Heat shock in Drosophila results in repression of most normal (non-heat shock) mRNA translation and the preferential translation of the heat shock mRNAs. The sequence elements that confer preferential translation have been localized to the 5'-untranslated region (5'-UTR) for Hsp22 and Hsp70 mRNAs (in Drosophila). Hsp90 mRNA is unique among the heat shock mRNAs in having extensive secondary structure in its 5'-UTR and being abundantly represented in the non-heat shocked cell. In this study, we show that Hsp90 mRNA translation is inefficient at normal growth temperature, and substantially activated by heat shock. Its preferential translation is not based on an IRES-mediated translation pathway, because overexpression of eIF4E-BP inhibits its translation (and the translation of Hsp70 mRNA). The ability of Hsp90 mRNA to be preferentially translated is conferred by its 5'-UTR, but, in contrast to Hsp22 and -70, is primarily influenced by nucleotides close to the AUG initiation codon. We present a model to account for Hsp90 mRNA translation, incorporating results indicating that heat shock inhibits eIF4F activity, and that Hsp90 mRNA translation is sensitive to eIF4F inactivation.

Stressful circumstances cause cellular and physiological damage, which when severe can lead to apoptosis and death (1, 2). All cells and organisms have developed responses that enhance their survival following stress. At the cellular level most metabolic processes are repressed by heat stress (3), likely to prevent the accumulation of damaged molecules that could irreversibly compromise cellular function. Concurrently, large amounts of a small group of proteins are newly synthesized. These proteins are termed the heat stress, or simply stress, proteins (Hsps), and function to prevent ongoing protein damage, to restore the activity of stress-damaged proteins, and to create a stress-resistant state to ameliorate future stress-based protein injury. Because stress inhibits gene expression at transcriptional and post-transcriptional steps, the induction of stress protein biosynthesis requires unique mechanisms to evade the general metabolic inhibition. A well characterized transcriptional response activates a latent transcription factor, heat shock transcription factor, which binds to conserved heat shock element sequences in the promoter of the Hsp genes and results in rapid, highly efficient transcription (4). Unique mechanisms also allow Hsp mRNAs to exit the nucleus, whereas the normal processing and transport of mRNAs is blocked (5).

In the cytoplasm, the Hsp mRNAs are efficiently translated. For example, polysome analysis of Hsp70 mRNA translation suggests that ribosome loading is near maximal (6). Concurrently, the non-heat shock, or normal, mRNAs are virtually excluded from translation, although they are neither degraded nor physically inactivated by any nucleotide modification (7). The basis for this translational discrimination has been extensively investigated, principally in Drosophila, because the extent of mRNA discrimination and preferential translation is accentuated in this poikilothermic organism. Most of the studies have focused on Hsp70 mRNA, in part because Hsp70 is the most abundantly synthesized Hsp, by about an order of magnitude. However, it has been noted that virtually all the Drosophila Hsp mRNAs share several common features (8, 9), which have been logical candidates as the regulatory feature(s) conferring their concurrent preferential translation (reviewed in Ref. 7).

First, the 5'-untranslated region (5'-UTR) of Hsp mRNAs is sufficient to confer efficient translation to a heterologous appended coding region (10, 11); and conversely, the Hsp coding sequence and 3'-UTR are unable to be translated during heat shock when a non-heat shock 5'-UTR, or a mutationally disabled Hsp 5'-UTR, precedes it (12, 13). Second, the common features found in virtually all Drosophila Hsp mRNA 5'-UTRs include: (i) long length (200–250 nucleotides); (ii) two conserved sequence segments, and positionally conserved nucleotides within the initial element; (iii) a high frequency of adenosine nucleotides (~50%); and (iv) a minimal extent of secondary structure (7). Investigations have been carried out to determine whether any or all of these features are necessary or sufficient for preferential translation.

Long length per se is not required, because ~170 nucleotides can be deleted with only a modest (30–50%) reduction in preferential translation (12). The remaining translational activity is still >10-fold higher than non-heat shock mRNA translation. Both conserved elements can be deleted, with no significant decrement in heat shock translation (12). Neither high adenosine content nor a paucity of secondary structure is sufficient to confer preferential translation, because scrambling the order of a tract of nucleotides abolishes translation while retaining adenosine content and minimal structure (14). On the other hand, the lack of structure is required. The introduction of a

This paper is available on line at http://www.jbc.org
modestly stable stem into the 5'-UTR of the Hsp70 mRNA causes no reduction in translation under normal, non-heat shock conditions, but virtually abolishes preferential translation during heat shock (13).

In this study we have initiated an investigation into the mechanism of Hsp90 mRNA translation during heat shock. The results indicate that this mRNA possesses several unique features that suggest that its translation, and especially its preferential translation during heat shock, occurs by a mechanism that distinguishes it from the other major Drosophila Hsp mRNAs. To date, the prevailing perspective has been that all Hsp mRNAs achieve preferential translation by a common mechanism, but this conclusion has been based on studies on a group of Hsp mRNAs (e.g. Hsp70 mRNA, Hsp22 mRNA) with properties distinct from Hsp90 mRNA.

Hsp90 is an abundant protein in Drosophila cells grown at their normal temperature. It plays multiple roles in protein folding, maturation, and the regulation of protein activities (15). Its mRNA is the only Hsp mRNA present in significant quantities in the non-stressed cell (16). Lines of evidence that suggest that its translation is uniquely regulated include: first, whereas virtually all major Hsp mRNAs lack significant 5'-UTR secondary structure, the 5'-UTR of Hsp90 mRNA contains significant structure, consistent with the extent observed in a typical non-heat shock mRNA (for illustration of these features, see Fig. 9A). Second, Hsp90 mRNA translation appears to be inhibited by eIF4F inhibition, whereas translation of the other Hsp mRNAs is not affected to any significant extent (17, 18). The presence of secondary structure and eIF4F dependence are likely related. It has been hypothesized that preferential translation, embracing both the inhibition of non-heat shock mRNAs and the high activity of Hsp mRNAs, lies in an ability of Hsp mRNAs to bypass an eIF4F activity lesion. However, this proposal presents a dilemma with respect to Hsp90 mRNA, because this mRNA possesses significant secondary structure, is translationally inhibited by eIF4F inhibition, yet is efficiently translated during heat shock. In this study we have investigated the basis for the preferential translation of Hsp90 mRNA, and uncovered several unexpected features that suggest that multiple mechanisms exist that lead to preferential Hsp mRNA translation in a single organism.

MATERIALS AND METHODS

**Chemicals**

Chemicals were purchased from Sigma unless otherwise indicated. All restriction enzymes were purchased from New England Biolabs. The Topo 2.1 vector used for ligation of PCR products was purchased from Invitrogen.

**Transfection of Drosophila S2 Tissue Culture Cells**

Drosophila S2 cells were cultured at 22–23 °C in Schneider’s Drosophila Medium (Invitrogen) containing 10% fetal calf serum, 20 mM l-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 mg/ml amphotericin B (Invitrogen). 24 h prior to transfection the cells were seeded at a density of 1–1.5 × 10^6 cells/ml in a T25 flask (Corning). Transfections were carried out as described (13). For the eIF4E-BP transfection, a total of 25 μg of plasmid was transfected in each case, made up of equal microgram amounts of eIF4E-BP and Gal4 plasmids (19), and 5 μg each of Hsp70Δ4C, and Hsp90Δ4C to retain the total amount of plasmids equally transfected in all cases, a copia (promoter)/β-galactosidase plasmid was added to some transfections.

**Heat Shock, [35S]Metionine Labeling, and Protein Extraction**

Drosophila S2 cells were scraped from T25 flasks, pelleted by brief centrifugation (3 min, 3000 rpm) in a clinical centrifuge (IEC), and resuspended in Grace’s media lacking methionine (Invitrogen). The cells were then transferred to 20-ml glass scintillation vials with a stir bar, and allowed to recover >15 min with stirring prior to heat shock (at heat shock or normal growth temperature (22–24 °C)). For heat shock, a portion of the cells was incubated in a 36 °C water bath with stirring for 15 min, at which time ~5 × 10^6 cells (1 ml of suspension) were labeled with 15–20 μCi of [35S]methionine/cysteine (ICN Biochemicals) for 15 min (i.e. total 30 min heat shock). For non-heat shock analysis, another equal portion of the same cell sample, maintained at normal growth temperature, was labeled concurrently as described above (for heat shock). At the end of labeling, cells were rapidly pelleted by centrifugation, resuspended in and washed twice at 4 °C (50 mM KCl, 15 mM MgSO₄, 4 mM CaCl₂, 3 mM KH₂PO₄, 10 mM dextrose, 8.4 mM HEPES, pH 7.2, and 20 μg/ml cycloheximide) by centrifugation. The cell pellet was lysed with (for two-dimensional gel analyses) 100–150 μl of Amphotolyse buffer (9.8 M urea, 5% 3.5–10 Biolytes (Bio-Rad), 3% Nonidet P-40, 1% β-mercaptoethanol), microcentrifuged for 3 min at top speed (Eppendorf), and the supernatant was recovered to give a final protein concentration of ~2 mg/ml. Protein concentration was measured by the Bradford assay (Bio-Rad), and radioactivity/μg of protein was measured by trichloroacetic acid precipitation. For samples to be analyzed by one-dimensional gel electrophoresis, washed protein pellets were lysed in hSDS (0.3% SDS, 50 mM Tris, pH 8.0, 1% β-mercaptoethanol, at ~98 °C). The pellet was disrupted by pipetting, 1/20 volume of RNase/DNase solution (5 mg/ml RNase A, 2.5 mg/ml RNase A, 500 μg/ml Tris, pH 7.0, 50 mM MgCl₂) was added for ~1 min, viscosity was reduced by pipetting, then 1/5 volume of 4× Laemmli formula SDS-PAGE buffer was added, and the samples were analyzed on one-dimensional slab gels as described below for the second dimension of the two-dimensional procedure.

**Analysis of Proteins by Two-dimensional Isoelectric Focusing/SDS-PAGE**

Two-dimensional IEF/SDS-PAGE was performed basically as described by O’Farrell (20), with modifications as described by Duncan and Hershey (21) to promote spot focusing. Gels were fixed, dried, and exposed to Kodak X-omat film for 4–15 days. Protein bands/spots were quantitated by densitometry (Bio-Rad VersaDoc 1000 imaging system/Ampholine software) or by LabWorks (UVP). Heat shock translation was calculated as the spot IOD at heat shock (numerator) divided by the spot IOD prior to heat shock (denominator). Transgene mRNA translational efficiency was also calculated as the protein synthesis rate (spot IOD) per unit transgene mRNA (determined by Northern blot analysis, autoradiography, and densitometry). Equal RNA loading was verified by methylene blue staining or endogenous Hsp70 hybridization, as described below. RNA analyses verified.
that the mRNA levels of the CuSO4-induced transgenes neither increase nor decrease during the 30-min heat shock interval. Similarly, protein synthesis-based analyses of the transgenes are unaffected by the inclusion of actinomycin D during the heat shock interval. Thus, comparative analysis of protein synthesis rates (spot darkness) before and after heat shock accurately quantifies heat shock preferential translation.

RNA Isolation, Analysis, and Quantitation
RNA was extracted from \(5 \times 10^6\) cells using TRIzol reagent (Invitrogen) as recommended by the manufacturer. The ethanol-precipitated RNA pellet was resuspended in a final volume of 25 \(\mu\)l of diethyl pyrocarbonate-treated water at \(\sim 2-3 \mu\)g/\(\mu\)l, as determined in a Beckman UV spectrophotometer. Samples were analyzed by Northern blotting as described (13), and bands on the film were quantified by densitometry using the Bio-Rad VersaDoc 1000 Imaging System/Quantity 1 software.

Construction of Plasmid Expression Vectors
General PCR Procedures—1 ng of template DNA was amplified for 22–25 cycles, gel purified using the GeneClean II Kit (Bio 101 Inc. (per the manufacturer’s instructions)), and verified by sequencing (University of Southern California Norris Cancer Center Microchemical Facility). The primers (Operon Technologies) used in the constructions are listed in Table I.

MT90-FL—The Drosophila Hsp90 gene encoded by plasmid pDm83 (gift of Dr. H. Lipschitz) was used as the PCR amplification target. The upstream 5’ primer (DU71) contains Hsp90 5’-UTR nucleotides +3 to +23 preceded by nucleotides that introduce an EcoRI restriction site at the start of transcription to facilitate subsequent plasmid construction. The 5’ end sequence of the transgene-expressed mRNA is GAAUUCUGA . . . ; the artificial 5’-UTR contains two extra 5’-terminal nucleotides, and the 4th nucleotide in the artificial 5’-UTR is U, whereas the corresponding nucleotide (position +2) in the authentic 5’-UTR is G. All other nucleotides are identical. The expressed mRNA containing the introduced EcoRI site is efficiently translated during heat shock (see Figs. 7 and 8), hence the EcoRI site does not impair heat shock translation. Similar observations were made for Hsp70 mRNA (13). The expressed mRNA contains the introduced EcoRI site at the initiator AUG (new sequence is CCCAUGG, which maintains the initiator AUG in an efficiently recognized context). The amplified sequence was ligated into the Topo 2.1 vector, blue colonies were identified, and accurate integrants were verified by sequencing (all other plasmids were prepared in like fashion). This plasmid was digested using EcoRI/Ncol, and inserted into an EcoRI/Ncol-digested pmthsp44-Ncol vector (13) that has been modified with the introduction of an Ncol site at the start of translation. The resulting MT90-FL vector contains the Drosophila metallothionein promoter precisely fused to the Drosophila full-length Hsp90 5’-UTR linked to an internally deleted Hsp70 coding region and Hsp70 3’-UTR. The expression construct leads to the synthesis of a unique \(\sim 44\)-kDa protein (12, 13). The sequences of all plasmids used in this study were verified by DNA sequencing.

Cap-proximal Deletions of MT-FL90
MT90-\(\Delta 40_{5'\text{UTR}}\)—Using MT90-FL plasmid as the amplification target, a 5’ upstream primer (DU86) was designed that hybridized to nucleotides +43 to +62 of the Hsp90 5’-UTR (numbering for this and subsequent constructions is based on the authentic Hsp90 5’-UTR; +1 represents the first transcribed nucleotide, an A); preceding (5’) the hybridizing nucleotides it contained in an EcoRI site. The downstream primer (DU89) hybridized at the unique SnaBI site in the coding region...
**FIG. 2.** Translational efficiency of Hsp90 mRNA is increased proportional to temperature. *Drosophila* S2 cells were placed in water baths equilibrated to 29–37 °C for 15 min, then pulse-labeled with [35S]methionine for 15 min. Cells were pretreated with actinomycin D for 10 min prior to immersion in the water bath (bottom rows, panel A), or not treated (top rows, panel A). Protein samples were prepared as described.
of the target. The PCR amplification product deletes the first 42 nucleotides of the authentic Hsp90 5'-UTR, but does reintroduce the EcoRI site nucleotides as +1 to +6 of the expressed 5'-UTR mRNA. The PCR product (~1160 nucleotides) was digested with EcoRI/SnaBI, and inserted into EcoRI/SnaBI-digested MT90-FL to yield MT90-Δ40CAP- 

MT90-Δ75CAP and MT90-Δ110CAP—The procedure was identical to that described above, except the upstream primers (DU87 and DU88, respectively) were designed to hybridize to nucleotides +77 to +96 and nucleotides +111 to +130, respectively, in the Hsp90 5'-UTR. The resultant expressed mRNAs have nucleotides +1 to +76 and +1 to +110, respectively, deleted.

**AUG-proximal Deletions of MT90-FL**

**MT90-Δ40**—The 5' upstream primer (DU68) hybridized at the start of the metallothionein promoter in MT90-FL, where there is a unique MfeI site. The downstream primer (DU85) hybridized to the Hsp90 5'-UTR nucleotides +86 to +105 and contained, at its 5' end, an NcoI site for cloning purposes. The MfeI/NcoI-digested PCR fragment was inserted into MfeI/NcoI-digested MT90-FL. The resulting plasmid MT90-Δ40 retained Hsp90 5'-UTR nucleotides +1 to +105.

**MT90-Δ75 and MT90-Δ110**—The procedure was identical to that described above, except the downstream primers (DU84 and DU83, respectively) were designed to hybridize to nucleotides +52 to +71 and +17 to +36, respectively, in the Hsp90 5'-UTR. The resultant expressed mRNAs retained Hsp90 5'-UTR nucleotides +1 to +71 and +1 to +36, respectively.

**Internal Deletion Mutant of MT90-FL**

**MT90-Δ40–110**—The procedure was identical to that described above for MT90-Δ40CAP, except the upstream primer (DU89) was designed to hybridize to nucleotides +112 to +128 in the Hsp90 5'-UTR. Preceding this hybridization segment the primer contained 5'-UTR nucleotides +1 to +37 (including the EcoRI site at the start of transcription). The resultant expressed mRNA has nucleotides +38 to +111 deleted.

**Coding Sequence Deletion Mutant of Hsp90**

**MT90Δ25**—The upstream primer (DUC3) was designed to hybridize to 5'-UTR nucleotides +138 to +149, and extend 18 nucleotides into the coding sequence. The downstream primer (DUC4) was designed to hybridize to nucleotides 1160 to 1185 in the Hsp90 coding sequence (based on the numbering in NM 079175 (Entrez nucleotide)). The primer appends an AflII site preceding the coding nucleotides (or, in the orientation of the mRNA, downstream of Hsp90 coding nucleotides) for plasmid construction purposes. The primers were used to amplify the 5'-UTR and coding sequence nucleotides using pDm83 as the amplification target. The PCR amplified DNA was digested with NcoI and AflII, and inserted into MT90-FL digested with the same enzyme pair. The resultant plasmid replaces the Hsp70Δ25 coding sequence with Hsp90Δ25. In MT90Δ25 we have replaced the "UAU" sequence within the CTTAAG AflII restriction site in-frame to constitute the stop codon. The resulting plasmid expresses ~840 nucleotides of the Hsp90 coding sequence, resulting in a ~30-kDa protein product (see Fig. 5).

**RESULTS**

**Heat Stress Increases the Translation of Hsp90 mRNA—**

Hsp90 mRNA is unique among the Drosophila Hsp mRNAs in being present in non-heat stressed cells in significant amounts. To investigate the translational characteristics of Hsp90 mRNA (throughout this report), S2 cells were pulse-labeled with [35S]methionine for 10–15 min, and the production of newly synthesized Hsp90 protein was assessed by gel electrophoresis, autoradiography, and densitometry.

The rate of synthesis of Hsp90 is rapidly increased by heat shock (Fig. 1A). There is a detectable increase when temperature is raised to 29 °C, a progressively larger induction as temperature is raised from 29 to 35 °C, and then its protein synthesis begins to decrease as temperature is further increased (to 37 °C in this experiment).

To assess whether this increase in protein synthesis represented increased translational efficiency (i.e., protein synthesis per mRNA), or simply occurred because there were more Hsp90 mRNAs, transcription was blocked by treatment with actinomycin D. In this case, there was still a significant increase in RNA samples were prepared as described (see “Materials and Methods”). Equal amounts of RNA (equal cell numbers) were loaded into each lane of the gel (based on A260, and confirmed by methylene blue staining of the nylon membrane after electrophoresis and transfer). Nylon membranes were probed using a 32P-labeled plasmid fragment for Hsp90 (A) or Hsp70 (B). Temperatures of heat shock are shown above the lanes. Dried membranes were exposed to film and labeled bands were detected by autoradiography. Panels shown for Hsp70, + actinomycin D, were prepared from equal amounts of RNA (verified by methylene blue staining) and exposed for the same interval. This analysis has been repeated in part or completely >5 times, with similar results. The apparent lower expression of Hsp90 mRNA seen at 34 °C in the top portion of panel A was not detected in other analyses.

The exposures shown are darker than the ones used to quantify expression to more fully reveal the spot patterns of proteins with a lower rate of synthesis. The darker exposures underestimate the expression differences because some of the spots shown, including the Hsps, are saturated in some panels.
maximum activity at 35 °C that is 3–4 times that observed at normal growth temperature (22–24 °C).

The efficacy of actinomycin D treatment can be observed in the inhibition of Hsp70 synthesis. Hsp70 mRNA is virtually absent in non-heat shocked cells. Hence, all its synthesis requires new, heat-induced transcription. The efficacy of treatment was also directly assessed by analysis of Hsp90 and Hsp70 mRNAs by Northern blotting. The heat shock-induced increase in Hsp90 mRNA was largely suppressed by actinomycin D treatment, and induction of Hsp70 mRNA was reduced by 95% (Fig. 3). This is similar to the extent of Hsp70 protein synthesis inhibition seen in Fig. 2.

The increase in Hsp90 synthesis as temperature is raised could theoretically be because of temperature generally activating the translational machinery (a "Q10-like" effect), or could reflect a shared characteristic of all the Hsp mRNAs. However, it is neither because of a general nor class-specific activation; first, there is no significant increase in the translation rate of numerous non-heat shock mRNAs at very mild heat shock temperatures (i.e. 30–32 °C) that do increase the synthesis of Hsp90 (see, for example, bands/spots in Figs. 1 or 2 representing synthesis of non-heat shock proteins (e.g. actin)). Second, the temperature-dependent translational activation is specific to Hsp90 mRNA because when Hsp70 mRNA was expressed at normal temperature (see Fig. 4, legend, for details), its translation did not increase with temperature (Fig. 4). Thus, there is no general increase in translation of Hsp mRNAs as the temperature is increased; Hsp90 mRNA possesses unusual characteristics that may extend to a unique pathway to preferential translation, as detailed below.

**Hsp90 mRNA Translation Is Cap-dependent, as Is Hsp70 mRNA Translation**—A potential unique pathway for Hsp90 mRNA translation would be IRES-mediated cap- (and eIF4F-) independent translation. Considering the sensitivity of Hsp90 mRNA translation in vitro to antibody-mediated eIF4F inhibition (17, 18), we wished to verify that the same sensitivity to eIF4F inhibition applied to in vivo translation, to more rigorously address the possibility that Hsp90 mRNA is translated via an IRES-mediated pathway under natural circumstances. To specifically inhibit cap-dependent translation in intact cells, Drosophila elF4E-BP was overexpressed (Fig. 5A) by transfection. Reporter genes for Hsp90 and Hsp70 mRNA translation were co-transfected. These mRNAs contain their respective Hsp full-length 5′-UTRs followed by their respective coding sequence, each containing an internal deletion to allow unique identification of the protein expression product (see McGarry and Lindquist (12) for Hsp70 mRNA, and see "Materials and Methods" for Hsp90 mRNA). Translation of the Hsp reporter mRNAs was measured by pulse labeling with [35S]methionine and two-dimensional IEF/SDS-PAGE. For quantification, the spot densities of both forms were summed.
transgenes. The influence of overexpressed eIF4E-BP on Hsp70 mRNA translation at heat shock was very similar to that observed at normal growth temperature (Fig. 5, D and E), whereas Hsp90 was significantly less affected; the extents of inhibition for Hsp70 and Hsp90 mRNA were 65 and 60%, respectively. The reduced sensitivity of Hsp90 mRNA to cap-dependent translation inhibition under heat shock conditions parallels results obtained using rapamycin in heat-shocked cells. Two distinct conclusions may be drawn from this analysis. First, the translation characteristics of Hsp90 mRNA are altered by heat shock to reduce its dependence on eIF4F. Second, and equally important, Hsp90 mRNA translation is cap-dependent, because its elevated resistance to moderate eIF4E-BP overexpression only results in partial translation, and its translation is completely abrogated by high level eIF4E-BP overexpression. These results showing Hsp mRNA preferential translation is cap-dependent at both normal and heat shock temperatures influence the model we propose for its translation (see "Discussion").

In addition to the mass effects of eIF4E-BP overexpression on its association with eIF4E and consequent protein synthesis inhibition, eIF4E-BP dephosphorylation can further increase its inhibitory effect by stabilizing its interaction with eIF4E. Our previous results had shown that mammalian eIF4E-BP transfected into Drosophila cells was dephosphorylated by heat shock (37 °C) (24), as well as showing that eIF4E-BP was dephosphorylated in mammalian cells by heat shock at temperatures ≥35 °C (24). To determine whether the effects of DmeIF4E-BP overexpression in heat shocked Drosophila cells included enhanced repression because of heat-induced dephosphorylation, the lower molecular weight region of the two-dimensional gels was examined. At 36 °C heat shock causes dephosphorylation of Drosophila eIF4E-BP, as seen by the reduction in the higher M, more acidic, phosphorylated variants (Fig. 6, arrows). The extent of phosphorylation at normal temperature is less than typically observed in mammalian cells (corroborated in numerous experi-
Hsp90 mRNA Translation during Heat Shock

**Fig. 5. Drosophila eIF4E-BP is dephosphorylated during heat shock.** Drosophila S2 cells were transfected with 4 plasmids, as described in the legend to Fig. 5. The lower region of the gels in which 5 \( \mu \)g of eIF4E-BP was transfected, labeled under normal temperature (panel A) or heat shock (panel B) conditions, is shown. The locations of the overexpressed eIF4E-BP are indicated with arrowheads. A spot of the lowest \( M_r \), which increases most significantly following heat shock, is indicated with a bold arrow in panel B. None of these spots was detected in the mock-transfected cells, and all increased in proportion to the amount of eIF4E-BP transfected (as shown in Fig. 5, panel A). The coordinates of three non-eIF4E-BP protein spots are indicated by asterisks in both panels, for orientation purposes. The positions of Hsp22 and Hsp23, which migrate at similar \( M_r \) and pl to certain eIF4E-BP variants, are shown in panel B (labeled H22 and H23). The isoelectric point of Hsp22 is virtually identical to actin, and the isoelectric point of the most basic eIF4E-BP variant is more basic than all Hsp70 variants, and is detected on the right (basic) edge of the sector shown in panels in Fig. 5. The pH gradient runs from more acidic to the left to more basic to the right. The most acidic eIF4E-BP variants migrate to the left border of the gel sectors shown in Fig. 5, which corresponds to the acidic terminus of the isoelectric focusing gel.

We have carried out a similar analysis to determine the location within the Hsp90 mRNA sequence of signals required for its preferential translation. First, the entire 5′-UTR was appended to a reporter coding sequence/3′-UTR to create expression plasmid MT90-FL. This coding body/3′-UTR cannot be translated during heat shock unless it has a preferential translation-promoting 5′-UTR (12, 13). mRNAs were expressed under the control of a metallothionein promoter. Expression was induced at normal growth temperature for 3 h using 500 \( \mu \)M CuSO\(_4\). Translation was assessed as above, using pulse labeling with \([^{35}S]\)methionine and quantification of reporter protein synthesis by two-dimensional IEF/SDS PAGE, autoradiography, and densitometry, at normal growth temperature and under heat shock conditions.

The 5′-UTR of Hsp90 mRNA is sufficient to confer translation during heat shock. The translation of MT90-FL mRNA remains high during heat shock, as evidenced by the robust production of transgene protein (Fig. 7, indicated with arrows). There was little to no decrease in translation rate relative to normal growth temperature (Figs. 7 and 8), mirroring results obtained when the Hsp70 5′-UTR is appended to this transgene (Refs. 13; Fig. 4). Thus, the full-length Hsp90 5′-UTR contains all the sequence information required for preferential translation. This observation parallels results regarding Hsp70 and Hsp22.

To identify which regions of the Hsp90 5′-UTR were necessary for preferential translation, two series of truncation mutants were constructed. In the first series 3 progressively longer blocks of nucleotides were removed from the cap end of the 5′-UTR, to create plasmids MT90-Δ40cap, MT90-Δ75cap, and MT90-Δ110cap. In the second series 3 progressively longer blocks of nucleotides were removed from the AUG-proximal end of the 5′-UTR, to create plasmids MT90-Δ40AUG, MT90-Δ75AUG, and MT90-Δ110AUG. In addition, a 5′-UTR comprised of the first 35 nucleotides linked to the last 35 nucleotides was created, MT90-Δ40–110I. All of these 5′-UTRs are diagrammed in Fig. 8. Translation ability during heat shock was determined as described above for MT90-FL.

mRNAs in which either 40 or 75 nucleotides have been deleted from the cap proximal region are translated relatively well during heat shock (Fig. 8). Translation rate decreases about 50%, which is similar to the decrement seen when similar lengths are truncated from the cap-proximal region of Hsp70 mRNA (14). The retained translation potency remains ~5–10-fold greater than the typical non-heat shock mRNA, which are inhibited by >90% on average (many examples can be seen in Fig. 7, comparing the spot intensities of non-shock mRNA and Hsp90 mRNA during heat shock). Deletion of 110 nucleotides from the cap results in severely compromised translation, typical of a non-heat shock mRNA. In summary, cap proximal nucleotides in Hsp90 mRNA influence preferential translation, but they can be deleted and significant preferential translation during heat shock is retained as long as a minimum amount of Hsp90 5′-UTR is present. Additionally, there are no required elements in internal nucleotides 38–110, because the internal deletion is translated relatively well during heat shock.

Deletions from the AUG-proximal region of Hsp90 5′-UTR suggest these nucleotides are required for significant heat shock translation. Deletion of 40 nucleotides reduced reporter gene translation to the minimal level characteristic of a non-heat shock mRNA (Fig. 8). Deletions of larger amounts from the AUG-proximal regions were consistent with this result, also showing very low synthesis of reporter protein during heat shock (Fig. 8). In all the AUG-proximal truncations, the nucleotides preceding and following the AUG are part of the NcoI site, which retains an adequate context for efficient translation (e.g. MT90-FL). These
results suggest that unique requirements and considerations apply to the mechanism of Hsp90 mRNA translation, because the strong dependence on AUG-proximal nucleotides has been unambiguously refuted for Hsp70 and Hsp22 mRNA translation during heat shock (10, 27).

**DISCUSSION**

Hsp90 mRNA and protein are abundant in *Drosophila* cells at normal growth temperature. Whereas virtually all mRNAs expressed under non-heat shock conditions are translationally repressed, synthesis of Hsp90 remains high, and the translational efficiency of Hsp90 mRNA even increases. Hsp90 mRNA translation is relatively inefficient at normal growth temperature and this inefficiency is relieved by heat shock. This heat-dependent activation of translation distinguishes Hsp90 from other Hsp mRNAs, such as Hsp70 mRNA, whose translation is very efficient at normal growth temperature and achieves preferential translation during heat shock by evading the global
inhibitory mechanism(s) induced by heat shock. This provides novel evidence that there exist two fundamentally different patterns, and likely pathways, for achieving preferential heat shock translation.

Several lines of evidence have conclusively excluded an IRES-mediated pathway for preferential translation of Hsp mRNA, including abrogation of its translation by appending nucleotides to its 5′-UTR terminus (12) or by the introduction of a stem-forming region proximal to the cap site (13). IRES-mediated translation would represent an obvious distinct pathway for Hsp90 mRNA heat shock translation, and no previous experiments have addressed this possibility. To investigate this possibility, the sensitivity of Hsp90 mRNA translation to eIF4E-BP overexpression was determined, because it has been consistently documented that IRES element-mediated translation is resistant to eIF4E-BP inhibition. The results clearly show that Hsp90 mRNA translation is suppressed by high-level overexpression of eIF4E-BP, indicating that this mechanism does not account for Hsp90 mRNA preferential translation. Other pathways must be entertained, and other molecular interactions determined.

Studies to identify the nucleotides that allow continued translation of Hsp90 mRNA during heat shock identified the 5′-UTR as sufficient to promote preferential translation, paralleling studies by others and ourselves investigating which portions of Hsp70 and Hsp22 mRNA confer preferential translation (10, 12, 13, 27). However, in distinction to those mRNAs, we find that the AUG-proximal nucleotides of Hsp90 mRNA are of critical importance, because their removal reduces reporter mRNA translation during heat shock to levels characteristic of a non-heat shock mRNA. These characteristics are featured in a model described in the following paragraphs.

Studies by Sierra and colleagues (17, 18) determined that Hsp mRNA translation in general is significantly resistant to
the inhibition of eIF4F activity. Along with other results directly measuring eIF4F activity and showing that it is reduced by heat shock (29, 30), this has led to the proposal that preferential translation of Hsp mRNA occurs via their ability to evade a heat-induced lesion in eIF4F activity. Our earlier results demonstrated that an Hsp70 mRNA variant in which the 5′-UTR is modified to contain a modest extent of secondary structure is efficiently translated at normal growth temperature, but completely loses its capacity to be translated during heat shock (13). This is wholly consistent with a model in which reduced eIF4F activity leads to inadequate unwinding activity for efficient translation of the stem-containing variant, whose secondary structure is similar to the extent found in non-heat shock mRNAs.

However, Sierra and colleagues (17, 18) also report the perplexing finding that Hsp90 mRNA translation is strongly inhibited by eIF4F inhibition. This is consistent with our analysis of the extent of secondary structure in Hsp90 mRNA relative to the other Hsp mRNAs, and relative to non-heat shock mRNAs, which show that the Hsp90 5′-UTR is characteristic of an eIF4F-dependent non-heat shock mRNA, and quite distinct from the other Hsp mRNAs (Fig. 9A). Yet, Hsp90 mRNA translation is demonstrably efficient during heat shock. Two alternative scenarios can account for this discrepancy. First, it is possible that eIF4F activity is not significantly compromised by heat shock. We believe this to be unlikely, insofar as the direct activity measurements and the stem-containing Hsp70 mRNA translation analysis, which shows that a modest stem structure in Hsp70 mRNA inhibits translation at normal temperature but not at heat shock (13), strongly suggests that a significant impairment of eIF4F activity occurs. Furthermore, it is unlikely that this scheme can be quantitatively modified to postulate that eIF4F inhibition occurs but its extent is insufficient to affect Hsp90 mRNA, because (i) the extent of secondary structure in Hsp90 mRNA is significantly greater than that in the inhibited Hsp70 mRNA stem-containing variant (pSL17.11) (13), and (ii) Hsp90 mRNA translation is not only resistant to heat shock inhibition but in fact enhanced by heat shock.

The alternative hypothesis is that the mechanism of Hsp90 mRNA translation is altered by heat shock such that it becomes less eIF4F-dependent. This heat-dependent transition leading to decreased eIF4F dependence would represent a novel path- way to achieve preferential heat shock translation, and would account for the inability to observe eIF4F independence in the in vitro analyses cited above (17, 18). A mechanism can be proposed that is wholly supported by our 5′-UTR deletion analyses, presents a strong parallel to mechanisms of Hsp mRNA preferential translation in prokaryotes (e.g. Ref. 31), and has parallels in the translation of mammalian Hsp70 mRNA (32). Folding analysis of the complete Hsp90 5′-UTR using Mfold (33) presents the theoretical structure shown in Fig. 9B. Notable features are the extensive regions of secondary structure, and specifically a long stem in the AUG proximal half of the UTR, as well as a short stem including the AUG initiation codon. We suggest that one or both of these regions of secondary structure comprises a heat-sensitive inhibitory element that impedes access to the initiation codon at normal growth temperature. Furthermore, the ability of ribosomal subunits to recognize this region could be heat enhanced, presumably through thermal destabilization of the stem. This model draws by analogy on studies that have elucidated a mechanism of bacterial heat shock preferential translation (28, 31). In that instance, a series of studies have shown that thermal melting of a stem-containing region including the Shine-Dalgarno region, and perhaps also a downstream box segment, allows mRNA base-pairing and ribosome recruitment only at elevated (heat shock) temperatures (28, 31). Whereas we do not yet have any direct evidence that a similar mechanism applies to Hsp90 mRNA translation in Drosophila, the concept that a prokary- otic mechanism of preferential translation might be retained as the foundation for a lower eukaryote is intriguing. Supporting evidence comes from studies of Hsp70 mRNA heat shock translation in human cells, where it has been shown that AUG-proximal sequences may recruit ribosomal subunits for shunting-mediated translation based on mRNA-rRNA base-pairing (32), analogous to the bacterial situation.

An aspect of this model must be its relative independence from eIF4F. We suggest that, in parallel to documented mechanisms in prokaryotes and human cells, the segment of nucleotides preceding the Hsp90 mRNA of AUG can recruit ribosomes during heat shock providing reduced eIF4F dependence. This may occur through a base-pairing mechanism, perhaps including a direct transfer of ribosomal subunits to the AUG from the cap-proximal region where they initially associate (i.e. shunting, as described for human Hsp70 mRNA). Assessing potential base pairing regions between the AUG-proximal Hsp90 5′-UTR and 18S rRNA is equivocal, insofar as potential regions can be identified (e.g. segments with 6 out 7 nucleotides paired), but in no case do these examples involve the terminal nucleotides of 18S rRNA with an mRNA segment close to the AUG, as occurs in the Shine-Dalgarno interaction. Our hypoth- esis predicts that there are two modes of Hsp90 mRNA trans- lation. Under normal temperature conditions, translation occurs by a typical scanning mechanism, is eIF4F-dependent, and relatively inefficient because of the secondary structure elements. During heat shock, translation shifts to a mode in which ribosomal subunits are more directly recruited to the AUG, promoted by direct mRNA-rRNA base pairing. This model predicts that deletion of the AUG proximal nucleotides would severely compromise heat-dependent translation, but have little effect on non-heat shock Hsp90 mRNA translation (which uses active eIF4F to unwind the AUG-proximal region, albeit inefficiently). This is exactly what we observe based on the experiments in this study. The AUG-proximal nucleotides could be a heat-activated IRES, promoting cap-independent translation, but we do not believe this is likely as discussed above. Thus, a model positing eIF4F-mediated cap-dependent ribosome subunit recruitment seems most consistent with our data; the initial eIF4F-mediated binding step may be tolerant of reduced eIF4F activity (allowing cap engagement during heat shock), whereas the subsequent eIF4F-dependent unwinding steps are bypassed. Further experiments are in pro- gress to provide direct evidence for this hypothetical model of Hsp90 mRNA translation in Drosophila. In conclusion, we sug- gest that certain types of preferential heat shock translation may reflect the adaptation of prokaryotic mechanisms to eu- karyotic cells.

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