

Translational Control of Protein Synthesis in Response to Heat Shock in *D. melanogaster* Cells

Robert V. Storti,* Matthew P. Scott,
Alexander Rich and Mary Lou Pardue

* Department of Biological Chemistry
University of Illinois at the Medical Center
Chicago, Illinois 60612 and
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

In response to elevated temperature, *Drosophila* cells synthesize a small set of proteins known as the heat-shock proteins, while synthesis of most other proteins ceases. In vitro translation has been used to demonstrate that the messenger RNAs encoding the normal (25°) spectrum of proteins are not broken down or irreversibly inactivated in response to the temperature change. During the heat shock only the heat-shock mRNAs plus a small number of preexisting mRNAs are translated, while most other messages are stored and can be reactivated upon return of the cells to their normal temperature. After recovery from heat shock, cells translate both the normal mRNA and the remaining heat-shock mRNA. The translational control operating in intact cells has been reproduced in cell-free translation systems directed by purified mRNA from normal and heat-shocked cells. Lysates prepared from heat-shocked *Drosophila* cells preferentially translated the heat-shock messages, while the lysate made from normally growing *Drosophila* cells indiscriminately translated both normal and heat-shock messages. Therefore there must be stable alterations in the translational components of heat-shocked cells which are capable of causing selective translation of the heat-shock messages. In addition there must be information encoded in the heat-shock messages that allows their selection.

Introduction

A number of organisms respond to elevated temperatures by preferential synthesis of a small number of proteins. While the most extensively studied case is the heat-shock response of *Drosophila* (reviewed by Ashburner and Bonner, 1979), similar phenomena occur in many other insect species (Vincent and Tanguay, 1979; M. P. Scott, unpublished observations), in Tetrahymena (Yuyama and Zimmerman, 1972; Hermolin and Zimmerman, 1976), in yeast (Miller, Xuong and Geiduschek, 1979), in *Naegleria* (Walsh, 1980), in chick fibroblasts and mammalian cell lines (Kelley and Schlesinger, 1978), in maize (Sachs and Freeling, 1978) and in *Escherichia coli* (Yamamori et al., 1978). The function of the induced proteins is unknown in most instances; however, the mechanisms of induc-

tion of this response are under intensive investigation because the proteins represent a small group of simultaneously synthesized products that may be coordinately regulated.

The heat-shock response in *Drosophila* was originally discovered as a result of transcriptional changes that occur in the polytene chromosomes of the larval salivary glands when the larvae are moved to higher temperatures (Ritossa, 1962). Another effect of heat shock was demonstrated by McKenzie, Henikoff and Meselson (1975) who observed that polysome breakdown occurs as an immediate response to heat shock. The heat-shock-specific pattern of polysomes develops somewhat later. This observation suggests that control of protein synthesis during heat shock might be mediated by a control acting at the level of mRNA translation as well as by the changes in transcription observed in nuclei. McKenzie (1976) has extended this idea by finding that a normal polysome profile reappears when the cells are returned to their normal (25°) temperature even if actinomycin is present to inhibit new transcription. These observations suggest that at least some part of the preexisting (25°) mRNA is retained during the heat shock and can be reutilized if the temperature is lowered. The results of studies employing cDNA probes to identify mRNA by hybridization (Biessmann et al., 1978a, 1978b) and in vitro translation of mRNA from heat-shocked cells (Mirault et al., 1978) have confirmed the presence of mRNAs coding for proteins other than heat-shock proteins in heat-shocked cells.

We present here an analysis of changes in translation and mRNA metabolism that occur in response to heat shock in cultured *Drosophila* cells. This analysis demonstrates that heat-shocked cells preferentially translate a subset of the mRNAs present in the cells. This preferential translation includes the heat-shock RNAs, the transcription of which is induced by the heat shock, but is not limited entirely to those mRNAs. The translational preference is maintained in cell-free protein-synthesizing lysates prepared from heat-shocked *Drosophila* cells, although similar lysates from control cells translate both control and heat-shock RNA very efficiently.

Results

The Effect of Temperature on Protein Synthesis in Vivo

The synthesis of most of the proteins produced by *Drosophila* cultured cells at their normal growing temperature of 25° is gradually reduced as the incubation temperature is raised. At the same time, synthesis of a new set of proteins, the heat-shock proteins increases (Tissieres, Mitchell and Tracey, 1974; Lewis, Helmsing and Ashburner, 1975). Synthesis of at least eight proteins is enhanced by increased temperature (Figure 1); all of these proteins are maximally pro-

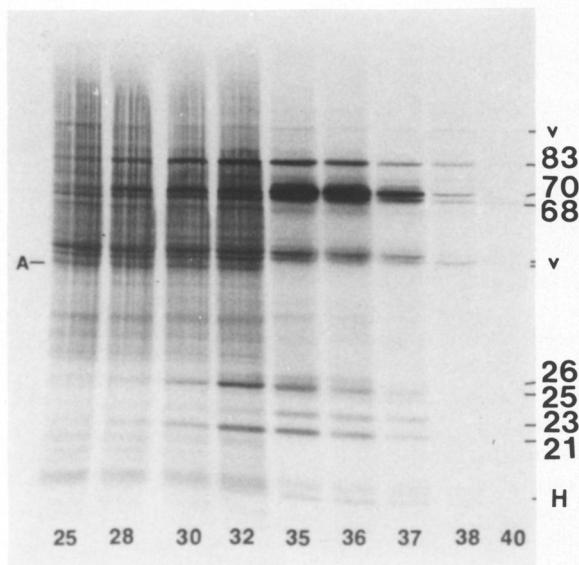


Figure 1. Autoradiogram Showing Proteins Synthesized in Cultured Drosophila Cells at Different Temperatures

For each lane, 6×10^5 cells were incubated at the indicated temperature for 45 min and then were labeled at that temperature for 30 min with 80 μ Ci/ml of 35 S-methionine. The cells were washed in saline and lysed in sample buffer. After a brief sonication, the labeled proteins were separated on a 9–14% gradient SDS-polyacrylamide gel. Equal numbers of cells were loaded in each lane: the TCA-precipitable cpm loaded on each lane are, from left to right, 36,000, 38,000, 44,000, 78,000, 34,000, 30,000, 13,500, 5900 and 1600. Autoradiograph exposed two days. A = actin, v = putative viral proteins; H = histones. Heat shock proteins are marked by lines.

duced at 35°. Most of the proteins made at 25° are produced at a barely detectable rate, if at all, at temperatures of 36° or higher. Hereafter, proteins synthesized at 25°, but not at 36°, will be referred to as "25° proteins". Synthesis of histones appears to be more resistant to repression by heat shock than is the synthesis of most other proteins made before the heat shock. These proteins continue to be synthesized even at fairly high temperatures. The messages encoding the histones are also synthesized during a heat shock, in contrast to most other nonmitochondrial mRNAs (Spradling, Pardue and Penman, 1977). Other resistant proteins are those labeled "v" in Figure 1; these are only observed sporadically and may be proteins of the virus(es) endogenous to these cell lines (Scott, Fostel and Pardue, 1980). The 83,000 dalton protein is generally considered a heat-shock protein, although it is synthesized at 25° in all our experiments, and on the basis of Coomassie blue staining of two-dimensional gels (data not shown), is an abundant protein in non-heat-shocked cells. The 83,000 dalton protein is found almost exclusively in the cytoplasmic fraction of cultured cells, and is also abundant in salivary glands, imaginal discs and brains (data not shown).

These changes in the pattern of protein synthesis induced by heat shock occur within 20 min of the

increase in temperature. Gels analyzing the time course of protein synthesis during a 36° heat shock are shown in Figure 2. Cells were heated by immersion in a 36° water bath and pulse-labeled for 10 min with 35 S-methionine before the temperature increase and at 15, 55 and 320 min after the initiation of the heat shock. Total cellular protein from equal numbers of cells was then electrophoresed on two-dimensional polyacrylamide gels.

Six heat-shock proteins are resolved on these gels (Figure 2C): one at 83,000 daltons, two at 70,000 daltons, one at 68,000 daltons and two small proteins with molecular weights of 23,000 and 22,000. Two other small heat-shock proteins with molecular weights of 26,000 and 27,000 have basic pIs and do not focus within the 5–7 pH range of the gels shown. On a gel with a 3–10 pH gradient, these proteins migrate as single species with a pI of approximately 7.5. The narrow pH range gels have been used in our experiments in order to increase resolution of other closely related proteins.

Twenty minutes after initiation of the heat shock, the rate of synthesis of heat-shock proteins is already close to maximum (Figure 2). Synthesis of characteristic 25° proteins has decreased considerably. After 60 min at 36°, synthesis of 25° proteins is barely detectable. These changes in protein synthesis coincide with the breakdown of preexisting polysomes and the appearance of new polysomes as reported by McKenzie et al. (1975).

The Retention of Translatable 25° mRNAs in Heat-Shocked Cells: *in Vivo* Analysis

Although polysomes containing 25° mRNA are broken down after heat shock, and synthesis of 25° proteins becomes barely detectable, significant amounts of translatable 25° mRNA are still present in heat-shocked cells. This 25° mRNA can be detected when cells are allowed to recover from heat shock under conditions that prevent transcription of new RNA. Such an experiment is illustrated in Figure 3. Cells were heat-shocked for 1 hr at 36° and split into three equal parts. One part was labeled with 35 S-methionine for 10 min at 36° and the proteins were harvested. The other two parts were returned to a 25° water bath and allowed to recover from heat shock. Actinomycin D (1 μ g/ml) was added to one sample before it was returned to 25°. This concentration of actinomycin is sufficient to inhibit more than 98% of 3 H-uridine incorporation within 1 min (data not shown). The remaining RNA synthesis is mitochondrial (Spradling et al., 1977). The two portions of cells were incubated at 25° until, at 1 and 4 hr after the return to 25°, aliquots were removed and pulsed for 10 min with 35 S-methionine. Figure 3A shows the proteins synthesized at the end of the 1 hr heat shock. Figure 3B displays the proteins made in the absence of actinomycin 4 hr after the end of the heat shock, while Figure 3C shows

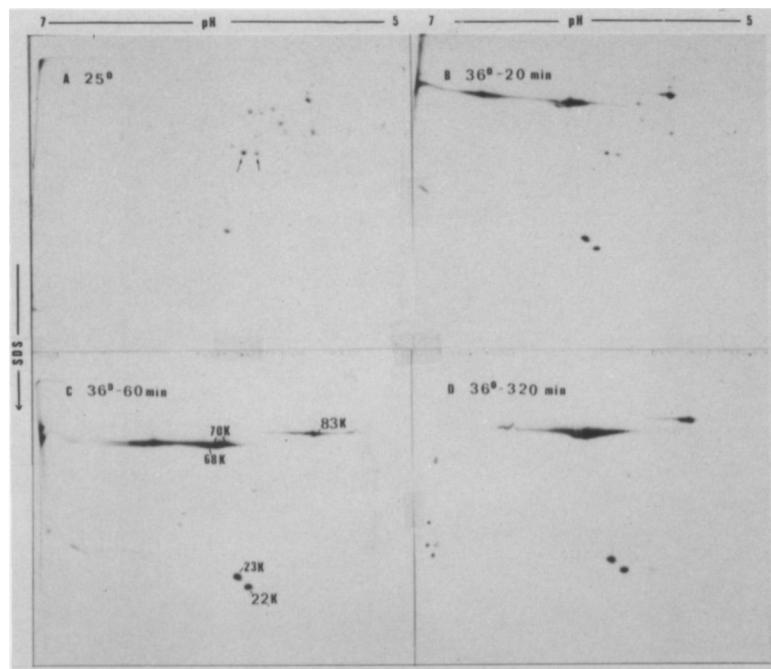


Figure 2. Two-Dimensional Polyacrylamide Gel Electrophoresis of Proteins Synthesized by Drosophila Cultured Cells at Different Times during Heat Shock

Schneider 2-L cells that had been at 36° for various lengths of time were pulse-labeled for 10 min with 100 μ Ci/ml of 35 S-methionine. Proteins synthesized during the pulse were analyzed by electrophoresis on two-dimensional polyacrylamide gels. The pH gradient in the isoelectric focusing dimension is approximately 4.7–7.0 (right to left). Electrophoresis in the second dimension is downward. A. Cells kept at 25°; B. cells kept at 36° for 20 min before labeling; C. cells kept at 36° for 1 hr before labeling; D. cells kept at 36° for 4 hr before labeling. 300,000 cpm were applied to each gel. Fluorography was for one day. The arrows in A identify the multiple forms of actin described previously (Storti et al., 1976). The heat-shock proteins have been indicated in C by their molecular weights. Because cells growing at 25° synthesize many different proteins, the 300,000 cpm applied to gel A are distributed among many faint spots instead of the few heavy spots seen on gels B–D. The actins are among the limited number of proteins synthesized heavily enough to be detected by a fluorographic exposure which also permits reasonable analysis of the prominent heat-shock proteins.

the proteins made after a 4 hr recovery in the presence of actinomycin. The samples taken after a 1 hr recovery were not detectably different from the 4 hr samples. It is clear from these gels that a substantial amount of 25° mRNA must be present during and after heat shock, because 25° proteins (see arrows in Figure 3B and 3C) are made upon return of the cells to 25°, even when transcription of new mRNA is prevented. It is also interesting that heat-shock proteins were still being synthesized 4 hr after the end of the heat shock even in cells in which new transcription of 25° RNA was not inhibited.

The Retention of Translatable mRNAs in Heat-Shocked Cells: Analysis by in Vitro Translation

In vitro translation provides another more direct way to detect translatable 25° mRNAs in heat-shocked cells (Mirault et al., 1978). RNA for our experiments was prepared as described in Experimental Procedures. Total cellular RNA was used because heat-shocked cells do not fractionate in the same way as normal cells. A large amount of cytoplasmic material adheres to heat-shock nuclei and is visible by light microscopy. Several fractionation techniques using nonionic detergents and/or hypotonic lysis have been tested, with no detectable improvement in the fractionation. Furthermore, in vitro translation of nuclear RNA has shown the presence of large amounts of translatable message in these preparations. To avoid preferential loss of mRNA in the heat-shocked cell fractionations, total cellular RNA has been used in

these studies. DNA is digested to small fragments with Pancreatic DNAase I. The total nucleic acid is fractionated on an oligo(dT)-cellulose column to separate poly(A)⁺ material from poly(A)⁻ material (nucleic acid containing no poly(A), or tracts of it insufficient in size to bind to oligo(dT)-cellulose).

RNA preparations were translated in vitro in reticulocyte lysate and wheat germ extract. Since cell-free protein-synthesizing systems may vary in total translational activity and perhaps also in the products synthesized from a particular RNA preparation, both in vitro systems were used. In each case a preliminary experiment was performed to measure protein synthesis in vitro as a function of RNA input. The amount of poly(A) in each RNA preparation was measured by hybridization to 3 H-poly(U). Maximum synthesis was obtained in both systems using approximately 0.5 μ g of poly(A)⁺ RNA, assuming the average length of the RNA to be approximately ten times that of the poly(A) segment. The amount of incorporation of 35 S-methionine into protein using amounts of RNA that gave maximal stimulation is presented in Table 1. In each of several experiments, significant amounts of 3 H-poly(U) hybridized to the poly(A)⁻ fraction, even after repeated cycles of oligo(dT)-cellulose purification. This 3 H-poly(U)-hybridizing material also failed to bind to affinity columns of poly(U) Sepharose (data not shown). The length of the poly(A) stretches in this "poly(A)⁻" RNA, and their terminal, as opposed to internal, location, have not been determined. However, the amount of poly(A) in the poly(A)⁻ fraction is

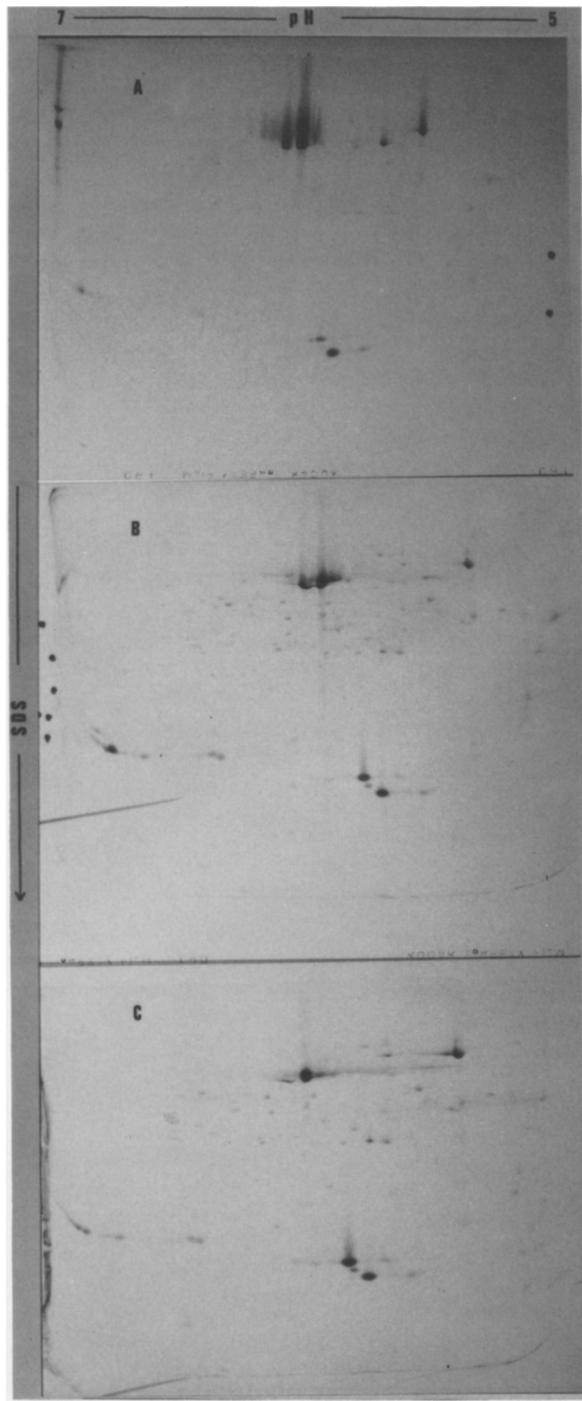


Figure 3. Two-Dimensional Gel Electrophoresis of Proteins Synthesized after Recovery from Heat Shock in the Presence or Absence of Actinomycin D

Cells were pulse-labeled with 100 μ Ci/ml of 35 S-methionine for 10 min. 200,000 cpm were loaded onto each gel. Electrophoresis conditions were as in Figure 2. Autoradiography was for 2 days. A. Cells kept at 36° for 1 hr and pulse-labeled; B. cells kept at 36° for 1 hr and shifted to 25° for 4 hr; C. cells kept at 36° for 1 hr and shifted to 25° for 4 hr in the presence of 1 μ g/ml of actinomycin D.

insufficient to account for the translational activity of that fraction if one were to assume that the translational activity is due to contaminating poly(A)⁺ RNA. In vitro translation of poly(A)⁻ RNA is 2–10 times greater than the activity of poly(A)⁺ RNA when expressed as amounts of 35 S incorporation as a function of nanograms of poly(A) input (Table 1, bottom). It is likely, therefore, that much of the translatable mRNA from these cells has little or no poly(A).

In vitro translation detects significant amounts of 25° mRNA in the RNA extracted from heat-shocked cells. Figure 4 shows the products of a reticulocyte lysate directed by RNA from 25° cells and from cells 20 and 60 min after initiation of a 36° heat shock. Subsaturating amounts of RNA were used in each of these reticulocyte lysate translations in order to minimize possible artifacts arising from competition between different RNA species for the available translation machinery. However, the same products were observed when saturating amounts of RNA were used (data not shown). Approximately equal amounts of acid-precipitable radioactivity were loaded onto each gel shown in Figure 4, which may be compared directly to Figure 2 since the same cell cultures were used for in vivo labeling and for the RNA extraction. The same RNA preparations were translated in wheat germ extract, with essentially the same results (data not shown).

In contrast to the in vivo labeling pattern, mRNAs for the normal 25° spectrum of proteins are detectable by in vitro translation after 1 hr of heat shock (Figure 4), well after the synthesis of these proteins becomes barely detectable in vivo. This is consistent with the actinomycin experiments shown in Figure 3; both experiments indicate the presence of untranslated mRNAs in heat-shocked cells. The actinomycin experiment suggests that the 25° mRNAs are retained for at least 4 hr of heat shock. The 25° RNAs are also stored in an untranslated form in heat-shocked *Drosophila* embryos (M. P. Scott, unpublished data).

Several other striking differences and changes are also apparent in Figure 4. For instance, the mRNAs coding for the 83,000 dalton protein and a 30,000 dalton protein are mostly in the poly(A)⁻ fraction in 25° cells (Figure 4B arrows), but after the heat shock, message for the 83,000 dalton protein (but not the other protein) appears in the poly(A)⁺ fraction as well as in the poly(A)⁻ fraction. Several of the mRNAs encoding 25° proteins are found in both poly(A)⁺ and poly(A)⁻ fractions 20 min after heat shock but become much more prominent in the poly(A)⁻ fraction 60 min after heat shock. Examples are identified by arrows in Figure 4F. While most of the proteins made in vitro are identical in molecular weight and isoelectric point to those made in vivo, some differ quantitatively. For example, the two proteins identified by arrows in Figure 4E are barely detectable in vivo but are prom-

Table 1. Incorporation of ^{35}S -Methionine in Cell-Free Protein-Synthesizing Systems Directed by RNA from Normal and Heat-Shocked Drosophila Cells

RNA*	System	Total cpm $\times 10^{-4}$	ng poly(A)	cpm/ng poly(A) ⁺	
				cpm/ng poly(A) ⁻	cpm/ng poly(A) ⁺
25°	Reticulocyte lysate:poly(A) ⁺	112.2	51	2.2	0.14
36°—20 min		159.1	27	5.9	0.15
36°—60 min		185.1	42	4.4	0.13
25°	Reticulocyte lysate:poly(A) ⁻	63.9	4.2	15.2	
36°—20 min		48.4	1.25	38.7	
36°—60 min		41.2	1.25	33.0	
25°	Wheat germ extract:poly(A) ⁺	357.0	51	7.0	0.65
36°—20 min		398.8	27	14.8	0.39
36°—60 min		366.2	42	8.7	0.25
25°	Wheat germ extract:poly(A) ⁻	45.6	4.2	10.9	
36°—20 min		46.8	1.25	37.5	
36°—60 min		43.6	1.25	34.9	

* Amounts of RNA used in each translation were those which gave maximal stimulation. Nanograms of poly(A) were determined by hybridization with ^3H -poly(U). 25°: RNA extracted from cells grown at 25°. 36°—20 min: RNA extracted from cells kept at 36° for 20 min. 36°—60 min: RNA extracted from cells kept at 36° for 60 min.

inent among the products made by reticulocyte lysates.

Translational Control of 25° and Heat-Shock mRNA in a Drosophila Cell-Free Protein-Synthesizing Lysate

The experiments presented above demonstrate that 25° mRNAs are present in heat-shocked cells in an untranslated form, but are not modified in a way that prevents their translation *in vitro*. The mechanism of the translational control operating during heat shock cannot be investigated *in vitro* using reticulocyte lysate or wheat germ extract since neither of these preparations exhibits the selective translation that occurs *in vivo* in heat-shocked cells. We have therefore developed a Drosophila cell-free protein-synthesizing system to study heat-shock translational control (Scott et al., 1979). Lysates made from normally growing cells were compared to lysates made from cells that had been kept at 36° for 75 min prior to preparation of the lysate.

A primary question about the lysate from heat-shocked cells is whether its optimum temperature for protein synthesis differs from that of the normal lysate. The normal lysate incorporates amino acids most actively at 28°. It is worth noting that although the L-2 cell line is maintained at 25°, maximum incorporation of ^{35}S -methionine occurs when the living cells are incubated at 28°. The 28° temperature optimum of the heat-shock lysate is identical to that of the 25° lysate and the intact cells. Qualitative differences in the proteins synthesized by the two lysates will be described below. Both lysates synthesize virtually no protein when incubated at 36°. The same results were observed using lysates made from Schneider L-2 cells, K_c cells (a second *D. melanogaster* cell line;

Echalier and Ohanessian, 1970) or *D. melanogaster* embryos. While it would be interesting if the translational machinery of heat-shocked cells were more resistant to heat than that of normal cells, it is useful that this is not the case. For comparative purposes both lysates can be incubated at the same temperature, eliminating any question about changed rates of synthesis or protein denaturation due merely to the different temperatures of incubation. The experiments shown below were all performed at the optimum temperature for protein synthesis, 28°.

When lysates made from normal or heat-shocked *Drosophila* cells are digested with micrococcal nuclease and then supplied with RNA from 25° or heat-shocked cells, the proteins synthesized are similar to the proteins synthesized by the corresponding living cells (Figure 5). The 25° lysate synthesizes both 25° proteins and heat-shock proteins, as do intact cells that have been heat-shocked and shifted back to 25°. The lysate from heat-shocked cells synthesizes very little protein when supplied only with RNA from 25° cells, but synthesizes the heat-shock proteins preferentially when supplied with heat-shock RNA (Figure 5). Since heat-shock RNA preparations contain messages encoding both the heat-shock proteins and the 25° proteins, the heat-shock lysate is reproducing the selection of heat-shock messages for translation that occurs *in vivo* during heat shock. The translational discrimination of lysates made from heat-shocked cells is also demonstrated by quantitative analyses. RNA from 25° cells and from heat-shocked cells directs approximately the same amount of protein synthesis in lysates from 25° cells. However, RNA from 25° cells stimulates very little protein synthesis in lysates from heat-shocked cells. RNA from a number of cultures of cells growing at 25° has never stimu-

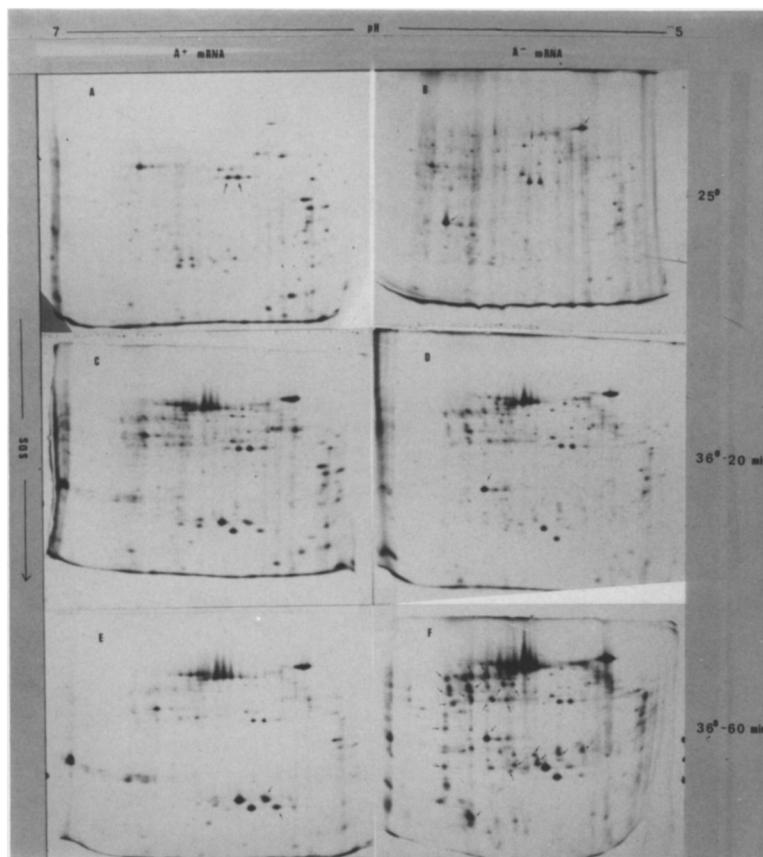


Figure 4. Proteins Synthesized in Reticulocyte Lysates Directed by RNA Isolated from Heat-Shocked and Control Drosophila Cells
 A and B are, respectively, products directed by poly(A)⁺ and poly(A)⁻ RNA from cells at 25°; C and D are, respectively, products directed by poly(A)⁺ and poly(A)⁻ RNA from cells after 20 min at 36°; E and F are, respectively, products directed by poly(A)⁺ and poly(A)⁻ RNA from cells after 1 hr at 36°. Electrophoresis conditions are as described for Figure 2. Half-saturating amounts of RNA were added to each translation mixture. Gels A, C, D, E and F contained 600,000 cpm, and gel B contained 480,000. Fluorography was for 2 days for A, E and F, 1 day for C and D, and 3 days for B. Arrows in A indicate the two actin species. Arrows in B indicate two protein products, the 83,000 dalton heat shock protein and a 30,000 dalton protein, encoded largely by the poly(A)⁻ RNA from 25° cells. Arrows in E indicate protein products encoded mostly by the poly(A)⁺ RNA fraction from heat-shocked cells. Arrows in F indicate proteins whose mRNA appears to be relatively enriched in poly(A)⁻ RNA in cells kept at 36° for 1 hr.

lated lysates from heat-shocked cells to incorporate more than 30% of the ³⁵S-methionine incorporation produced by heat-shock RNA.

In two-dimensional gel electrophoresis the 70,000 dalton cluster of heat-shock proteins frequently appears as a long streak (Figure 6A). The streaking occurs sporadically when *in vivo* labeled proteins are analyzed (compare Figures 2 and 3) and is always seen after electrophoresis of the products of *in vitro* translation in reticulocyte or Drosophila cell lysates. The effect is specific for the 70,000 dalton protein cluster. If the translation mix is treated with RNAase before electrophoresis, the streak is resolved into a pattern of spots identical to the pattern most commonly produced by *in vivo* labeled proteins (Figure 6B). Several different RNAases as well as alkaline treatment eliminate the streaking of the 70,000 dalton cluster. Treating the translation products with DNAase has no effect. Because of this streaking phenomenon, all translation reactions reported in this paper were treated with RNAase before electrophoresis unless otherwise noted.

Discussion

Translational control of protein synthesis can be defined as any mechanism or difference operating at the

level of mRNA, ribosomes, soluble factors or cell compartmentalization that results in the preferential translation of certain mRNAs during the course of cell growth or differentiation. In the context of this definition, in heat-shocked cells a large set of mRNAs is subject to translational control. These mRNAs code for 25° proteins and can be translated in heterologous cell-free systems but are selectively inactivated in heat-shocked cells. The histone mRNAs apparently are not included in the set of repressed messages. Thus there are a number of mRNA molecules that are actively translated during heat shock: those encoding the heat-shock proteins, the histones and certain viral proteins (Scott et al., 1980). A detailed structural analysis of these RNAs might reveal common characteristics responsible for their continued translation, but this information is not yet available.

Several mechanisms could account for selective inactivation of translation of specific mRNAs or groups of mRNAs. A selective 5', 3' or internal modification of mRNA could prevent translation. Such a modification would be expected to prevent translation *in vitro* as well as *in vivo*. Farmer et al. (1978) have reported mRNA inactivation in unattached 3T3 cells; however, the mRNA from these suspended cells was inactive both *in vivo* and *in vitro*. The situation is quite different for the 25° mRNAs in heat-shocked cells. They are

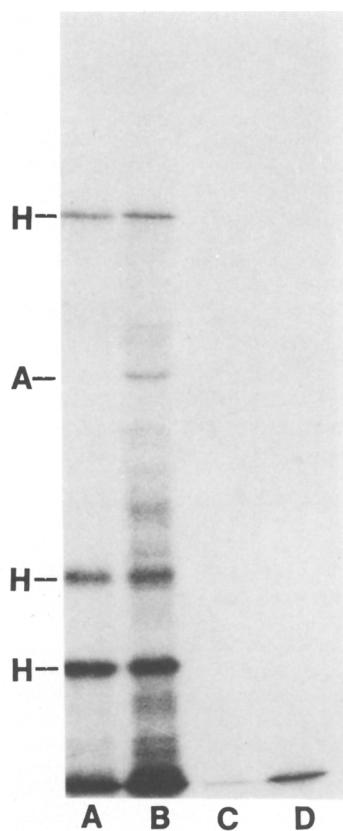


Figure 5. Preferential Translation of Heat Shock mRNA in Lysates of Heat-Shocked Drosophila Cells

Autoradiogram of an SDS-polyacrylamide gel displaying ^{35}S -labeled polypeptides synthesized in lysates prepared from cells heat-shocked at 36° for 1 hr (A) or cells kept at 25° (B). Both lysates were directed by a mixture of heat shock and control poly(A)* RNA. The RNA was prepared from 12–18 hr Drosophila embryos kept at 23° (control) or held for 1 hr at 36° (heat-shocked). Equal amounts of the RNA mixture (approximately half-saturating) were added to each lysate. C displays polypeptides made by the heat-shock lysate without added RNA. D displays polypeptides made by the control lysate in the absence of added RNA. Equal amounts of each reaction were loaded on each gel. Heat shock proteins (H) and actin (A) are indicated.

actively translated in vitro and show no evidence of modification.

Other possible mechanisms of translational control could involve changes in specific mRNA recognition factors in response to heat shock, either the repression of one or more factors required for translation of 25° mRNAs or the induction of one or more factors that positively select heat-shock mRNAs. Alternatively, heat-induced conformational changes in recognition proteins could selectively alter translational properties. If heat-induced conformational changes are involved in the heat-shock translational control, these changes must be stable after removal of the inducer. Lysates prepared from heat-shocked cells preferentially translate heat-shock mRNA although the translations are done at 28°. Lysates from control

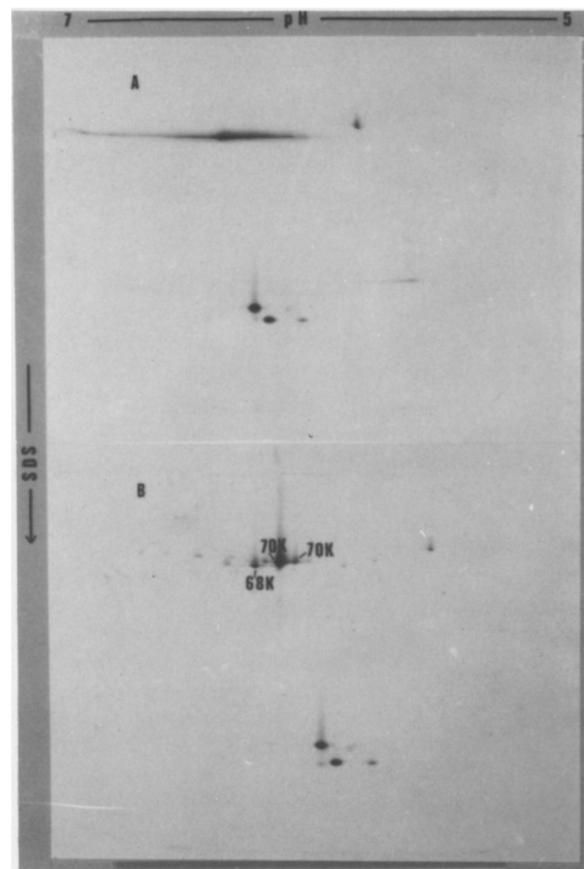


Figure 6. Two-Dimensional Gel Electrophoresis of Endogenous Protein Synthesis in Lysates of Heat-Shocked Drosophila Cells, Untreated with Micrococcal Nuclease

The endogenous products were electrophoresed directly (A) or digested with 5 $\mu\text{g}/\text{ml}$ of pancreatic RNAase for 10 min at room temperature before electrophoresis (B). The 70,000 dalton heat-shock protein cluster forms a large streak when the translation products are applied directly to the gel (A). After the products are treated with RNAase, the cluster is resolved into the pattern of spots normally seen with *in vivo*-labeled proteins (B). The conditions of electrophoresis are as described in Figure 2. The gels contained approximately 200,000 cpm and autoradiography was for 2 days.

cells, also at 28°, translate both 25° mRNA and heat-shock mRNA efficiently.

Spradling, Penman and Pardue (1975) have shown that the first hour of heat shock is characterized by the synthesis of at least six new mRNA species. This correlates in time with the breakdown of old polysomes and the appearance of new polysomes containing heat shock mRNA (McKenzie et al., 1975). The new RNA species hybridize to specific "puffed" regions of the polytene chromosomes, and several have been shown to code for heat-shock proteins (Spradling et al., 1977; Livak et al., 1978; Mirault et al., 1978; Ashburner and Bonner, 1979). The synthesis of this RNA and of the heat-shock proteins correlates with the appearance during the first 20 min of mRNA coding for heat-shock proteins as detected by

our *in vitro* translations (Figure 4). Another effect of heat shock is the gradual loss of up to 80% of the total cytoplasmic poly(A)⁺ sequences during the first hour (Spradling et al., 1977). That this loss of poly(A)⁺ material may represent changes in the poly(A) content of specific mRNAs is shown in Figure 4. A large fraction of the mRNA species synthesized in 25° cells is also present in cells that have been heat-shocked for 20 min. After 20 min of heat shock most of this mRNA has stretches of poly(A) large (or accessible) enough to bind to oligo(dT)-cellulose. After 1 hr of heat shock, however, many of the same polypeptides encoded by poly(A)⁺ RNA in the earlier samples have become prominent translation products of the poly(A)⁻ RNA fraction. This result suggests that much of the poly(A) content of the 25° mRNA sequences has been reduced or eliminated so that these sequences are found predominantly in the oligo(dT)-cellulose-unbound mRNA fraction (Figure 4C and 4F). The conversion of poly(A)⁺ mRNA into poly(A)⁻ mRNA has not, however, been directly determined. It is also possible that the apparent loss of poly(A)⁺ RNA sequences from the cytoplasm might be due to an increased tendency of these sequences to fractionate with the nuclei during heat shock. An alternative but not mutually exclusive explanation for the results of Spradling et al. (1977) is that the mRNAs for the 25° proteins are less stable at the higher temperature (37°) used for their experiments than at the 36° temperature used in the experiments shown above. Lindquist (1980) has presented data supporting the idea that heat-shocking cells to a temperature higher than that yielding maximal heat-shock protein synthesis causes the degradation of preexisting messages. This result suggests that the heat-shock proteins may have a role in stabilizing mRNA.

The significance of the large amounts of ³H-poly(U)-hybridizing material in all of the oligo(dT) unbound fractions is unknown. This RNA also fails to bind to affinity columns of poly(U)-Sepharose. It seems probable that the ³H-poly(U) binding to fractions that do not bind to affinity columns is due to RNA having 3' or internal poly(A) stretches long enough to hybridize to ³H-poly(U) but too short to allow retention under the conditions of the column chromatography.

Of the assorted mRNAs found in the poly(A)⁻ fraction, the mRNA coding for the major 83,000 dalton protein is of particular interest. Substantial amounts of this protein are made and accumulated in 25° cells (Figure 2A). Unlike the other heat-shock proteins, which have not been localized uniquely in any specific subcellular fraction, the 83,000 dalton protein is found exclusively in the cytoplasm (M. P. Scott, unpublished data). The polyadenylation of the 83,000 dalton protein mRNA also differs from the remaining heat-shock mRNAs. In 25° cells it is found predominantly in the poly(A)⁻ mRNA fraction. After heat shock the distribution of this mRNA in poly(A)⁺ and poly(A)⁻ fractions

is similar to other heat-shock mRNAs suggesting that the heat shock has induced either a change in the mRNA polyadenylation or a second, independently regulated mRNA.

The 68,000–70,000 dalton protein complex also shows some unusual features that may reflect on its mechanism of either synthesis or function. The streaking phenomenon shown in Figures 2 and 6 has been observed as a regular occurrence in the products of all cell-free translation experiments, although it occurs only sporadically on gels of proteins labeled *in vivo*. The susceptibility of the streaking to RNases, alkali, and heat suggest some kind of transient association with nucleotides or RNA, perhaps of peptidyl-tRNA nature. Perhaps these heat-shock proteins interact in some way with either their own or other mRNA.

Experimental Procedures

Cell Growth and Labeling

The Schneider line 2-L cells are Schneider line 2 D. melanogaster cultured cells (Schneider, 1972) that have been adapted to spinner culture and defined medium (Lengyel, Spradling and Penman, 1975). Cells were grown at 25° in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.5% lactalbumin hydrolysate, MEM nonessential amino acids, 50 u/ml penicillin, and 50 µg/ml streptomycin (all from Gibco). These cells double about every 25 hr, growing at 2.5–8 × 10⁶/ml. For labeling of 25° and 36° cells with ³⁵S-methionine, cells were centrifuged at low speed and were resuspended in the same medium without methionine or lactalbumin hydrolysate. The fetal calf serum used in this medium was dialyzed extensively against phosphate-buffered saline and was sterilized by filtration. The cells were centrifuged after the wash and resuspended at 5–10 times their normal concentration in the methionine-free medium. The cells were split into equal aliquots for further growth at 25° or 36°. Cells were labeled by adding 50 µCi/ml ³⁵S-methionine (Amersham) to the medium for the times indicated in the figure legends. The incubation bath temperatures were maintained to within 0.1°C.

Cell Fractionation

For analysis of proteins, cells were grown in ³⁵S-methionine. After labeling, the cells were washed in ice-cold phosphate-buffered saline and pelleted at 1000 × g for 4 min. The cell pellets were lysed directly into 100 µl of isoelectric focusing sample buffer [9.5 M urea, 2% NP-40, 2% ampholine (80% pH 5–7, 20% pH 3.5–10), 0.5% SDS].

For RNA extraction, the cell pellet obtained by centrifugation at 1000 × g for 4 min was resuspended in lysis buffer (100 mM NaCl, 10 mM CaCl₂, 30 mM Tris, pH 7.4, 1% Diethylpyrocarbonate added just prior to use). Triton X-100 was then added to 0.5% and the cells were lysed by brief agitation in a Vortex mixer. Nuclei were removed by centrifugation at 4000 × g for 4 min. The cytoplasmic supernatant was made 0.5% SDS and 10 mM EDTA. The nuclear pellet was resuspended in 0.5 M NaCl, 10 mM Tris, 5 mM Mg(OAc)₂, pH 7.4, and digested for 15 sec at 37° with 40 µg/ml pancreatic DNAase. We used 0.5% SDS and 10 mM EDTA to stop the digestion, after which the nuclear and cytoplasmic fractions were pooled.

RNA Extraction and Fractionation

Lysed cells were repeatedly extracted with phenol and chloroform/isoamyl alcohol (99:1) as described by Spradling et al. (1977). The nucleic acid was precipitated with two volumes of ethanol, then resuspended in 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4, 0.5% SDS, and digested with 500 µg/ml Proteinase K for 30 min at 25° C. The mixture was again phenol-chloroform extracted and ethanol-precipitated. The resulting pellet was dissolved in binding

buffer (500 mM NaCl, 10 mM Tris; pH 7.4, 1 mM EDTA, and 0.5% SDS). RNA containing and lacking poly(A) was separated by affinity chromatography on oligo(dT)-cellulose. The bound RNA was eluted with 10 mM Tris, pH 7.4, 1 mM EDTA, 0.1% SDS. Unbound RNA was recycled over the column several times. Three ethanol precipitations were used to remove SDS each time the RNA (and DNA) was redissolved in 0.2 M NaAc, pH 5. RNA was stored frozen in distilled water.

The concentration of RNA and DNA was determined from absorbancy at 260 nm assuming 20 A₂₆₀ units to be equal to 1 mg/ml nucleic acid. The amount of poly(A)-containing RNA was determined by hybridization to ³H-poly(U) in 2 × SSC at 45° for 15 min, followed by digestion with 100 µg/ml RNAase A. Poly(A) content was determined from a standard curve prepared using commercially available poly(A) (Collaborative Research).

Cell-Free Protein Synthesis

RNA has been translated in cell-free systems made from wheat germ embryos, rabbit reticulocytes, and *Drosophila* 2-L cultured cells. Wheat germ extracts were prepared according to the procedure of Roberts and Paterson (1973). Reaction mixtures were incubated at 25° for 3 hr. We prepared mRNA-dependent rabbit reticulocyte lysates treated with micrococcal nuclease according to the method of Pelham and Jackson (1976). The procedure was modified by lysing the reticulocytes in water containing 40 µg/ml hemin. The assay system in a final volume of 25 µl was as described (Scott et al., 1979) using 0.64 mM Mg(OAc)₂ and 80 mM KAc. Incubation was at 37° for 1 hr. The mRNA-dependent cell-free system made from *Drosophila* 2-L cultured cells has been fully described elsewhere (Scott et al., 1979). Briefly, washed cells were lysed in 10 mM Hepes, 6 mM 2-mercaptoethanol, pH 7.6, with 20% rat liver supernatant (RLS) to inhibit endogenous ribonuclease activity. The supernatant from a 20 min, 40,000 × g centrifugation was used for translation. The standard reaction mixture in a final volume of 25 µl consisted of: 10 µl of lysate, 3 µl of a mixture of 0.25 mM amino acids minus methionine, 66.6 mM creatine phosphate, 10 mM dithiothreitol, 5 mM spermidine and 20 mM Hepes, pH 7.6. ³⁵S-Methionine (20–40 µCi, Amersham), RNA, and water were added to give a final volume of 25 µl. Incubation was at 28° for 1 hr. Lysates from heat-shocked cells were prepared in the same way from cells kept at 36° for 75 min. These reactions were also incubated at 28°.

Protein synthesis in each cell-free system was monitored by the incorporation of ³⁵S-methionine into trichloroacetic acid(TCA)-precipitable material. One µl aliquots were spotted onto Whatman 3MM filter paper discs, washed 5 min in 10% TCA, 5 min in 5% TCA and 10 min in boiling 5% TCA. The discs were rinsed with ethanol and ether and dried. Samples were counted in Liquifluor/toluene scintillant.

Two-Dimensional Polyacrylamide Gel Electrophoresis

For electrophoresis, translation reactions were digested with 5 µg/ml pancreatic RNAase for 10 min at room temperature unless indicated otherwise. Appropriate amounts for electrophoresis were dried in vacuo and resuspended in isoelectric focusing sample buffer.

Two-dimensional polyacrylamide gel electrophoresis was by a modification of the procedure of O'Farrell (1975). Samples (25–50 µl) were electrophoresed at 500 V for 16–20 hr in 2 × 130 mm glass tubes containing gels with a pH gradient of 5–7. After electrophoresis, the isoelectric focusing gels were equilibrated for 20 min in 10% glycerol, 0.1 M dithiothreitol, 0.0625 M Tris-HCl, pH 6.8, and either frozen in dry ice-ethanol and stored at –80° C or loaded directly onto the second dimension gel.

Second dimension electrophoresis was in 12% SDS-polyacrylamide slab gels according to Laemmli (1970), except that the stacking gel contained 2.5 M urea as described previously (Storti, Coen and Rich, 1976). The addition of urea facilitated overlaying the stacking gel with 0.1% SDS. The isoelectric focusing gel was layered on top of the stacking gel and sealed with 1% agarose in equilibration buffer. The gels were electrophoresed as described previously (Storti et al., 1976) and either dried for autoradiography or fluorographed (Bonner and Laskey, 1974) using XR-5 X-ray film.

Acknowledgments

This work was supported by grants from the National Institutes of Health (to M.L.P., A.R. and R.V.S.). We thank Karen Traverse for her many contributions to this work.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 26, 1980

References

- Ashburner, M. and Bonner, J. J. (1979). The induction of gene activity in *Drosophila* by heat shock. *Cell* 17, 241–254.
- Biessmann, H., Levy, W. B. and McCarthy, B. J. (1978a). In vitro transcription of heat shock-specific RNA from chromatin of *Drosophila melanogaster* cells. *Proc. Nat. Acad. Sci. USA* 75, 759–763.
- Biessmann, H., Wadsworth, S., Levy, W. B. and McCarthy, B. J. (1978b). Correlation of structural changes in chromatin with transcription in the *Drosophila* heat shock response. *Cold Spring Harbor Symp. Quant. Biol.* 42, 829–834.
- Bonner, W. M. and Laskey, R. A. (1974). A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46, 83–88.
- Echalier, G. and Ohanessian, A. (1970). In vitro culture of *Drosophila melanogaster* embryonic cells. *In Vitro* 6, 162–172.
- Farmer, S. R., Ben-Ze'ev, A., Benecke, B.-J. and Penman, S. (1978). Altered translatability of messenger RNA from suspended, anchorage-dependent fibroblasts: reversal upon cell attachment to a surface. *Cell* 15, 627–637.
- Hermolin, J. and Zimmerman, A. M. (1976). RNA synthesis in division-synchronized tetrahymena macronuclear and cytoplasmic RNA. *J. Protozool.* 23, 594–600.
- Kelley, P. M. and Schlesinger, M. J. (1978). The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. *Cell* 15, 1277–1286.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature* 227, 680–685.
- Lengyel, J., Spradling, A. and Penman, S. (1975). Methods with insect cells in suspension culture II. *Drosophila melanogaster*. *Meth. Cell Biol.* 10, 195–208.
- Lewis, M., Helmsing, P. J. and Ashburner, M. (1975). Parallel changes in puffing activity and patterns of protein synthesis in salivary glands of *Drosophila*. *Proc. Nat. Acad. Sci. USA* 72, 3604–3608.
- Lindquist, S. (1980). Varying patterns of protein synthesis in *Drosophila* during heat shock: implications for regulation. *Dev. Biol.* 77: 463–479.
- Livak, K. F., Freund, R., Schweber, M., Wensink, P. C. and Meselson, M. (1978). Sequence organization and transcription at two heat shock loci in *Drosophila*. *Proc. Nat. Acad. Sci. USA* 75, 5613–5617.
- McKenzie, S. L. (1976). Protein and RNA synthesis induced by heat treatment in *Drosophila melanogaster* tissue cultures cells. Ph.D. thesis, Harvard University, Cambridge, Massachusetts.
- McKenzie, S. L., Henikoff, S. and Meselson, M. (1975). Localization of RNA from heat-induced polysomes at puff sites in *Drosophila melanogaster*. *Proc. Nat. Acad. Sci. USA* 72, 1117–1121.
- Miller, M. J., Xuong, H.-H. and Geiduschek, E. P. (1979). A response of protein synthesis to temperature shift in the yeast *Saccharomyces cerevisiae*. *Proc. Nat. Acad. Sci. USA* 76, 5222–5225.
- Mirault, M.-E., Goldschmidt-Clermont, M., Moran, L., Arrigo, A. P. and Tissieres, A. (1978). The effect of heat shock on gene expression in *Drosophila melanogaster*. *Cold Spring Harbor Symp. Quant. Biol.* 42, 819–827.
- O'Farrell, P. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 25, 4007–4021.

- Pelham, H. R. B. and Jackson, R. J. (1976). mRNA dependent translation system from reticulocytes. *Eur. J. Biochem.* 67, 247–256.
- Ritossa, F. M. (1962). A new puffing pattern induced by heat shock and DNP in *Drosophila*. *Experientia* 18, 571–573.
- Roberts, B. E. and Paterson, B. (1973). Efficient translation of tobacco mosaic virus RNA and rabbit globin RNA in a cell-free system from commercial wheat germ. *Proc. Nat. Acad. Sci. USA* 70, 2330–2334.
- Sachs, M. M. and Freeling, M. (1978). Selective synthesis of alcohol dehydrogenase during anaerobic treatment of maize. *Mol. Gen. Genet.* 161, 111–115.
- Schneider, I. (1972). Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* 27, 353–365.
- Scott, M. P., Fostel, J. M. and Pardue, M. L. (1980). A New Type of Virus from cultured drosophila cells: characterization and use in studies of the heat-shock response. *Cell* 22, 929–941.
- Scott, M. P., Storti, R. V., Pardue, M. L. and Rich, A. (1979). Cell-free protein synthesis in lysates of *Drosophila melanogaster* cells. *Biochemistry* 18, 1588–1594.
- Spradling, A., Penman, S. and Pardue, M. L. (1975). Analysis of *Drosophila* mRNA by *in situ* hybridization: sequences transcribed in normal and heat shocked cultured cells. *Cell* 4, 395–404.
- Spradling, A., Pardue, M. L. and Penman, S. (1977). Messenger RNA in heat-shocked *Drosophila* cells. *J. Mol. Biol.* 109, 559–587.
- Storti, R. V., Coen, D. M. and Rich, A. (1976). Tissue-specific forms of actin in the developing chick. *Cell* 8, 521–527.
- Tissieres, A., Mitchell, H. K. and Tracey, U. M. (1974). Protein synthesis in salivary glands of *Drosophila melanogaster*: relation to chromosome puffs. *J. Mol. Biol.* 84, 389–398.
- Vincent, M. and Tanguay, R. M. (1979). Heat-shock induced proteins in the cell nucleus of *Chironomus tentans* salivary gland. *Nature* 281, 501–503.
- Walsh, C. (1980). Appearance of heat shock proteins during the induction of multiple flagellae in *Naegleria gruberi*. *J. Biol. Chem.* 255, 2629–2632.
- Yamamori, T., Ito, K., Nakamura, Y. and Yura, T. (1978). Transient regulation of protein synthesis in *Escherichia coli* upon shift-up of growth temperature. *J. Bacteriol.* 134, 1133–1140.
- Yuyama, S. and Zimmerman, A. M. (1972). RNA synthesis in tetrahymena. Temperature-pressure studies. *Exp. Cell. Res.* 71, 193–203.