mechanism by which Ω-derived sequences enhance gene expression? Increased expression of a gene containing Ω is not a result of promoter activity from within the Ω sequence itself. In the absence of the *trp* promoter derivative, very little GUS expression can be detected from Ω–GUS reporter gene constructs (D.R.G., unpublished observations). This is consistent with *in vitro* observations where translation of SP6-generated Ω-containing mRNAs in an *in vitro E. coli* translational system was enhanced posttranscriptionally (11). There is also no correlation between Ω-associated enhancement and increased mRNA stability *in vitro* (11, 28, 29). However, transcripts devoid of any ribosomes can be unstable, as a result of endonucleolytic cleavage within the ribosome binding site (30, 31); therefore, any feature of an mRNA that promotes ribosome–mRNA interaction might increase mRNA stability indirectly through the process of translation. The effect of Ω on mRNA stability *in vivo* remains to be investigated. Little is known about the actual means by which Ω might promote ribosome–mRNA interaction. Recent evidence suggests that the sequence 5'-ACAAUUAC-3' (repeated three times in Ω) and the number of copies present in a tandem array determine the level of translational enhancement (D.R.G., unpublished results). If this is so, each copy may act as an independent entry point for the 30S ribosomal subunit.

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1. Smiley, B., Lupski, J., Svec, P., McMacken, R. & Godson, G. N. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4550–4554.
2. Pirrotta, V. (1979) *Nucleic Acids Res.* **6**, 1495–1508.
3. Ptashne, M., Backman, K., Humayun, M., Jeffrey, A., Maurer, R., Meyer, B. & Sauer, R. T. (1976) *Science* **194**, 156–161.
4. Shine, J. & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1342–1346.
5. Steitz, J. A. (1980) in *Ribosomes: Structure, Function, and Genetics*, eds. Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L. & Nomura, M. (University Park Press, Baltimore), pp. 479–495.
6. Steitz, J. A. & Jakes, K. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4734–4738.
7. Gold, L. D., Pribnow, D., Schneider, T., Shinedling, S., Singer, B. S. & Stormo, G. (1981) *Annu. Rev. Microbiol.* **35**, 365–403.
8. Gold, L., Stormo, G. & Saunders, R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7061–7065.
9. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1988) *Nucleic Acids Res.* **16**, 883–893.
10. Roberts, T. M., Kacich, R. & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 760–764.
11. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1987) *Nucleic Acids Res.* **15**, 3257–3273.
12. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1987) *Science* **236**, 1122–1124.
13. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1987) *Nucleic Acids Res.* **15**, 8693–8711.
14. Simon, R., Priefer, U. & Puhler, A. (1983) *Bio/Technology* **1**, 784–791.
15. Rogowsky, P., Close, T., Chimera, J. A., Shaw, J. J. & Kado, C. I. (1987) *J. Bacteriol.* **169**, 5101–5112.
16. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
17. Close, T. J. & Rodriguez, R. L. (1982) *Gene* **20**, 305–316.
18. Sleat, D. E., Gallie, D. R., Jefferson, R. A., Bevan, M. W., Turner, P. C. & Wilson, T. M. A. (1987) *Gene* **60**, 217–225.
19. Morrison, D. A. (1979) *Methods Enzymol.* **68**, 326–331.
20. Kado, C. I., Heskett, M. G. & Langley, R. A. (1972) *Physiol. Plant Pathol.* **2**, 47–57.
21. Jefferson, R. A., Burgess, S. M. & Hirsh, D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8447–8451.
22. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
23. Ganoza, M. C., Kofoid, E. C., Marliere, P. & Louis, B. G. (1987) *Nucleic Acids Res.* **15**, 345–360.
24. Stormo, G., Schneider, T. & Gold, L. (1982) *Nucleic Acids Res.* **10**, 2971–2996.
25. Gheysen, D., Iserentant, D., Derom, C. & Fiers, W. (1982) *Gene* **17**, 55–63.
26. Shepard, H. M., Yelverton, E. & Goeddel, D. (1982) *DNA* **1**, 125–131.
27. Jay, G., Khoury, G., Seth, A. & Jay, E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5543–5548.
28. Sleat, D. E., Hull, R., Turner, P. C. & Wilson, T. M. A. (1988) *Eur. J. Biochem.* **175**, 75–86.
29. Gallie, D. R., Walbot, V. & Hershey, J. W. B. (1988) *Nucleic Acids Res.* **16**, 8675–8694.
30. Schneider, E., Blundell, M. & Kennell, D. (1978) *Mol. Gen. Genet.* **160**, 121–129.
31. Belasco, J. G., Nilsson, G., von Gabain, A. & Cohen, S. N. (1986) *Cell* **46**, 245–251.