Role of the Leader Sequence during Thermal Repression of Translation in Maize, Tobacco, and Carrot Protoplasts

Letizia Pitto, Daniel R. Gallie, and Virginia Walbot

Department of Biological Sciences, Stanford University, Stanford, California 94305

ABSTRACT

The 5'-untranslated leader of maize (Zea mays) heat-shock protein (hsp) 70 mRNA is required for translational competence during heat shock in protoplasts. When the β-glucuronidase gene was used as a reporter mRNA, expression at elevated temperatures increased more than 10-fold when the hsp70 leader constituted the 5'-untranslated region. The hsp70 leader did not affect the physical half-life of the mRNA and, therefore, does not function at the level of transcript stability. The maize hsp70 leader was required to escape thermal repression in both maize and tobacco (Nicotiana tabacum) but was less effective in carrot. In addition, mRNAs containing the tobacco mosaic virus untranslated leader (Ω) were also efficiently translated during heat shock, data suggesting that the presence of the Ω sequence enables the transcript to escape the translational repression that occurs during thermal stress.

When organisms as diverse as mammals, bacteria, and plants are exposed to thermal stress, they respond by synthesizing a set of proteins, the hsp's (12, 18). This phenomenon is under complex regulatory controls exerted at both the transcriptional and translational levels. As a consequence of thermal stress, the transcription of heat-shock genes, which were previously either silent or expressed at low levels, is increased both dramatically and rapidly, whereas other genes are transcriptionally repressed. Moreover, heat-shock messages are preferentially translated over the preexisting cellular mRNAs at elevated temperatures (12).

The transcriptional regulation of heat-shock genes has been highly conserved throughout eukaryotes, including plants (9, 16, 18, 19). All heat-shock genes investigated to date contain several copies of a 14-bp HSE upstream of the TATA box of the promoter that was first described for the Drosophila hsp70 gene (15). The HSE functions as the binding site for a transcription factor that is activated in response to heat shock and is necessary and sufficient to confer thermoinduction on a promoter. The maize (Zea mays) hsp70 gene also contains multiple copies of the HSE and undergoes thermoinduction both in maize (1) and in transgenic petunia (16), suggesting that transcriptional regulation in heat-shock genes has been conserved between monocotyledonous and dicotyledonous species.

In Drosophila, efficient translation during heat shock is dependent on the leader. The leaders for hsp70 and hsp22 mRNAs are necessary for the mRNA to escape the translational repression induced by heat shock (10, 14). Apparently, this function is not conserved in plants because the Drosophila hsp70 gene directs active transcription but not translation in transgenic tobacco (Nicotiana tabacum) during heat shock (20). Few studies, however, have focused on translational regulation during the heat-shock response in plants. Schöffl et al. (19) investigated both the transcriptional and translational regulatory elements of a soybean hsp gene. This study suggested that the leader may be required for efficient expression during heat shock. Deletions of sequences that included the leader resulted in a reduction in expression during thermal stress. However, deletions within the leader resulted in a lower steady-state level of mRNA, making it difficult to draw a conclusion concerning the role of the leader in posttranscriptional regulation.

In this paper, we report that the presence of the maize hsp70 leader in a heterologous mRNA construct enables the continued translation of the transcript in heat-shocked maize and tobacco protoplasts. We show this using DNA constructs electroporated into protoplasts using the maize hsp70 promoter to drive mRNA synthesis in vivo. However, to eliminate any influence that the leader might have on transcription or mRNA processing, in vitro synthesized chimeric mRNAs containing the hsp70 leader were electroporated into protoplasts and the effect of the leader on translation and mRNA stability was tested directly during conditions of heat shock. We also tested the TMV leader, Ω, and demonstrate that it can functionally substitute for an hsp leader.

MATERIALS AND METHODS

Materials and Cell Cultures

Restriction enzymes were purchased from Gibco-BRL and New England Biolabs. The sources of the protoplast isolation enzymes used were: pectinase (Pectolyase Y23), Seishin Pharmaceutical; cellulase (CELF), Worthington Biochemical; cy-
tolyase TM917, Genencor. The *Zea mays* L. Black Mexican Sweet (American Type Culture Collection No. 54022) cell suspension was a gift from P. Okubara (Staufier Chemical Co.).

**Plasmid Construction**

All DNA manipulations were performed using standard DNA techniques as described by Maniatis et al. (13). p70-GUS and p70-Luc plasmids are derived from the previously described plasmid, pshp70-CAT-NOS (1), in which the CAT gene was replaced by the *Escherichia coli* GUS and firefly Luc genes, respectively. An *XbaI* fragment containing the hsp70 promoter-GUS-NOS was transferred from p70-GUS to pUC119, resulting in p70ΔX (Fig. 1A), for subsequent manipulation. An *XbaI* site was created at the hsp70 transcriptional start site to produce p70Xba-GUS (Fig. 1B). Expression characteristics from p70Xba-GUS were identical with p70ΔX, p70-GUS, p70ΔX, and p70Xba-GUS all contain the 5′-terminal 83 bases of the 107-base hsp70 leader. To generate a construct lacking all but 10 bases of the hsp70 leader, the sequence between the BamHI and the newly created *XbaI* site of p70Xba-GUS was deleted, resulting in p70ΔL-GUS. p70ΔGUS was created by replacing the hsp70 leader of p70Xba-GUS with the Ω sequence from Ω-GUS-A50.

The GUS-A50 and Ω-GUS-A50 constructs have been described previously (6). The 17-base control leader of GUS-A50 is m6GppppGCUAGGCGUUCGAAGGAAGUAGCAGGGGGAUC-NGCGUGAGGGGUUCGAAAGAAGUAGCAGGGGGAUC-NGCCCAUG. The hsp70 leader (HSL) construct, HSL-GUS-A50 (Fig. 1C), was created by replacing Ω-GUS from Ω-GUS-A50 with HSL-GUS from p70Xba-GUS. The HSL leader sequence is m6GppppGCUAGGCGUUCGAAGGAAGUAGCAGGGGGAUC-NGCGUGAGGGGUUCGAAAGAAGUAGCAGGGGGAUC-NGCCCAUG.

**In Vitro Transcription**

Capped (m6GpppG) GUS mRNAs (Fig. 1C) were generated in vitro as described by Gallie et al. (6). All mRNAs were capped concomitantly with transcription, and more than 90% are capped under the conditions used.

**Protoplast Isolation and Electroporation**

Protoplasts were isolated as described previously (5) from 1-d-old suspension cultures from maize or 2-d-old suspension cultures for tobacco (*Nicotiana tabacum*) and carrot (*Daucus carota*). The only exception was that 1% cytolysin was used in place of rhamozyme in the protoplast digestion medium. The same enzyme mixture was used for all species. Electroporation conditions for both DNA and RNA constructs were: for tobacco and maize, 180 V, 10 ms, and 1550 μF capacitance; for carrot, 260 V, 10 ms, and 1550 μF capacitance.

Duplicate electroporations using 10 μg of mRNA were carried out at 4°C using a Promega X-cell TM 450 electroporator. After 5 min on ice, the protoplasts were divided into three aliquots, added to the protoplast growth medium (5), and incubated for 5 h at the test temperatures (25, 37, and 40°C for dicots and 25, 40, and 42°C for maize). The levels of reporter gene expression resulting from each set of duplicate electroporations were averaged.

For DNA transformation, 50 μg of test DNA (and 20 μg of p70-Luc, the internal control) were used for electroporation at room temperature. Electroporated protoplasts were allowed to recover in protoplast growth medium for 12 h at 25°C before the application of heat shock.

An appropriate heat-shock response was monitored by [35S]Met incorporation into nascent protein synthesized during the last hour of incubation. Hsps were identified using SDS-PAGE.

**GUS and Luc Assays**

GUS activity was measured as described by Gallie et al. (6). GUS-specific activity was determined as pmol of substrate metabolized per min per mg of protein.

Luc activity was measured by first adding 10 to 50 μL of cell extract to 200 μL of assay buffer (825 mM Tricine, 15 mM MgCl₂, 5 mM ATP, and 500 μg/mL BSA). Photon detection was carried out following the addition of 100 μL of 0.5 mM luciferin using a Monolight 2001 luminometer (Analytical Luminescence Laboratories).

**Physical mRNA Half-Life Determination**

Aliquots of protoplasts electroporated with 5 μg of each GUS mRNA construct were removed at specific time intervals. Pelleted cells were resuspended in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 100
**RESULTS**

Heat Inducibility of hsp70 DNA Constructs in Electroporated Maize, Tobacco, and Carrot Protoplasts

The thermal induction of expression of the CAT gene under the control of the maize hsp70 promoter in electroporated maize protoplasts demonstrated that maize protoplasts respond appropriately to heat shock (1). To dissect the translational control of the heat-shock response, the CAT gene in the hsp70 construct, phsp-CAT-NOS (1), was replaced by the GUS gene to take advantage of the greater sensitivity of the GUS reporter gene. Following p70-GUS plasmid delivery using electroporation, the protoplasts were allowed to recover for 12 h at room temperature before being heat shocked. The hsp70 promoter was induced at elevated temperatures in maize and tobacco but not in carrot (Fig. 2). Similar results were obtained from the equivalent Luc construct, p70-Luc, at 37 and 40°C. However, the loss of Luc activity (10-fold) in extracts from protoplasts incubated at 42°C compared with 40°C suggested that the Luc gene product (RNA and/or protein) was unstable at elevated temperatures (data not shown). As a result, the GUS gene was chosen for subsequent constructs.

**Effect of the hsp70 Leader on Translational Control during the Heat-Shock Response**

In plants, the mechanism by which heat-shock messages are selectively translated during thermal stress remains unclear. To test whether the maize hsp70 leader might be required to escape the thermal repression of translation, GUS mRNAs, with or without the hsp70 leader, were generated in vitro and delivered to protoplasts via electroporation before the application of a heat shock. The effect of the leader on the translation and stability of GUS mRNA could be measured directly by using RNA-based constructs. An XbaI site was introduced at the hsp70 transcriptional start site so that the fragment containing the hsp70 leader-GUS gene could be moved to a T7-based vector for subsequent in vitro RNA synthesis. The resulting mRNA construct, HSL-GUS-A50 (Fig. 1C), was synthesized as a polyadenylated transcript as a result of a poly(A)<sub>50</sub> tract present in the T7 vector.

Figure 3 demonstrates the variations of GUS activity in extracts of protoplasts electroporated with in vitro synthesized HSL-GUS-A50 mRNA compared with the control. The

---

Figure 2. GUS activity in maize (■), carrot (○), and tobacco (□) protoplast extracts as a function of the protoplast incubation temperature after electroporation with p70-GUS.

Figure 3. Effect of the presence of the hsp70 leader on the translational efficiency of GUS mRNA in electroporated maize protoplasts. A, Schematic diagrams of the constructs used. B, Protoplasts were electroporated with GUS-(A)<sub>50</sub> (■) or HSL-GUS-A50 (□) mRNA.
control GUS-A50 mRNA, containing a 17-base polylinker leader, was subject to thermorepression observed for non-heat-shock mRNAs (12). Translation was more efficient at 25°C when the hsp70 leader was present, and as the temperature increased, the translational efficiency of HSL-GUS-A50 remained high. By 40°C, the hsp70 leader conferred a substantial (12-fold) advantage on the translation of GUS mRNA. In carrot, translation benefited from the presence of the hsp70 leader at 25 and 37°C, but at 40°C, the advantage of the hsp70 leader was lost (Fig. 4). We ruled out the possibility that the advantage conferred by the hsp70 leader at elevated temperatures was due to a selective increase in mRNA stability (see below); therefore, we conclude that the hsp70 leader functions as a translational regulatory element during heat shock.

We confirmed the essential regulatory role of the hsp70 leader in maize during thermal stress by removing all but 10 bases of the hsp70 leader from p70Xba-GUS to produce p70ΔL-GUS (Figs. 1B and 5A). The truncation of the hsp70 leader had no effect on expression at 25°C (Fig. 5B). At 37 and 40°C, the translation of the truncated hsp70 leader construct was 10-fold less efficient than the construct containing the intact hsp70 leader. By 42°C, the difference in translational efficiency between the two constructs increased 100-fold. These results suggest that selective translation during heat shock is dependent on the hsp70 leader itself and not on sequences within the GUS gene.

TMV Untranslated Leader Can Functionally Replace the hsp70 Leader in Monocots and Dicots

The untranslated leader from TMV RNA (referred to as Ω), is a translational enhancer in both eukaryotes and prokaryotes at normal growth temperatures (7). One characteristic of TMV is that it is initially more aggressive at elevated temperatures (32°C) compared with normal growth condi-

![Figure 4](image)

Figure 4. The role of the hsp70 leader in thermal regulation of GUS mRNA translation in carrot. Protoplasts were electroporated with GUS-l(A)50 (□) or HSL-GUS-A50 (●) mRNA.

tions (11). Moreover, during heat shock, TMV-infected tobacco continues to express viral proteins in addition to the nascent synthesis of hsp7s (3). Therefore, it was of interest to test whether Ω was responsible for the continued translation during thermal stress.

To test this hypothesis, we substituted Ω for the hsp70 leader sequence in p70Xba-GUS to result in p70Ω-GUS (Fig. 1B). Ω did confer a translational advantage at elevated temperatures, and its regulatory profile between 25 and 42°C was similar to that of the hsp70 leader-containing construct, p70Xba-GUS, in maize (Fig. 6A) and tobacco (Fig. 6C). Although the hsp70 leader did not function well in carrot (see Figs. 6B and 2), the presence of the Ω sequence did provide a translational advantage at moderate (37 and 40°C) heat-shock temperatures.

We also examined the impact of Ω on translation during heat shock in maize and carrot using mRNA-based constructs. As observed in Figure 3, the control GUS-A50 mRNA was subject to increasing thermorepression with increasing temperature (Fig. 7). However, the presence of Ω not only prevented thermorepression but significantly increased translational efficiency with increasing temperature.
TRANSLATIONAL CONTROL DURING HEAT SHOCK

Effect of Ω and hsp70 Leader on mRNA Stability

Ω increases translational efficiency of an mRNA without increasing transcript stability (6). To determine whether the impact of hsp70 leader on expression during heat shock was solely at the translational level or may selectively increase mRNA stability, the physical half-life for each of these mRNAs was measured at both physiological and heat-shock temperatures (40°C). Aliquots of tobacco protoplasts electroporated with GUS mRNA containing either Ω, the hsp70 leader, or the control leader were removed at time intervals, and total RNA was extracted and displayed on a denaturing formaldehyde-agarose gel (Fig. 8). Physical half-life measurements were determined by following the decay kinetics of the full-length form of the mRNA (6). The half-life of the control GUS-A50 mRNA at 24°C was not significantly altered when hsp70 leader was substituted. Moreover, Ω did not increase GUS mRNA stability, in good agreement with previous observations (6). Similarly, the presence of either the hsp70 leader or Ω did not significantly increase GUS mRNA stability at 40°C. These results demonstrate that these leaders that confer a translational advantage at elevated temperatures do not involve changes in mRNA stability.

DISCUSSION

Our results demonstrate that the presence of hsp70 leader in a transcript does permit its continued translation under conditions that normally result in thermorepression. Although the hsp70 leader functioned in tobacco and maize, it was less effective in carrot. Although it is unexpected that the magnitude of the heat-shock response is more similar between a dicot (tobacco) and a monocot (maize) than between the two dicot species (tobacco and carrot), the extent to which species respond to thermal stress may be more indicative of common environmental stresses to which they may be subjected than their degree of relatedness. Both tobacco and maize grow in climates in which elevated temperatures are not uncommon. Whether this or some other aspect of the heat-shock response is the basis for difference between the species investigated in this study will be determined when additional species are examined.

Deletion of all but 10 bases of the hsp70 leader resulted in the loss of thermoregulation to the extent that the truncated hsp70 leader construct functioned like the 17-base polylinker control construct. The effect of the deletion was most pronounced at 42°C. The loss of translational competence at elevated temperatures was not due to short leader length. This is consistent with a regulatory role for the hsp70 leader: the more severe the stress, the greater the translational repression and, therefore, the greater the regulatory impact of the hsp70 leader.

TMV RNA is not translationally repressed under conditions of heat shock (3). The ability of Ω, the leader sequence of the TMV genomic RNA, to confer translational competence at elevated temperatures was demonstrated using both DNA- and RNA-based constructs. It is interesting that the translational competence of Ω-containing mRNAs improved with increasing temperature. In contrast, the presence of the hsp70 leader permitted continued translation but did not signifi-

Because both the 84-base Ω (the 67-base Ω leader plus 17 bases of polylinker) and the 94-base HSL (84 bases of the HSL plus 10 bases of polylinker) were significantly longer than the 17-base control leader, it was necessary to investigate the possibility that continued translation of Ω or HSL constructs during heat shock was a function of leader length and not due to the actual sequence of the leader. A 74-base control leader, m7GppGUAAUUUGUCGACACUGUAG, and a 74-base Ω construct (the 67-base Ω leader plus 7 bases of polylinker) were compared with the 17-base control and HSL constructs in maize (Table 1). At 25°C, in addition to the high level of expression from the Ω and HSL leader constructs, expression from the 74-base leader control was greater than that from the 17-base control. Recently, studies of the effect of leader length on expression in carrot determined that leader length up to 74 bases (the longest leader tested) does have a small but positive effect on expression (8). During heat shock, however, thermorepression was observed for both the 17- and 74-base control leader constructs. In contrast, expression from the Ω construct, and to a lesser extent, the HSL construct, increased. These data suggest that primary sequence and not just the length of Ω and HSL is responsible for continued translation during heat shock.

Figure 6. Examination of the TMV leader, Ω, as a functional heat-shock leader in maize (A), carrot (B), and tobacco (C) using DNA-based constructs. Protoplasts were electroporated with p70XbaGUS (Ω) or p70B-GUS (B). Copyright © 1992 American Society of Plant Biologists. All rights reserved.
Significantly enhance translation during conditions in which translation of non-heat-shock mRNAs was repressed.

Schoffl et al. (19) demonstrated that a portion of the cauliflower mosaic virus 35S mRNA leader could functionally replace the leader of a soybean heat-shock gene. However, not only was a portion of the leader present in the construct but the core 35S promoter was present as well; consequently, nuclear and cytoplasmic regulatory events might both be involved. Delivery of mRNA using electroporation enabled us to determine whether the hsp70 leader functioned during heat shock to regulate translation or increase mRNA stability. That the hsp70 leader permitted continued translation during thermal stress but did not increase mRNA stability suggests that it is a translational regulator only. Linkage between the translatability and the stability of transcript has been observed (21). It might be expected, therefore, that the reduction in translational activity of the control constructs at elevated temperatures might result in greater instability for that mRNA. However, the lack of change in transcript stability at heat-shock temperatures is in agreement with observations in other species in which non-heat-shock messages are translationally repressed but not destabilized during heat shock (12).

Table 1. Effect of Leader Length on GUS mRNA Translation in Maize Protoplasts during Heat Shock

<table>
<thead>
<tr>
<th>Leader</th>
<th>Leader Length (bases)</th>
<th>GUS-Specific Activity at 25°C</th>
<th>GUS-Specific Activity at 42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>Control</td>
<td>74</td>
<td>0.52</td>
<td>0.18</td>
</tr>
<tr>
<td>Ω</td>
<td>74</td>
<td>2.1</td>
<td>10.0</td>
</tr>
<tr>
<td>HSL</td>
<td>94</td>
<td>0.94</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Figure 7. Examination of Ω as a functional heat-shock leader in maize (A) and carrot (B) protoplasts using mRNA-based constructs. Protoplasts were electroporated with GUS-A50 mRNA (●) or Ω-GUS-A50 mRNA (□). Insets, The effect of the 17-base control leader (■), HSL (○), and Ω (□) on the translational efficiency of GUS mRNA. Values are reported as relative percentages of expression at 25°C.

Figure 8. Effect of the hsp70 leader on mRNA stability in electroporated tobacco protoplasts. Bands represent the full-length form of each mRNA at indicated times following RNA delivery. Control GUS-A50 mRNA: A, 24°C; B, 40°C. Ω-GUS-A50 mRNA: C, 24°C; D, 40°C. HSL-GUS-A50 mRNA: E, 24°C; F, 40°C.
Secondary structure within the leader can lower the requirement for the cap-binding complex, eIF-4F (17). The degree of phosphorylation of eIF-4F changes as a consequence of heat shock and may influence translational efficiency (4). A leader with little structure may be an advantage in the translation of a message under conditions in which the eIF-4F activity is diminished. Although the cap dependence of the hsp70 leader remains to be determined, the relatively structureless Ω is dependent on the cap for function (6). Simply the length or the lack of structure within a leader, however, is not sufficient to explain the escape from thermorepression because both the 17- and 74-base control leader constructs were translationally repressed during heat shock.

No conserved elements within the leaders of Drosophila heat-shock genes have been found, although leaders from Drosophila are required for continued translation during thermal stress (10, 14). Although mutational analysis within the Drosophila hsp70 leader failed to identify any regulatory elements (14), the first 26 bases of the hsp22 leader from Drosophila were essential for escaping thermorepression (10). It is interesting to note that this 26-base region is an AC-rich sequence in a way not unlike a 25-base AC-rich region present within Ω. We recently identified the AC-rich region within Ω as part of the core regulatory element responsible for translation enhancement at nonheat-shock temperatures (8). Whether this same element within Ω is responsible for the continued translation observed at heat-shock temperatures remains to be determined. However, the ability of Ω to optimize ribosome-mRNA interactions at elevated as well as physiological temperatures may be a consequence of the evolutionary need for the leader to function under conditions of stress, i.e. viral infection. At the translational level, stress as a result of pathogen attack may be similar to that generated by extreme heat. Under conditions of stress, whether pathogen or heat induced, in which many processes in the cell become less efficient, any mRNA regulatory element, such as a leader that improves ribosome loading, will result in a great advantage for that gene. Understanding how an enhancer of translation can escape thermorepression will contribute to our overall understanding of gene regulation during the heat-shock response.

ACKNOWLEDGMENT

We thank Frederique Planckaert for skilled technical assistance.

LITERATURE CITED