Mechanism of Inhibition of Polypeptide Chain Initiation in Heat-shocked Ehrlich Cells Involves Reduction of Eukaryotic Initiation Factor 4F Activity*

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Almost all living organisms studied respond to elevated temperature with a marked inhibition of overall protein synthesis but increased synthesis of a specific set of proteins, the so-called heat-shock proteins. We have prepared a cell-free protein synthesizing system (lysate) from heat-shocked Ehrlich ascites tumor cells that reflects the inhibition of protein synthesis in intact cells at elevated temperatures. We have isolated and partially purified a stimulator of the heat-shocked cell lysate from Ehrlich cells. Through four purification steps, the stimulator is chromatographically identical to eukaryotic initiation factor 4F (eIF-4F), an initiation factor which specifically binds mRNA cap structure. Therefore, we have tested the effects of highly purified reticulocyte eIF-4F on the heat-shocked cell lysate. Protein synthesis is strongly stimulated by addition of highly purified eIF-4F. Synthesis in the heat-shocked lysate is more inhibited at high (70 mM) KCl concentrations, than at lower concentrations, and stimulation by eIF-4F is correspondingly greater at higher KCl concentrations, so that the rate of protein synthesis is returned to control (non-heat-shocked lysate) levels at all KCl concentrations. Furthermore, at 70 mM KCl, in heat-shocked lysates, synthesis of the 68-kDa heat-shock protein is much less inhibited than synthesis of the bulk of non-heat-shock proteins, and eIF-4F stimulates synthesis of 68-kDa protein to a much lesser extent than non-heat-shock proteins. Thus, addition of purified eIF-4F reverses the effects of elevated temperatures on Ehrlich cells that are reflected in lysates. Therefore, we propose that the inhibition of translation in heat-shocked Ehrlich cells is the result of inactivation of eIF-4F function.

Almost all living organisms studied respond to elevated temperatures by increasing the rate of synthesis of a small set of proteins, the so-called heat-shock proteins, through a very rapid induction of synthesis of mRNA for these proteins (1–4). An equally universal, but less emphasized, response is a rapid induction of synthesis of mRNA for these proteins (1–4). An equally universal, but less emphasized, response is a qualitative change in the protein synthesis apparatus such that a factor is inactivated which is required specifically for translation of non-heat-shock mRNAs and is not required for heat-shock protein mRNAs (13, 14).

Recently we reported that the level of 40 S initiation complexes is reduced in heat-shocked Ehrlich ascites tumor cells concomitant with inhibition of polypeptide chain initiation (8). We report here that cell-free protein synthesis in lysates isolated from heat-shocked cells is inhibited and that synthesis is restored by the addition of eIF-4F. Furthermore, addition of eIF-4F preferentially stimulates translation of non-heat-shock mRNAs. Eukaryotic initiation factor 4F binds specifically to mRNA cap structure and is required for efficient binding of mRNA to 40 S ribosomal subunits (15). The factor probably consists of three nonidentical subunits of 220, 45, and 24 kDa and is thought to be identical to cap binding protein II (16, 17). It restores translation of capped mRNAs in extracts of poliovirus-infected HeLa cells. Although heat-shock mRNA is not known to be uncapped, this does at least show that differential translation is possible by modulating eIF-4F activity.

We conclude that inactivation of eIF-4F in lysates of heat-shocked cells is responsible for the decreased rate of protein synthesis in these lysates and for the preferential translation of heat-shock mRNAs. Inactivation of eIF-4F could, therefore, explain the alterations in translation in intact heat-shocked cells.

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The abbreviations used are: eIF, eukaryotic initiation factor; Mops, 3-(N-morpholino)propanesulfonic acid; GEF, GDP/GTP exchange factor; hsp, heat-shock protein, SDS, sodium dodecyl sulfate. 9648
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EXPERIMENTAL PROCEDURES

Materials—The radioactive amino acids [35S]methionine (1400 Ci/mmol) and [14C]leucine (283 Ci/mol) were obtained from Amersham International and New England Nuclear, respectively.

Cell Culture—Ehrlich cells were grown in spinner suspension culture as described previously (18). Briefly, stock cultures were diluted routinely to 2 × 10^6 cells/ml, and the volume was kept constant at 100 ml in antibiotic-free minimal essential medium modified for spinner culture and supplemented with 10% (v/v) calf serum and 20 mM Mops, pH 7.3. Under these conditions the cells grew with a doubling time of approximately 14 h. When large cultures were required, this stock was expanded.

Preparation of 37 and 44 °C Cell Lysates (19)—Suspension cultures (3 liters) at 6 × 10^6 cells/ml centrifuged at 500 × g for 10 min at room temperature and cells were resuspended in minimal essential medium supplemented with 1% calf serum and 20 mM Mops, pH 7.3, at a density of 3 × 10^6 cells/ml. The cultures were brought rapidly to either 37 or 44 °C, as described (8), and incubated for 20 min. The cells were rapidly cooled and sedimented by centrifugation at 500 × g for 7 min and washed twice with cold phosphate-buffered saline. The final cell pellets were resuspended in an equal volume of hypertonic buffer (20 mM Mops, pH 7.5, 10 mM KCl, 2.5 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mM EDTA) and cells were lysed with a Dounce homogenizer. The homogenates were made supramen 1% (v/v) sucrose and 20 mM Mops, pH 7.4, and 80 mM KCl, 2.5 mM magnesium acetate, 0.5 mM dithiothreitol. The excluded fractions were combined, divided into aliquots, and kept under liquid nitrogen.

Preparation of Ribosomal Salt Wash—Ehrlich cell postmitochond rial supernatant was centrifuged at 200,000 × g for 1.5 h in a Beckman Ti70 rotor. Pelleted ribosomes were suspended in high salt buffer (20 mM Mops, pH 7.4, 500 mM KCl, 5 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mM EDTA, 8% (v/v) sucrose) and centrifuged for 1.5 h at 200,000 × g. Postribosomal supernatant (ribosomal salt wash) was stored under liquid nitrogen.

Purification of Ehrlich Cell Stimulator—Ribosomal salt wash (15 ml) was dialyzed for 15 h against buffer A0 (20 mM Mops, pH 7.6, 10% (v/v) glycerol, 0.25 mM dithiothreitol, 0.1 mM EDTA, 0.1 M KCl (subscript)) and applied to a 35-ml column of CM-Sepharose equilibrated in A0 (20). Stimulatory activity eluted with the flow-through fractions which were pooled (25 ml) and applied to a DEAE-cellulose column (1 × 8 cm) equilibrated in A0. Bound material was gradient-eluted (60 ml) between 0.1 and 0.3 M KCl. Activity eluted at 0.22 M KCl and pooled fractions (11.5 ml) were dialyzed against buffer A1 (16 ml) and dialyzed against A1 for 16 h and applied to a phosphocellulose column (1 × 5 cm) equilibrated in A1. Bound material was eluted stepwise; stimulatory activity eluting with the buffer A2 wash. Active fractions were pooled (3.5 ml), dialyzed against A3, and frozen at −80 °C. Activity was assayed throughout by adding 10 µl of fractions to the heat-shocked cell-free system described below (50 µl final volume), adjusting the assay KCl concentration to 70 mM.

Preparation of Initiation Factors—GEF and eIF-2 were prepared 50% pure from Ehrlich cells as described (20). Eukaryote initiation factors eIF-4A, eIF-4B, and eIF-4F were purified from rabbit reticulocytes as described (15).

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (21). Equal amounts of radioactive material were applied to the gels. Gels containing protein labeled in the cell-free system (6 × 10^6 cpm/lane) were autoradiographed for 5 days, while gels with proteins labeled in intact cells (10^6 cpm/lane) were autoradiographed for 3 days. Where indicated, lanes from x-ray plates were scanned at 633 nm for optical density with an Ultrospec laser densitometer (LKB).

Assay of Lysates for Protein Synthesis—Protein synthesis in the cell-free systems was followed as described (19). Briefly, 50-µl reactions contained 12.5 µl (25%) of lysate as stated in figure legends, 20 mM Mops, pH 7.6, 1.76 mM magnesium acetate, 0.25 mM dithiothreitol, 3.2 mM ATP, 0.3 mM GTP, 3 mM phosphoenolpyruvate, 40 µg/ml of pyruvate kinase, 19 amino acids at 40 µM, and 5 µCi/ml (16.6 µM) [35S]leucine. KCl was added to the concentrations indicated in the figures or figure legends. Samples, 20 µl, were removed from incubations which were at 37 °C, and spotted onto Whatman No. 3MM filter-paper discs. The discs were boiled in 5% trichloroacetic acid, washed with 10% trichloroacetic acid, ethanol, and acetone, dried, and counted for radioactivity. Unless otherwise indicated, radioactive counts represent incorporation of [35S]leucine into protein for 30 min.

RESULTS

Characterization of Cell-free Systems—Lysates were prepared from cells incubated at 37 and 44 °C. They were preincubated to run off pre-existing polyribosomes, and endogenous amino acids were removed by gel filtration. In the cell-free protein-synthesizing system, the two lysates had similar requirements for Mg2+ (data not shown) and both incorporated [14C]leucine linearly for at least 30 min. The 37 °C lysate exhibited a KCl optimum typical of mammalian cell-free systems (70 mM) (22, 23) and had a relatively broad optimum (Fig. 1). At 70 mM KCl the incorporation by the 44 °C lysate was below that in the 37 °C lysate, thus mimicking the in vivo situation, in which protein synthesis was inhibited by about 90% in the cells at 44 °C. The low synthesis by the heat-shocked lysate proved to be because it had an unusually low and sharp KCl optimum (Fig. 1). Both cell-free systems were incubated at 37 °C so that this change reflects an effect of elevated temperatures on the intact cells from which the lysates were prepared. The sharp KCl optimum indicates strong inhibition at higher KCl concentrations. Inhibition was much less when K+ acetate was substituted for KCl, suggesting that Cl− is the major inhibitory ion (Fig. 2). Elongation was ruled out as the inhibition site at higher KCl concentrations through measurements of rate of elongation at different KCl concentrations, as follows. The lysates were

FIG. 1. Effect of KCl on protein synthesis in 37 and 44 °C cell lysates. Lysates were incubated at 37 °C as described under "Experimental Procedures." Incubations contained 25% (v/v) lysate, so that the minimum KCl concentration attainable was 20 mM. ○, 37 °C cell lysate; ●, 44 °C cell lysate.
incubated under conditions which produced similar numbers of polyribosomes and then edeine (a specific inhibitor of initiation) was added to prevent further initiation. Elongation by the preformed polyribosomes was followed at different KCl concentrations by the addition of [14C]leucine. In both the 37 and 44 °C lysates the rate of elongation was relatively insensitive to KCl, rates being similar at 40 and 80 mM KCl (data not shown).

Incubation of Ehrlich cells above 44 °C resulted in the partial degradation of mRNA so that lysates prepared from these cells were partially dependent on added mRNA. However, they responded to addition of KCl in a similar manner to the lysates used for the work presented here which have no mRNA dependence (data not shown).

**Purification of a Stimulator of Heat-shocked Cell-free Systems**—Small amounts of 37 °C lysate were strongly stimulatory when added to the 44 °C lysate. We, therefore, fractionated the 37 °C lysate to isolate the factor lacking in the 44 °C lysate, using as an assay the ability to stimulate protein synthesis in a heat-shocked cell-free system at the higher KCl concentration (70 mM). Ribosomal salt wash that had been dialyzed against A₁₀₁ (20 mM Mops (KOH), pH 7.6, 100 mM KCl, 0.25 mM dithiothreitol, 0.1 mM EDTA, 10% (v/v) glycerol) was found to contain a stimulator (Table I). This was fractionated by CM-Sephadex chromatography; the stimulatory activity eluted with the flow-through fractions at 0.1 M KCl (Table I). The pooled fractions were further fractionated on DEAE-cellulose and the stimulator eluted as a single

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**TABLE I**

| Purification from Ehrlich cells of a stimulator of protein synthesis in heat-shocked cell lysates |
|---|---|---|---|
| Addition | Specific activity | Purification | Yield |
|---|---|---|---|
| Ribosomal salt wash | 34 | 5650 | 1.0 |
| CM-Sephadex (0.1 M KCl) | 35 | 5250 | 2.0 |
| DEAE-cellulose (0.22 M KCl) | 260 | 2350 | 7.8 |
| Phosphocellulose (0.3 M KCl) | 960 | 1050 | 29 |

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**TABLE II**

| Effect of initiation factors on cell-free protein synthesis in heat-shocked cell lysate |
|---|---|---|
| Factor added | Amount | Protein synthesis |
|---|---|---|
| 0 | µg protein | cpm | -fold |
| eIF-2 | 2 | 5,530 | 1 |
| GEF | 3 | 4,270 | 0.77 |
| eIF-4A | 2 | 3,020 | 0.55 |
| eIF-4B | 2 | 7,240 | 1.30 |
| eIF-4F | 2 | 6,502 | 1.17 |
| Ehrlich cell stimulator | 6.4 | 9,180 | 1.55 |

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**FIG. 2.** Comparison of K⁺ acetate and KCl in 44 °C cell lysate. 44 °C lysate (25%, v/v) was incubated as described under "Experimental Procedures." Reactions contained 20 mM KCl contributed by the lysate, and K⁺ concentration was varied by addition of K⁺ acetate (○) or KCl (■).

**FIG. 3.** Effect of Ehrlich cell stimulator on the KCl dependence of protein synthesis in heat-shocked cell lysate. Lysates were incubated at 37 °C as described under "Experimental Procedures" without (○) or with (■) 6.4 µg of stimulator (purified through the phosphocellulose step). Fold stimulation is the ratio of synthesis with/without stimulator.
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Fig. 4. Effect of eIF-4F on the KCl dependence of protein synthesis in heat-shocked cell lysates. A, control (37 °C) cell lysates (25%) and B, heat-shocked cell lysates (25%) were incubated at 37 °C as described under "Experimental Procedures" without (○) or with (●) eIF-4F (1 µg).

Effect of eIF-4F and Other Initiation Factors on the Heat-shocked Cell-free System—Comparison of the chromatographic properties of the Ehrlich cell factor to those of known initiation factors showed that eIF-4F had the greatest resemblance. Eukaryotic initiation factor 4F is purified from the ribosomal salt wash, elutes from DEAE-cellulose at 0.22 M KCl, elutes from phosphocellulose between 0.15 and 0.45 M KCl, and behaves similarly to the Ehrlich cell factor in gel filtration (15). A number of initiation factors were tested for stimulatory activity in the heat-shocked system (Table II). Only highly purified eIF-4F had any significant stimulatory effect. Other factors, eIF-2, GEF, and eIF-4B, had no stimulatory effect at any KCl concentration; however, eIF-4A stimulated by 30%. Further, eIF-4F has greater stimulatory activity on the heat-shocked lysate at higher KCl concentrations (Fig. 4B) and only minimal effect on the 37 °C lysate (Fig. 4A). The reason for the inhibition by eIF-2 and GEF is unknown; these factors have no effect on control cell lysates (data not shown).

Effect of KCl and eIF-4F on Heat-shocked Protein mRNA Translation—The heat-shocked system was examined for the synthesis of heat-shock protein. Ehrlich cells induce at least one heat-shock protein (68 kDa); others are not detectable in the one-dimensional electrophoresis system shown in Fig. 5. The induction is prevented completely by actinomycin D (Fig. 5, lane 4). The heat-shocked cell lysate contains heat-shock mRNA so that the relative efficiency of translation at different KCl concentrations could be studied. Densitometric scans (Fig. 6) of autoradiographs of SDS gels of [35S]methionine-labeled protein from lysates incubated at 30 and 70 mM KCl were analyzed for synthesis of hsp 68 and actin. The results are expressed in Table III as the per cent of total protein synthesized. Essentially, a constant percentage of actin is synthesized under different conditions while total protein synthesis is inhibited by 70% at the higher KCl concentration. However, hsp 68 synthesis is less inhibited at higher KCl concentrations so that it represents a greater percentage of total protein synthesis. Addition of eIF-4F stimulated total protein synthesis and actin synthesis by 2.6-fold at 70 mM KCl; however, hsp 68 synthesis was stimulated 1.5-fold only.

Fig. 4. Effect of eIF-4F on the KCl dependence of protein synthesis in heat-shocked cell lysate. A, control (37 °C) cell lysates (25%) and B, heat-shocked cell lysates (25%) were incubated at 37 °C as described under "Experimental Procedures" without (○) or with (●) eIF-4F (1 µg).

Discussion
We have shown that lysates prepared from cells incubated at elevated temperatures have a striking alteration in their response to KCl when incubated in the cell-free protein-synthesizing system at 37 °C. At the optimum KCl concentration (30-40 mM) there is no apparent inhibition in lysates from heat-shocked cells. However, heat-shocked cell lysates
are severely inhibited by KCl concentrations (60–70 mM) that are optimal for 37 °C lysates. The inhibition is not due to a reduction in elongation, which is independent of KCl concentration in the range used. Premature termination is excluded because the pattern of proteins being synthesized is the same at different KCl concentrations (Fig. 6) and is the same as that found with pulse-labeled whole cells. We conclude that the reduced rate of protein synthesis in 44 °C lysates at high KCl concentrations is caused by inhibition of initiation reflecting the inhibition in heat-shocked cells (8). Further, heat-shock mRNA is preferentially translated at the high KCl compared to low KCl concentrations in the 44 °C lysates. Others have observed in intact cells that heat-shock protein mRNAs are more efficiently translated than non-heat-shock protein mRNA at elevated temperatures (5–7). The inhibition of initiation in heat-shocked cell lysates at high KCl concentrations can be reversed by a factor purified from Ehrlich cell ribosomal salt wash and by highly purified rabbit reticulocyte eIF-4F. The two factors have indistinguishable chromatographic properties and identical effects on the response of heat-shocked lysates to KCl. The Ehrlich cell factor is presumably eIF-4F, and it can be concluded that an alteration of eIF-4F or an altered requirement for eIF-4F produces changes in the response to KCl of heat-shocked lysates. However, it is unknown whether the eIF-4F added to our lysates is acting catalytically or stoichiometrically. We propose that the inhibition of initiation in heat-shocked cells is due to reduction of eIF-4F activity which results from either an alteration of eIF-4F or an altered response of the Ehrlich cell translation apparatus to eIF-4F. This conclusion is strengthened by the observation that translation of non-heat-shock mRNA is stimulated more than hsp 68 mRNA by addition of eIF-4F to cell lysates, indicating that the loss of activity of this mRNA binding factor probably plays a major role in the preferential

**Fig. 6.** Densitometer scans of SDS-gel autoradiographs showing the effect of eIF-4F on 68-kDa heat-shock protein synthesis. Heat-shock cell lysate was incubated at different KCl concentrations with or without eIF-4F for 60 min with 200 μCi/ml of [35S]methionine, instead of [3H]leucine, as described under “Experimental Procedures.” Equal amounts of radioactive protein were applied to an SDS-polyacrylamide gel; autoradiographs were prepared and scanned for optical density as described under “Experimental Procedures.” A, 30 mM KCl. B, 30 mM KCl, 2 μg of eIF-4F. C, 70 mM KCl. D, 70 mM KCl, 2 μg of eIF-4F. The positions of 68-kDa hsp (h), actin (a), and marker carbonic anhydrase (c) are shown.

| KCl concentration | eIF-4F added | Total protein synthesis | hsp 68 | actin |
|-------------------|--------------|------------------------|--------|-------|
| mM                | μg           | cpm x 10^3             | %      | %     |
| 30                | 0            | 170                    | 2.5    | 13    |
| 30                | 2            | 230                    | 2.2    | 11.6  |
| 70                | 0            | 52                     | 12     | 12    |
| 70                | 2            | 155                    | 6      | 10.5  |
translation of hsp 68 mRNA in cells at elevated temperatures. (This well-documented effect of heat shock (9-11) is in addition to the striking induction of transcription of hsp mRNAs.) Ray et al. (24) have concluded previously that eIF-4F (cap binding protein II) is an mRNA discriminating factor. They observed that addition of eIF-4F to a Krebs ascites cell-free translation system relieved competition between large concentrations of globin and reovirus mRNAs. Therefore, it is possible that hsp 68 mRNA outcompetes other mRNAs for a limited amount of eIF-4F in our heat-shocked cell-free system so that hsp 68 synthesis is less inhibited than non-heat-shock protein synthesis. Addition of eIF-4F to the heat-shock cell lysate relieves this competition (Fig. 6). Further, synthesis of heat-shock proteins in poliovirus-infected HeLa cells is more resistant to inhibition than normal host proteins (25). Poliovirus infection results in loss of eIF-4F activity (26) (possibly by degradation of the 220-kDa subunit) which is consistent with our hypothesis that heat-shocked Ehrlich cells also have reduced eIF-4F activity. We have found no apparent inhibition of any other factor in our heat-shocked cell lysate. Duncan and Hershey (12) have reported reduced eIF-2, eIF-4B, and eIF-3 and/or eIF-4F activity. However, it is possible that elevated temperatures reduced eIF-4F activity. We have found no apparent inhibition of any other factor in our heat-shocked cell lysate. Duncan and Hershey (12) have reported reduced eIF-2, eIF-4B, and eIF-3 and/or eIF-4F activity. However, they have found that protein synthesis in HeLa culture is less sensitive to inhibition by elevated temperatures than cells grown in suspension culture. Further, they used a reconstituted in vitro assay to identify changes in initiation factor activity. Therefore, differences in culturing conditions, cell lines, in vitro assay conditions, or all three may explain differences in our findings.

We have previously reported that 40 S initiation complex levels are reduced in heat-shocked Ehrlich cells (8). This is surprising because inactivation of the cap binding protein eIF-4F would, at first sight, be expected to cause inhibition of mRNA binding to 40 S initiation complexes (40 S-GTP·eIF-2·Met-tRNA) and cause an accumulation of free 40 S initiation complexes. We now have evidence that addition of eIF-4F restores 40 S initiation complex levels in heat-shocked lysates, suggesting that, in an as yet unexplained way, eIF-4F can affect eIF-2 function.2

The evidence presented here is consistent with a reduction in eIF-4F activity at higher KCl concentrations resulting in reduced rates of initiation and preferential synthesis of 68-kDa hsp. However, it is possible that elevated temperatures have other effects on the translation apparatus which are not expressed in our Ehrlich cell-free system.

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