© 1996 Oxford University Press

Nucleic Acids Research, 1996, Vol. 24, No. 12 **2441–2449**

Sequence and structure determinants of *Drosophila* Hsp70 mRNA translation: 5′-UTR secondary structure specifically inhibits heat shock protein mRNA translation

Mark A. Hess and Roger F. Duncan*

Department of Molecular Pharmacology and Toxicology, University of Southern California School of Pharmacy and Department of Molecular Microbiology and Immunology, School of Medicine, 1985 Zonal Avenue, Los Angeles, CA 90033, USA

Received October 30, 1995; Revised and Accepted April 9, 1996

ABSTRACT

Preferential translation of *Drosophila* heat shock protein 70 (Hsp70) mRNA requires only the 5′-untranslated region (5′-UTR). The sequence of this region suggests that it has relatively little secondary structure, which may facilitate efficient protein synthesis initiation. To determine whether minimal 5′-UTR secondary structure is required for preferential translation during heat shock, the effect of introducing stem–loops into the Hsp70 mRNA 5′-UTR was measured. Stem–loops of –11 kcal/mol abolished translation during heat shock, but did not reduce translation in non-heat shocked cells. A –22 kcal/mol stem–loop was required to comparably inhibit translation during growth at normal temperatures. To investigate whether specific sequence elements are also required for efficient preferential translation, deletion and mutation analyses were conducted in a truncated Hsp70 5′-UTR containing only the cap-proximal and AUG-proximal segments. Linker–scanner mutations in the cap-proximal segment (+1 to +37) did not impair translation. Re-ordering the segments reduced mRNA translational efficiency by 50%. Deleting the AUG-proximal segment severely inhibited translation. A 5′-extension of the full-length leader specifically impaired heat shock translation. These results indicate that heat shock reduces the capacity to unwind 5′-UTR secondary structure, allowing only mRNAs with minimal 5′-UTR secondary structure to be efficiently translated. A function for specific sequences is also suggested.

INTRODUCTION

Drosophila cells subjected to a sudden heat shock rapidly suppress the translation of existing mRNAs and efficiently synthesize the heat shock proteins (Hsp; particularly Hsp70) from newly synthesized transcripts. Early investigations of the *Drosophila* Hsp mRNAs demonstrated that only their 5′-untranslated regions (5′-UTR) are required for efficient translation during heat shock (1–4). Sequence analysis of the *Drosophila* Hsp mRNA leaders has revealed several distinguishing characteristics. They are relatively long, ranging from ~112 to 250 nt, they have a biased sequence composition of ~50% adenine, resulting in a paucity of secondary structure, and there are two conserved regions, beginning at nucleotides +2 and +93 (5). Truncated leaders retaining only 72 (3) to as few as 37 nt (4) conferred a significant measure of preferential translation, suggesting that length *per se* has limited importance. A synthetic leader with an adenine-rich base composition was not sufficient to confer preferential translation (6), suggesting that functional sequence elements not present in the synthetic leader were required. However, neither the conserved sequences nor any other specific region appears necessary, based on sequential deletion of overlapping 20–60 nt blocks from the start to the end of the leader (7). Progressive deletion from the 5′-end of the Hsp70 mRNA leader correlated with a progressive decrease in translation during heat shock (6), suggesting that some as yet ill-defined but critical signals occur in this region. In sum, a variety of analyses have provided clues to, but thus far have failed to precisely define, the critical sequence or structural features required to impart preferential translation during heat shock.

Eukaryotic mRNA 5′-UTRs influence the rate limiting initiation step in protein synthesis (8) and thereby determine the efficiency of mRNA translation. As described by the scanning model (9), initiation requires binding of eIF4F to the mRNA 5′ cap to create a target for 5′-end-dependent 40S ribosomal subunit loading. 3′ Scanning of the subunits leads to initiation at the first AUG codon occurring in the appropriate context (9). Migration of the 40S subunits is dependent on the melting of 5′-UTR secondary structure (10), in keeping with a linear mode of scanning. Extensive secondary structure leads to inefficient translation (11–13) that can be relieved by overexpression of eIF4E (11,13), presumably through enhanced unwinding activity. Minimal secondary structure may therefore confer highly efficient initiation by facilitating scanning, perhaps by reducing the requirement for eIF4F-mediated unwinding. This is consistent with eIF4F-dependent steps being rate limiting for most mRNA translation (14).

Several reports have shown that protein synthesis initiation factors eIF4E and eIF4F are altered by heat shock (15–22, reviewed in 23). In *Drosophila* embryos, Zapata *et al.* (15) found decreased amounts of the trimeric cap binding protein complex eIF4F in lysates prepared from heat shocked cells. Furthermore, depletion of the eIF4F subunits 4E and 4G from heat shocked/recovered embryo cell-free translation lysates inhibited non-heat shock but not Hsp

* To whom correspondence should be addressed

mRNA translation (15,16), suggesting preferential Hsp mRNA translation could result from eIF4F inhibition. Heat shock inactivation of eIF4F in *Drosophila* does not appear to involve decreased eIF4E phosphorylation (17), whereas in mammalian cells both eIF4E dephosphorylation and eIF4F dissociation occur during heat shock (18–21). Supplementation of heat shock cell-free translation lysates with eIF4E and eIF4F has confirmed that eIF4F activity is suppressed during heat shock and that this restricts non-heat shock but not heat shock mRNA translation (22,24). Furthermore, reducing eIF4E levels by expression of antisense mRNA inhibits non-heat shock protein synthesis while Hsp mRNAs are induced and efficiently translated (25). In sum, these studies imply that Hsp mRNAs can efficiently translate with little or no eIF4F activity.

Recent studies have identified mRNAs that translate by cap- and eIF4F-independent, internal entry of the 40S ribosomal subunit at an internal ribosome entry site (IRES), contrary to the standard scanning model of initiation. Picornaviral polycistronic mRNAs, including poliovirus, encephalomyocarditis virus and rhinovirus, fall into this class (26–28). Evidence suggests that certain cellular mRNAs, including those coding for *Drosophila* Antennapedia and human Grp78, bFGF and IGF-1, initiate through a similar mechanism (29–33). Since Hsp mRNAs translate efficiently with greatly reduced eIF4F activity, this alternative cap-independent pathway has emerged as a likely mechanism by which the Hsp leaders promote translation during heat shock (see for example 25,34).

To identify features of the Hsp70 mRNA 5′-UTR critical for translation during heat shock, possibly by an eIF4F- and cap-independent internal initiation pathway, we altered the Hsp70 5′-UTR by *in vitro* mutagenesis. To determine whether the paucity of secondary structure is required for efficient heat shock translation, stem–loops of varying stability were introduced at two locations within the wild-type 5′-UTR. To identify critical sequence elements, shortened leaders were subjected to fine scale mutagenesis. To address whether the specific sequence at the 5′-terminus has a disproportionate influence on preferential translation, its location within the 5′-UTR was changed and extraneous sequence was appended. Based on these manipulations, we find that minimal secondary structure emerges as a key determinant of preferential translation, that the specific location of the cap-proximal portion of the leader influences initiation and that a cap-independent mode of translation is unlikely.

MATERIALS AND METHODS

Construction of plasmid expression vectors

pmthsp44. Plasmid pDM301 contains a complete Hsp70 gene with a coding sequence deletion resulting in a 44 kDa expressed Hsp70 variant ($Hsp70_{\Delta C}$) (3). pmths70 expresses a full-length Hsp70 under the control of the metallothionein promoter (35). Its 5′-UTR contains nucleotides +1 to +55 of the metallothionein mRNA 5′-UTR, followed by Hsp70 5′-UTR nucleotides +2 to +242. pmthsp44 was constructed to place the $Hsp70_{\Delta C}$ transcription unit (from pDM301) under metallothionein promoter control, using *Eco*RI sites introduced at the transcription initiation sites of pDM301 and pmths70 via PCR primers. The mutagenesis introduces a G residue as +1 of the transcript preceding the A transcription initiation nucleotide in pDM301, but this does not affect heat shock translation (see Fig. 1). All DNA ligations were carried out as described by King and Blakesley (36), except that a vector:insert ratio of 1:3 was used. All changes to the Hsp70 leader were confirmed by sequencing (in this and later described constructions).

Table 1. Nucleotide sequences of 5′-UTRs analyzed by transfection/translational efficiency

Stem–loops beginning at nucleotide +17	
‘Wild-type’	gaatt caatt caaac aagca aagtg aacac gtcgc taagc gaaag
SL17.11	gaatt caatt caaac aagca aagtg aacac TtcAc tTTgc gaaag
SL17.6	gaatt caatt caaac aaCGa aagtg aacac TtcAc tTTgc gaaag
SL17.c	gaatt caatt caaac aagca aagtg aacac TtcAc tTTgc gaaag
The first nucleotide shown is the first transcribed nucleotide (+1). It is a non-Hsp, appended G introduced for cloning purposes. Its presence in the wild-type leader causes no reduction in heat shock-specific translation (Fig. 1). The first nucleotide in the stem is Hsp 5′-UTR nucleotide +17, or +18 in the G-appended 5′-UTR.	
Stem–loops beginning at nucleotide +60	
‘Wild-type’	a gcgca gctga acaag ctaaa caatc tgcag taaag tgcaa gtta
SL60.22	a gcgca gctga acaag ctaGT TCaGc tgcGC taaag tgcaa gtta
SL60.11	a gcgca gctga acaag ctaGT TCaGc tgGaA taaag tgcaa gtta
SL60.16	a gcgca gctga acaag cta Gc tgcGC taaag tgcaa gtta
The first nucleotide shown is Hsp 5′-UTR nucleotide +60. The base pair adjacent to the loop in the –16 kcal mutant is a G–U. The –11 kcal/mol stem–loop has nucleotide +64 as the first stem base, but is referred to as one of the +60 group for simplicity.	
Scanning mutatations in the cap-proximal portion of the Hsp70 5′-UTR	
Wild-typeW	aattc aattc aaaca agcaa agtga acacg tcgct
H1	aaGAT CTttc aaaca agcaa agtga acacg tcgct
H2	aattc aatAG aTCTa agcaa agtga acacg tcgct
H3	aattc aattc aaaca aAGaT CTtga acacg tcgct
H4	aattc aattc aaaca agcaa agtAG aTCTg tcgct
Nucleotides +1 to +35 of the plasmid Hsp transcript are shown. The *Bgl*II sites are underlined.	

The mutated bases are shown in upper case and the predicted regions of base pairing in the stem–loop containing 5′-UTRs are underlined. Theoretical secondary structure folding analysis of each complete 5′-UTR using the GCG FOLD program (37) identified the intended stem–loop structures in the modified 5′-UTR.

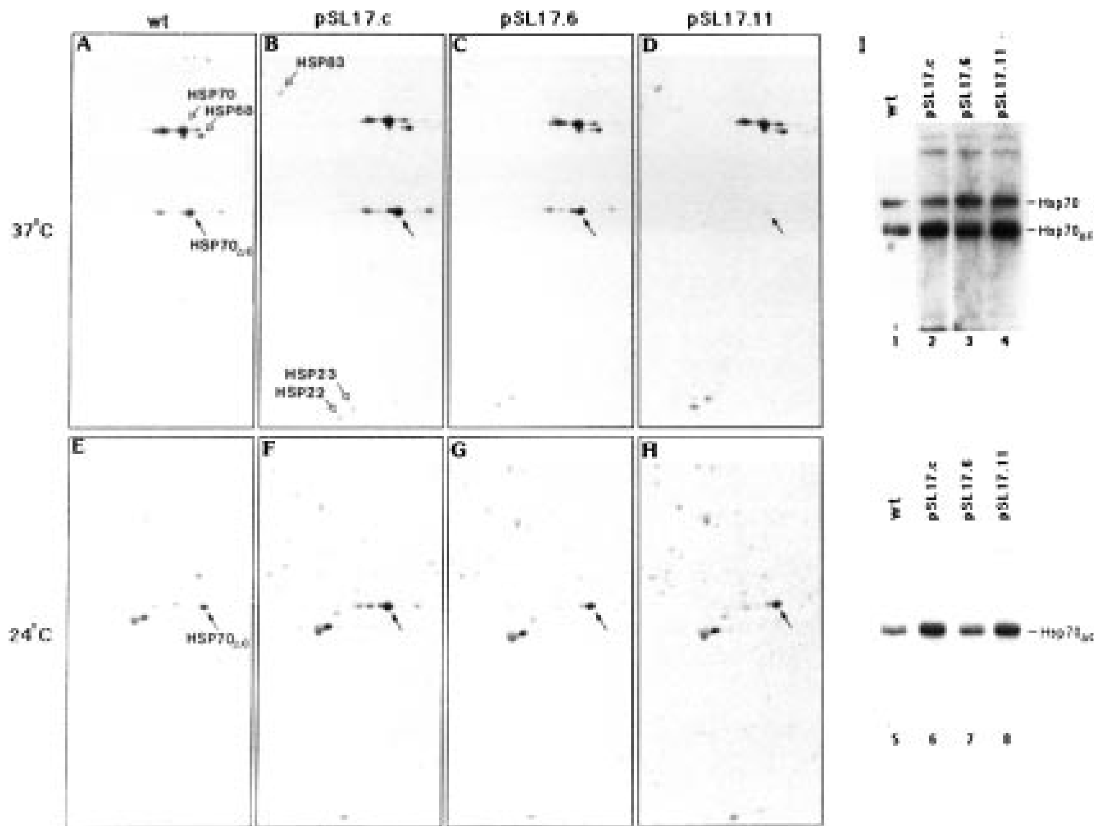

Figure 1. Effect of increased 5′-UTR secondary structure on Hsp70 mRNA preferential translation. *Drosophila* SL2 cells were transfected with $Hsp70_{\Delta C}$ genes mutated to form stem–loops in the mRNA 5′-UTR. Forty eight hours after transfection cells were treated with $CuSO_4$ for 3 h to induce transgene expression. Half of each culture was then heat shocked at 37°C for 60 min. Cells were pulse-labeled with [^{35}S]methionine for 15 min at 3 h 45 min after copper induction (at both 22–24 and 37°C). Protein samples were prepared as described (see Materials and Methods). Equal amounts of protein (equal cell numbers) were loaded on each first dimension gel (verified by Coomassie Brilliant blue staining after gel electrophoresis). Dried gels were exposed to film at room temperature for 2–5 days. (**A**–**D**) Cells subjected to heat shock; (**E**–**H**) cells maintained at 24°C. The location of the main $Hsp70_{\Delta C}$ protein spot is indicated by an arrow in each panel. In some analyses, such as the ones shown here, multiple $Hsp70_{\Delta C}$ protein spots (and endogenous Hsp70 forms) were resolved and their amounts summed. The locations of the endogenous Hsp70 and Hsp68 are indicated by open-headed arrows in (A) and the locations of Hsps 83, 22 and 23 in (B). In (A) and (E) only the portion of the gel between ~30 and 85 kDa is shown. In all panels, only the central region of the gel covering protein pI values between ~5.0 and 6.0 is shown. (**I**) RNA was extracted from heat shocked and non-heat shocked cells at 3 h 55 min after copper induction (55 min after heating) as described in Materials and Methods. Hsp70 and $Hsp70_{\Delta c}$ RNAs were quantified by hybridization with a ^{32}P-labeled Hsp70 coding sequence probe. Exposure was at –80°C for 2–4 days. Lanes 1–4, RNAs from heat shocked (37°C) cells; lanes 5–8, RNAs from non-heat shocked (22–24°C) cells. Differences in RNA loading per lane were corrected for by normalizing the $Hsp70_{\Delta C}$ mRNA band darkness (c.p.m.) to the actin and/or endogenous Hsp70 mRNA hybridization signals. wt, wild-type; pSL17.c, plasmid expressing the leader with mutations that do not permit secondary structure formation; pSL17.6, plasmid expressing the leader with introduced –6 kcal/mol stem–loop; pSL17.11, plasmid expressing the leader with introduced –11 kcal/mol stem–loop. A representative result is shown. The experiment was repeated two to three times and the averaged results are reported in Figure 2.

5′-UTR mutants of pmthsp44. (i) Stem–loop mutants at position +17 in the Hsp70 5′-UTR. pmthsp44 was digested with *Eco*RI and *Cla*I. The Hsp70 5′-UTR-containing fragment was PCR amplified using the mutagenic primers listed below, which allow stems with free energies of –6 and –11 kcal/mol to form. The upstream PCR primers contained four complementary nucleotides to tack down the end, the introduced G base (described above) and then nucleotides +1 to + 45 of the Hsp70 5′-UTR, with four base changes at positions +30, +33, +36 and +37 for both the –6 and –11 stems, as well as at +17 and +18 for the –6 stem. The wild-type and all mutant sequences are listed in Table 1. The mutagenic primers eliminate an *Afl*III site. A control mutagenic primer also introduced four base changes at positions +30, +33, +36 and +37, but they do not lead to predicted stem–loop formation. The downstream primer annealed to coding sequence nucleotides +249 to +272 (overlapping a *Cla*I site at +260). The PCR products were digested with *Eco*RI/*Cla*I and used to replace the wild-type 5′-UTR by insertion into *Eco*RI/*Cla*I-digested pmthsp44. Putative mutants were identified by the loss of the *Afl*III site and confirmed by sequencing. The resultant plasmids were named pSL17.11, pSL17.6 and pSL17.c.

(ii) Stem–loop mutants at position +60 in the Hsp70 5′-UTR. pmthsp44 DNA was linearized by digestion with *Sma*I and in a separate reaction 'windowed' by double digestion with *Xho*I and *Eag*I to remove a region where the mutagenic oligomer could hybridize. Aliquots of 100 ng each of linearized DNA and deleted DNA were mixed with 12.5 pmol of mutagenic phosphorylated oligonucleotide (sequence listed in Table 1, representing nucleotides +66 to +104 inclusive) in 20 mM Tris, pH 7.5, 10 mM $MgCl_2$ and 50 mM NaCl (38), boiled for 3 min, incubated at 37°C for 15 min and then 30°C for 15 min. To this was added 4.5 U T4 DNA ligase and either 3 U T4 DNA polymerase or 2.5 U Klenow enzyme, followed by incubation at 37°C for 2 h and 75°C for 15 min. Following bacterial transformation and growth, pooled plasmid DNA was isolated (39) and digested with *Pst*I (which the mutagenic oligonucleotides were designed to eliminate in

successfully mutated plasmids). If plasmids lacking one of three *Pst*I sites (removed by mutagenesis) were present in the pool, individual colonies were picked, grown and the plasmid isolated. The mutagenic oligomers introduce seven base changes. One allows a perfectly base paired 8 nt stem with a free energy of –11 kcal/mol to form (pSL60.11), while the second allows a perfectly base paired 13 nt stem with a free energy of –22 kcal/mol to form (pSL60.22). A –16 kcal/mol stem–loop-containing mutant was fortuitously obtained as an aberrant deletion/point mutant and has lost leader nucleotides +79 to +83 (pSL60.16; see Table 1).

Scanning block mutations of pDM306 (pH1–pH4). Plasmid pDM306 contains an $Hsp70_{\Delta C}$ gene with a 5′-UTR deletion that removes nucleotides +38 to +205 (3). Four oligomers were designed to introduce *Bgl*II sites at staggered locations throughout the first 30 nt of the $Hsp70_{\Delta C}$ 5′-UTR. The mutation-creating oligomers spanned nucleotides –14 to +25 (H1), –8 to +30 (H2), –4 to +35 (H3) and +4 to +43 (H4). Windowing mutagenesis was carried out as described for the +60 stem–loop constructions and the putative mutants were identified by the presence of the introduced *Bgl*II site. Plasmids were named pH1–pH4 based on the mutagenic oligomer.

Shortened +1 to +37 leader (pH5). Nucleotides +206 to +242 were deleted from pDM306 using the windowing procedure with an oligomer (spanning nucleotides +23 to +37, followed by +243 to +263) that caused these nucleotides to loop out. *Mut*S competent cells were transformed and individual clones selected based on size reduction of the leader region and sequenced to verify that nucleotide +37 was apposed to nucleotide +243 (the A in the ATG).

pDM306 leader rearrangement (H6). Splice overlap extension PCR (41) was used to create a modification of pDM306 in which the order of the two Hsp70 5′-UTR segments was reversed. In two separate PCR reactions: (i) the Hsp70 promoter region was amplified using a downstream primer that appended Hsp70 5′-UTR nucleotides +206 to +242 as the first transcribed nucleotides (tgtgtgtgagttcttctttctcggtaacttgttgaaagtcgctccgtagacgaag; metallothionein promoter complementary nucleotides listed in upper case); (ii) plasmid H5 was amplified using an upstream primer that hybridized to 5′-UTR nucleotides +1 to +16 and that appended Hsp70 5′-UTR nucleotides +223 to +242 to the 5′-end of the PCR product (gaaagaagaactcacacacaaattcaattcaaacaagcaa). The downstream primer hybridized to the *Cla*I-proximal region (annealing site detailed above). Secondly, the two segments were co-amplified in the presence of the outer flanking primers (M13 sequencing primer, upstream; *Cla*I-proximal primer, downstream), the resultant DNA was double digested with *Hin*dIII and *Cla*I and substituted for the analogous fragment in *Hin*dIII/*Cla*I-digested pDM306.

Wild-type leader 5′-extension. pmt44hs is an $Hsp70_{\Delta C}$ variant containing the complete $Hsp70_{\Delta C}$ transcription unit preceded by metallothionein (Mt) 5′-UTR nucleotides +1 to +55. This plasmid was originally constructed by Peterson and Lindquist (42), but its heat shock translational efficiency has not been reported. It was recreated here by replacing the Mt promoter–Hsp70 5′-UTR coding sequence region in pmthsp 44, with the analogous Mt promoter 55 nt 5′-UTR–Hsp70 5′-UTR-cds fragment from pmt70hs.

Leader replacements with non-Hsp leader nucleotides. The Hsp70 5′-UTR was replaced with two non-Hsp 5′-UTRs. Plasmid pH0 was first constructed, which eliminates the Hsp70 5′-UTR from $Hsp70_{\Delta C}$. It contains a single *Bgl*II site immediately following the –1 Hsp promoter base, followed by leader nucleotides +37 and +38 and then the AUG start codon. Plasmid pH7 was created by inserting the inverse complement of Hsp70 5′-UTR nucleotides +1 to +85 at the *Bgl*II site in plasmid pH0 by PCR amplification of nucleotides +1 to +85 of the Hsp70 5′-UTR. Plasmid pH8 was created by inserting a 47 nt arbitrary DNA fragment (obtained from Dr A.S.Lee) into plasmid pHO following sequential *Bgl*II and Klenow treatment. The sequence of the arbitrary DNA fragment is 5′-ctcgagccgcttcgaatcggactatcgactagcttgctacggtcgac-3′.

Protein labeling and quantitation

Drosophila S2 cells were removed from T25 flasks, pelleted by brief centrifugation in a clinical centrifuge, resuspended in Dulbecco's MEM lacking methionine (Gibco) and transferred to 20 ml glass scintillation vials, with a stir flea, for experimental manipulations and analyses (17,43). Induction of the transgenes under metallothionein promoter control was by induction with copper sulfate for 3 h. Heat shocked cells were incubated in a 37°C water bath, with stirring, for 45 min, at which time ~5×10^6 cells were labeled with 15–20 µCi [^{35}S]methionine (NEN EXPRE^{35}S^{35}S) for 15 min. Protein extraction and analysis by one- and two-dimensional gel electrophoresis were as described (43,44). Gel regions were excised from the dried gels, rehydrated briefly in water, incubated overnight at 37°C in 3% protosol/PPO/POPOP/toluene and counted. Transgene mRNA translational efficiency was calculated as the protein synthesis rate (^{35}S c.p.m. in the excised IEF/SDS–PAGE transgene protein spot) per unit transgene mRNA (^{32}P c.p.m. on the Northern blot, normalized to actin or endogenous Hsp70 hybridization; see below). Translational efficiencies are reported relative to pmthsp44 (wild-type 5′-UTR) in most cases.

RNA isolation and quantitation

RNA was extracted from 10^7 non-heat shocked or heat shocked (at 55 min) cells using a guanidinium isothiocyanate-based buffer, as described by Chomczynski and Sacchi (45) with modifications (46). RNA was resuspended in 0.5% SDS, 2 mM EDTA and displayed on formaldehyde denaturing gels (40) using 1/3× formaldehyde. RNA was transferred to NYTRAN (Schleicher and Schuell) by capillary transfer, fixed by UV irradiation (Stratagene Stratalinker) and stained with 0.5% methylene blue (40). Blots were probed with random hexamer-primed ^{32}P-labeled DNA probes corresponding to Hsp70 (which hybridizes to a region common to both the endogenous and transfected genes) and *Drosophila* actin. Following hybridization and washing, probe-labeled bands were identified by autoradiography and directly counted by excision. Some blots were quantified by scanning densitometry (Millipore BioImage).

Transfection of tissue culture cells

Drosophila S2 cells were propagated in Schneider's medium supplemented with 10% fetal calf serum (Gibco BRL). Approximately 18 h prior to transfection, cells were seeded at a density of $1–1.5 \times 10^6$/ml in T25 flasks (Corning). For each flask, 8–10 µg plasmid DNA were mixed with 0.5 ml 250 mM $CaCl_2$ and added slowly to 0.5 ml 2× HEPES-buffered saline (1) with constant aeration. The DNA/phosphate solution was added to the cell culture within 5 min of preparation. At 16–24 h post-transfection, the culture medium was replaced and cells were grown for an additional 1–3 days.

Stem-loop Location	Plasmid	Stability in ΔG (kCal/mol)	5'UTR structure	Translational Efficiency (% wild type 5'UTR) 24°C	37°C
None (Wild-type)	pmthsp44	-4	AUG	100	100
+17	pSL17.6	-6	AUG	55	128
	pSL17.11	-11	AUG	80	<1
	pSL17.c	-4	AUG	88	128
+60	pSL60.11	-11	AUG	83	4
	pSL60.16	-16	AUG	127	9
	pSL60.22	-22	AUG	8	4

Figure 2. Schematic representation and translational effects of stem–loop structures in the mutated Hsp70 5′-UTR. The 5′-UTRs are summarized in diagrammatic form. Stem–loops located at +17 and +60 have these nucleotides as the first (5′) stem base (see Materials and Methods regarding pSL60.11). pSL17.c has four single base changes (open circles) that do not lead to predicted stem–loop formation. The thermodynamic stabilities of base paired regions were determined using the FOLD program (45). Translational efficiency was calculated as described (Materials and Methods) by dividing the c.p.m. in the Hsp70Dc spot(s) by the transgene mRNA/cell equivalent. pmthsp44, containing the wild-type leader, was assigned a value of 100% at each temperature. Values reported are the average of at least three replicate transfections for the +60 stem–loops and two replicates for the +17 stem–loops.

RESULTS

Minimal secondary structure in the Hsp70 mRNA 5′-UTR is necessary for preferential translation

Analysis of the *Drosophila* Hsp mRNA 5′-UTRs has identified several features that may be necessary for efficient translation during heat shock (6,23), as outlined in the Introduction. Among these is a biased sequence composition of 46–50% adenine that decreases the likelihood of secondary structure formation (6). To determine if a minimum of secondary structure is necessary for efficient preferential translation of Hsp mRNAs, stem–loops of varying stability were introduced into the Hsp70 5′-UTR by *in vitro* mutagenesis. The reporter gene in all cases was an Hsp70 variant deleted in the coding region (Hsp70$_{\Delta C}$) that produces a novel ~44 kDa protein product readily detectable by pulse-labeling proteins followed by two-dimensional IEF/SDS–PAGE and autoradiography (Fig. 1A). All transfected genes in this part of the study were placed under control of the *Drosophila* metallothionein promoter, so that their translation under non-heat shock and heat shock conditions could be compared. Following transient transfection, transgene expression was induced with CuSO$_4$ at 24°C for 3 h. Transgene protein synthesis was assayed during subsequent 37°C heat shock and during maintenance at 24°C. Comparison of pulse-labeled proteins from heat shocked (Fig. 1A) and non-heat shocked (Fig. 1E) cells demonstrates the ability of the wild-type leader Hsp70$_{\Delta C}$ mRNA to retain efficient and preferential translation following heat shock, when protein synthesis of the normal repertoire of proteins is severely inhibited.

Stem–loop structures of varying stabilities were created at two positions, beginning either at nucleotide +17 or +60. The sequences of the wild-type and mutated 5′-UTRs are listed in Table 1 and depicted in Figure 2. A modestly stable –11 kcal/mol stem beginning at +17 inhibited translation during heat shock by >90%, relative to the wild-type leader (Fig. 1A, wild-type, versus D, –11 kcal/mol stem). [In all transfections transgene mRNA abundance was concurrently measured (detailed below) so that protein synthesis rates could be converted into translational efficiencies, as reported in Fig. 2.] This –11 kcal/mol stem and loop structure did not significantly inhibit translation at 24°C (Fig. 1E versus H), possessing 80% wild-type activity (Fig. 2). Thus, the non-heat shocked translational machinery can unwind the stem, whereas the heat shocked translational machinery cannot. This defines a specific functional lesion induced by heat shock. A stem of –6 kcal/mol stability did not inhibit translation at either temperature (Figs 1C and G and 2). The modestly reduced translational efficiency of this construct's mRNA at 24°C likely reflects experimental variability, since insertion of the more stable –11 kcal/mol stem did not decrease mRNA translational efficiency under non-heat shock conditions. To ensure that the protein synthesis rate differences detected by IEF/SDS–PAGE reflected translational efficiency differences, the transgene Hsp70$_{\Delta C}$, endogenous Hsp70 and actin mRNA abundances were concurrently determined in all analyses (Fig. 1I; the actin mRNA quantitation is not shown) and used to calculate the translational efficiency reported in Figure 2 (see Materials and Methods for details). The slightly reduced amounts of Hsp70$_{\Delta C}$ mRNA detected in lanes 1, 3 and 5 were paralleled by reduced amounts of actin and/or endogenous Hsp70, indicating that the transgene mRNA expression per cell was approximately equal in each case and therefore the protein expression data in panels A–H reflects the translational efficiencies of the respective mRNAs.

The –11 kcal/mol stem was created by changing four bases in the Hsp70 leader, which potentially allows folding into an 8 bp stem. To provide evidence that formation of the stem–loop structure rather than the base changes caused inhibition of preferential translation, a different set of four base changes that do not permit stem formation (detailed in Materials and Methods) were introduced at the same positions in the leader. This leader construct translated as well as the wild-type leader under heat shock conditions (Fig. 1A versus B), indicating that mutation of these four bases does not impair translation unless they create the potential to form secondary structure.

The cap-proximal location of the stem–loop structure could in theory inhibit heat shock translation by impeding cap-directed initiation events, rather than by stem-dependent inhibition of ribosomal subunit scanning. To distinguish between these possibilities, a second set of stem–loop structures that begin with nucleotide +60 was created (Table 1). A –11 kcal/mol stem at this position was functionally equivalent to the cap-proximal –11 kcal/mol variant, specifically inhibiting translation by >90% during heat shock (quantitation reported in Fig. 2). The –11 and –16 kcal/mol stems did not significantly impair translation at 24°C. A more stable stem of –22 kcal/mol inhibited translation at 24°C by >90% (Fig. 2), exceeding the unwinding capacity of the non-heat shock *Drosophila* translational machinery. The latter results are in agreement with previous studies that show that single stem–loops in the mRNA 5′-UTR can impair normal translation (8), though the minimum stability causing inhibition is less than has been reported for mammalian cells (10). The observation that a –11 kcal/mol stem–loop had comparable inhibitory effects at +17 and +60 during heat shock suggests that the heat shock translational machinery is incapable of translating mRNAs containing stem–loops of this stability regardless of their location in the proximal third of the leader.

Specific sequences in the cap-proximal region of the Hsp70 5′-UTR are not required for preferential translation

A second series of experiments was performed to investigate whether sequence-specific elements, in addition to minimal secondary structure, are required for efficient Hsp70 mRNA translation during heat shock. A survey of the *Drosophila* Hsp mRNA 5′-UTRs identified highly conserved sequence elements located at +2 and +93 (5), but a translation-promoting role for these has not been determined. A series of 20–60 nt scanning deletions that covered the entire Hsp70 5′-UTR, including both conserved regions, failed to identify any specific region necessary for preferential translation (3,7). While these results argue against a function for specific sequence elements, they are also consistent with the presence of a functionally redundant motif distributed throughout the 5′-UTR.

In attempting to identify specific functional sequence elements, we reasoned that in a shortened leader, the number of redundant elements would be reduced, revealing a more robust translational effect when the remaining copy or copies were mutated. We initially used plasmid pDM306 (3), which has a large 5′-UTR internal deletion spanning nucleotides +39 to +205 (retaining only ~70 5′-UTR nucleotides) and translates relatively efficiently during heat shock; between 40 (this study, see below) and 60% (3) of the full-length leader. Both values are ≥10 times the efficiency of a non-heat shock leader-containing mRNA (as quantified below). Since evidence suggests that at least one of these elements resides in the first 35 nt of the 5′-UTR (1,4,6), our fine scale mutational analysis was focused on this region. Plasmids used in this series of experiments contained the heat-inducible Hsp70 promoter and mRNA expression was induced by a 60 min, 37°C heat shock. Translational efficiencies were determined as above, using IEF/SDS–PAGE and Northern blot analyses to quantify protein synthesis rate and RNA abundance, respectively.

Groups of 6 nt beginning at +2 and scanning progressively to +29 were converted to *Bgl*II sites (Table 1 and Fig. 3). In each group, three or five bases of the wild-type sequence were changed. Each of the *Bgl*II mutant mRNAs translated as efficiently as pDM306 mRNA during heat shock (pH1–pH4, Fig. 3). We were thus unable to identify specific sequence determinants of preferential translation in this test leader. pH4 mRNA consistently translated as well as or better than pDM306. The significance of this observation awaits further study. To investigate the possibility that redundant sequence elements in the distal portion of the pDM306 leader (+206 to +242) compensated for the 5′-proximal mutations, we deleted +206 to +242 (plasmid pH5). However, pH5 mRNA translated very poorly during heat shock, though slightly better than mRNA with a non-heat shock 5′-UTR (Fig. 3, compare to pH7 and pH8), precluding further mutational analysis.

As an alternative test of functional sequence elements in the cap-proximal portion of the 5′-UTR, we reversed the order of the two pDM306 segments, +1 to +37 and +206 to +242 (pH6, Fig. 3). The translational efficiency of this mRNA addresses whether a location-dependent sequence element occurs in the wild-type cap-proximal segment (+1 to +37), since pDM306 and pH6 have very similar nucleotide compositions and predicted free energies of folding (based on GCG FOLD analysis; 37). A modest 40–50% reduction in translational efficiency occurred (Fig. 3, pH6 versus pDM306). However, there is substantial sequence similarity between the two segments, which suggests pH6 could have partially recreated the putative location-dependent element.

Two non-Hsp sequences were inserted between the Hsp70 promoter and the $Hsp70_{\Delta C}$ coding sequence. When an arbitrary sequence was the sole 5′-UTR sequence (pH7), it did not support heat shock translation (Fig. 3). Similarly, insertion of the inverse complement of +1 to +85 of the wild-type Hsp70 5′-UTR, resulting in a T-rich sequence (pH8), creates an mRNA which did not translate during heat shock (Fig. 3). Both mRNAs were translated in cells recovering from heat shock, approximating non-heat shock translational conditions, indicating that their 5′-UTRs do not contain generally inhibitory elements. Both pH7 and pH8 mRNAs can fold into relatively stable stem–loop-containing structures, which may account for the absence of heat shock translation.

A cap-independent, internal initiation pathway for Hsp mRNA translation is unlikely

The constructs containing a stem–loop at +17 suggest that an internal initiation pathway is not available to the Hsp mRNA, since the majority of the 5′-UTR, including the putative IRES, remains present and unaltered. However, the cap-proximal stem–loop could have interacted with internal 5′-UTR sequences, thereby interfering with putative IRES function. To further investigate whether an IRES exists in the Hsp70 5′-UTR, we

5' UTR Modification	Plasmid	Sequence	Translational Efficiency (% wild type 5'UTR)
None (Wild-type)	pDM301	+1 … +242 AUG	100
	pDM306	+37 … +206 AUG	41
pDM306 scanning mutations	pH1	AUG	48
	pH2	AUG	46
	pH3	AUG	49
	pH4	AUG	71
pDM306 deletion	pH5	AUG	13
pDM306 rearrangement	pH6	+206 +242 … +1 +36 AUG	23
Arbitrary sequence	pH7	AUG	<5
Inverse complement	pH8	AUG	<5
5' Extension	pmt44hs	AUG	13

Figure 3. Schematic representation and translational effects of Hsp70 5′-UTR sequence manipulations. The 5′-UTRs are summarized in diagrammatic form. The construction and sequences of these plasmids are described in Materials and Methods. Nucleotide numbering (+1 equals the cap site) are reported above the 5′-UTRs. Open rectangles (pH1–pH4) represent nucleotides mutated to create *Bgl*II sites. Open arrows (at +206) represent a *Bam*HI linker introduced into the original construction of pDM306 (3). The thin lines in plasmids pH7 and pH8 represent non-Hsp sequence used to create test leaders. The striped box in plasmid pmt44hs represents metallothionein 5′-UTR nucleotides +1 to +55 which precede the full-length Hsp70 leader. Translational efficiency during heat shock was calculated as described (see Materials and Methods) and is reported as a percentage of the wild-type 5′-UTR carried in plasmid pDM301 (3). The average translational efficiency of at least three replicate transfections is reported, except for pH6, which was measured twice relative to pDM306 and its translational efficiency is reported relative to pDM301. Translation of pH7 and pH8 was evaluated under non-heat shock conditions by inducing expression for 60 min at 32°C, followed by return to non-heat shock temperature and assay at 15–30 min of recovery. Their mRNAs translated at ≥50% the wild-type pDM306 rate during this recovery from heat shock. In each of five replicates, pH4 translated better than pDM306. pmt44hs transcription was induced by copper from the *Drosophila* metallothionein promoter. The remaining Hsp70 mutant genes were controlled by the *Drosophila* Hsp70 promoter.

determined the translational efficiency of an Hsp70$_{\Delta C}$ reporter mRNA with 55 nt of the *Drosophila* metallothionein 5′-UTR preceding the full-length Hsp70 5′-UTR (42). This mRNA translated very poorly during heat shock (Fig. 3) (but efficiently at 22–24°C), indicating that Hsp70 5′-UTR sequences must be present in the cap-proximal region for them to promote heat shock preferential translation. These results argue against the presence of an IRES in the Hsp70 5′-UTR, as do similar experiments by McGarry and Lindquist (3) showing that an unrelated 30 nt 5′-extension to the Hsp70 5′-UTR suppressed heat shock translation.

DISCUSSION

We have investigated the role of 5′-UTR secondary structure and primary sequence in directing preferential translation of Hsp70 mRNA during heat shock. Our results indicate that a relative absence of secondary structure is imperative for efficient Hsp70 mRNA translation. The creation of 5′-UTR stem–loops effectively abolished translation in heat shocked cells, but did not impede translation in unstressed cells. A –11 kcal/mol stem–loop suppressed heat shock translation when located at either +17 or +60, indicating stem–loop structures inhibit in both a cap-proximal as well as an internal location. Heat shock therefore reduces the capacity of the translational machinery to unwind 5′-UTR secondary structure. This observation is in agreement with previous studies showing that hypertonic stress dramatically enhances the inhibitory effect of an mRNA 5′-UTR stem–loop in COS cells (47) and could be explained by competition among mRNAs for limiting factors involved in 5′-UTR unwinding. Hsp mRNAs are resistant to translational suppression induced by hypertonic stress in *Drosophila* KC 161 cells (48), consistent with the idea that their unstructured 5′-UTRs promote translation when stress inhibits initiation factor-dependent unwinding activity.

The apparent loss of unwinding activity that occurs during heat shock presumably results from a reduction in one or more initiation factor activities. Heat shock reduces the amount of trimeric eIF4F complex (15,16,19–21), such that cap recognition activity likely becomes reduced. Heat shock also results in dephosphorylation of the eIF4E subunit, which may also impair

eIF4F activity (18–21). We attempted to restore heat shock translation to the –16 kcal/mol stem mRNA by overexpressing mammalian eIF4E, but this strategy proved unsuccessful, perhaps because the mammalian initiation factor complements inefficiently in *Drosophila* cells or eIF4F activity is impaired at multiple levels.

The observation that heat shock inhibits eIF4F has led to the suggestion that the Hsp mRNAs are translated via a cap-independent, internal initiation pathway (25,34). In support of this view, Grp78 mRNA, a member of the Hsp70 family, can translate in this manner (29). However, our observation that both cap-proximal stem–loop structures and a 5′-extension of the Hsp70 5′-UTR severely compromise heat shock translation argues against this proposition. Another 5′-extension of the 5′-UTR with Hsp70 promoter sequence also blocks heat shock translation (3). Furthermore, heat shock inhibits IRES-mediated, internally initiated translation of the *Drosophila* Antennapedia mRNA (H.-J.Song and R.F.Duncan, unpublished results), suggesting that this initiation pathway is not available during heat shock. Even the relatively unstructured Hsp 5′-UTRs appear to require some eIF4F activity, since efficient translation of Hsp70 and Hsp83 mRNA is impaired when eIF4F-dependent cap recognition is inhibited (15,16,49). It follows that the reduced levels of eIF4F present during heat shock are nonetheless sufficient for efficient initiation of Hsp mRNA.

The implication that minimal secondary structure alone might be sufficient for preferential translation led us to compare the free energy of folding and predicted structure of 5′-UTR sequences of heat shock and non-heat shock mRNAs (from >30 non-heat shock mRNAs), using the FOLD and StemLoop programs in the GCG software package (37). *Drosophila* Hsp 5′-UTRs as a class tend to have the lowest overall stabilities, the exception being Hsp83, whose overall stability is among the highest 20% of all mRNAs. Most significantly, several non-heat shock leaders, including for example actin and catalase 5′-UTRs, have less average overall stability than the Hsp70 5′-UTR. However, the single most stable stem–loop could be the critical factor determining whether a 5′-UTR contains inhibitory secondary structure. When the folded structures of the non-heat shock 5′-UTRs (Squiggles plots; 37), including actin and catalase, were re-examined no individual stable stem–loops (with stabilities equal to or greater than those which inhibited Hsp70 mRNA translation) were detected. In summary, minimal secondary structure appears to be necessary, but not sufficient, for preferential translation. This conclusion is strengthened by empirical studies showing that 50 or 100 nt, adenine-biased sequences, when substituted for nucleotides +1 to +37 in the pDM306 Hsp70 5′-UTR, failed to confer efficient heat shock translation (6), despite retaining a low free energy of folding similar to that of the native sequence.

Since minimal 5′-UTR secondary structure is not sufficient to confer preferential translation, other features, such as specific sequence elements or length, are presumably necessary. We did not find any evidence for sequence elements in the +1 to +37 cap-proximal region of the Hsp70 5′-UTR: *Bgl*II block mutations of this region had no deleterious effects on translation, despite the restriction on functional redundancy imposed by using the truncated pDM306 leader (nucleotides +1 to +37 linked to +206 to +242). It is nonetheless conceivable that nucleotides +206 to +242 contain copies of an important sequence and that these compensated for the effects of our mutations. Unfortunately, deletion of this region reduced heat shock translation to an extent that precluded meaningful analysis. Moreover, this does not imply that the +206 to +242 sequence is necessary or that the +1 to +37 sequence is insufficient, since reducing 5′-UTR length below 50 nt has been shown to substantially decrease the rate of initiation (50). On the other hand, a severely truncated Hsp22 5′-UTR, containing only ~38 nt interrupted by a *Kpn*I linker, conferred efficient translation in heat shocked *Drosophila* (4). This discrepancy warrants further investigation. It is clear that length *per se* has little significance down to ~70 nt in the Hsp70 5′-UTR, since the pDM306 leader confers between 40 and 65% of the full-length efficiency. We suspect that Hsp 5′-UTR length enhances heat shock mRNA translation as it does non-heat shock mRNA translation, but the threshold for a significant effect needs to be rigorously evaluated.

The possibility remains that specific sequences are required for preferential translation, but what are they and why have previous scanning deletions throughout the leader (7) failed to provide any hint of their identity? We favor the idea that there is some redundancy of functional elements throughout the Hsp leader. If this model is correct, the observation that deletion of a region fails to reduce translation does not prove that the region lacks functional sequence elements, only that they are dispensable. Repeated motifs may have arisen through duplication of a shorter ancestral leader, perhaps favored by increases in heat shock mRNA translation. Our results and others (3) indicate that the cap-proximal positioning of the first ~35 nt is critical to heat shock translation, suggesting a specific location dependence. Alignment of the cap-proximal leader sequence (~35 nt) with the remainder of the leader reveals evidence for a repeating, degenerate motif [based on alignment scores (Intelligenetics Suite) compared to several random, base composition-matched sequences]. If amplification of the 5′ cap-proximal sequence leads to the current Hsp70 5′-UTR structure, then functional determinants in the cap-proximal region may have retained the optimal sequence, whereas cap-distal copies partially lost the sequence-specific element(s) while retaining low secondary structure. This model is consistent with the observation that deletions in the first 100 nt of the Hsp70 5′-UTR are substantially more inhibitory than similar length deletions of the AUG-proximal region (3). An intriguing possibility is that the sequence motifs in the cap-proximal portion of the 5′-UTR can preferentially recruit ribosomal subunits by a Shine–Dalgarno-like base complementarity, perhaps facilitated by its relatively unstructured nature (51). Substantial base pairing can occur between the Hsp70 5′-UTR +1 to +35 and the 3′-terminal 100 nt of the 18S ribosomal subunit, but the existence and significance of this potential interaction remains to be addressed.

Based on the investigations reported here and elsewhere (3,6,7), a straightforward investigation should establish or refute the sufficiency of the cap-proximal region and identify its salient features. The replacement of nucleotides +1 to +37 with adenine-rich sequences does not permit translation during heat shock (6), showing that specific sequence elements present in +1 to +37, but absent from random, adenine-rich sequences, are sufficient to impart preferential heat shock translation. A converse substitution, using the +1 to +37/+206 to +242 leader and replacing the +206 to +230 segment with random, adenine-biased sequences, will provide a critical test of whether the +1 to +37 nucleotides are sufficient to recruit ribosomes efficiently during heat shock. Point mutation analysis of regions shown to be

both necessary and sufficient could precisely define the sequence(s) necessary for heat shock translation.

In summary, the ~50% adenine content of the Hsp mRNA leaders is likely important for preferential translation to the extent that it prevents formation of significant 5′-UTR secondary structure. As we have shown, a single stem–loop can severely impair translation during heat shock, while having little or no effect on the non-stressed translational machinery. The minimum adenine content ensuring sufficiently low secondary structure is unknown, but the Hsp83 leader promotes efficient heat shock translation with as little as 38% adenine and a relatively high overall secondary structure. The existence of a necessary sequence motif is also likely and the evidence to date is consistent with its first occurrence in the cap-proximal region of the Hsp70 5′-UTR, with fewer copies and/or extensive degeneration of the sequences nearer the AUG-proximal region.

ACKNOWLEDGEMENTS

We thank D.Ann for critical reading of the manuscript. We are grateful to Dr S.Tahara for material and critical support, to Drs J.Shih and G.Cortopassi for equipment support and especially to Dr S.Lindquist for providing the critical plasmids pDM301, pDM306 and pmt44hs. This work was supported by NIGMS 42644 and the University of Southern California School of Pharmacy.

REFERENCES

1 Di Nocera,P.P. and Dawid,I. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7095–6098.
2 Bonner,J.J., Parks,C., Parker-Thornberg,J., Mortin,M.A. and Pelham,H.R.B. (1984) *Cell*, **37**, 979–991.
3 McGarry,T.J. and Lindquist,S. (1985) *Cell*, **42**, 903–911.
4 Hultmark,D., Klemenz,R. and Gehring,W.J. (1986) *Cell*, **44**, 429–438.
5 Holmgren,R., Corces,V., Morimoto,R., Blackman,R. and Meselson,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3775–3778.
6 Lindquist,S. and Petersen,R. (1990) *Enzyme*, **44**, 147–166.
7 Lindquist,S. (1987) In Ilan,J. (ed.), *Translational Regulaton of Gene Expression*. Plenum Press, New York, NY, pp. 187–207.
8 Kozak,M. (1992) *Annu. Rev. Cell Biol.*, **8**, 197–225.
9 Kozak,M. (1983) *Cell*, **34**, 971–978.
10 Kozak,M. (1989) *Mol. Cell. Biol.*, **9**, 5134–5142.
11 Fagan,R.J., Lazaris-Karatzas,A., Sonenberg,N. and Rozen,R. (1991) *J. Biol. Chem.*, **266**, 16518–16523.
12 Manzella,J.M. and Blackshear,P.J. (1990) *J. Biol. Chem.*, **265**, 11817–10822.
13 Koromilas,A.E., Lazaris-Karatzas,A. and Sonenberg,N. (1992) *EMBO J.*, **11**, 4153–4158.
14 Rhoads,R.E., Joshi-Barve,S. and Rinker-Schaeffer,C. (1993) *Prog. Nucleic Acids Res. Mol. Biol.*, **46**, 183–219.
15 Zapata,J.M., Maroto,F.G. and Sierra,J.M. (1991) *J. Biol. Chem.*, **266**, 16007–16014.
16 Zapata,J.M., Martinez,M.A. and Sierra,J.M. (1994) *J. Biol. Chem.*, **269**, 18047–16052.
17 Duncan,R.F., Cavener,D.R. and Qu,S. (1995) *Biochemistry*, **34**, 2985–2997.
18 Duncan,R., Milburn,S.C. and Hershey,J.W.B. (1987) *J. Biol. Chem.*, **262**, 380–388.
19 Duncan,R. and Hershey,J.W.B. (1989) *J. Cell Biol.*, **109**, 1467–1181.
20 Lamphear,B.J. and Panniers,R. (1990) *J. Biol. Chem.*, **265**, 5333–5336.
21 Lamphear,B.J. and Panniers,R. (1991) *J. Biol. Chem.*, **266**, 2789–2794.
22 Panniers,R., Stewart,E.B., Merrick,W.C. and Henshaw,E.C. (1985) *J. Biol. Chem.*, **260**, 9648–9653.
23 Duncan,R.F. (1995) In Hershey,J.W.B., Mathews,M. and Sonenberg,N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. In press.
24 Maroto,F.G. and Sierra,J.M. (1988) *J. Biol. Chem.*, **263**, 15720–15725.
25 Joshi-Barve,S., De Benedetti,A. and Rhoads,R.E. (1992) *J. Biol. Chem.*, **267**, 20338–20343.
26 Pelletier,J. and Sonenberg,N. (1988) *Nature*, **334**, 320–325.
27 Jang,S.K., Krausslich,H.-G., Nicklin,M.J.H., Duke,G.M., Palmenberg,A.C. and Wimmer,E. (1988) *J. Virol.*, **62**, 2636–2643.
28 Meerovitch,K., Pelletier,J. and Sonenberg,N. (1989) *Genes Dev.*, **3**, 1026–1034.
29 Sarnow,P. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5795–5799.
30 Macejak,D.G. and Sarnow,P. (1991) *Nature*, **353**, 90–94.
31 OH,S.-K., Scott,M.P. and Sarnow,P. (1992) *Genes Dev.*, **6**, 1643–1653.
32 Teerink,H., Kasperaitis,M.A.M., De Moor,C.H., Voorma,H.O. and Thomas,A.A.M. (1994) *Biochem. J.*, **303**, 547–553.
33 Vagner,S., Gensac,M.-C., Maret,A., Bayard,F., Amalric,F., Prats,H. and Prats,A.C., (1995) *Mol. Cell. Biol.*, **15**, 35–44.
34 Lindquist,S. (1993) In Ilan,J. (ed.), *Translational Regulaton of Gene Expression*. Plenum Press, New York, NY, Vol. II, pp. 187–207.
35 Solomon,J.M., Rossi,J.M., Golic,K., McGarry,T. and Lindquist,S. (1991) *New Biologist*, **3**, 1106–1020.
36 King,P.V. and Blakesley,R.W. (1986) *Focus*, **8**, 1–3.
37 Genetics Computer Group (1995) *Wisconsin Sequence Analysis Package Data Files*, Release 8. Genetics Computer Group, Madison, WI.
38 Deng,W.P. and Nickoloff,J.A. (1992) *Anal. Biochem.*, **200**, 81–88.
39 Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513.
40 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
41 Horton,R., Hunt,H., Ho,S., Pullen,J. and Pease,L. (1989) *Gene*, **77**, 61–68.
42 Peterson,R. and Lindquist,S. (1888) *Gene*, **72**, 161–168.
43 Hess,M.A. and Duncan,R.F. (1994) *J. Biol. Chem.*, **269**, 10913–10922.
44 Li,D. and Duncan,R.F. (1995) *Eur. J. Biochem.*, **231**, 454–465.
45 Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
46 Duncan,R.F. (1995) *Eur. J. Biochem.*, **233**, 784–792.
47 Kozak,M. (1988) *Mol. Cell. Biol.*, **8**, 2737–2744.
48 Jackson,R.J. (1986) *Eur. J. Biochem.*, **158**, 623–634.
49 Song,H.-J., Gallie,D.R. and Duncan,R.F. (1995) *Eur. J. Biochem.*, **232**, 778–788.
50 Kozak,M. (1991) *Gene Expression*, **1**, 117–125.
51 Sonenberg,N. (1988) *Prog. Nucleic Acids Res. Mol. Biol.*, **35**, 173–207.