# Modulation of Heat-Shock Polypeptide Synthesis in HeLa Cells during Hyperthermia and Recovery†

Eileen D. Hickey* and Lee A. Weber

ABSTRACT: In HeLa cells incubated at 42 °C, the rate of protein synthesis undergoes a rapid reduction, followed by a gradual increase over the next few hours [McCormick, W., & Penman, S. (1969) *J. Mol. Biol. 39*, 315]. In the present study, we show that the recovery of protein synthesis during hyperthermia corresponds to the increased synthesis of three size classes of heat-shock polypeptides ($M_r$ 27 000, 70 000, and 80 000). The 70 000 and 27 000 proteins are resolved into multiple components by two-dimensional gel electrophoresis. The 80 000 protein appears to be a single polypeptide and is synthesized at a substantial rate at 37 °C. Induction of the heat-shock polypeptides, and the accumulation of mRNA coding for in vitro polypeptides with the same electrophoretic mobility, can be blocked with actinomycin D, indicating that new RNA synthesis is required for induction. Heat-shock polypeptides are produced at high rates for only the first 4–6 h of hyperthermia, after which the rate of synthesis is greatly reduced. A similar decline is observed when cells subjected to 2 h at 42 °C are returned to 37 °C. In both cases, the change in synthesis is paralleled by a loss of translatable heat-shock mRNA. Experiments using cycloheximide to inhibit the rate of polypeptide chain elongation indicate that the concentration of heat-shock mRNA alone cannot account for the relative rate of heat-shock polypeptide synthesis at 42 °C. Synthesis of heat-shock proteins is much more resistant than that of other polypeptides to inhibition of initiation brought about by hypertonic stress. Thus, it appears that the modulation of heat-shock protein synthesis during hyperthermia and recovery involves the accumulation and subsequent inactivation of highly efficient mRNAs. The heat-shock mRNAs are translated preferentially under the conditions of reduced polypeptide chain initiation that occur at elevated temperatures.

The effects of hyperthermia on the rate of protein synthesis have been well characterized in HeLa cells by Penman and co-workers (McCormick & Penman, 1969; Goldstein & Penman, 1973; Goldstein et al., 1974). Amino acid incorporation is rapidly inhibited upon shifting the incubation temperature from 37 to 42 °C (McCormick & Penman, 1969). Protein synthesis recovers when the cells are transferred back to 37 °C. If cells are maintained at 42 °C for several hours, the rate of protein synthesis at the higher temperature gradually increases. Polyribosome size and number, which are initially reduced at 42 °C, increase during the recovery of protein synthesis although control levels are not completely restored. The increase in protein synthesis and re-formation of large polyribosomes at 42 °C does not occur when RNA synthesis is inhibited by actinomycin D, while recovery of protein synthesis following return to 37 °C occurs even in the presence of the inhibitor. These results indicate that hyperthermia causes a specific lesion in protein synthesis at the level of polypeptide chain initiation, which can be reversed either by restoration of normal temperature or by RNA synthesis at 42 °C.

In the present study, we show that the recovery of protein synthesis during incubation at 42 °C corresponds to the increased synthesis of specific heat-shock polypeptides (HSP)[1] similar to those found in *Drosophila* [reviewed by Ashburner & Bonner (1979)] and other eukaryotes (Kelley & Schlesinger, 1978; Giudice et al., 1980; Barnett et al., 1980; Loomis & Wheeler, 1980; Walsh, 1980). Most other cellular proteins continue to be synthesized at low rates. The HSP comprise 20–40% of the total cellular amino acid incorporation activity after 2 h of heat treatment. However, HSP synthesis rapidly declines after 3–6 h at 42 °C and also following a return to 37 °C.

Experiments presented here were directed toward understanding how HSP synthesis is modulated during prolonged hyperthermia and recovery at 37 °C. The relationship between the rate of HSP synthesis and the level of cytoplasmic mRNA encoding the polypeptides was studied by in vitro translation. We also present evidence that HSP mRNA is translated preferentially relative to other cellular messages at 42 °C and suggest that this is a consequence of the reduced rate of initiation at elevated temperatures.

## Experimental Procedures

*Cell Culture and Heat Shock.* HeLa cells were maintained in mid-log phase in Spinner culture in Jokliks modified Eagle's medium plus 5% calf serum. Heat shock was effected by submerging culture flasks with agitation in a 46 °C water bath and following the temperature change on a thermometer immersed in the culture. Small cultures of 50–200 mL reach 42 °C in less than 2 min. A few experiments required heat shock of 1-L cultures, which required 6–8 min to reach 42 °C. Temperatures were maintained in a water bath placed on a magnetic stirrer. The cells used were consistently at a concentration of (3–5) × $10^5$ cells/mL and had been fed the evening before use. Viable cell number was determined by Trypan blue exclusion.

*Analysis of Polypeptide Patterns by Polyacrylamide Gel Electrophoresis.* For assay of proteins synthesized in vivo, cells were concentrated to (2–3) × $10^6$ cells/mL by centrifugation and resuspension in prewarmed leucine- or methionine-free medium plus 5% dialyzed calf serum and containing 10 μCi/mL either of [$^{35}$S]methionine (NEN) or of [$^3$H]leucine (RPI). [$^{35}$S]Methionine labeling produced the highest quality autoradiograms for densitometry. [$^3$H]Leucine was the label of choice for most experiments, however, because the low

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[1] Abbreviations: HSP, heat-shock polypeptide(s); $Cl_3AcOH$, trichloroacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$ (SDS in the figures), sodium dodecyl sulfate.

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molecular weight heat-shock polypeptide did not incorporate detectable amounts of methionine in vivo. Incubation was carried out in a water bath with agitation for 30 min. Duplicate 25- or 50-$\mu$L samples were spotted on filter paper for analysis of $Cl_3AcOH$-precipitable incorporation (Weber et al., 1975). The assay was terminated by dilution of the remainder of the incubation mixture with 10 volumes of ice-cold saline. The cells were pelleted and resuspended in 0.2 mL of sample buffer for polyacrylamide gel electrophoresis, boiled 6 min, and analyzed on 12.5% polyacrylamide slab gels according to Laemmli (1970). Two-dimensional polyacrylamide gel electrophoresis of $NaDodSO_4$-denatured samples was performed according to O'Farrell (1975). For the first dimension, ampholytes with a range of pH 5–7 (Bio-Rad) were used. Gels were stained with Coomassie blue and impregnated with En$^3$Hance (NEN) before drying for fluorography at −70 °C on prefogged Kodak XR-5 XOmat film (Laskey & Mills, 1975). Gels containing $^{35}S$ radioactivity were dried and autoradiographed directly at room temperature by using Kodak SB-5 film. Autoradiograms and fluorograms were analyzed with a Gelman ACD-18 automatic computing densitometer. Care was taken to assure that exposures were within the linear range of the film.

*Partial Proteolytic Digestion.* Lysates were prepared from cells labeled with 10 $\mu$Ci/mL [$^{35}S$]methionine for 1 h and subjected to one-dimensional polyacrylamide gel electrophoresis as described above. Each lane contained either 800 000 cpm of control cell protein or 400 000 cpm of protein from heat-shocked cells. A sample prepared from cells incubated for 24 h at 42 °C was run in parallel. The gels were briefly stained with Coomassie blue. The regions corresponding to the stained 70- and 80-kilodalton heat-shock proteins visible in the 24-h heat-shock sample were excised with a scalpel from each of the lanes containing radioactive sample. Accuracy was verified by subsequent autoradiography of the remainder of the gel. The excised gel bands were placed in the wells of 15% acrylamide gels and subjected to limited proteolysis with staphylococcal V8 protease and electrophoresis according to Cleveland et al. (1977). The gels were fixed in 10% $Cl_3AcOH$, stained, and fluorographed as described above.

*Isolation of RNA.* For preparation of total or polyadenylated cytoplasmic RNA, cultures of 50–100 mL were used. After treatment of the cultures as indicated, 5–10-mL samples were concentrated for labeling of polypeptides in vivo. The remainder were washed 3 times in ice-cold saline and lysed by 10 strokes of the tight-fitting pestle of a Dounce homogenizer in ice-cold lysis buffer [10 mM KCl, 1.5 mM $Mg(OAc)_2$, 20 mM Hepes, pH 7.4, and 0.5 mM dithiothreitol]. Nuclei were removed by low-speed centrifugation, and the supernatant was adjusted to contain 1% $NaDodSO_4$ and subjected to extraction with phenol–chloroform–isoamyl alcohol, 25:24:1, as described (Weber et al., 1979). RNA was precipitated with ethanol and separated into polyadenylated and nonpolyadenylated fractions by chromatography on oligo(dT)–cellulose (Pemberton et al., 1975). Preliminary experiments showed that the mRNAs for the heat-shock proteins were found only in the polyadenylated fraction. In most experiments total RNA was translated. The total cytoplasmic RNA, or the polyadenylated fraction, was repeatedly precipitated from ethanol before use in translation assays.

*Cell-Free Protein Synthesis.* Control or heat-shock RNAs were translated in the nuclease-treated reticulocyte cell-free protein synthesizing system (Pelham & Jackson, 1976). The RNA concentrations in translation assays were always within the range at which protein synthesis was proportional to RNA input. Use of subsaturating amounts of RNA was important to minimize effects of RNA competition. Assays were diluted 5-fold with Laemmli gel sample buffer and boiled for 2 min prior to analysis of the translation products by polyacrylamide gel electrophoresis.

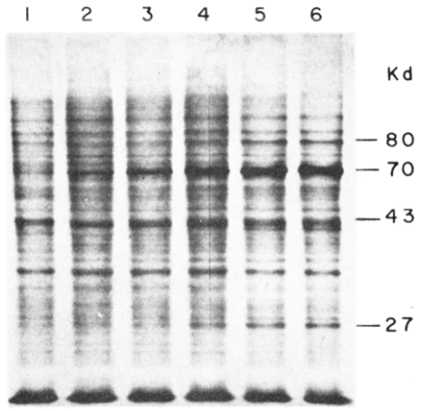


FIGURE 1: Pattern of polypeptide synthesis during heat shock. HeLa cells at $5 \times 10^5$ cells/mL were rapidly brought to 42 °C. At zero time (37 °C), and at intervals at the elevated temperature, 10-mL samples of cells were concentrated 10-fold in leucine-free Jokliks medium with 5% dialyzed calf serum and 10 $\mu$Ci/mL [$^3H$]leucine and incubated for 30 min at 37 °C (zero time) or 42 °C. Analysis of polypeptide synthesis by electrophoresis and fluorography was performed as described under Experimental Procedures. Equal amounts of radioactivity were applied to each well (20 000 cpm). Lane 1, zero time, cells incubated at 37 °C; lanes 2, 3, 4, 5, and 6, cells incubated at 42 °C for 10 min, 30 min, 1 h, 2 h, and 3 h, respectively. The band marked p43 is a major product of protein synthesis at 37 °C and is noted for comparison.

*Sources.* Actinomycin D and cycloheximide were obtained from Sigma, and V8 protease was obtained from Miles Biochemicals. Molecular weight markers consisted of a mixture of phosphorylase kinase containing three subunits of 145 000, 128 000, and 45 000 (a gift from Dr. Gerald Carlson) and bovine albumin (66 000), egg albumin (45 000), pepsin (34 700), trypsinogen (24 000), $\beta$-lactoglobulin (18 400), and lysozyme (14 300) from Sigma.

## Results

*Polypeptide Synthesis in HeLa Cells during Heat Shock.* In preliminary experiments (data not shown) we established that when HeLa cells were transferred from 37 to 42 °C, incorporation of either methionine or leucine was reduced to 10–20% of control levels within 10 min. During continued incubation at 42 °C, incorporation gradually increased to between 30% and 50% of the control rate. Upon return of the cells to 37 °C after 1 h at 42 °C, protein synthesis was restored to control levels within 30 min. Following longer incubation times at the elevated temperature, the time required to recover control levels of incorporation at 37 °C increases. These results are in agreement with those published by McCormick & Penman (1969).

The recovery of protein synthesis at 42 °C corresponds to the increased labeling of three major polypeptide bands as resolved by one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 1 shows the pattern of polypeptides labeled for 30 min with [$^3H$]leucine in vivo at intervals during the first 3 h of heat shock. Equal amounts of radioactivity were applied to each lane to facilitate comparison of relative rates of synthesis of different polypeptides. Thus, lane 2 contains 5-fold more protein than lane 1. The similar intensity of labeling of the polypeptide bands in lanes 1 and 2 reflects the inhibition of synthesis of most cellular polypeptides (hereafter called 37 °C polypeptides) at 42 °C. Labeling of

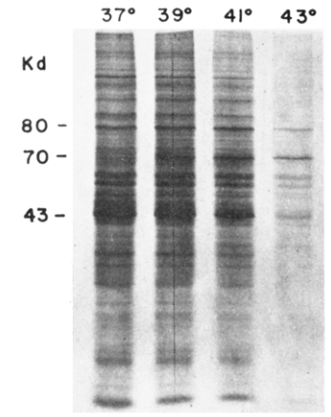


FIGURE 2: Minimum temperature for induction of heat-shock polypeptide synthesis. HeLa cells at $3 \times 10^5$ cells/mL were incubated at the temperatures indicated for 1 h. Cells were concentrated 10-fold in methionine-free Jokliks medium supplemented with 5% dialyzed calf serum and 10 $\mu$Ci/mL [$^{35}$S]methionine and labeled at the individual incubation temperature for 30 min. Analysis of polypeptide products by electrophoresis and autoradiography was as described under Experimental Procedures. Ten-microliter samples were applied to each well and contained the following: for 37 °C, 151 000 cpm; for 39 °C, 146 000 cpm; for 41 °C, 99 000 cpm; for 43 °C, 28 000 cpm.

polypeptides at $M_r$ 80 000, 70 000, and 27 000 gradually increases during the 3 h at 42 °C. These polypeptides will be called heat-shock polypeptides (HSP) p80, p70, and p27. Detectable induction of synthesis of p70 is already apparent during the earliest labeling interval (from 10 to 40 min; lane 2). After the first hour of hyperthermia, the HSP are major products of cellular protein synthesis. The intensity of labeling of the heat-shock bands after 2 h at 42 °C can vary as much as 2-fold among different groups of cells. The basis of this variation is not yet understood.

Increased synthesis of HSP is detected when cells are incubated at temperatures only slightly above normal. Figure 2 shows the pattern of polypeptides labeled with [$^{35}$S]-methionine following a 60-min incubation at various temperatures. The HSP p27 is not evident on this autoradiogram because it does not incorporate methionine significantly. Enhanced synthesis of HSP p70 can be seen at 39 °C, a temperature well within the range of common fever. Inhibition of synthesis of 37 °C polypeptides does not occur until higher temperatures are reached. We have consistently found that amino acid incorporation is first inhibited (by 20%) at 40 °C and drops rapidly at higher temperatures, until at 45 °C no incorporation is detectable (data not shown).

For determination of whether HSP are synthesized to any extent at 37 °C, labeled polypeptides were resolved by two-dimensional polyacrylamide gel electrophoresis as described by O'Farrell (1975). As shown in Figure 3b, HSP p80 migrates as a single spot in this system. However, p70 is resolved by the first dimension into a cluster of five components with different isoelectric points. The most basic and most acidic members of the cluster are labeled to a lesser extent than the members with intermediate isoelectric points. The three intermediate members of the p70 cluster are each labeled as intensely as any other polypeptide displayed on the fluorogram. HSP p27 is resolved into two components which have distinctly different isoelectric points, with the most basic polypeptide labeled more strongly. Relative synthesis of another polypeptide ($M_r \cong 76\,000$) with an isoelectric point slightly more acidic than p80 also increases at 42 °C (Figure 3). The intensity of labeling of this polypeptide (marked r in Figure 3) is not affected by inhibition of RNA synthesis during heat

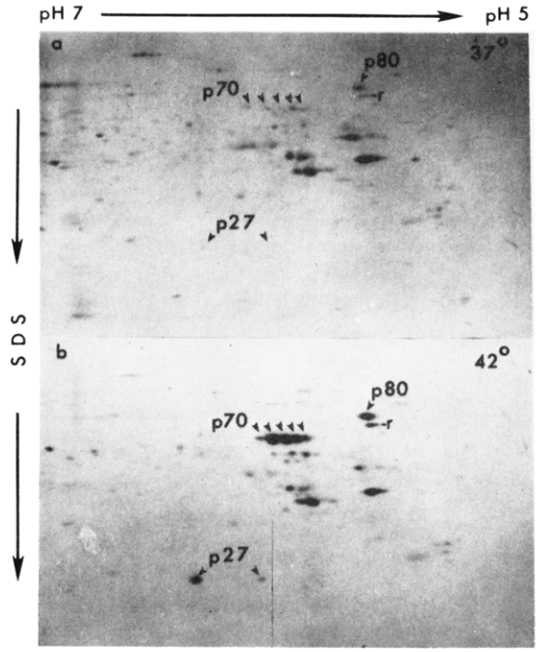


FIGURE 3: Two-dimensional electrophoretic pattern of polypeptides synthesized by HeLa cells at 37 and 42 °C. HeLa cells at $3 \times 10^5$ cells/mL were sampled either at 37 °C or after 3 h at 42 °C, concentrated 10-fold, labeled with [$^3$H]leucine, and prepared for polyacrylamide gel electrophoresis as described in Figure 1. Samples were brought to 9 M urea and diluted with 1 volume of O'Farrell lysis buffer (O'Farrell, 1975), before isoelectric focusing in the range of pH 5–7. The electrophoresis in the second dimension and fluorography were as described under Experimental Procedures. Equal amounts of radioactivity were applied to both gels. (a) 37 °C cells; (b) 42 °C cells. Arrows: HSP or their corresponding positions on the fluorogram of the 37 °C cells. r: see the text.

shock (see below). Therefore, polypeptide r is not considered an HSP but rather a 37 °C polypeptide whose synthesis is relatively resistant to inhibition by hyperthermia. Polypeptides which behave similarly to r during heat shock have been described in *Drosophila* (Storti et al., 1980; Scott et al., 1980).

Analysis of polypeptides labeled at 37 °C (Figure 3a) reveals a weakly labeled spot at the same position as the more basic p27. A faint group of polypeptides with identical electrophoretic coordinates as some of the p70 cluster is also labeled at 37 °C. However, not all members of the cluster are detectably labeled at 37 °C. The single p80 polypeptide is labeled to a greater extent at 37 °C than the other HSP. A major Coomassie blue stained polypeptide is located at the same coordinates as p80 on both gels fluorographed in Figure 3 (data not shown). Thus, this analysis suggests that most of the HSP are synthesized to some extent at 37 °C and that p80 is an abundant cellular protein under normal growth conditions.

The identity of p80 and p70 synthesized in control and heat-shocked cells was established by partial proteolytic digestion with V8 protease according to Cleveland et al. (1977). Proteins were labeled with [$^{35}$S]methionine in either 37 °C or hyperthermic cultures and subjected to one-dimensional electrophoresis. The 70 000 and 80 000 regions were excised and subjected to a second round of electrophoresis in the presence of different concentrations of protease. As shown in Figure 4, the 80-kilodalton polypeptide made in control cells and the HSP p80 yield identical patterns of peptide fragments. In contrast, digestion of the 70-kilodalton region of the gel containing control cell proteins yields a diffuse pattern of fragments which would be expected from a mixture of heterogeneous proteins with similar molecular weights. However,

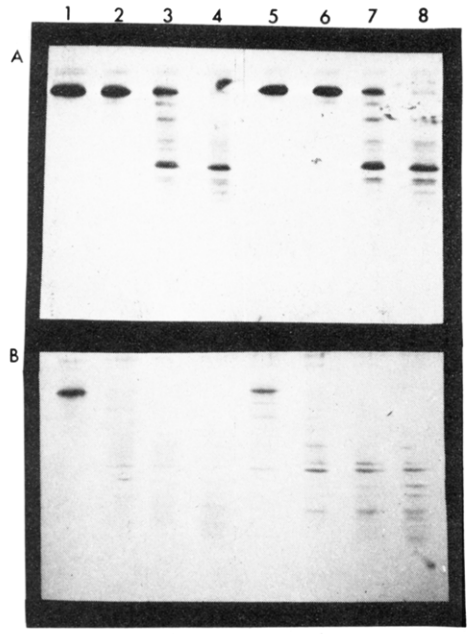


FIGURE 4: Partial proteolytic digestion pattern of p80 and p70. HeLa cells at $3 \times 10^5$ cells/mL were incubated at 37 °C or at 42 °C for 90 min and then concentrated 5-fold and labeled at the appropriate temperature for 60 min with 10 $\mu$Ci/mL [$^{35}$S]methionine. 800 000 cpm of 37 °C sample and 400 000 cpm of 42 °C sample were each loaded into eight separate wells and subjected to one-dimensional polyacrylamide gel electrophoresis as described. Following light staining with Coomassie blue, the 80- and 70-kilodalton regions of each lane were excised. These bands were then subjected to V8 proteolysis during electrophoresis according to Cleveland et al. (1977). (Panel A) Bands from the 80-kilodalton region. Lanes 1–4, sample from 37 °C cells; lanes 5–8, sample from 42 °C cells. Lanes 1 and 5, no V8 protease; lanes 2 and 6, protease at 1 ng/well; lanes 3 and 7, 25 ng/well; lanes 4 and 8, 100 ng/well. The fluorograph was exposed for 10 days. (Panel B) Bands excised from the 70-kilodalton region. Lanes 1–4, sample from 37 °C cells; lanes 5–8, sample from 42 °C cells. Lanes 1 and 5, no V8 protease; lanes 2 and 6, protease at 25 ng/well; lanes 3 and 7, 100 ng/well; lanes 4 and 8, 500 ng/well. The fluorograph was exposed for 7 days. The partial digestion apparent in lane 5 is due to diffusion of the protease from the adjacent well.

several peptide fragments with the same mobility as the HSP p70 fragments are apparent. The protease digestion allows visualization of discrete polypeptide fragments derived from the most abundant proteins in the mixture. The results demonstrate clearly that HSP p80 and at least some of the p70 family of HSP are synthesized at significant rates at 37 °C. The heat-shock response, therefore, involves a dramatic increase in the synthesis of proteins which are normal cellular constituents.

*Induction of HSP Synthesis Requires the Accumulation of Specific mRNAs.* In several organisms, induction of HSP synthesis has been shown to be inhibited when RNA synthesis is blocked by actinomycin D (Ashburner & Bonner, 1979; Kelley & Schlesinger, 1978). In Figure 5, evidence is presented showing that actinomycin D treatment prevents both the onset of HSP synthesis and the appearance of translatable mRNA encoding HSP. Cells were preincubated at 37 °C for 10 min in the presence or absence of actinomycin D and then incubated for 90 min at 42 °C. Total cytoplasmic RNA was isolated from both groups of cells as well as from control cells kept at 37 °C and translated in vitro in a rabbit reticulocyte translation system (Pelham & Jackson, 1976). The pattern of [$^3$H]leucine-labeled translation products was compared with that of polypeptides labeled in aliquots of the same cells in vivo (Figure 5). The results clearly show that RNA from heat-shocked cells encodes polypeptides with the same electrophoretic mobilities as p27, p70, and p80 synthesized in intact

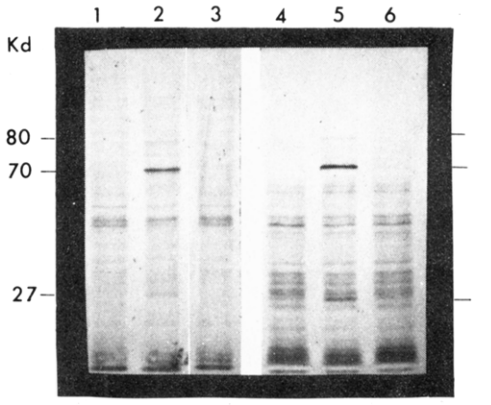


FIGURE 5: Effect of actinomycin D on appearance of translatable RNA for heat-shock polypeptides. HeLa cells at $5 \times 10^5$ cells/mL were incubated at 42 °C in the presence or absence of 5 $\mu$g/mL actinomycin D for 90 min or at 37 °C without actinomycin D. Cells were labeled with [$^3$H]leucine in vivo or used for preparation of RNA as described. Total cytoplasmic RNA was translated in the mRNA-dependent reticulocyte lysate at a concentration of 100 $\mu$g of RNA/mL. Equal radioactivity (27 000 cpm) was applied to each well, and electrophoresis and fluorography were as described for Figure 1. Lanes 1, 2, and 3: polypeptides labeled in vivo at 37 °C in the absence (lane 1), at 42 °C in the absence (lane 2), or at 42 °C in the presence (lane 3) of actinomycin D. Lanes 4, 5, and 6: translation products of poly(A)-containing RNA from cells incubated at 37 °C in the absence (lane 4), at 42 °C in the absence (lane 5), or at 42 °C in the presence (lane 6) of actinomycin D.

cells. Translation products of mRNA isolated from cells heat shocked in the presence of actinomycin D do not show increased amounts of these polypeptides. Rather, the pattern of in vitro polypeptides is identical with that of the translation products of mRNA from 37 °C control cells. These results indicate that the increase in HSP synthesis during hyperthermia depends upon the rapid accumulation of newly synthesized mRNA. Kelly et al. (1980) have reported similar increases in mRNAs encoding the major HSP in chicken embryo fibroblasts following heat stress.

The patterns of polypeptides translated in vitro indicate that control and heat-shocked cells contain similar amounts of mRNA coding for abundant 37 °C proteins. These data explain the observation that actinomycin D does not prevent the recovery of protein synthesis when heat-shocked cells are returned to 37 °C (McCormick & Penman, 1969; E. Hickey, unpublished observation). Apparently, the majority of mRNAs coding for 37 °C polypeptides are stable during heat shock but are translated at a reduced rate.

It should be noted that subsaturating concentrations of RNA were translated in the experiment shown in Figure 5. The concentration of RNA which maximally stimulates protein synthesis was first determined in a preliminary experiment. We find that total cytoplasmic RNA from control or heat-shocked cells stimulates equal [$^3$H]leucine incorporation per microgram of RNA. The concentration of RNA translated for polypeptide analysis is half the concentration which yields maximum protein synthesis. In this way we minimize the effects of competition among different mRNAs for translation (Storti et al., 1980). Nonetheless, the reticulocyte lysate does not translate all mRNAs with equal efficiency. There is a bias toward synthesis of the lower molecular weight polypeptides in vitro. This accounts in part for the more intense labeling of the in vitro polypeptides below 70 kilodaltons in Figure 5, despite the fact that equal amounts of radioactivity were applied to each lane of the gel.

*Synthesis of HSP and 37 °C Polypeptides during Prolonged Hyperthermia and Recovery at 37 °C.* HeLa cells survive

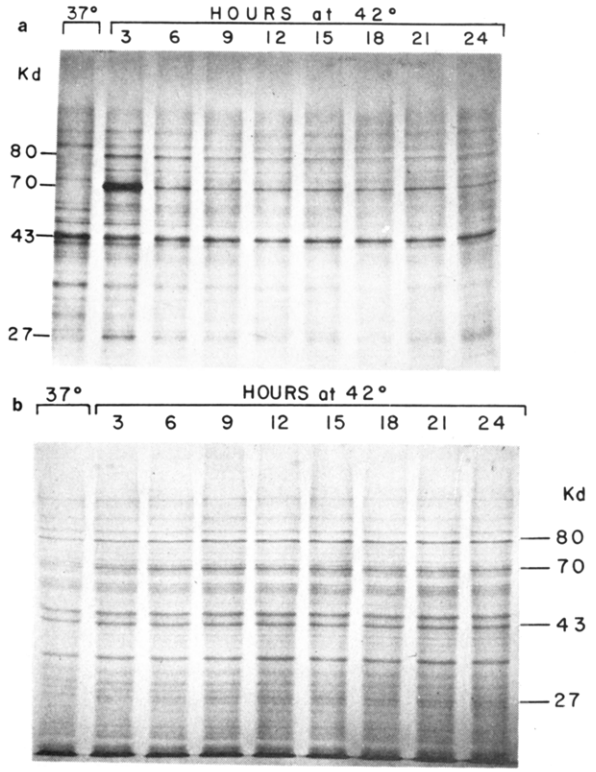


FIGURE 6: Synthesis of heat-shock polypeptides during 24 h at 42 °C. (a) HSP synthesis during 24 h at 42 °C. HeLa cells at $5 \times 10^5$ cells/mL were rapidly brought to 42 °C. 37 °C control cells were labeled as in Figure 1. At 3-h intervals, 10-mL aliquots of 42 °C cells were concentrated, labeled with [$^3$H]leucine, and electrophoresed as in Figure 1. Equal amounts of radioactivity (55 000 cpm) were applied to each well, and the gel was fluorographed as in Figure 1. (b) Coomassie blue stained proteins of HeLa cells during 24 h at 42 °C. Equal aliquots of the samples prepared as described for panel a were subjected to polyacrylamide gel electrophoresis as described. The gel was fixed for 30 min in 10% $Cl_3AcOH$ and then fixed and stained in 0.075% Coomassie blue in 10% acetic acid–50% methanol overnight. The gel was destained and photographed wet.

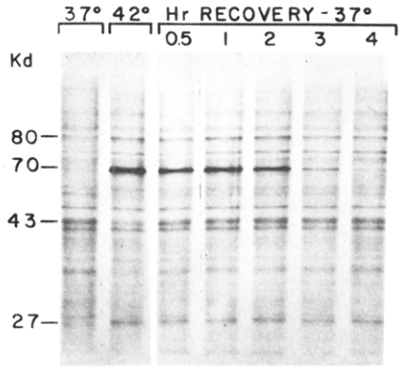


FIGURE 7: HSP synthesis during recovery at 37 °C. HeLa cells were incubated at $3 \times 10^5$ cells/mL for 90 min at 42 °C. Ten milliliters was removed for labeling at 42 °C, and the remaining cells were returned to 37 °C. Ten-milliliter samples were taken for labeling at the times indicated, labeled, electrophoresed, and fluorographed as above. 55 000 cpm was applied to each track of the gel.

incubation at 42 °C for periods of at least 24 h (Waroquier & Scherrer, 1969; E. Hickey, unpublished observation). The number of viable cells after such long-term heat shock is reduced by no more than 20%. Growth is arrested for about 1 day after return of these cells to 37 °C and then resumes at a normal rate (our unpublished results). Analysis of the pattern of polypeptides labeled in vivo for 30 min at intervals during 24 h at 42 °C shows that labeling of the HSP, particularly p70, diminishes between 3 and 6 h of heat shock (Figure 6a) and proceeds at a greatly reduced rate. This occurs without any change in the rate of amino acid incorporation for the duration of the experiment. The Coomassie blue staining pattern of the gel shown in Figure 6b indicates accumulation of a polypeptide coincident with p70. The intensity of the stained band does not increase after 6 h at 42 °C. Furthermore, newly synthesized p70 labeled during a 30-min pulse with [$^{35}$S]methionine remains completely stable during a chase with excess unlabeled methionine for a minimum of 3 h at 42 °C (data not shown). Therefore, the reduction in labeling of p70 during prolonged hyperthermia is a consequence of reduced synthesis rather than instability of the protein. Thus, HSP synthesis in HeLa cells is under temporal regulation at elevated temperatures.

HeLa cells continue to synthesize HSP for several hours following return to 37 °C. Figure 7 shows the pattern of polypeptides labeled following 90 min at 42 °C and at intervals during the first 4 h of recovery. Synthesis of p70 and p80 decreases to near control levels between 2 and 4 h after return to 37 °C. Labeling of p27 decreases only slightly during this time. On the other hand, synthesis of 37 °C polypeptides is restored rapidly at 37 °C. Incorporation of label into the 37 °C polypeptides increases relative to that of the HSP within the first 60 min of recovery. This can most easily be seen by comparing the intensity of the major 37 °C polypeptide of $M_r$ 43 000 (p43) labeled at 42 °C and after 0.5 h of recovery.

The reductions in HSP synthesis which occur during prolonged incubation at 42 °C and following 3 h of recovery at 37 °C correlate with decreases in the concentrations of functional HSP mRNAs. Total cytoplasmic RNA was isolated at hourly intervals from 42 °C cultures and from cultures returned to 37 °C after 90 min at 42 °C. Identical half-saturating amounts of each RNA preparation were translated in the reticulocyte cell-free system. We assume that distortion in relative translation of different mRNAs due to characteristics of the translation system will be constant among all assays. The translation products were analyzed by polyacrylamide gel electrophoresis, fluorography, and subsequent densitometry. The areas under the individual HSP peaks and the p43 peak were determined as percent of total radioactivity in each lane and compared with the relative synthesis of the same polypeptides in vivo. In Figure 8, the relative synthesis of the HSP p70 in vivo and in vitro during 7 h at 42 °C is compared. In Figure 9a, the percents of radioactivity found in p70 and the 37 °C polypeptide p43 are compared during heat shock and recovery in vivo, whereas in Figure 9b, incorporation into these polypeptides in vitro is shown. The results show clearly that the rate of HSP synthesis at 42 °C correlates with the amounts of translatable HSP mRNAs. It is interesting to note that the amount of translatable mRNA for p43 does not vary significantly during heat shock and recovery (Figure 9b). In vivo, however, there is much less p43 synthesized at 42 °C than at 37 °C (Figure 9a). Relative synthesis of the HSP p70 in vivo is reduced by 20% immediately upon return of heat-shocked cells to 37 °C and continues to diminish over the next 3 h. This can be seen in Figure 9a, which shows the proportions of p70 and p43 labeled at either 42 °C (bars 2a) or 37 °C (bars 2b), immediately following 90 min at 42 °C. The amount of translatable p70 mRNA, however, remains essentially the same during the first 2 h of recovery at 37 °C and is only reduced after this time (Figure 9b). The mRNA for p70 therefore appears to be translated preferentially at 42 °C. These results show that, although long-term regulation of HSP synthesis involves accumulation and loss of functional HSP mRNAs, HSP syn-

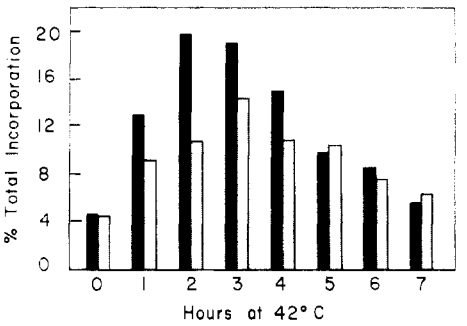


FIGURE 8: Comparison of p70 synthesis in vivo and in translation products of RNA isolated from cells during 7 h at 42 °C. HeLa cells at $5 \times 10^5$ cells/mL were sampled at 37 °C (zero time), brought to 42 °C, and sampled hourly for 7 h thereafter. At each time point, 10-mL aliquots of cells were labeled for 30 min with [$^3$H]leucine and analyzed by polyacrylamide gel electrophoresis and fluorography as described in Figure 1. At the same time points, 50-mL samples of the same cultures were taken, and total cytoplasmic RNA was isolated from the cells as described under Experimental Procedures. The RNA was translated at a concentration of 100 μg/mL as described, and the translation products were analyzed by polyacrylamide gel electrophoresis and fluorography. Fluorograms of HSP synthesized in vivo and in vitro were analyzed by densitometry, and the percent of total radioactivity on the gel in p70 regions of the gel was determined. The dark shaded bars represent incorporation into p70 in vivo, while the light bars represent percent incorporation into p70 among the in vitro translation products of total cellular RNA from the same cells.

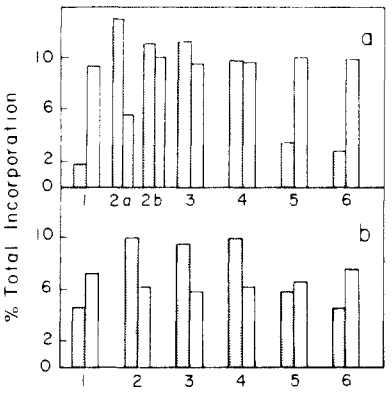


FIGURE 9: Comparison of p70 and p43 synthesis in vivo and in translation products of RNA isolated from cells during heat shock and recovery at 37 °C. HeLa cells at $3 \times 10^5$ cells/mL were incubated at 42 °C for 90 min and then returned to 37 °C. At the times given below, 10-mL aliquots of cells were labeled for 30 min in vivo, and total cytoplasmic RNA was isolated from 50-mL aliquots of cells as described for Figure 6. Incorporation into polypeptides in vivo and into translation products in vitro was analyzed as in Figure 6. The percent incorporation into the HSP p70 region of the gel (shaded bar) and the 37 °C polypeptide p43 region (white bar) was calculated. (Panel a) Percent radioactivity in p70 and p43 in vivo. Lane 1, 37 °C control cells; lane 2a, cells incubated 90 min at 42 °C, labeled at 42 °C; lane 2b, cells incubated as in lane 2a, labeled at 37 °C; lanes 3, 4, 5, and 6, cells incubated as in 2a, returned to 37 °C for 1, 2, 3, and 4 h, respectively, and labeled at 37 °C. (Panel b) Percent radioactivity in p70 and p43 among translation products of RNA isolated from cells incubated as in panel a. Lane numbers correspond to treatments in panel a. In lane 2, RNA was isolated from a single sample of cells taken immediately following 90 min at 42 °C. This corresponds to the RNA present in the cells labeled in vivo in both lanes 2a and 2b in panel a.

thesis must be regulated at the translational level as well.

*Preferential Translation of HSP mRNA at Elevated Temperatures Is Related to the Reduced Rate of Polypeptide Chain Initiation.* The reduction in the overall rate of protein synthesis that occurs at 42 °C is caused by a lesion at the initiation step of mRNA translation (McCormick & Penman, 1969). The synthesis of certain viral and cellular polypeptides has been shown to be less sensitive to inhibition of initiation than that of other proteins (Lodish & Nathan, 1972; Nuss et al., 1975; Nuss & Koch, 1976; Sonenschein & Brawerman, 1976). The mRNAs coding for these resistant polypeptides are believed to have a high affinity for some limiting component(s) of the initiation process. Under conditions of reduced polypeptide chain initiation, the mRNAs with the highest intrinsic efficiencies for initiation outcompete less efficient mRNAs and are translated preferentially (Lodish, 1974; McKeehan, 1974; Golini et al., 1976; Kabat & Chappell, 1977). Therefore, the preferential synthesis of HSP at 42 °C might reflect characteristics of the HSP mRNAs which allow efficient translation under conditions of reduced initiation.

Table I: Relative Synthesis of HSP in the Presence of Cycloheximide or in Hypertonic Medium

| inhibitor | % incorpn into HSP[a] | % incorpn into p43 | % of control incorpn[b] |
|---|---|---|---|
| cycloheximide | | | |
| 0 | 37.5 | 2.9 | 100 |
| 0.1 μg/mL | 33.8 | 3.4 | 94 |
| 0.5 μg/mL | 28.5 | 6.2 | 71 |
| 1.0 μg/mL | 23.3 | 6.4 | 65 |
| added NaCl | | | |
| 0 | 30.6 | 5.0 | 100 |
| 60 mM | 44.4 | 5.0 | 79 |
| 100 mM | 48.7 | 2.7 | 35 |
| 140 mM | 51.4 | 2.6 | 35 |

[a] HSP = p80 + p70 + p27. [b] (cpm/50-μL cells with inhibitor)/(cpm/50-μL cells without inhibitor) × 100.

Drugs, such as cycloheximide, which specifically inhibit the rate of polypeptide chain elongation, can be used to distinguish among mRNAs with different initiation efficiencies. As the rate of elongation is progressively inhibited by increasing concentrations of drug, a point is approached at which the rate of initiation is limited only by available ribosome binding sites on mRNA (Lodish, 1974). Ultimately, the availability of initiation components is no longer limiting, and competition among different mRNAs for translation is diminished. Thus, cycloheximide preferentially inhibits synthesis of polypeptides encoded by mRNAs with high initiation efficiencies when these mRNAs are present at low concentration. The drug has less of an effect on synthesis of polypeptides encoded by abundant mRNAs with lower initiation efficiencies (Lodish, 1971; Lodish & Desalu, 1973; Sonenschein & Brawerman, 1976; Jen et al., 1978).

Hypertonic stress, brought about by introducing excess NaCl into the culture medium, has been shown to decrease the rate of initiation in intact HeLa cells (Robbins et al., 1970; Wengler & Wengler, 1972; Saborio et al., 1974) and preferentially inhibit synthesis of polypeptides encoded by mRNAs with low initiation efficiency (Nuss et al., 1975; Opperman & Koch, 1976). Thus, it is possible to assess the relative initiation efficiencies and abundance of mRNAs coding for HSP and 37 °C polypeptides by comparing the effects of cycloheximide and hypertonic stress on their synthesis.

In one experiment, cells were subjected to heat shock for 2 h and then split into four groups which were preincubated with increasing concentrations of cycloheximide for 10 min. In a second experiment, a different group of cells were heat shocked in a similar manner and divided, and each group was subjected to different degrees of hypertonic stress induced by excess NaCl. The cells were then labeled for 30 min with [$^3$H]leucine at 42 °C in the presence of inhibitors. The labeled polypeptides were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, fluorography, and densitometry. The results are presented in Table I. It can be seen that as

Table II: Effect of Hypertonic Medium on the Pattern of Polypeptide Synthesis in Control, Heat-Shocked, or Recovering HeLa Cells

| mM added NaCl | control (37 °C) | | | heat shock (42 °C) | | | recovery (37 °C) | | |
|---|---|---|---|---|---|---|---|---|---|
| | % incorpn into | | total [$^{35}$S]Met incorpd (%)[b] | % incorpn into | | total [$^{35}$S]Met incorpd (%)[b] | % incorpn into | | total [$^{35}$S]Met incorpd (%)[b] |
| | HSP[a] | p43 | | HSP | p43 | | HSP | p43 | |
| 0 | 10.2 | 10.6 | 100 | 29.9 | 5.1 | 37 | 23.4 | 8.0 | 50 |
| 60 | 13.9 | 6.5 | 20 | 32.4 | 4.0 | 20 | 30.6 | 5.5 | 35 |
| 100 | 15.2 | 6.3 | 7 | | | 3 | 33.7 | 3.6 | 10 |

[a] HSP = p80 + p70 (p 27 does not incorporate detectable amounts of methionine). [b] The total [$^{35}$S]methionine incorporation in each group of cells is expressed as a percent of the incorporation into 37 °C control cells with no added NaCl.

the rate of elongation is progressively inhibited with cycloheximide, the synthesis of HSP relative to that of other polypeptides diminishes. Conversely, the fractional synthesis of the major 37 °C polypeptide p43 increases over 2-fold. Precisely the opposite effect occurs under conditions of increasing inhibition of initiation by hypertonic stress. As the overall rate of protein synthesis is inhibited by excess NaCl, the fractional synthesis of HSP increases while that of p43 is reduced. These results indicate that the mRNAs encoding the HSP are translated at rates in excess of their relative frequency among cellular mRNAs. Furthermore, their translation is less sensitive to inhibition of initiation than that of the majority of 37 °C mRNAs. We propose, therefore, that the HSP mRNAs initiate translation more efficiently than most 37 °C mRNAs under the conditions of reduced polypeptide initiation existing at 42 °C.

Following a period of heat shock, cells continue to synthesize HSP for several hours at 37 °C (Figure 7). Therefore it was possible to examine the effect of inhibition of initiation by hypertonic stress on HSP synthesis at the control temperature. The effect of hypertonic stress on the pattern of polypeptides synthesized was determined in control cells, cells incubated for 90 min at 42 °C, and cells which were allowed to recover for 30 min at 37 °C following 90 min of heat shock. Samples of each group were preincubated with increasing concentrations of excess NaCl for 10 min and then labeled with [$^{35}$S]-methionine for 30 min. Labeled polypeptides were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, autoradiography, and densitometry, and the results are presented in Table II. Protein synthesis in control cells is proportionally more sensitive to hypertonic stress than in either heat-shocked or recovering cells. However, synthesis of polypeptides with similar electrophoretic mobilities to HSP p80 and p70 is unmasked by inhibiting initiation in 37 °C cells. The relative labeling of p43 again decreases. Hypertonic stress again increases the fractional synthesis of HSP in heat-shocked cells. Interestingly, 60 mM excess NaCl reduces overall protein synthesis in recovering cells to the same level seen in heat-shocked cells without added NaCl (37% of control). In these cells, the relative synthesis of HSP and p43 is nearly the same as in the heat-shocked cells incubated at 42 °C without NaCl. Further inhibition of initiation in recovering cells causes an even greater fractional synthesis of HSP. These results suggest that the preferential translation of the HSP mRNAs at 42 °C is a consequence of inhibition of initiation at that temperature.

## Discussion

The induction of heat-shock proteins in cells of vertebrate origin was first reported by Kelley & Schlesinger (1978). Exposure of chicken embryo fibroblasts, mouse L cells, and baby hamster kidney cells to 45 °C caused increased synthesis of polypeptides with apparent molecular weights of 95 000, 76 000, and 22 000. A similar set of polypeptides is induced in chicken embryo fibroblasts treated with certain amino acid analogues (Kelley & Schlesinger, 1978), sulfhydryl reagents (Levinson et al., 1980), and arsenite (Johnston et al., 1980). Induction was shown to require new RNA synthesis and the accumulation of specific mRNAs which code for the proteins in vitro (Kelley et al., 1980; Johnson et al., 1980). The major HSP induced in murine cells (mouse myeloma and 3T3 fibroblasts) show the same electrophoretic mobility as HeLa HSP in our gel system (data not shown). Variations in the $M_r$ reported are a consequence of the electrophoretic systems and molecular weight markers used. Apparently, the sizes of the major HSP are highly conserved among vertebrates.

The heat-shock phenomenon has been extensively studied in *Drosophila* [reviewed by Ashburner & Bonner (1979)]. Shifting the temperature from 24 to 37 °C causes an abrupt series of changes in gene expression which leads to the synthesis of at least seven HSP. After 1 h of heat shock, the HSP are synthesized almost to the total exclusion of other cellular proteins. The $M_r$ values of the *Drosophila* HSP resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis are generally reported as 82 000, 70 000, 68 000, 27 000, 26 000, 23 000, and 22 000 (Ashburner & Bonner, 1979). Similarities exist between the most intensely labeled *Drosophila* HSP (82, 70, and 26 kilodaltons) and the HeLa HSP. In both *Drosophila* and HeLa cells, the major HSP has an apparent molecular weight of 70 000 and is resolved into five components by isoelectric focusing (Mirault et al., 1978; Figure 2b). Substantial amounts of the 82-kilodalton *Drosophila* HSP are made and accumulated in cells at the control temperature (Storti et al., 1980). This polypeptide migrates as a single component in two-dimensional electrophoresis (Mirault et al., 1978; Storti et al., 1980). The HeLa HSP p80 also consists of a single electrophoretic component (Figure 2b) and is an abundant protein in cells prior to heat shock. In addition, p80 is the only HeLa HSP found exclusively in the postmitochondrial subcellular fraction (E. Hickey, unpublished results). A similar cytoplasmic localization of the 82-dalton Kd HSP in *Drosophila* has also been reported (Storti et al., 1980).

The molecular events involved in the induction of the HSP have been studied in some detail in *Drosophila*. The presence of polytene chromosomes in *Drosophila* salivary gland tissue allows experimental approaches not feasible in mammalian systems. Increasing the temperature from 24 to 37 °C causes almost complete inhibition of transcription at most polytene chromosome loci and induction of transcription at several new loci, called the heat-shock puffs (Ritossa, 1962; Lewis et al., 1975). In situ hybridization of polyribosomal mRNA from heat-shocked cells (McKenzie et al., 1975; Spradling et al., 1977) as well as genetic evidence (Ish-Horowicz et al., 1977) has demonstrated that these loci contain the structural genes for several of the HSP. Thus, there is clear evidence for regulation of HSP synthesis at the level of transcription in *Drosophila*.

Induction of HSP synthesis and accumulation of HSP mRNA in HeLa cells can be blocked with actinomycin D.

This alone, however, is insufficient evidence that synthesis of HSP is under transcriptional control. It is possible that RNA processing or stability of HSP mRNAs are the steps in gene expression affected by hyperthermia. Further experiments are necessary to determine whether transcription of DNA sequences coding for HSP is stimulated at 42 °C in HeLa cells.

HeLa cells held at 42 °C down regulate HSP synthesis after significant quantities have accumulated. Mirault et al. (1978) have reported that *Drosophila* cells reduce HSP synthesis to about 50% of the maximum during 8 h of heat shock at 37 °C. It is not clear, however, whether this is a consequence of a regulatory mechanism or the result of cell death. *Drosophila* do not survive after more than a few hours at 37 °C (Lewis et al., 1975).

We have shown that the decrease in HSP synthesis in HeLa cells during prolonged heat shock or following return to 37 °C is correlated with a loss of translatable HSP mRNA. Either of two mechanisms might explain this decline in HSP mRNA concentrations. One possibility is that the stability of the HSP mRNA may change during the heat-shock response in a manner similar to that described for histone mRNA during the cell cycle (Perry & Kelley, 1973). This mechanism would not need to be associated with a change in the rate of transcription of HSP genes. Alternatively, HSP mRNA may have a short half-life and rapid synthesis may only occur during the first few hours of heat shock. The latter mechanism is supported by analogy to the heat-shock response of *Drosophila*, in which the heat-shock puffs regress after the first 30 min of heat shock (Lewis et al., 1975).

The rapid accumulation of HSP mRNAs appears to be responsible for the significant recovery of protein synthesis observed in HeLa cells during the first hour at 42 °C. The re-formation of large polyribosomes at this time reported by McCormick & Penman (1969) coincides with the onset of HSP synthesis. The mRNAs coding for the HSP have properties which allow them to form initiation complexes despite the general inhibition of initiation which occurs during heat shock. Goldstein et al. (Goldstein & Penman, 1973; Goldstein et al., 1974) compared the rates of initiation in cell-free protein synthesizing systems prepared from control and heat-shocked cells. Incorporation of [$^{35}$S]methionine introduced as methionyl-tRNA$_f$ into polypeptides translated from endogenous mRNA was found to be higher in extracts from heat-shocked cells than in extracts from control cells. The increase in initiation activity could be prevented by blocking RNA synthesis with actinomycin D during incubation at 42 °C. Thus, they proposed that initiation was regulated by a new species of RNA synthesized during heat shock. It is likely that HSP mRNA was responsible for this effect. The same characteristics of HSP mRNAs which allow preferential translation during heat shock would also permit them to initiate efficiently in HeLa cell extracts, which have limited ability to reinitiate polypeptide chains (Reichman & Penman, 1973; Goldstein et al., 1974). Thus, extracts from heat-shocked cells might be expected to show higher initiation rates than extracts from control cells simply as a result of the presence of more efficient mRNAs rather than because of differences in the activity of the translational machinery.

The translational control mechanism operating during heat shock might be more complex in *Drosophila* where the HSP mRNAs are ultimately almost the only messages that are translated (Tissieres et al., 1974; Lewis et al., 1975; McKenzie et al., 1975). Unlike the case in vertebrate cells, the overall rate of protein synthesis in *Drosophila* is unaffected, or slightly increased, during heat shock (Moran et al., 1978; Mirault et al., 1978; Lindquist, 1980). It may nonetheless be argued that increased competition among mRNAs for translation occurs in *Drosophila* cells incubated at 37 °C. Despite similar rates of amino acid incorporation at 24 and 37 °C, the number of polyribosomes is much lower at the higher temperature (McKenzie et al., 1975). Reduction of the fraction of ribosomes in polyribosomes without a concomitant effect on the overall rate of polypeptide synthesis is most easily explained by a decrease in ribosome transit time. According to the kinetic model of mRNA translation of Lodish (1974), and more recently refined by Bergmann & Lodish (1979), enhancement of the rate of polypeptide chain elongation can increase mRNA competition for initiation by making a greater proportion of ribosome binding sites available at any instant. Although the rate of elongation has been estimated for heat-shock polypeptides in *Drosophila* at 37 °C (Lindquist, 1980), no measurements of elongation rates at control temperatures have been reported.

The role of the HSP in cellular physiology remains a mystery. Nonetheless, it is remarkable that so similar a response to temperature stress is shared by essentially all eukaryotic organisms. It remains to be determined precisely how this response is regulated and whether the regulatory mechanisms are also conserved through evolution.

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# Activity of the 2′ and 3′ Isomers of Aminoacyl Transfer Ribonucleic Acid in the in Vitro Peptide Elongation on *Escherichia coli* Ribosomes†

Thomas Wagner, Friedrich Cramer, and Mathias Sprinzl*

ABSTRACT: Properties of Phe-tRNA$^{Lys}$-CpCp3′dA and Lys-tRNA$^{Lys}$-CpCp2′dA from *Escherichia coli* were studied in partial reactions of protein biosynthesis using the *E. coli* in vitro translation system. These aminoacyl-tRNAs carry the aminoacyl residue on either the 2′- or 3′-hydroxyl group of the terminal adenosine. The migration of the aminoacyl residue is restricted by the absence of the vicinal hydroxyl group. Both aminoacyl-tRNA$^{Lys}$ analogues interact with the elongation factor Tu. EF-Tu stimulates the binding of both isomers to 70S ribosomes. However, Phe-tRNA$^{Lys}$-CpCp3′dA exhibited a higher level of EF-Tu-dependent binding to the ribosomal A site as compared to Lys-tRNA$^{Lys}$-CpCp2′dA. The nonenzymatic binding was considerably more efficient in the case of the latter analogue, in which the aminoacyl residue is attached to the 3′ position. Lys-tRNA$^{Lys}$-CpCp2′dA is active as a peptide acceptor and $Ac_2$Lys-tRNA$^{Lys}$-CpCp2′dA as a peptide donor. The corresponding 2′ isomers derived from tRNA$^{Lys}$-CpCp3′dA do not form dipeptides under these conditions. The peptidyl-tRNA$^{Lys}$-CpCp2′dA binds directly to the ribosomal P site, as indicated by the absence of stimulation of the puromycin reaction by the elongation factor G. The aminoacyl- or peptidyl-tRNAs derived from tRNA$^{Lys}$-CpCp2′dA are active in all partial reactions of the protein elongation cycle investigated; however, Lys-tRNA$^{Lys}$-CpCp2′dA is still not able to participate on the in vitro poly(A)-dependent synthesis of poly(Lys). There must therefore be a particular step or several steps in the elongation process in which the aminoacyl-tRNA either adopts an unknown intermediate structure, different from a 2′ or a 3′ isomer, or where the presence of the 2′-hydroxyl group is absolutely required.

An aminoacyl-tRNA exists in two isomeric forms. This is due to the fact that the aminoacyl residue can be attached to either the 2′ or the 3′ position of the terminal adenosine. Since the migration of the aminoacyl residue between the two vicinal *cis*-hydroxyl groups occurs spontaneously at standard physiological conditions (Griffin et al., 1966), there are potentially two substrates available for each particular step of the ribosomal peptide elongation process, namely, the 2′- or the 3′-aminoacyl-tRNA. The rate of isomerization exceeds the in vivo rate of the peptide bond formation by several orders of magnitude. It is therefore not possible to determine by direct chemical analysis which isomer of aminoacyl-tRNA is functional in the elongation process. To approach this problem, analogues of aminoacyl-tRNAs were prepared, in which the migration of the aminoacyl residue between the vicinal *cis*-hydroxyl groups is restricted by a chemical modification of the 3′-terminal adenosine of the tRNA. This can be achieved

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen, Federal Republic of Germany (T.W. and F.C.), and the Lehrstuhl für Biochemie der Universität Bayreuth, Bayreuth, Federal Republic of Germany (M.S.). *Received August 27, 1981.* Dedicated to the memory of the late Professor Dr. František Šorm, Institute of Organic Chemistry and Biochemistry of the Czechoslovac Academy of Sciences, Prague, Czechoslovakia.

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