Protein Synthesis and Protein Phosphorylation during Heat Stress, Recovery, and Adaptation

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Abstract. Incubating cells at elevated temperatures causes an inhibition of protein synthesis. Mild heat stress at 41-42°C inhibits the fraction of active, polysomal ribosomes from >60% (preheating) to <30%. A return to 37°C leads to an increase in protein synthesis, termed "recovery." Continuous incubation at 41-42°C also leads to a gradual restoration of protein synthesis (>70% of ribosomes reactivated by 2-4 h), termed "adaptation." Protein synthesis inhibition and reactivation in prestressed, recovered cells that contain elevated levels of the heat stress proteins occur to the same extent and at the same rate as in "naive" cells. The adaptation response requires transcription of new RNA whereas recovery does not. A large number of phosphorylation changes are induced by severe heat stress and occur with kinetics similar to the inhibition of protein synthesis. These include phosphorylation of eukaryotic protein synthesis initiation factor (eIF)-2α and dephosphorylation of eIF-4B and eIF-4Fp25 (eIF-4E). However, the extent to which the modification occurs is proportional to the severity of the stress, and, under mild (41-42°C) heat stress conditions, these initiation factor phosphorylation changes do not occur. Similarly, under conditions of severe heat stress eIF-2α and eIF-4B frequently recover to their prestress phosphorylation state before the recovery of protein synthesis. eIF-4E dephosphorylation likewise does not occur under mild heat stress conditions. Therefore, these changes in phosphorylation states, which are thought to be sufficient cause, are not necessary for the inhibition of protein synthesis observed.

SUBJECTING mammalian cells to elevated temperatures (termed heat shock or heat stress) inhibits translation of most cellular mRNAs (McKenzie et al., 1975; Hickey and Weber, 1981; Tissieres et al., 1974). A few mRNAs, including the heat stress mRNAs (McKenzie et al., 1975; Hickey and Weber, 1982; Tissieres et al., 1974), can escape inhibition for reasons as yet unknown. Studies in Drosophila have demonstrated that sequences in their 5′ untranslated regions are necessary and sufficient to confer translation at elevated temperatures (McGarry and Lindquist, 1985; Klementz et al., 1983; Bonner et al., 1984). The translational lesion in heat stressed cells principally occurs at the initiation step of translation (Hickey and Weber, 1982; Duncan and Hershey, 1984a; McKenzie et al., 1975). Protein synthesis initiation factors may regulate the repression since the phosphorylation state of eukaryotic protein synthesis initiation factor (eIF)-2, eIF-4B, and eIF-4Fp25 (eIF-4E) changes (Duncan and Hershey, 1984a; Ernst et al., 1982; DeBenedetti and Baglioni, 1986; Duncan et al., 1987) and the activities of these same factors are inhibited (Duncan and Hershey, 1984a; Ernst et al., 1982; DeBenedetti and Baglioni, 1986; Duncan et al., 1987).

In this report we continue our examination of the characteristics and mechanism of the heat stress-induced inhibition of protein synthesis. We set two goals: (a) to characterize in more detail the heat-induced inhibition of protein synthesis and its restoration in HeLa cells and (b) to investigate what molecular mechanisms might be causal. Based on the previous findings of ourselves and others, we have focused on reversible phosphorylation changes in the protein synthesis initiation factor proteins. Characterization of heat stress effects on protein synthesis will have specific pertinence to the heat stress response and general relevance towards understanding of molecular mechanisms of translational control.

Our results suggest that recovery and adaptation are distinct phenomena and that heat stress proteins are not sufficient to provide thermoprotection in HeLa cells. Furthermore, initiation factor protein modifications are not required for the inhibitory response.

Materials and Methods

Materials

[35S]Methionine (>1,000 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). [32P]Phosphate was purchased from New England Nuclear (Boston, MA).

Cell Cultures

HeLa S3 cells were propagated as suspension cultures in exponential growth.
phase (between 2.7 x 10^6 cells/ml) by frequent dilution with fresh Joldik's modified essential medium containing 8% calf serum (Eichison et al., 1982). For individual experiments, aliquots were removed from the growth stock and either transferred to smaller spinner bottles (200–1,000 ml of culture) or transferred to 35-mm tissue culture dishes (3 ml of culture) and allowed to attach for 2 h. Medium was then removed and replaced with 1.5 ml fresh medium containing 8% calf serum. As discussed elsewhere, this transfer to monolayer culture causes no detectable differences in the parameters we measure.

**Cell Physiology**

A survey of metabolic characteristics indicates that our HeLa cells demonstrate a healthy physiology, minimal stress at 37°C, and a heat stress response as described by others (Thomas et al., 1982; Slater et al., 1991; Watowich and Morimoto, 1988) and ourselves (Duncan and Hershey, 1984a). The following metabolic features document their integrity. The cells contain 80–90% of their ribosomes in polysomes in exponentially growing cells. The nonstressed cell cultures exhibit a rapid cell growth rate (24 h doubling time). The abundance of two heat-inducible stress proteins, HSP 72 (constitutive but heat enhanced) and HSP 73 (undetectable in 37°C cells), are not elevated in exponentially growing cells (their IEF/SDS-PAGE patterns appear as described by Thomas et al. [1982], Watowich and Morimoto [1988], and ourselves [Duncan and Hershey, 1984a] for HeLa cells) and they increase when heat stress is applied. Heat stress inhibition and adaptation occur as described by others (McCormick and Penman, 1969); complete restoration of protein synthesis rates due to recovery or adaptation indicates an intact response capacity, necessarily requiring positive function of numerous molecular components.

**Heat Stress Methods**

For heat stress of large cell cultures (experiments described in Tables I and II), 300–400 ml of suspension culture of HeLa cells (~5 x 10^6 cells/ml) in a 1-liter bottle was transferred to a heated water bath and incubated with magnetic stirring. Heat stress intervals were measured from the time of immersion. The volume of cells, the volume of heated water, the calibration thermometer, and the circulating heater setup were the same for most analyses described; exceptions are noted. For cells in the recovery protocol, cells were heated for 30 min and then returned to the air incubator. Whereas more rapid temperature equilibration during heat stress induction and recovery could be accomplished by centrifugation and resuspension or adding additional thermostatically controlled medium, we wished to reduce experimental manipulations and sources of variability to a minimum. For the thermoprotection protocol involving prestress and recovery, the culture was heated as above but at 45°C for 30 min and then allowed to recover for 3 h at 37°C. For heat stress of small culture volumes (experiments described in Figs. 3–6), 1–3 ml of monolayer-cultured HeLa cells (~1.5 x 10^6 cells/35-mm dish) was transferred to a heated air incubator. In kinetic experiments, each replicate culture was analyzed individually so that temperature fluctuations due to opening and closing the incubator were not a variable. The temperature of heat stress was varied in individual experiments (see text) between 40 and 50°C. Three temperature intervals are distinguishable (between 2-7 x 10^5 cells/ml) by frequent dilution with fresh Joldik's modified essential medium containing 8% calf serum (Eichison et al., 1982). For individual experiments, aliquots were removed from the growth stock and either transferred to smaller spinner bottles (200–1,000 ml of culture) or transferred to 35-mm tissue culture dishes (3 ml of culture) and allowed to attach for 2 h. Medium was then removed and replaced with 1.5 ml fresh medium containing 8% calf serum. As discussed elsewhere, this transfer to monolayer culture causes no detectable differences in the parameters we measure.

**Cell Labeling**

For protein labeling with [35S]methionine, monolayer cells in 35-mm plates in complete medium were pulse labeled with 50–100 μCi/ml for 60 min, and the protein was rapidly extracted into two-dimensional IEF/SDS-PAGE lysis buffer (Duncan and Hershey, 1983, 1984b). Incorporation was measured by mixing an aliquot of the lysate into 5% TCA, incubating on ice for 10 min, collecting on GF/C filters by filtration, and counting by liquid scintillation. For protein labeling with [32P]phosphate, monolayer cells in 35-mm plates in medium lacking phosphate were incubated with 1 mCi/ml [32P] for 20 min. Heat stress cells were then transferred to a 45 or 46°C air incubator for stress intervals of 5, 10, 15, 20, 25, and 30 min. Plates were individually heat-stressed to exclude temperature fluctuations due to opening and closing the incubator door. Labeling was rapidly stopped by removing the media and adding 2 ml ice-cold Earl's saline and 100 ml 10% trichloroacetic acid. After three washes, protein was extracted into two-dimensional IEF/SDS-PAGE lysis buffer (Duncan and Hershey, 1983, 1984b) and analyzed as described (Duncan and Hershey, 1983, 1984a). Similar phosphorylation patterns were detected at both temperatures.

**Polysome Analysis**

30-ml aliquots (~1.5 x 10^6 cells) were removed and poured over frozen crushed Earl's salts containing 300 μg cycloheximide (to give a final concentration of 0.10 μg/ml). Cells were washed three times with Earl's salts and then the cell pellet was resuspended and homogenized in 1 ml polysome buffer (100 mM KCl, 3 mM MOPS, 20 mM HEPES, 7 M 1,10-phenanthroline, 10 μg/ml cycloheximide, and 0.5% NP-40). An S10 was prepared by centrifugation for 10 min at 8,000 rpm at 4°C (SS-34 rotor; Sorvall Instruments Div., Newton, CT). The supernatant was layered on a 20–40% sucrose density gradient made of polysome buffer without NP-40 and centrifuged for 120 min at 36,000 rpm at 4°C (SW-40 rotor; Beckman Instruments, Inc., Fullerton, CA). Gradients were analyzed by continuous flow scanning at 254 nm. Quantitations were determined by copying the chart tracings onto standard weight paper, cutting out regions corresponding to 40, 60, and 80 S and polysomes (disomes and larger), and weighing each.

**Transit Time Measurements**

The following protocol is formally analogous to that described by Fan and Penman (1970), but differs in the way that the data points are obtained. The following methodology circumvents difficulties frequently encountered in differentiating subcellular compartments by centrifugation and pelleting (Nielsen and McConkey, 1980). Cells were cultured with 1.6 μCi/ml [3S]methionine (10 μCi/ml [3H]leucine was used in other experiments with indistinguishable results). At 6, 8, and 10 min of labeling, a 30-ml aliquot of cell culture was removed and rapidly processed as described above. A 100-μl aliquot of the S10 was removed before polysome centrifugation to determine total incorporation. Other analyses of amino acid incorporation into protein in rapidly lysed, wholly unfractinated lysates by ourselves and others have established that the rate is linear from the initiation of the labeling period. The line determined by the 6-, 8- and 10-min labeling aliquots removed from the S10 was linear and extrapolated to zero at time zero, indicating that differential losses do not bias our measurements of total incorporation. The remainder of the S10s were fractionated on gradients and separated into ~15–20 fractions. Based on the A254 scan tracing, the fractions corresponding to the polysomes (disomes and greater), subpolysomes (~40 S), and pellet were separately pooled. The radioactivity in each pooled fraction was measured by TCA precipitation. The amounts of radioactivity in polysomes at each labeling time were used to generate a second line representing the amount of radioactivity in free protein chains. This line was generated by subtracting the counts per minute in polysomes from the corresponding total incorporation value, thus producing a second set of three points. Since the counts per minute in polysomes is constant, as is required by the experiment if the polysomes are in a steady-state situation and the labeling interval exceeds a transit time, the lower line is parallel to the upper. These two lines are identical with those described and characterized by Fan and Penman (1970), wherein it was established that the transit time equals twice the time axis intercept of the lower line. This quantity was estimated graphically and is reported in Table I. The totaled counts per minute in all gradient fractions plus pellet were also summed to provide an independent, confirming determination of the total protein incorporation which was principally measured by the S10 aliquot.

**Immunoblot Analysis**

For monolayer cells, at the indicated times, a plate was removed and the medium was rapidly removed and replaced with 2 ml of 4°C Earl's salts (wash medium). After three washes, extracts were prepared and analyzed by IEF/SDS-PAGE and immunoblotting as described (Duncan and Hershey, 1983, 1984a). The gel regions containing eIF-2α, eIF-2β, and eIF-4B were excised and treated with affinity-purified antibodies to eIF-2α and eIF-2β or with anti-eIF-4B antiserum. For suspension-cultured cells, cell lysates were prepared as for polysome analysis (see above) with the addition that 0.2 mM PMSF and 0.3 μg/ml aprotinin were added to the polysome extraction buffer. At the point where the S10 was ready for gradient loading, an aliquot was removed for analysis by IEF/SDS-PAGE immunoblotting. The IEF sample was prepared by adding 30 mg urea and 16 μl IEF buffer (Duncan and Hershey, 1984b) to ~30 μl of lysate. Some lysates were prepared with phosphate inhibitors (50 mM NaF and/or 50 mM β-glycerophosphate) present. The initiation factor phosphorylation extent was not detectably greater in the inhibitor-containing buffer; thus, there is no evidence for factor dephosphorylation during lysate preparation.
m'GTP-Sepharose Analysis of eIF-4E

The preparation and analysis of heat stress samples for eIF-4E was as described in Duncan et al. (1987). Briefly, ~500 ml of cell suspension (2.5 x 10^6 cells) was poured over frozen, crushed Ear's salts, collected by centrifugation, washed three times, and lysed in affinity column-binding buffer (100 mM KCl, 20 mM Heps, pH 7.5, 0.2 mM EDTA, 10% glycerol, 7 mM β-mercaptoethanol, and 0.2 mM PMSF) containing 0.5% NP-40 and 2 U/ml aprotinin (Sigma Chemical Co., St. Louis, MO). The sample was passed over an mGTP-Sepharose 4B column (Pharmacia Fine Chemicals, Piscataway, NJ) (~1 ml every 5 min) and then washed sequentially with binding buffer and binding buffer plus 100 μM GDP. Bound proteins were eluted with binding buffer containing 75 μM mGTP, precipitated with 4 vol of acetone, resuspended in IEF buffer (also called lysis buffer; Duncan and Hershey, 1983, 1984b), and analyzed by IEF/SDS-PAGE and silver staining (Morrissey, 1981).

Results

Definitions and Methodology

To facilitate descriptions of the results, it will be helpful to precisely define some frequently used terms. Severity is used to denote the stress temperature, ranging from mild (41-42°C) to moderate (43-44°C) to severe (>45°C). Restoration of protein synthesis refers to the reactivation of inhibited ribosomes and mRNA. Restoration can occur via several distinct pathways. Recovery specifically refers to changes accompanying the transfer of the heated culture back to 37°C. Adaptation refers to restoration that occurs when cells are maintained at heat stress temperatures. Thermoprotection (also referred to by others as thermotolerance or acquired thermotolerance) refers to alterations conferring resistance to heat-induced inhibition of protein synthesis. It is usually produced as a consequence of heating and may occur in adapting or recovering cells. As we shall describe, this multiplicity of terms seems to be required because each of the above listed processes/states possesses unique or distinct features.

The protocols that we have used to probe heat stress effects on protein synthesis are summarized in Fig. 1, A and B, which shows recovery and adaptation, respectively. In the first section we have focused on whether thermoprotection can influence the protein synthesis rate in either protocol. Thermoprotection was assessed using a 43°C preheat stress.

Figure 1. Protocols used to measure heat stress effects on protein synthesis. The three stress protocols used are recovery, adaptation, and thermoprotection (A-C, respectively). Two varieties of thermoprotection are depicted. Both begin with a priming heat stress followed by a recovery interval. Subsequently, cells are either analyzed by the recovery protocol or the adaptation protocol. The length of time required to reach temperature equilibrium (dashed lines) varies depending on the cell culture volume and the method of heating and cooling; see individual experiments and Materials and Methods for details. In some analyses, DRB, an inhibitor of RNA synthesis, was added at the indicated times.
followed by recovery at 37°C and a second mild heating, as shown in Fig. 1 C. The preheat and experimental heat were kept in the mild range to more closely parallel situations encountered by homeotherms during life. Preheating clearly induced the accumulation of heat stress proteins. Based on Coomassie blue staining of total cytoplasmic proteins separated by IEF/SDS-PAGE, the amount of HSP 70 and HSP 90 proteins in the prestressed cells approximately doubled in 3 h (data not shown) and showed slight subsequent increase up to 12 h after recovery. This extent of induction closely parallels previously reported values in Chinese hamster ovary cells (Scandra and Subjeck, 1984).

Mild stress conditions cause different sorts of molecular alterations than severe heat, as we will document below. We would emphasize in advance that there is no basis to expect that results obtained under milder conditions should parallel previously reported thermoprotection results obtained using harsher protocols (this will be considered further in the Discussion).

**Alterations in the Number of Active Ribosomes during Heat Stress, Recovery, and Adaptation**

The rate of protein synthesis can be expressed as the number of active ribosomes multiplied by the rate at which the ribosomes transit the mRNAs. Our goal is to use the number of polysomal (active) ribosomes as a comparative measure of protein synthetic activity in 37°C vs. heat stressed cells. As we document below, we find no evidence that heat stress polysomal ribosomes are elongation blocked, so using polysomal localization as the criterion for active ribosomes seems warranted. The fraction of ribosomes in polysomes can be readily determined by sucrose density gradient centrifugation of cytoplasmic lysates (see Materials and Methods).

To convert the comparative numbers of active ribosomes to comparative protein synthesis rates, the transit times in each condition must be measured. These can also be readily determined. We have used a modification of the Fan–Penman determination method (see Materials and Methods for a detailed description). We measure equivalent transit times of ~1.5 min in 37°C cells and 42°C heat stressed cells (Table I). The transit time at 37°C is in good accord with previous determinations for HeLa cells (Fan and Penman, 1970; Nielsen and McConkey, 1980). It is difficult to predict a priori what the heat stress transit time would be expected to be, but we feel that an equivalent time is reasonable. On the one hand, it is well documented that raising the temperature from ~10 to 37°C results in progressively faster ribosome movement, as predicted on Q10 considerations. At temperatures >40°C, however, protein activities begin to become thermally inhibited, and the balance between activations and inhibitions among the >100 components in the protein synthetic machinery cannot be predicted. We have not attempted to assess the detailed basis for our results showing equivalent transit times at 37 and 42°C, but presumably the inhibitions and activations balance out. The equivalent rates establish that the number of active ribosomes will be directly proportional to the protein synthetic rate in the comparative experiments presented below.

In this section, we focus on the role of heat stress proteins in the thermoprotection of protein synthesis. Specifically, does the accumulation of heat stress proteins, or of any other heat-induced component, provide thermoprotection? The specific events assayed are: (a) the extent of heat-induced ribosome disaggregation; (b) the rate and extent of recovery; and (c) the rate or extent of adaptation. The results of these experiments are summarized in Table II.

Control cells (Table II, line 1), without a preheat step, show polysome disaggregation when heated to 41 (line 2) or 42°C (line 3). At 41°C the fraction of ribosomes in polysomes decreases to ~25% within 10 min and remains relatively constant at 15 and 20 min after heating. At 42°C, the inhibition is roughly the same, perhaps a bit more extensive (17%). When cells heated to 41°C are maintained at that temperature, adaptation occurs. Polysomes progressively reform over the next 4 h (line 2), as originally described by McCormick and Penman (1969). From a disaggregated value of ~22% at 20 min, polysomes reform to ~35% at 1 h, 47% at 2 h, and 51% at 4 h, the longest interval examined. A similar adaptation also occurs with cells maintained at 42°C (line 3).

When cells heated for 20–30 min at 42°C are returned to 37°C, polysomes rapidly reform to ~90% of their preheat value at 15 min (63 vs. 57%) and to ~100% by 30 min (line 4). This illustrates the process termed recovery. Polysome profiles are shown in Fig. 2 A; these exemplify data used to obtain the numbers reported in Table II.

The specific proteins synthesized during adaptation were monitored by pulse labeling for 60 min and PAGE. At every time, >90% of the incorporation was into "37°C" proteins (Fig. 2 B). Thus, adaptation constitutes a mechanism to reactivate the translation of the 37°C mRNAs. During adaptation, the amount of incorporation into the HSP 70 and 90 rises substantially, but it remains a minor fraction of the total. The massive accumulation and preferential translation of heat stress mRNAs observed in *Drosophila* is not observed in HeLa cells (Fig. 2 B; Hickey and Weber, 1982), hamster cells (Scandra and Subjeck, 1984), or mouse cells (Duncan, R., unpublished results).

The same kinds of measurements were performed in cells that had been prestressed by heating at 43°C for 30 min and then allowed to recover at 37°C for 3 or 10 h. The second heating at 41°C produces an extent of inhibition roughly equivalent to control cells after either the 3-h (22% when treated for 20 min) or the 10-h recovery (30% when treated for 20 min) (Table II, lines 5 and 6, respectively). The rate and extent of recovery at 37°C in preheated cells is roughly equivalent to control cells (line 7 vs. line 2; 15 and 30 min of treatment). And, finally, the rate and extent of adaptation at 41°C in preheated cells is roughly equivalent to control cells (lines 5 and 6 vs. line 2). In summary, preheating followed by a recovery interval provides no thermoprotective effect for any of the measured parameters.

Table II, lines 8–11, documents a further set of experiments probing two points: first, do recovery and adaptation require

| Culture condition | Transit time |
|-------------------|-------------|
| 37°C              | 1.5 min ± 0.3 |
| 42°C              | 1.5 min ± 0.3 |

* See Materials and Methods for experimental protocol and method of analysis.
Table II. Percent Ribosomes in Polysomes in Heat Stressed Cell Cultures*

| Experimental treatment/ cell condition | Minutes of treatment |
|---------------------------------------|----------------------|
|                                       | PRE/0 | 5 | 10 | 15 | 20 | 30 | 60 | 120 | 240 |
| Control cells                         |       |   |    |    |    |    |    |     |    |
| (1) 37                                | 63 (9)| % | %  | %  | %  | %  | %  | %   | %  |
| Adaptation                            |       |   |    |    |    |    |    |     |    |
| (2) 37/41                             | 63±   | 55 | (4) | 24 | (6) | 27 | (4) | 22 | (8) |
| (3) 37/42                             | 63±   | 55 | (4) | 24 | (6) | 27 | (4) | 22 | (8) |
| Recovery                              |       |   |    |    |    |    |    |     |    |
| (4) 37/42/37                          | 17±   | 57 | (1) |    | 64 | (2) |    | 73 | (1) |
| Thermotolerance                       |       |   |    |    |    |    |    |     |    |
| (5) 37/43/37                          | 68 | (2) | 22 | (4) |    | 37 | (3) | 52 | (3) |
| (6) 37/43/37                          | 73 | (2) |    | 30 | (2) | 44 | (1) | 44 | (1) |
| (7) 37/43/37                          | 17   |    |    |    |    | 64 | (1) |    |    |
| DRB-treated cells                     |       |   |    |    |    |    |    |     |    |
| (8) 37                                | 76 | (1) | 81 | (1) | 78 | (1) | 78 | (1) | 65 | (1) |
| (9) 37/41/11                          | 63± | (9) | 30 | (2) | 44 | (1) | 44 | (1) | 40 | (1) |
| (10) 37/42/37/37                      | 12 | (1) | 30 | (2) | 44 | (1) | 44 | (1) | 40 | (1) |
| (11) 37/43/37/37                      | 12 | (1)| 61 | (1) | 63 | (1) |    |    |    |    |
|                                       |       |   |    |    |    |    |    |     |    |

* Lysates were analyzed by sucrose density centrifugation and an A260 tracing was produced. The tracing was photocopied and polysomal and subpolysomal (40, 60, and 80 S) regions were cut out and weighed. Values are polysomal weight/total weight. Numbers in parentheses give the number of gradients profiles measured.
† Value taken from line 1.
‡ Combined results from three experiments: the first measured 10 and 30 min after heating, the second measured 20 min after heating, and the third measured 30 min after heating. Percents were 32, 17, 14, and 12, respectively.
† One experiment was performed by heating for 20 and 30 min, respectively, recovery results have been combined.
§ Value taken from line 7.
** Heating was for 30 min and recovery was at 37°C for 3 h.
†† Heating was for 30 min and recovery was at 37°C for 10 h.
††† Second heating was for 30 min.
II DRB added immediately before heating.
††† The two specific cell cultures used in these experiments began with 68% ribosomes in polysomes (65 and 70%, respectively) and had 30% (28 and 31%, respectively) at 60 min. Disaggregation between 10 and 30 min was not measured, but predicted disaggregation is to 15-30% (see footnote †††). Actual disaggregation can be estimated from the 1-h adaptation value (30%) to be in the 15-30% range since DRB does not significantly reduce polysome percents over this interval (see line 8).
*** Heating was for 30 min and DRB was added immediately before the inception of heating.
††† Data reported in lines 4-7 indicate 15-30% ribosomes in polysomes after 41-42°C heating; the greater disaggregation is probably not due to DRB since the coanalyzed control had a similarly extensive disaggregation (footnote †, experiment 3).

RNA synthesis and, second, does preheating in any way abrogate the requirement. McCormick and Penman (1969) reported that actinomycin D blocked heat adaptation, but subsequent results demonstrated that actinomycin D can act as a direct inhibitor of protein synthesis (Singer and Penman, 1972). Thus, the failure to adapt becomes equivocal. We have repeated the analysis using dichlororibofuranosylbenzimidazole (DRB), an RNA synthesis inhibitor without this drawback. DRB alone has no effect on polysome content after 1 h (line 8); in analyses between 2 and 4 h there is an ~20% drop in polysome content, presumably due to the degradation of rapidly turning over mRNAs.

Adaptation does not appear to occur in the presence of DRB, as documented on line 9. Instead, a roughly constant polysome fraction is measured with, perhaps, a small decrease with time. If adaptation could occur, the numbers should have risen, even accounting for the inhibition predicted due to direct DRB effects (predicted to rise to ~40% [80% of 47-51%, the control values]). This conclusion that DRB blocks adaptation must be considered soft, based on the imprecision of the quantitation vs. the numerical spread; however, since the results confirm previously "established" findings, we have not performed numerous replicates to obtain statistically significant numbers. Adaptation in pre-stressed cells treated with DRB concurrent with second heating (line II) likewise suggests that preaccumulation of heat stress proteins or heat stress mRNAs does not thermoprotect protein synthesis, with the same reservations noted above. To close on a positive note, the ability of cells to recover in DRB was also examined (line 10) and, in this case, no deleterious effect was observed. We conclude that restoration due to recovery and to adaptation probably proceed by different mechanisms.

Phosphorylation Changes during Heat Stress

The Induction of Heat Stress. In this section we have investigated what molecular alterations might contribute to or cause the heat stress inhibition of protein synthesis. We and others have hypothesized that initiation factor protein covalent modifications are likely to play a role in this regard (Duncan and Hershey, 1984a; Bonanou-Tzedaki et al., 1981; Ernst et al., 1982; Panniers and Henshaw, 1984; Panniers et al., 1985; DeBenedetti and Baglioni, 1986). Attention has been focused principally on the role of increased eIF-2α phosphorylation since phosphorylation is known to inhibit eIF-2 function (Matts and London, 1984; Proud, 1986). Also of interest are eIF-4B, eIF-4Fp25 (eIF-4E), and ribo-
Figure 2. Alterations in polysomal ribosomes and protein synthesis during heat stress and recovery. (A) HeLa S3 cells were heat stressed, and polysome profiles were analyzed as described in Materials and Methods. (a) Exponentially growing, never heated (naive) cells at 37°C; (b) naive cells after 30 min at 42°C; (c) naive cells heated as in b 30 min after return to 37°C (recovery); and (d) cells treated as in c, except instead of naive cells this culture was preheated for 30 min at 43°C and returned to 37°C for 3 h before use. (B) HeLa S3 cells were transferred to 41°C. Proteins were labeled for 1 h with [35S]methionine, extracted, and analyzed by one-dimensional SDS-PAGE. The number above the lane indicates the hour at higher temperature and the end of the labeling interval. Control proteins were labeled for 1 h at 37°C. Equal counts per minute of radioactive protein were loaded per lane; thus, the radioactivity per lane does not represent the relative rates of protein synthesis at each interval. Very similar results were obtained at 42°C. Molecular weight markers are shown on the left. The arrows point to HSP 70 and HSP 90.

dosomal protein S6, whose phosphorylation states (Duncan and Hershey, 1984a; Duncan et al., 1987; Glover, 1982; Burdon et al., 1982) and activities (Duncan and Hershey, 1984a; Panniers et al., 1985) are also altered by heat stress. The following experiments probe in detail the kinetic correlation between initiation factor protein covalent modification and protein synthesis inhibition and restoration. We initially describe our results using monolayer-cultured cells that were heat stressed by transfer into a hot air incubator. These conditions follow those used in our previous work (Duncan and Hershey, 1984a). The analyses provide sufficient material for immunoblot analysis, though not for polysome analysis. They are also advantageous because numerous replicate analyses can be performed easily and the small volumes of culture medium used (~1 ml) facilitate rapid temperature equilibrations.

The kinetics of inhibition were compared with the kinetics of initiation factor protein covalent modification. Protein synthesis rate was measured by amino acid incorporation at 5-min intervals using continuous labeling or 5-min pulses. Protein synthesis between 0 and 5 min after transfer into a 45°C air incubator occurred at the 37°C control rate, but was
Figure 3. Kinetics of changes in initiation factor phosphorylation induced by heat stress. Monolayer HeLa cells were heat stressed by transfer into a 45°C air incubator. Cell cultures were removed and processed as described in Materials and Methods. 

(A-C) Gel sectors reacted with affinity-purified antibodies to eIF-2α and eIF-2β at 0, 5, and 10 min after heat stress inception; (D-F) gel sectors reacted with anti-eIF-4B at 0, 5, and 10 min after heat stress inception. Note that the separation of eIF-2α and eIF-2α(P) is small in C so that the two forms appear as a dumbbell. The location of the most basic eIF-4B form in this and subsequent panels is indicated by an arrow; the acidic forms are indicated with a bracket in D.

reduced to <10% of the control rate between 5 and 10 min of heat stress. The covalent modification status of initiation factor proteins eIF-2α, eIF-2β, and eIF-4B in cell lysates at parallel times were evaluated by IEF/SDS-PAGE and immunoblot analysis. As shown in Fig. 3, the phosphorylation of eIF-2α and dephosphorylation of eIF-4B show little change after 5 min at 45°C, but in the sample taken 10 min after heating they have become evident. The extent of eIF-4B dephosphorylation between experiments is variable and was relatively low in this experiment.

Protein phosphorylation changes during the induction of heat stress were also measured by [32P]phosphate labeling (Fig. 4). These analyses likewise show that the phosphorylation of eIF-2α and dephosphorylation of eIF-4B commence between 5 and 10 min after heating. Little change in the pattern was detectable at 5 min (data not shown), whereas at 10 min the phosphorylation of eIF-2α was evident, as was enhanced phosphorylation of HSP 28 (Fig. 4 B). Additional samples were removed at intervals up to 25 min after transfer. For most proteins, including eIF-2α and eIF-4B, the major phosphorylation changes were complete by 10 min, though for a few, notably HSP 28, labeling continued to increase (data not shown). The kinetic correlations between protein synthesis inhibition and covalent modification changes in the initiation factor proteins are consistent with a causal relationship under these heat stress conditions.

The Relationship between Heat Stress Severity and eIF Protein Phosphorylation Changes. Our original heat stress analyses (Duncan and Hershey, 1984a) were performed at 45°C. This temperature has been reported to be a useful, adequate choice to cause a reproducible inhibition of protein synthesis (Slater et al., 1981; Mizzen and Welch, 1988). Based on the observations reported above documenting an extensive inhibition of protein synthesis at 41°C in suspension culture, we decided to more thoroughly characterize the effects of mild heat stress on monolayer cultures. Polysome analyses using cells in 60-mm dishes established that 41°C inhibited ribosomes in polysomes to >30%, parallelizing the suspension culture results. Analyses of the covalent modification status of initiation factor proteins were carried out in cultures heat stressed at a range of increasing temperatures. Little or no alteration in eIF-2α phosphorylation is detected at temperatures <43°C (Fig. 5, compare A and B [41°C] and C and D [42°C], which shows two independent cell cultures). Very minor, but detectable, eIF-4B dephosphorylation is variably observed during mild stress. In Fig. 5 it is detectable in the 41°C culture but not in the 42°C culture. Protein synthesis, as monitored by polysome disaggregation, becomes inhibited by >70% at temperatures of 41–42°C. The eIF protein modification changes begin to be detected at temperatures >43°C and become more pronounced only when higher temperatures are reached (Fig. 5, E–G); in other words, only during severe heat stress. Greater than 20% of eIF-2α becomes phosphorylated, the most acidic eIF-4B variants disappear, and the abundance of the central group becomes skewed towards the basic end. In summary, these results indicate that the inhibition of protein synthesis does not require a change in the covalent modification status of either eIF-2α or eIF-4B; it thus seems unlikely that either of these changes represents the primary molecular mechanism by which heat inhibits protein synthesis (this question is further addressed in the Discussion). eIF-2β was also analyzed
Figure 4. $^{32}$P-protein phosphorylation changes during the induction of heat stress. Cells were heat stressed by transfer into a 46°C air incubator and processed as described in Materials and Methods. $^{32}$P-Labeled proteins from cells (A) before heat stress and (B) after 10 min of heating. The locations of eIF-4B, eIF-2α(P), and HSP 28 are indicated.
Figure 5. Induction of initiation factor protein modification changes at different severities of heat stress. HeLa cells were heat stressed at different temperatures for 30 min and then proteins were extracted and analyzed by IEF/SDS-PAGE and immunoblotting. Analyses from three separate experiments are shown. (A and B) 37°C and heat stress at 41°C, respectively; (C and D) 37°C and heat stress at 42°C, respectively; (E-G) heat stress at 44, 46, and 48°C, respectively. The left column shows the eIF-2 analyses and the right column shows the eIF-4B analyses. The position where the unmodified form of eIF-2α runs in each grouping is indicated with a bar.
in these studies. No consistent changes were detected. In some samples, eIF-2β reacts poorly, particularly during more severe heat stress, consistent with enhanced proteolytic activity in heat stressed cells or extracts.

The Recovery from Heat Stress. In previous work we observed that after 2 h of recovery from a 45°C heat stress, protein synthesis had completely recovered (Duncan and Hershey, 1984a), and, likewise, the phosphorylation changes in eIF-2α and eIF-4B were reversed. These observations were consistent with the factor modification changes contributing to or causing the heat stress inhibition.

To investigate the correlation in greater detail, kinetic comparisons of protein synthesis rate and covalent modification state during recovery were made at earlier times and at different stress temperatures. When cells were given a mild to moderate heat stress (41-43°C) for 30 min, recovery occurred within 1-2 min (i.e., the shortest time interval we could evaluate) and initiation factor modification changes during heat stress induction or recovery were undetectable or very minor (as described above). In cells stressed at 44-45°C, recovery also occurred rapidly, and, in all instances, eIF-2α dephosphorylation and eIF-4B rephosphorylation occurred concurrently. We always observe the phosphorylations of these factors returning to their 37°C status when protein synthetic activity becomes restored. As more severe heat stresses (>45°C) are applied, we begin to detect slower measurable kinetics of recovery. In many of these cell samples, initiation factor protein modifications are completely reversed at a time when protein synthesis remains inhibited by 30-70%. Shown in Fig. 6 is an example of a culture very severely heat stressed at 49°C for 30 min. Immuno-blot analysis of extracts removed over the ensuing 4 h of recovery at 37°C shows that phosphorylation changes reverse rapidly within 30 min (compare Fig. 6, first two rows) while protein synthesis remains 60% inhibited. 32P-labeling analyses likewise indicated that the initiation factor phosphoryla-

| RECOVERY [MIN at 37°C] | 2 | 4B | INCORPORATION [% CONTROL] |
|------------------------|---|----|----------------------------|
| 0                      |   |    | 10                          |
| 30                     |   |    | 40                          |
| 90                     |   |    | 60                          |
| 180                    |   |    | 90                          |

Figure 6. Changes in initiation factor protein modification during recovery from heat stress. Cells were prepared for analysis as described in Materials and Methods. Cells were heat stressed by transfer to an air incubator at 49°C for 30 min and then transferred back to a 37°C air incubator. Replicate tissue plates were removed at the intervals indicated. The left column shows the eIF-2 analyses and the right column shows the eIF-4B analyses. The time of recovery is indicated to the left and the amino acid incorporation rate of a parallel sample relative to the before stress 37°C sample is indicated to the right.
Phosphorylation Changes during Inhibition, Recovery, and Adaptation in Suspension-cultured Cells. Examinations by others of the heat stress inhibition of protein synthesis in HeLa cells have used suspension-cultured cells (McCormick and Penman, 1969; Hickey and Weber, 1982; Slater et al., 1981; Mizzen and Welch, 1988). To assess whether a better correlation between initiation factor modification changes and protein synthesis inhibition occurred in suspension-cultured cells, some of the above experiments were repeated. The characteristics of polysome disaggrega-

Figure 7. Phosphorylation changes in suspension-cultured cells during heat stress (adaptation protocol) at 41°C. Cells were heat stressed in a water bath set at 41°C as described in Materials and Methods. The left column shows the eIF-2 analyses and the right column shows the eIF-4B analyses.

tion states are reversed rapidly after severe heat stress (data not shown). It is important to point out that severe heat stress has not simply incapacitated the protein synthesis machinery, as evidenced by the fact that by 3 h protein synthesis has recovered to ~90% of the control rate. The initiation factor proteins remain in their active states (Fig. 6, lower rows). This leads to the conclusion that other elements capable of inhibiting protein synthesis must exist. These unknown or unidentified factors require a longer interval to be restored to their prestress, active state.
tion, recovery, and adaptation in mildly stressed suspension-cultured cells have been detailed above. Samples were removed from these suspension cultures at various times, and the covalent modification status of the initiation factor proteins was monitored. In accord with the results obtained with the monolayer-cultured cells, during heat stress (adaptation protocol) at 41°C there is little or no detectable alteration in eIF-2α or eIF-4B protein modification (Fig. 7; a very minor eIF-4B dephosphorylation is detected, but the majority of the most acidic variants remain and the acidic members of the central group remain the most abundant). Thus, during this sequence of protein synthesis inhibition and restoration there is no evidence for the involvement of eIF-2α or eIF-4B covalent modification changes.

These results suggest that we must direct attention toward discovering new molecular alterations that may account for the inhibition of the protein synthesis machinery. The small subunit of eIF-4F, termed eIF-4E or the cap-binding protein, exists in 37°C cells in two forms. The phosphorylated variant comprises 30-50% of the mass (Rychlik et al., 1986; Duncan et al., 1987). During heat stress at 44-45°C, dephosphorylation occurs such that virtually all of the eIF-4E lacks phosphate (Duncan et al., 1987). We ask whether this modification is a candidate for the change induced at 41-42°C that leads to inhibition during mild stress. The phosphorylation state of eIF-4E was examined by passing crude cytoplasmic lysates (S10s) over an m7GTP-Sepharose affinity column to isolate eIF-4E. Analysis of eIF-4E retained by the column by IEF/SDS-PAGE and silver staining shows that transfer of 37°C cells to 41°C for 30 min does not cause dephosphorylation (Fig. 8, A and B). This corresponds to the time of maximum protein synthesis inhibition. eIF-4E analyzed from cells incubated at 41°C for 4 h, after adaptation has occurred, likewise has the same distribution of forms (Fig. 8 C). A parallel cell culture heat stressed at 44°C shows a virtually complete loss of the phosphorylated form spot (Fig. 8 D) as previously reported. Thus, we conclude that eIF-4E changes cannot be involved in the mild stress inhibitory mechanism.

Discussion

Heat stress provides a powerful means to investigate mechanisms of translational control (e.g., Ernst et al., 1982; DiDomenico et al., 1982; Duncan and Hershey, 1984a; Panniers and Henshaw, 1984; Panniers et al., 1985). Polysome profile analyses of heat stressed HeLa cells provide a clear documentation of the 41°C inhibition of protein synthesis and establish this as a useful system. Similar results have been reported by McCormick and Penman (1969). Examination of many initiation factor proteins for covalent modification or mass changes suggests that neither mediates protein synthesis repression under these conditions. This contrasts with results obtained at more severe heat stress temperatures, where several modification changes occur (Ernst et al., 1982; Duncan and Hershey, 1984a; DeBenedetti and Baglioni, 1986; Duncan et al., 1987; Scorsone et al., 1987).

Protein synthesis adapts to mild heat stress temperatures. Cells maintained at 41°C increase protein synthesis rates over several hours, ultimately approximating the control rate. Similar adaptation results were previously reported by McCormick and Penman (1969) for HeLa cells and, likewise, occur in many other cell types (e.g., Neidhardt et al., 1984; McMullin and Hallberg, 1986; Altschuler and Mascarenhas, 1982). The ability of heat stress proteins or other heat-induced changes to provide thermoprotection was examined. Two main features of protein synthesis regulation were unaffected: (a) the inhibition upon transfer to mild heat temperatures occurred to an equal extent and (b) adaptation proceeded with equal kinetics and extent. Thus, heat stress protein accumulation cannot be the sole basis for adaptive restoration. In a similar vein, RNA synthesis appears to be required for adaptation, but prestress to preload cells with putative heat stress–induced RNAs is not sufficient to allow adaptive restoration.

The reason why no thermoprotection is observed is not resolved, but we can eliminate several basic critical possibilities. Heat stress proteins were synthesized in amounts and
with kinetics previously described to confer thermotolerance. The most complete documentation of these parameters may be found in Scandra and Subjeck (1984), where Chinese hamster ovary cells were used. Our amounts and kinetics closely parallel theirs. Using an assay based on recovery after a moderate to severe heat stress, Scandra and Subjeck found 80–90% maximum thermotolerance is acquired during 3 h of recovery from prestress (100% by 4 h). Similarly, Mizzen and Welch (1988) found thermoprotection develops within 4 h of recovery from prestress. We detect no evidence for thermoprotection at 3 h and, likewise, no evidence for it after 10 h of recovery, even though our heat stress protein accumulation profiles are similar. We hypothesize that our results differ because adaptation to heat is a fundamentally different process from protection from lethal heat-induced molecular alterations, as elaborated below.

The inability of the prestress protocol to provide thermoprotection implies that the thermoprotected state doesn't persist at 37°C. Contrast two cell cultures, both assayed for protein synthesis activity 3.5 h after temperature shift: one culture was raised to 41°C and maintained there (adapted cells) and synthesizes protein rapidly at 41°C; the other culture was raised to 41°C for 30 min and then returned to 37°C for 3 h and cannot synthesize protein at 41°C. That is, the challenge 41°C heat stress at 3.5 h after the initial heating causes extensive polysome disruption. The simplest interpretation is that the molecular changes conferring heat-resistant translation decay rapidly at 37°C. RNAs that are rapidly induced during heating and rapidly degraded during recovery have been identified (Krauss et al., 1987). This would also apply to other potential, as yet unidentified, components of the adaptive response. The precise kinetics of thermotolerance acquisition and decay should be measurable using the adaptation protocol and might provide a basis for the isolation and characterization of the active component(s).

The adaptation process can be clearly differentiated from recovery because the component(s) inhibited by mild heat stress is readily reactivated upon return to 37°C without new RNA synthesis. In contrast, in cells adapting to 41°C growth, maximal protein synthesis restoration requires ongoing RNA synthesis. This suggests that there are two events required for adaptation: reactivation (or bypass) of the inhibited translational component and RNA synthesis. It is not clear how the two events are linked or if there is no link at all. Identification of the translational lesion at 41–42°C will provide a useful focus to approach these questions.

Adaptation to mild heat produces different molecular changes and has different thermoprotection characteristics via a vis moderate to severe heat stress. We believe that the adaptation protocol provides a valuable model for the sorts of heat stress encountered by organisms in their environment. It addresses a physiologically relevant form of thermoprotection. The more commonly used protocol for assessing thermotolerance (or "acquired thermotolerance") involves an initial priming stress followed by a recovery interval and then a relatively severe, usually transient, challenge heat stress. This protocol has experimental usefulness since thermoprotection (thermotolerance) of many cell processes, including protein synthesis (Petersen and Mitchell, 1981; Subjeck et al., 1982; Subjeck and Scandra, 1984), cell viability (Hahn and Li, 1982; Li, 1983; Li and Zerb, 1982), and cell morphology (the inhibition of phenocopies [Mitchell et al., 1979]), can be demonstrated. A principal rationale for this protocol is that the severe challenge heat stress emphasizes differences.

It seems likely, however, that this protocol may muddle specific molecular pathways. For example, our results indicate that different inhibitory pathways regulate protein synthesis at different heat stress severities (expanded upon below). Which, if any, of these is protected by this protocol? We hypothesize that thermoprotection to a severe challenge stress may occur principally by the elaboration of global, nonspecific thermoprotective agents (e.g., HSP 70 and HSP 90 functioning as molecular "nurses" for wounded molecules [Pelham, 1986]). This form of thermoprotection is not sufficient to provide function at the severe heat stress temperature; it, however, indiscriminately protects function, regardless of pathway, and facilitates rapid recovery. This is clearly a very different form of thermoprotection from adaptation, where specific molecular pathways are altered to provide function at a normally nonpermissive temperature.

**Covalent Modification Changes during Heat Stress and Restoration**

The second focus of this project is to characterize molecular alterations that contribute to or cause the heat stress inhibition. The results suggest that a novel unrecognized mechanism must be involved. Previous results from this and other laboratories have focused on heat stress-induced phosphorylation of eIF-2α (Ernst et al., 1982; Duncan and Hershey, 1984a; DeBenedetti and Baglioni, 1986). However, we find that less severe heat stress that inhibits protein synthesis by >70% does not elicit this change. Similarly, Mariano and Siekierka (1986) have concluded that 42°C heat stress of HeLa cells does not induce eIF-2α phosphorylation, using a different and complementary assay procedure. Results reported here also indicate that the eIF-4B and eIF-4E dephosphorylations observed during severe heat stress are not detected at the mild temperatures.

It seems inescapable that other alterations in the translational machinery must be occurring that are capable of repressing translation and that may constitute the primary cause of heat stress–induced inhibition. An attractive candidate for a regulatory factor is eIF-2B, the guanine nucleotide exchange factor. Its protein composition has been well characterized, defined functional assays exist, certain subunits are known to be phosphorylated (Konieczny and Safer, 1983; Dholakia and Wahba, 1988), phosphorylation influences activity in vitro (Dholakia and Wahba, 1988), and evidence suggests that eIF-2B activity is limiting in certain translationally repressed situations (Person et al., 1984).

The question of what is the "primary cause" of the heat stress inhibition poses a semantic problem. The phosphorylation of eIF-2α that we and others have observed almost cer-
certainly is sufficient to inhibit the translation of normal mRNAs in the severely heat stressed cell. Similarly, heat stress leads to the dephosphorylation of eIF-4B and eIF-4E (Duncan and Hershey, 1984c; Duncan et al., 1987), and the activities of these factors are also thought to be inhibited (Panniers et al., 1985; Duncan and Hershey, 1984a). These dephosphorylations may also contribute to or be sufficient to cause the inhibition of protein synthesis at the mRNA-binding step in severely heat stressed cells. In the experiments described here, we provide evidence for a second, as yet undefined, inhibitory mechanism. We cannot assess whether the component(s) inhibited at 41°C remains inhibited at 45°C until we understand the molecular mechanism involved. Thus, the question of primary cause during severe heat stress cannot yet be resolved, but, since the mild stress response is activated at lower temperatures, it seems best considered as primary cause.

Finally, we should ask if neither eIF-2α phosphorylation nor eIF-4B or eIF-4E dephosphorylation are necessary for translational repression, what is the physiological significance of these protein modifications in the more severely heat stressed cell? On the one hand, the modifications almost certainly are sufficient to produce severe protein synthesis inhibition based on in vitro assays (Matts and London, 1984; Clemens et al., 1982). Observations made in vivo correlate such changes with inhibition of protein synthesis induced by serum depletion (Duncan and Hershey, 1985), serum removal (Duncan and Hershey, 1987), amino acid depletion (Clemens et al., 1987), or mitosis (Bonneau and Sonenberg, 1987). Furthermore, an inhibitory role for eIF-2α phosphorylation by the double-stranded RNA-regulated eIF-2α kinase has been demonstrated in vivo by using cells transfected with a cDNA encoding a mutant form of eIF-2α that cannot be phosphorylated (Kaufman et al., 1989). We therefore believe that the heat-induced changes in eIF-2α, eIF-4B, and eIF-4E are physiologically relevant. How the primary cause mechanism complements or makes redundant the second mechanism involving eIF-2α, eIF-4B, and eIF-4F is an interesting problem whose solution will require a better characterization of these apparently independent pathways. One hypothesis to explain why the second severe heat stress mechanism exists is that it provides another layer of translational control.

If we assume that the function of the heat-induced inhibition is to prevent the accumulation of aberrant proteins, then the adaptation response implies that mechanisms exist to compensate for mild heat-induced translation errors. If these compensatory changes are unable to ensure fidelity during severe stress, then the cell would require a superceding inhibitory path way that cannot be "adapted." The eIF-2α phosphorylation pathway, and perhaps the eIF-4B and 4E pathways, admirably fill this role.

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