Visualizing mRNA Expression in Plant Protoplasts: Factors Influencing Efficient mRNA Uptake and Translation

Daniel R. Gallie,[a] William J. Lucas,[b] and Virginia Walbot[a,1]

[a] Department of Biological Sciences, Stanford University, Stanford, California 94305-5020
[b] Department of Botany, University of California, Davis, California 95616

In this paper we demonstrate that RNA sequences present upstream and downstream of a reporter gene coding region play an important role in determining the amount of protein produced from an mRNA. A translational enhancer, Ω, derived from tobacco mosaic virus, when present at the 5'-end of β-glucuronidase mRNA increased the efficiency of translation 16-fold to 18-fold in electroporated tobacco or carrot protoplasts, and threefold to 11-fold in maize or rice protoplasts. The presence of Ω did not alter the half-life of the mRNA in vivo. We also demonstrate for the first time that a minimum polyadenylated tail length of 25 adenylate residues is sufficient to substantially increase the expression and half-life of the reporter mRNA in plants. When in vitro-produced mRNAs were synthesized such that extra sequence was added to the 3'-end of the poly(A) tail, however, the final level of expression was decreased up to 80%. Ω, the translational enhancer, and a poly(A) tail function independently of each other; their combined effect on translation, when both are present in an mRNA, is the multiplication of their individual effects. Histochemical analysis for the presence of β-glucuronidase in tobacco established that virtually all viable cells receive mRNA during electroporation. Video image analysis of tobacco protoplasts electroporated with luciferase mRNA demonstrated that there is a wide range in the level of expression of this marker. Carrier RNA, when present during electroporation, had only a modest effect on increasing mRNA uptake. Reporter mRNA expression in electroporated protoplasts was directly proportional to the input mRNA up to at least 30 μg/ml.

INTRODUCTION

Among those factors acting posttranscriptionally that determine gene expression is the requirement for a cap [m⁷G(5')ppp(5')N] at the 5'-terminus of an mRNA, the 5'-untranslated and 3'-untranslated sequences, mRNA transport and half-life, and protein turnover. Virtually all eukaryotic mRNAs examined, with the exception of a few viral mRNAs, contain the 5'-terminal 7-methylguanosine. This terminal nucleotide is associated with the cap binding protein complex (reviewed in Moldave, 1985) and is essential for efficient expression in vitro (Muthukrishnan et al., 1975; Wodnar-Filipowicz et al., 1978) and in vivo (Drummond et al., 1985; Callis et al., 1987; Gallie et al., 1987b). The cap has been proposed as the site at which the 43S initiation complex first attaches to the mRNA (Moldave, 1985).

The mRNA untranslated leader sequences can also regulate translation. Mechanisms include primary or secondary sequence structures to which specific regulatory proteins bind (Hentze et al., 1987; Leibold and Munro, 1988); small open reading frames, the translation products of which may (Werner et al., 1987), or may not (Williams

et al., 1988) be directly involved in the regulatory process; or internal initiation in the absence of the 7-methylguanosine cap (Trono et al., 1988). The 5'-untranslated leader sequence can also play an important part in determining the efficiency of translation. In the scanning model for initiation, once the 40S subunit binds to the 5'-terminus, it moves along the message in a 5'→3' direction until the first AUG in an appropriate context is encountered (Kozak, 1986). The requirements for efficient scanning are thought to be a leader with little or no secondary structure and leader length (Kozak, 1988). The leader also provides the 5' context for potential initiation codons; the sequence AACAAUGGC has been proposed for plants (Lutcke et al., 1987).

In addition to secondary structure and length, the sequence of the leader can contribute significantly to expression. The untranslated leaders from tobacco mosaic virus (TMV) RNA (Gallie et al., 1987a), as well as alfalfa mosaic virus RNA4 and brome mosaic virus RNA3 (Gallie et al., 1987b; Jobling and Gehrke, 1987) act to increase the expression of reporter mRNAs in vitro and in vivo in plant-derived translational systems. The ability of the TMV leader, a 67-base sequence, referred to as Ω, to enhance

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[1] To whom correspondence should be addressed.

expression may, in part, explain the preferential translation of TMV RNA in in vitro competititon assays (Herson et al., 1979) and may reflect the ability of the virus to efficiently capture cellular translational machinery once it has entered the host cell (Gallie et al., 1987c; Plaskitt et al., 1988).

The 3'-untranslated region also plays an essential role in influencing expression. All mRNAs that have been examined, with the exception of cell-cycle regulated histones (reviewed in Birnstiel et al., 1985), terminate in poly(A). Although a poly(A) tail of approximately 30 adenylate residues is sufficient to result in a substantial increase in expression (Nudel et al., 1976; Hoffman and Donaldson, 1988) poly(A) tails are generally greater in length (Edmonds and Caramela, 1969; Edmonds et al., 1971). Moreover, in *Dictyostelium*, mRNAs with longer poly(A) tails are recruited more efficiently onto polysomes (Shapiro et al., 1988) and are thereby more highly expressed. The mechanism by which this posttranscriptional addition is thought to act is by slowing 3'→5' exonucleases, although some reports suggest that polyadenylation may influence the efficiency of translational initiation (reviewed in Brawerman, 1981). Little has been reported on the requirements of poly(A) tail length for efficient expression in plants or the mechanism by which it functions.

In this paper, we investigated the role untranslated sequences play in determining the final level of expression in plants. By generating reporter mRNA in vitro, we were able to control exactly the length and type of sequence present at the 5'-untranslated and 3'-untranslated regions of an mRNA. Delivery of the mRNA, via electroporation-mediated transfer to protoplasts, made it possible to directly access translation. This also served to avoid complications that might occur from transcriptional and post-transcriptional events if DNA-based constructs had been used for the in vivo translational expression analysis.

RESULTS

Construction of β-Glucuronidase (GUS) mRNAs Containing Ω and Poly(A) Tails and Their Effect on Translational Expression

As discussed above, Ω probably functions to enhance the rate of translational initiation. A similar role in promoting translational efficiency has been demonstrated for the poly(A) tail in a few cases (Palatnik et al., 1984; Shapiro et al., 1988). Therefore, it was of interest to establish how these two structural features of mRNA might interact when both were present in an mRNA. To study these 5' and 3' structural features, GUS reporter gene constructs were introduced into a T7-based vector. In vitro transcription allowed production of well-defined mRNAs that could be used for electroporation into protoplasts. Figure 1 illustrates the sequences present at the termini for the mRNAs discussed.
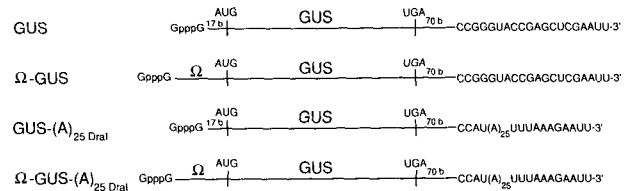

Figure 1. GUS mRNA Constructs With or Without an Ω and Poly(A) Tail.

Capped mRNAs are shown as synthesized in vitro before delivery into protoplasts. The GUS coding region is not shown to scale; 3'-terminal region is displayed to show the exact sequence.

Four GUS mRNAs were introduced into two dicotyledonous species (tobacco and carrot) and two monocotyledonous species (maize and rice). Table 1 shows that, in two separate experiments, the level of GUS expression from capped GUS mRNA that contained neither an Ω sequence nor a poly(A) tail was below the limit of detection in all four species. Consequently, in tobacco, Ω enhanced this expression at least 16-fold to 18-fold, similar to earlier findings with this species (Gallie et al., 1987b). The presence of 25 adenylate residues at the 3'-terminus was sufficient to result in 21-fold to 34-fold increase in GUS expression. The presence of both an Ω sequence and a poly(A) tail in the GUS mRNA was the multiplication of the individual effects on translation. This suggests that the two sequences function independently. Similar results were obtained with the other dicot species, carrot; however, in this case, the contribution by Ω in increasing GUS activity was equal to the contribution by a poly(A) tail. The combination of the two resulted in at least a 500-fold to 600-fold increase over that seen for the GUS mRNA without an Ω or poly(A) tail.

Ω was much less effective in enhancing translation in the monocot species tested. In maize protoplasts, Ω-associated enhancement was at least eightfold to 11-fold and in rice, less than that. In contrast, the presence of a poly(A) tail was very effective, as it resulted in approximately a 20-fold increase in GUS activity in both maize and rice. Again, as in the dicot species tested, the combination of an Ω and poly(A) tail was better than either alone. We conclude that the leader and poly(A) tail act separately in both monocots and dicots to boost reporter gene expression.

Level of Expression Is Proportional to Poly(A) Tail Length

It has been proposed that the stability, or, in some cases, the translational competence of an mRNA is influenced by the length of the poly(A) tail. We tested whether the level

Table 1. Effect of Ω and Poly(A) Tail on Expression in Dicot and Monocot Protoplasts Electroporated with GUS mRNA

| mRNA | Specific Activity[a] | | | | | | | |
| | Tobacco | | Carrot | | Maize | | Rice | |
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
GUS	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ω-GUS	0.16	0.18	0.23	0.18	0.08	0.11	0.03	0.09
GUS-A_{25}	0.21	0.34	0.22	0.20	0.20	0.23	0.11	0.43
Ω-GUS-A_{25}	5.62	5.71	5.95	4.96	2.19	1.73	0.39	1.73

[a] Specific activity is reported in nanomoles of substrate metabolized per minute per milligram of protein.

of expression in plant protoplasts could be influenced by the length of the poly(A) tail. To introduce additional copies of the poly(A) oligonucleotide (described in "Methods"), a Dral site and an EcoRI site were included just downstream of the poly(A) tract, which, once restricted, would allow easy insertion of a second copy of the poly(A)$_{25Dral}$ oligo. Because the vector used contained additional Dral sites, a new poly(A)$_{25}$ oligonucleotide was synthesized that incorporated an Hpal site where the Dral site had been. The 3'-terminus of GUS mRNA containing the poly(A)$_{25Hpal}$ oligo is 5'-GpppG-Ω-GUS-A_{25}-GUUAACGAAUU-3' (when linearized with EcoRI).

Once one copy of the poly(A)$_{25Hpal}$ was introduced downstream of the GUS gene, a copy of the poly(A)$_{25Dral}$ was introduced in the Hpal/EcoRI sites. This resulted in a poly(A) tail that contained two 25-adenylate tracts separated from each other by a GUU sequence, half of the Hpal site remaining after the introduction of the second poly(A) oligonucleotide. After linearization with EcoRI, the Ω-GUS-A_{50} mRNA synthesized in vitro contained a 3'-terminus as follows: 5'-GpppG-Ω-GUS-A_{25}-GUU-A_{25}-UUUAAAGAAUU-3'.

GUS mRNA with or without an Ω and containing either a poly(A)$_{25Dral}$ or poly(A)$_{25Hpal}$ or the poly(A)$_{50}$ tract was synthesized and delivered to protoplasts of the four plant species. Table 2 shows that both the poly(A)$_{25Dral}$ and poly(A)$_{25Hpal}$ 3'-tail yielded equivalent increases in expression. In all cases, a poly(A) tail length of 50 was better than a length of 25. For example, with tobacco and carrot, the increase in poly(A) tail length from 25 to 50 increased the level of GUS expression an additional 300%. Interestingly, when Ω was present, doubling the poly(A) tail length, resulted in only a 50% to 100% increase in GUS expression.

Additional Sequences 3' to the Poly(A) Tail Are Detrimental to Expression

We investigated whether there was a functional reason why a poly(A) tail is present at the 3'-terminus of eukaryotic

mRNAs other than poly(A) polymerase's requirement for a free 3'-terminus to which adenylate residues are added. Using pΩ-GUS-A_{25} and linearizing the plasmid at various restriction sites downstream of the poly(A) tract, it was possible to generate GUS mRNAs in vitro that contained increasing lengths of sequence 3' to the poly(A) sequence. As a control, the same reactions were carried out on pΩ-GUS to examine the effect that increasing the length of the 3'-untranslated region (lacking poly[A]) would have on GUS expression. Figure 2 illustrates that when pΩ-GUS-A_{25} was linearized with BamHI, which cuts immediately upstream of the poly(A) sequence and therefore results in an Ω-GUS mRNA being synthesized with no poly(A) tail, the level of expression achieved in tobacco protoplasts was equivalent to EcoRI-linearized pΩ-GUS. When pΩ-GUS-A_{25} was linearized with either Dral, which cuts immediately downstream of the poly(A) tract, or EcoRI, which cuts 8 bp further downstream than Dral, similar high levels of GUS were detected. The presence of the extra 11 bases 3' to the poly(A) tail in the EcoRI-linearized construct did not impair the function of the poly(A) tail. When pΩ-GUS-A_{25} was linearized with Pvull, resulting in an additional 101 bases being present 3' to the poly(A) sequence, GUS expression was only 19% of that seen for Dral-linearized pΩ-GUS-A_{25} in tobacco.

Table 2. Effect of Poly(A) Tail Length on the Utilization of GUS mRNA

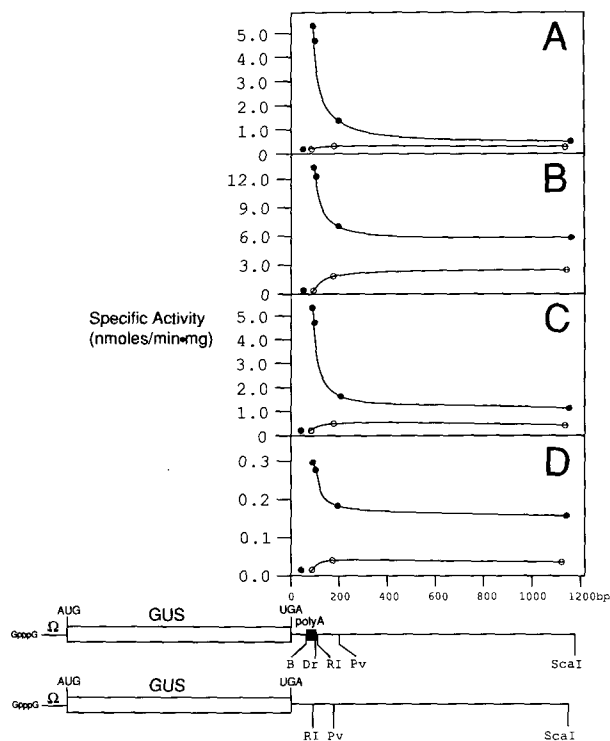| mRNA | Specific Activity | | | |
	Tobacco	Carrot	Maize	Rice
GUS	<0.01	<0.01	<0.01	<0.01
GUS-A_{25Dral}	0.34	0.30	0.57	0.27
GUS-A_{25Hpal}	0.36	0.35	0.72	0.24
GUS-A_{50}	0.93	1.03	2.28	0.81
Ω-GUS	0.29	0.30	0.17	0.03
Ω-GUS-A_{25Dral}	10.6	12.5	3.22	0.88
Ω-GUS-A_{25Hpal}	9.9	12.5	2.78	0.71
Ω-GUS-A_{50}	18.5	20.0	5.49	1.43

Figure 2. Effect of Additional Sequence 3' to the Poly(A) Tail on GUS Expression.

Ω-GUS-poly(A) and Ω-GUS constructs are illustrated. The region 3' to the GUS coding region is drawn to the scale directly above the constructs. The GUS coding region is not drawn to scale. The poly(A) sequence is represented by the black box. Restriction sites present within the 3'-region used to produce mRNAs with various 3'-untranslated regions are B, BamHI; Dr, DraI; RI, EcoRI; Pv, PvuII; and ScaI. Species of protoplast tested were A, tobacco; B, carrot; C, maize; D, rice. ●, Ω-GUS-A_{25}-based constructs; ○, Ω-GUS-based constructs. Five micrograms of each GUS mRNA construct was used for electroporation.

This result demonstrates that the poly(A) tail functions best if positioned close to, if not quite at, the 3'-terminus. This observation was supported by the results of mRNA synthesized from ScaI linearized pΩ-GUS-A_{25}, where a 3'-untranslated region of 1050 bases was present downstream of the poly(A) sequence. The GUS expression from this mRNA in tobacco protoplasts was only 7.5% that seen for DraI-linearized pΩ-GUS-A_{25}. In the control plasmid, the presence of increasingly larger 3'-untranslated regions had only a moderate effect (twofold to threefold) on increasing GUS expression. It should also be noted that, although additional 3' sequence had a detrimental effect on the poly(A)-mediated increase in GUS activity, the construct containing the poly(A) tract outperformed (twofold to fourfold) the construct that contained no poly(A) sequence

regardless of how much additional 3' sequence was present. Similar results were obtained with the protoplasts of the other three species tested. In carrot, the presence of additional sequence 3' to the poly(A) sequence was less detrimental than in tobacco, reducing expression by approximately 50%. Moreover, larger 3'-untranslated regions resulted in greater GUS expression (13-fold to 17-fold) for GUS mRNA containing no poly(A) sequence. Additional sequence 3' to the poly(A) sequence was severely detrimental in maize protoplasts and had only a moderate effect in rice.

Effect of an Ω or Poly(A) Tail on mRNA Stability

It has been demonstrated previously in vitro that the phenomenon of Ω-mediated enhancement is not a result of increased mRNA half-life (Sleat et al., 1988). Indeed, mRNAs containing Ω were more quickly inactivated in in vitro translation assays in the presence of micrococcal nuclease (Gallie et al., 1988). Of course, in vivo conditions may not mirror those found in vitro and it was necessary, therefore, to determine the effect of Ω on mRNA half-life in plant protoplasts.

The role of a poly(A) tail remains unclear. It has been shown to increase the half-life of an mRNA in many cases (Brawerman, 1981; Birnstiel et al., 1985). Alternatively, in *Dictyostelium* (Palatnik et al., 1984; Shapiro et al., 1988), it has been established that the poly(A) tail does not contribute to the mRNA half-life but, in fact, acts to increase translational efficiency.

To determine the role, if any, that Ω or a poly(A) tail might play in the half-life of an mRNA, radioactively labeled GUS mRNAs with or without an Ω or a poly(A)$_{50}$ tail were electroporated into tobacco protoplasts, and aliquots were removed over time. Total RNA was extracted from each aliquot and analyzed on a denaturing formaldehyde-agarose gel. Figure 3 shows the results of this analysis. The half-life of GUS mRNA containing neither an Ω nor a poly(A) tail was determined to be 30 min. When an Ω was present, the half-life remained virtually unchanged at 33 min. GUS mRNA containing a poly(A)$_{50}$ tail did exhibit a longer half-life of 87 min. A poly(A) tail also contributed to a longer half-life for the Ω-GUS-A_{50} mRNA (69 min).

In tobacco, the presence of even small lengths of sequence 3' to the poly(A) were substantially detrimental to the poly(A)-associated increase in GUS expression (Figure 2). To establish whether this extra sequence acts to lower the expression of GUS by changing mRNA half life, pΩ-GUS-A_{50} was digested with PvuII to generate a template with 101 bases 3' to the poly(A)$_{50}$ sequence; labeled mRNA synthesized from this construct was electroporated into tobacco protoplasts. When the mRNA was analyzed, the half-life (39 min) was less than that of Ω-GUS-A_{50} but greater than that determined for Ω-GUS. These data parallel the GUS activity results where the poly(A)$^+$ form of
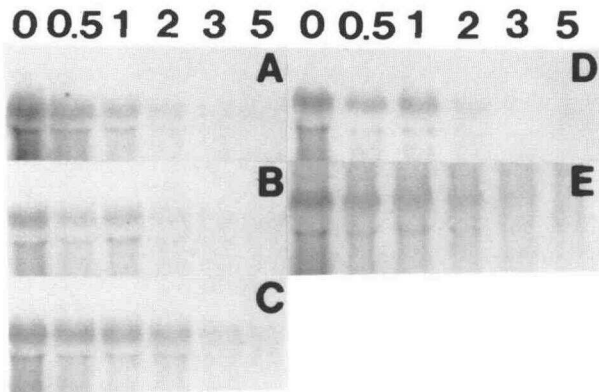
0 0.5 1 2 3 5   0 0.5 1 2 3 5

Figure 3. Effect of an Ω and a Poly(A) Tail on the Stability of GUS mRNA in Electroporated Tobacco Protoplasts.

Two micrograms of each GUS mRNA was delivered to protoplasts; at the time intervals (hr) indicated above each lane, total RNA was extracted. As a result of the time required for the initial wash of the protoplasts following electroporation, and subsequent collection, the zero time point represents protoplasts 30 min after electroporation. A, GUS mRNA; B, Ω-GUS mRNA; C, GUS-poly(A)$_{50}$ mRNA; D, Ω-GUS-poly(A)$_{50}$ mRNA; E, PvuII Ω-GUS-poly(A)$_{50}$ mRNA.

GUS mRNAs always outperformed the poly(A)$^-$ form regardless of the amount of 3' sequence present. It should be noted that, although the presence of the poly(A)$_{50}$ tail did increase the half-life of GUS mRNA by 2.9-fold, this does not completely account for the 21-fold to 34-fold increase observed in GUS expression when the protoplasts are analyzed for GUS activity.

Analysis of Cap Requirements for Efficient in Vivo Expression

The requirement of a cap structure of the 5'-terminus of an mRNA for efficient translation in eukaryotes is well known (Furuichi et al., 1977; Moldave, 1985). There is, however, some variation in response to a cap among translation assay systems. We sought to establish whether the protoplasts were responsive to the capped form of the mRNA synthesized from our T7-based vector. Table 3 shows that when 1 μg of uncapped Ω-GUS-A$_{25}$ mRNA was used for electroporation into tobacco or carrot protoplasts, the resulting GUS activity was just above the limit of detection, whereas for maize and rice, it was below this limit. When the same mRNA construct was synthesized to contain the unmethylated cap (GpppG), substantial increases in GUS expression were observed for all four protoplast systems. This clearly demonstrated the need for capped RNA to achieve a detectable level of expression.

Although most plant mRNA cap structures are mono-methylated, the role of this methylation is unknown. To determine whether a methylated cap may result in greater translational efficiency than the unmethylated form, Ω-GUS-A$_{25}$ mRNA was synthesized with each cap type. One microgram of each of the capped GUS mRNAs was introduced into protoplasts of the four plant species. Table 4 illustrates that, in all cases, the methylated form of the cap resulted in higher GUS expression than that observed for the unmethylated cap, although the increase in translational efficiency was moderate (50% to 100%).

Other Parameters Affecting Transiently Expressed mRNA

Although the half-life for the various forms of GUS mRNAs was determined by following the degradation kinetics of the full-length form of the mRNA, minor degradation by exonuclease activity at the 3'-terminus or 5'-terminus would not be detected by this method. For example, the loss of the cap alone would be undetectable but would effectively inactivate the expression of the mRNA at the translational level. Therefore, we carried out a functional examination of the kinetics of translation by following the appearance of GUS activity over time. Figure 4 illustrates the functional fate of GUS mRNA with or without an Ω or poly(A) tail when electroporated into tobacco and maize protoplasts.

The data presented in Table 1 demonstrated that GUS-A$_{25}$ mRNA resulted in higher GUS activity than Ω-GUS mRNA in tobacco and maize protoplasts when assayed 8 hr after electroporation. Figure 4 illustrates that, during the initial phase of translation in tobacco, however, Ω-GUS mRNA resulted in detectable levels of GUS activity by 30 min following electroporation, whereas GUS-A$_{25}$ mRNA did not result in detectable levels of activity until 60 min. First detection of GUS activity from Ω-GUS mRNA in maize occurred at 60 min, and a similar level of activity was not detected until 120 min when GUS-A$_{25}$ mRNA was used. These data support the model that Ω serves to enhance the rate of ribosomal loading onto the mRNA. Interestingly,

Table 3. Effect of a Cap (GpppG) on Ω-GUS-A$_{25}$ mRNA Utilization

| Species | Specific Activity | | Increase |
	Uncapped mRNA	Capped mRNA	
			-fold
Tobacco	0.03	1.87	64
Carrot	0.02	2.22	111
Maize	<0.01	0.68	>68
Rice	<0.01	0.13	>13

Table 4. Effect of Cap Methylation on Ω-GUS-A$_{25}$ mRNA Utilization

Species	Specific Activity		
	GpppG	m^{7}GpppG	Increase
			-fold
Tobacco	3.21	4.78	1.5
Carrot	5.23	10.7	2.0
Maize	1.19	2.37	2.0
Rice	0.78	1.65	2.1

translation of GUS-A$_{25}$ mRNA continued for a longer period of time than for Ω-GUS mRNA, and eventually surpassed Ω-GUS in the final level of GUS expression achieved. This result supports the conclusion that the poly(A) tail serves to increase GUS mRNA half-life.

We then examined the relationship between input mRNA amount and GUS activity in the four species. Figure 5 shows the results of using increasing amounts of Ω-GUS-A$_{25}$ mRNA for electroporation into protoplasts. In all the species tested, the amount of GUS activity was directly proportional to the input mRNA from 0.20 μg to 30 μg. There was a slight plateau at the highest concentrations of mRNA. As observed in the previous analyses, carrot and tobacco protoplasts were approximately equal in GUS activity, followed by maize and rice.

This linear relationship between input mRNA and expression extended to even very small quantities of mRNA where one might expect to see significant degradation prior to electroporation of the mRNA. The effect of carrier RNA was examined to determine whether it might be beneficial in the uptake or protection of the GUS mRNAs. Table 5 illustrates the effect of adding increasing amounts of total yeast RNA to the electroporation medium on the final expression of 1 μg Ω-GUS-A$_{25}$ input mRNA in tobacco and maize. Although the presence of the carrier RNA did result in higher levels of GUS activity in both species, the effect was only moderate, needing at least 400 μg of the yeast RNA to bring about a 50% increase in GUS expression. The effects of such large quantities of yeast tRNA, rRNA, and mRNA on translation in protoplasts are unknown.

Analysis of the Percentage of the Protoplast Population Receiving Reporter mRNA during Electroporation and the Range of Expression

From this and prior work (Callis et al., 1987; Gallie et al., 1987b), it is clear that mRNA can be transiently expressed after electroporation of protoplasts. Unknown, however, was the percentage of the protoplast population that ac-

tually receives and expresses mRNA. It was possible that mRNA is delivered to only a very small fraction of the protoplasts in the electroporation chamber or that only a subpopulation of the protoplasts could express the new message. To address these questions, histochemical analysis of tobacco and maize protoplasts, electroporated with 10 μg of Ω-GUS-A$_{25}$ mRNA, was carried out. With this type of GUS assay, those protoplasts that have expressed sufficient quantities of GUS turn blue when analyzed histochemically. Figure 6A demonstrates that >90% of the electroporated tobacco cells are blue; therefore, these cells both received and expressed the Ω-GUS-A$_{25}$ mRNA. As a result of the electroporation conditions used for tobacco protoplasts, approximately 10% of the cells die immediately (data not shown) and would not be translationally competent. Therefore, we conclude that virtually all viable tobacco cells were successfully electroporated.

In contrast, only 1% to 2% of the maize protoplasts are blue in color in histochemical analysis (Figure 6B). The histochemical assay for GUS requires considerable GUS activity for adequate color formation. The insensitivity of this assay, when low GUS activity is present, was also observed for tobacco protoplasts electroporated with either the Ω-GUS or GUS-A$_{25}$ forms of the mRNA. Constructs lacking either the Ω or the poly(A) tail yielded such low GUS expression that no blue cells were found (data not shown). As electroporation conditions were identical to those for Ω-GUS-A$_{25}$ mRNA, we conclude that this GUS assay is simply not sensitive enough to score electroporation efficiency with these constructs. In the case of the
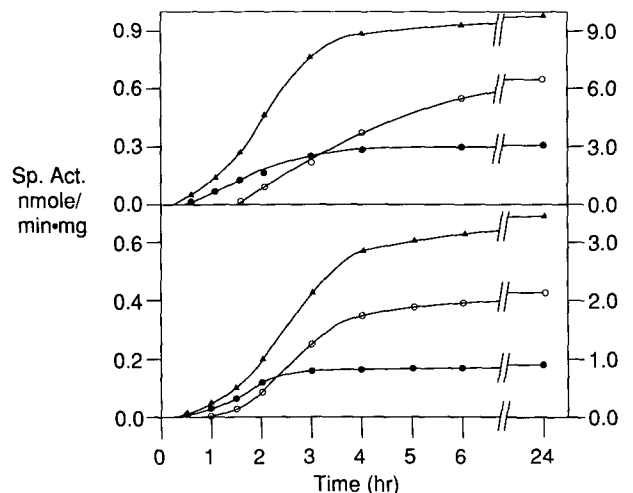

Figure 4. Time Course Following the Expression of GUS mRNAs in Electroporated Tobacco (Top Graph) and Maize (Bottom Graph) Protoplasts.

Aliquots of the protoplasts were removed at regular time intervals following electroporation. Five micrograms of each mRNA were used for the electroporation. Left scale: ●, Ω-GUS mRNA; O, GUS-poly(A)$_{25}$ mRNA; Right scale: ▲, Ω-GUS-poly(A)$_{25}$ mRNA.
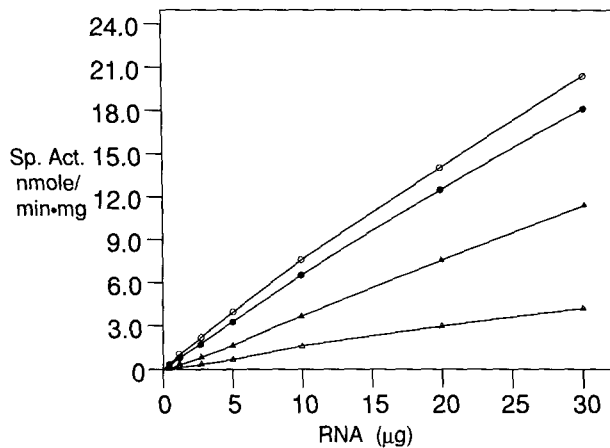
Figure 5. Effect of Increasing Input Ω-GUS-poly(A)$_{25}$ mRNA in Electroporation of Protoplasts.

O, carrot; ●, tobacco, ▲, maize; △, rice.

maize protoplasts, it is probable that the 1% to 2% blue cells represent protoplasts at the high range of GUS expression for this species. We propose that electroporation successfully delivers mRNA to more protoplasts but that most protoplasts expressed too little enzyme for ready detection.

Although we were able to gather qualitative data on individual protoplasts with this type of analysis, there is no assay for GUS that easily allows quantitative analysis of individual protoplasts. To pursue this, we used a combination of the luciferase (LUC) gene from firefly (de Wet et al., 1987), to produce Ω-LUC-A$_{25}$ mRNA that could be used in electroporation, and a video image analyzer that enabled us to quantitate the light production (as a result of Ω-LUC-A$_{25}$ mRNA translation) from individual protoplasts. Figure 6C shows tobacco protoplasts that have been stained with fluorescein diacetate 2 days after electroporation. Only those that take up the fluorescein diacetate and enzymatically cleave the molecule to produce the fluorescence are viable. Figure 6D shows the luciferase activity exhibited by the same field of protoplasts shown in Figure 6C. Because the luciferase reaction requires ATP, only living cells are capable of producing light. Nonviable cells that may have received Ω-LUC-A$_{25}$ mRNA and expressed it, but died before this analysis was carried out, would not be capable of producing light. This is in contrast to the GUS assay, in which protoplasts containing GUS will turn blue when assayed, whether they are viable or not at the time of the assay. Comparing Figure 6C to Figure 6D, not all the viable protoplasts produce detectable levels of light. Although 10 μg of GUS and LUC mRNA was used for the experiments in Figure 6, A and D, differences in half-life between GUS and LUC mRNA as well as detection sensitivity may account for this result. Because of the results with Ω-GUS-A$_{25}$ mRNA in tobacco protoplasts, it is probable that Ω-

LUC-A$_{25}$ mRNA was delivered to all protoplasts. A wide range in expression was observed in those cells that produced detectable levels of light. Figure 7 displays the data as a histogram. Cell volume, metabolic activity, translational competence, as well as the amount of mRNA received are among the factors that probably contribute to this range in expression.

DISCUSSION

We have described those features of an mRNA essential for optimizing translational efficiency and have demonstrated the utility of mRNA electroporation. The use of in vitro synthesized mRNA allows the precise control of the sequence at the 5'-termini and 3'-termini of an mRNA. In addition to the easy manipulation of the terminal sequence composition, controlling the modification state of the cap structure is also possible.

A poly(A) tail length of 25 residues was sufficient to bring about a substantial increase in the level of GUS expression in electroporated tobacco, carrot, maize, and rice protoplasts. The increase ranged from 16-fold to 40-fold. When the poly(A) tail length was increased to 50 residues, a further 50% to 300% increase was observed, demonstrating that the addition of the first 25 adenylate residues makes up the largest contribution to the poly(A) tail effect. In yeast and in mammals, 25 to 27 adenylate residues per poly(A)-binding protein molecule were required for tight binding (Baer and Kornberg, 1980; Sachs et al., 1987). Our results suggest that plants contain a protein with a similar requirement for tail length. Short tails (<15 adenylate residues) are ineffective in plants (J.R. de Wet, unpublished observations).

No previous experiment has explored whether a poly(A) tail must be at the 3'-end of an mRNA to function. Due to the availability of appropriate restriction sites downstream of the poly(A) sequence, a poly(A)$_{25}$ tail containing only

Table 5. Effect of Carrier RNA Present during Electroporation on Ω-GUS-A$_{25}$ mRNA Expression

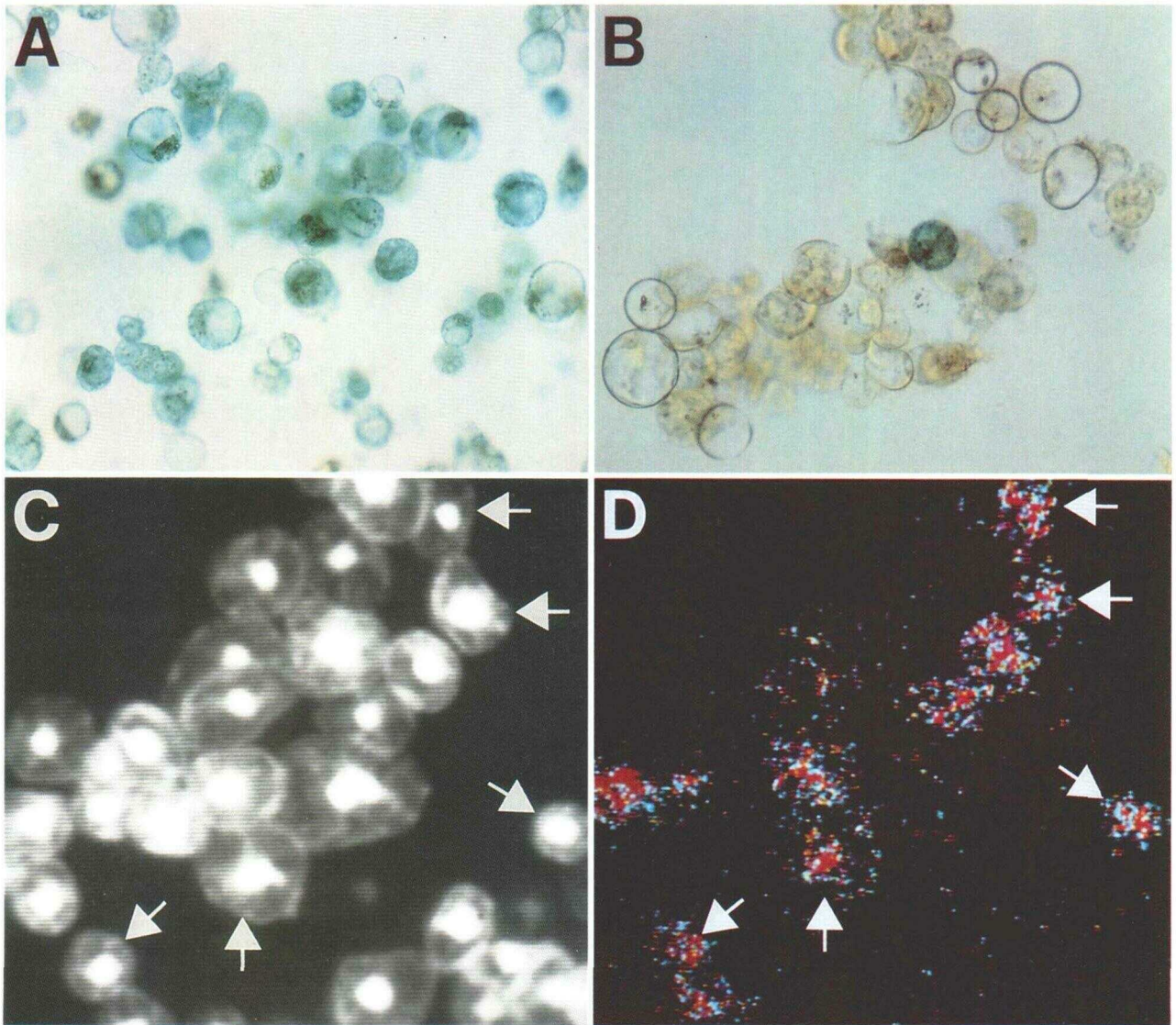| Yeast RNA Added	Carrot		Maize	
	Specific Activity	Relative % Expression	Specific Activity	Relative % Expression
μg				
0	1.58	100	0.695	100
5	1.44	91	0.841	121
10	1.57	99	0.812	117
25	1.86	118	0.779	112
50	1.94	123	0.868	125
100	1.58	100	0.918	132
200	2.5	158	1.06	153
400	2.26	143	1.04	150

Figure 6. In Situ Analysis of β-Glucuronidase and Luciferase mRNA Expression in Electroporated Protoplasts.

GUS histochemical analysis of tobacco (**A**) and maize (**B**) protoplasts electroporated with 10 μg of Ω-GUS-poly(A)$_{25}$ mRNA. Protoplasts were collected from the incubation medium and resuspended in a 50 mM potassium phosphate buffer containing 0.3 M mannitol 5 hr after electroporation. The cells turned blue within 3 hr of the assay but were photographed after 18 hr.
(**C**) Fluorescein diacetate-stained tobacco cells at ×160 magnification, 1 day after electroporation with 10 μg of Ω-LUC-poly(A)$_{25}$ mRNA.
(**D**) False color analysis of video-imaged photon counts of the same field as in (**C**). Light emitted from the cells was counted over a 4-min period. The colors correspond to the light intensity such that red is the greatest, and blue, the least amount of light. The white arrows in (**C**) point to FDA-stained protoplasts which can be used in (**D**) as points of reference for the video image analysis. The analysis is described in greater detail in "Methods."

three extra non-adenosine bases at the 3'-terminus could be synthesized. Little change in the level of expression was observed when 11 extra bases were present at the 3'-terminus, suggesting that the poly(A)$_{25}$ tail can continue to be recognized by the poly(A)-binding protein if small lengths of sequence are present downstream. Up to 80% of the enhancement of expression associated with the presence of the poly(A)$_{25}$ tail was lost, however, when a 101-base sequence was added to the 3'-end of the poly(A)$_{25}$ tail. This result indicates clearly that there is a
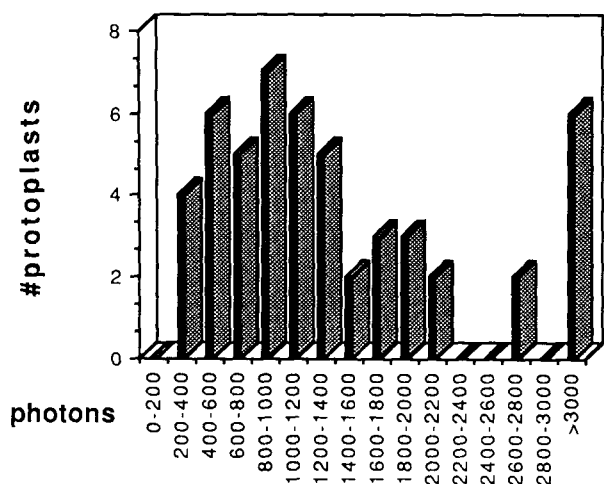
Figure 7. Histogram Analysis of Light-Producing Tobacco Protoplasts Electroporated with 10 μg of Ω-LUC-poly(A)$_{25}$ mRNA.

Photons were counted within a 225 pixel window centered over each protoplast for 4 min. Data were collected for 51 individual cells 4 days following electroporation.

limit to how much additional 3' sequence the poly(A) tail will tolerate before losing the ability to function. The sensitivity of the poly(A) tail to extra sequence varied among the four protoplast systems tested, being least sensitive in carrot and rice, and most sensitive in tobacco and maize, suggesting that the difference in sensitivity does not correlate with the monocot/dicot division. The mechanism by which sequences 3' to the poly(A) tail adversely affect function is unknown. One hypothesis is that the poly(A)-binding protein requires terminal adenylate residues, perhaps to allow the protein to "cap" the 3'-end. Because we found that 3 to 11 extra nucleotides of mixed sequence did not eliminate poly(A) function, the poly(A)-binding protein most likely does not recognize a terminal adenylate residue.

The poly(A) tail was shown to contribute, in vivo, to a longer mRNA half-life, but the increase in half-life could not completely account for the enhancement in expression associated with the presence of a poly(A) tail. It is possible that the poly(A) tail has a role in increasing the efficiency of translation. It is also possible that there is more than one pool of an mRNA: one that is associated with the polysomal fraction and one that is not. These two pools might differ in half-life or translational efficiency, as has been suggested for some mRNAs in *Dictyostelium* (Shapiro et al., 1988). Our results do not support a role for the poly(A) tail in the transport of mRNA from the nucleus to the cytoplasm, since it is within the cytoplasm, into which the mRNA is delivered via electroporation, that the poly(A) tail acts to increase expression.

The TMV-derived untranslated leader sequence, Ω, worked better as a translational enhancer in the two dicot

species tested. The two monocot species were only moderately responsive, suggesting that there may be, in general, a fundamental difference between dicots and monocots in the degree to which the translational machinery responds to Ω. The presence of an Ω sequence did not result, in vivo, in an increase in mRNA half-life. The presence of both an Ω and a poly(A) tail resulted in a combined effect on expression that was the multiplication of their individual contributions. If the poly(A) tail does influence the efficiency of translation, it probably does so at a different point in the initiation process than Ω.

Although the presence of a cap at the 5'-terminus of the mRNA was essential for detectable expression (providing up to an 111-fold increase), methylation of the cap stimulated expression only a further 50% to 100%. This is in contrast to the in vitro translation (wheat germ lysate) studies with reovirus mRNA (Furuichi et al., 1977), in which the presence of an m^{7}GpppG cap resulted in efficient expression but the unmethylated form, GpppG, did little to increase expression over that seen for the uncapped form of the mRNA. This apparent discrepancy highlights the importance of in vivo-based assays in determining the appropriate role of mRNA structural features.

METHODS

Plasmid Constructs

The vector used for all constructs was pBluescript in which the 445-bp PvuII fragment containing the T7/T3 promoters and polylinker region was replaced with the corresponding 322-bp polylinker-containing PvuII fragment from pUC19. Into the HindIII/SalI sites of this modified vector, a 36-bp oligonucleotide containing the sequence for the T7 promoter was introduced:

```
      T7 promoter          StuI   HindIII   SalI
5'-AGCTCATTAATACGACTCACTATAGGCCTAAGCTTG-3'
    3'-GTAATTATGCTGAGTGATATCCGGATTCGAACAGCT-5'
```

The 5'-end of this oligonucleotide was designed such that it contained the appropriate sticky end for ligation to the HindIII site within the vector but did not restore a HindIII site. As a result, the HindIII site downstream from the T7 transcriptional start site (underlined base) is unique.

To introduce a 25-bp poly(A) sequence into this T7 vector, a oligonucleotide containing 25 adenylate residues, followed by a DraI site (or an HpaI site for the HpaI-containing poly[A] oligonucleotide) and an EcoRI site,

```
                                      DraI    EcoRI
5'-ATAAAAAAAAAAAAAAAAAAAAAAAAAATTTAAAG-3'
    3'-TATTTTTTTTTTTTTTTTTTTTTTTTTTAAATTTCTTAA-5'
```

was introduced into the SmaI/EcoRI sites of the pUC19 polylinker.

The GUS gene was introduced into the SalI site as a SalI fragment. The Ω-GUS gene construct was introduced into the HindIII/XbaI sites as a HindIII/XbaI fragment.

Oligonucleotides were synthesized on an Applied Biosystems 380B DNA Synthesizer.

In Vitro Transcription

The T7-based, GUS-containing constructs were linearized with EcoRI (or in some cases with DraI or HpaI, depending on the poly[A] oligonucleotide present) and in vitro transcription of linearized plasmid DNA was carried out using bacteriophage T7 RNA polymerase as described (Schenborn and Mierendorf, 1985). Capped transcripts were obtained by modifying the published reaction conditions to include 200 μM GTP and 1.5 mM G(5')ppp(5')G (Pharmacia LKB Biotechnology Inc.). Labeled mRNAs were synthesized by including α-[^{32}P]UTP. RNA integrity was determined by formaldehyde-agarose gel electrophoresis as described (Melton et al., 1984).

Preparation and Electroporation of Protoplasts

Protoplast media and isolation methods for cell suspensions of maize (Black Mexican Sweet) (Fromm et al., 1987) and rice (Taipei 309) (Kyozuka et al., 1987) were as described. The protoplast isolation protocol for tobacco (Xanthi) and carrot (Redwood City Wild Carrot) was identical to that for maize except no pectinase was used.

Electroporation conditions for tobacco were 180 V, 10 msec, 1550 μF capacitor; for carrot, 270 V, 10 msec, 1550 μF capacitor; for maize, 180 V, 10 msec, 1550 μF capacitor; and for rice, 270 V, 10 msec, 1550 μF capacitor. mRNA (0.25 μg to 30 μg) was added to the protoplasts followed by immediate electroporation using an X-cell TM 450 Electroporation System (Promega Biotec). After 10 min on ice, the protoplasts were allowed to recover 8 hr to 24 hr before collection for analysis.

mRNA Stability

Aliquots of tobacco protoplasts electroporated with radiolabeled GUS mRNAs were removed over time and immediately mixed with phenol/chloroform. Five micrograms of carrier tRNA was added to the aqueous portion and the RNA was precipitated. After resuspension, the RNA was denatured and loaded onto a formaldehyde-agarose gel. The gel was washed in 1% glycine for 30 min and subsequently stained with 1 μg/ml ethidium bromide to confirm that equivalent amounts of total RNA were present in each lane. The gel was then dried and autoradiographed. The region of the gel corresponding to the full-length form of the GUS mRNA was cut out for each lane, solubilized, and counted, to quantitate band intensities and determine mRNA half-life.

Analysis of GUS Activity

Protoplasts were collected by centrifugation and then resuspended and sonicated in 0.5 ml of buffer (50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 1 mM EDTA). 4-Methylumbelliferyl-β-D-glucuronide (Sigma) was added to 1 mM to aliquots which were then incubated at 37°C for 15 min to 180 min; the reaction was terminated by the addition of 0.2 M sodium carbonate. Fluorescence was measured by excitation at 365 nm and emission at 455 nm in a TKO 100 DNA Fluorometer (Hoefer Scientific). GUS-specific activity was determined as nanomoles of substrate metabolized per minute per milligram of protein.

For histochemical analysis, protoplasts were collected 5 hr after electroporation and resuspended in 50 mM sodium phosphate,

pH 6.7, 0.3 M mannitol and incubated at 25°C for 4 hr in the presence of 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (Clontech Laboratories, Palo Alto, CA).

Video Image Analysis of Protoplasts Expressing Luciferase Activity

Immediately following electroporation, protoplasts were plated on black cellulose (Millipore, Type AA, 0.8 μm pore size) that was placed on top of a presterilized absorbant pad resting on solid medium. At the time of analysis, a small section of the protoplast/cellulose/absorbant pad was placed onto a glass slide, and the preparation was overlaid with 0.1 mg/ml fluorescein diacetate (Sigma) in protoplast medium. This was immediately followed by a 1 mM luciferin solution (K$^+$ salt, Analytical Luminescence Laboratory, San Diego, CA) prepared with protoplast medium. The protoplasts were observed under a Leitz Orthoplan Photomicroscope with epi-illumination using a halogen light source. The fluorescence image produced (Leitz Ploempac filter cube, exitation filter, 390 nm to 490 nm; barrier filter, 515 nm) was acquired and processed using a Hamamatsu C1966 AVEC/VIM (video-intensified microscope) photonic camera and image processing system, which was attached to the Leitz microscope. To quantitate luciferase activity, photon counting over a 4-min period was carried out in complete darkness. Images stored in a Sony videocassette recorder (model VO-5800) were processed through the Hamamatsu C1966 system and false color analysis was displayed on a Sony Trinitron color video monitor (model PVM-1271Q) from which photographs could be taken directly.

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