Heat Shock Induced Alterations in Polyadenylate Metabolism in *Drosophila melanogaster*†

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ABSTRACT: We have studied the effect of heat shock on poly(adenylic acid) [poly(A)] metabolism in *Drosophila melanogaster* cells by using hybridization of ribonucleic acid (RNA) samples to [^{3}H]poly(uridylic acid). Shortly after heat shock begins, cytoplasmic poly(A) decays rapidly. Two components were detected. Component I constitutes 46% of the total cytoplasmic poly(A) and decays with a half-life of 10 min. Component II (54% of total) is more stable. A half-life was not estimated for component II because new synthesis was not blocked. Studies on the size of cytoplasmic poly(A) indicate component I is completely degraded while component II remains essentially unchanged. Nuclear poly(A) increases rapidly, peaking at 2 or 3 times the normal level after 90 min of heat shock. Studies on the size of nuclear poly(A) indicate the increase is not due to addition of poly(A) to existing poly(A) segments nor appreciably to newly synthesized RNA unless transit time is markedly increased. We conclude that nuclear poly(A) is added in large part to already existing nonadenylated primers. Studies with RNA and protein synthesis inhibitors show RNA and protein synthesis are not required for the changes in poly(A). As the temperature is raised above 34–35 °C, the effect on poly(A) metabolism becomes more severe. Changes in external pH, without increased temperature, also alter poly(A) levels. Poly(A) changes may therefore be a rapid response to a variety of physiological factors.

When cultured cells, larvae, and adult flies of *Drosphila melanogaster* are transferred from their normal growth temperature of 25 °C to 37 °C a series of regulatory changes rapidly take place [for reviews, see Leenders et al. (1974) and Ashburner & Bonner (1979)]. Chromosomal puffs recede and seven to nine new puffs appear (Ritossa, 1962; Ashburner, 1970). These new puffs are sites where [^{3}H]uridine incorporation occurs (Tissieres et al., 1974; Bonner & Pardue, 1976) and RNA[1] polymerase accumulates (Plagens et al., 1976; Jamrich et al., 1977a,b) suggesting that new transcription occurs. The precursors to 5S and 19S–26S ribosomal RNAs are no longer processed (Ellgaard & Clever, 1971; Rubin & Hogness, 1975; Jacq et al., 1977).

Translation is also altered during heat shock. Overall protein synthesis is markedly decreased. Within 10–20 min following heat shock, preexisting polysomes disappear and are later replaced by new polysomes containing heat shock specific RNAs (McKenzie et al., 1975; Beissmann et al., 1978). Six to nine new polypeptides are synthesized, and, of the proteins normally made at 25 °C, only the histones continue to be synthesized (Lewis et al., 1975; Tissieres et al., 1974; McKenzie et al., 1975; Koninkx, 1976).

Little is known about preexisting mRNA[1] after the temperature change. Is it degraded or does it remain intact in the cell? Using hybridization to [^{3}H]poly(U), Spradling et al., (1977) demonstrated that 40–60 min after temperature change 80% of the poly(A) in the cytoplasm is lost, suggesting RNA degradation. However, Mirault et al., (1978) found that RNA isolated from heat-shocked cells or normal cells coded for similar proteins in an in vitro system. Mirault et al. (1978) interpret their results as indicating that mRNA is stable in heat-shocked cells.

Another feature of heat-shock RNA synthesis and utilization which is interesting is that histone mRNA continues to be transcribed and translated (Spradling et al., 1977). Thus the major poly(A$^-$) mRNA in the cell escapes the regulatory processes which act at the level of transcription and translation. It is tempting to speculate that the 3′-poly(A) may play a role in the regulatory events, perhaps targeting some mRNAs for destruction.

We have studied the fate of poly(A) in *Drosophila* cells at 25 and 37 °C in an attempt to determine the fate of preexisting mRNA and the role of polyadenylation and deadenylation of RNA during heat shock. Our results show that 46% of the cytoplasmic poly(A) decays with a $t_{1/2}$ of 10 min and is completely degraded at 37 °C. Nuclear poly(A) accumulates 2–3-fold above 25 °C levels. Both temperature and pH affect poly(A) levels, and neither protein nor RNA synthesis is required for the effects. The effects are induced at 34 °C and become more severe with increasing temperature.

Experimental Procedures

Cell Culture and RNA Labeling Conditions. Schneider's cell line 2 adapted for growth in spinner culture (Lengyel et al., 1975) was used for these studies except where indicated. Cells were grown in Joklik modified Eagle's medium (Microbiological Associates) supplemented with 1% 100X nonessential amino acids (Grand Island Biological Co., GIBCO), 5% lactalbumin hydrolysate (GIBCO), and 10% fetal calf serum (GIBCO) at 25 °C. They were maintained at a density of 1×10^6–4×10^6 cells/mL and had a doubling time of 24 h. Echalier's KC_0 cell line (Echalier & Ohanessian, 1970) was grown in suspension in D_{22} medium and maintained at a density of 0.8×10^6–1.2×10^6 cells/mL. Under these conditions, the generation time was 30–35 h at 25 °C. For labeling of RNA, cells were concentrated 4-fold and incubated at 25 °C for 30 min to allow recovery. Following recovery, [^{3}H]uridine was added to a concentration of 40 μCi/mL. The

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[1] Abbreviations used: RNA, ribonucleic acid; mRNA, messenger RNA; rRNA, ribosomal RNA; poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); poly(A$^-$) mRNA, mRNA lacking a 3′-poly(A) sequence; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piparazine-*N*′-2-ethanesulfonic acid; oligo(dT), oligodeoxythymidylate; ATP, adenosine triphosphate; DEPC, diethyl pyrocarbonate; SSC, standard saline citrate; Cl_3CCOOH, trichloroacetic acid; DNA, deoxyribonucleic acid.

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Table I: Inhibition of Macromolecular Synthesis[a]

inhibitor	% inhibition[b,c] 25 °C	37 °C
actinomycin D	99	98
cyclohexamide	92	95
puromycin	89	22
chloramphenicol	22	32

[a] Cells were concentrated 4-fold, incubated in media containing 12.5 mM Hepes, pH 6.8, at 25 °C for 30 min, and preincubated with drugs as described under Experimental Procedures. After 5 min at the indicated temperatures, cultures were labeled with 10 μCi of [^{3}H]uridine (actinomycin experiments)/mL or 200 μCi of [^{3}H]leucine/mL for 30 min. Cells were then washed in ISB and lysed in $NaDodSO_4$ buffer. Aliquots were assayed for trichloroacetic acid precipitable radioactivity. [b] [cpm (no inhibitor, 25 °C) − cpm (with inhibitor, 25 °C)]/cpm (no inhibitor, 25 °C) × 100. [c] [cpm (no inhibitor, 37 °C) − cpm (with inhibitor, 37 °C)]/cpm (no inhibitor, 37 °C × 100.

cells were heat shocked as follows. Cultures (100 mL) were placed in a water bath at the appropriate temperature. Aliquots (20 mL) were removed every 30 min, separated into nuclei and cytoplasm, and assayed for poly(A). After removal of the 0-min sample, the remaining culture reached 37 °C in 5 min.

Inhibition of RNA and Protein Synthesis. Inhibitors were added at the following concentrations: cyclohexamide, 20 μg/mL; actinomycin D, 10 μg/mL; chloramphenicol, 200 μg/mL; puromycin, 200 μg/mL. A 20-min preincubation was used for all inhibitors except puromycin (2 min). For half-life experiments at 25 °C, 10 μg/mL actinomycin D was added immediately before the experiment. Table I shows the effect of the various inhibitors on macromolecular synthesis at 25 and 37 °C in Schneider's line 2 cells.

Cell Fractionation. Aliquots of cells were pelleted by centrifugation for 10 min at 1000 rpm in an IEC CRU-5000 refrigerated centrifuge. Pellets were washed once in ice-cold insect standard buffer (ISB, 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 10 mM $CaCl_2$) and were then resuspended in 1 mL of ISB with 1% NP40 to lyse the cells. Nuclei were separated by centrifugation at 2000 rpm for 10 min. The supernatant (cytoplasm) was removed, and nuclei were resuspended in 1 mL of ISB with 1% NP40. $NaDodSO_4$, EDTA, and EGTA were added to nuclear and cytoplasmic fractions at final concentrations of 1%, 5 mM, and 20 mM, respectively. Extracts were either assayed immediately or frozen at −20 °C and stored overnight. Storage of the extracts did not result in any difference in the amount of poly(A) assayed.

Hybridization Assay for Poly(A). The procedure of Milcarek et al. (1974) was used with minor modifications. It is not necessary to deproteinize samples prior to assay with [^{3}H]poly(U) when $NaDodSO_4$ is present, as determined by control experiments adding known amounts of poly(A) to extracts. Cycloplasmic or nuclear extracts equivalent to 3.3×10^5–6.6×10^5 cells were added to 200 μL of 2 × SSC plus 0.2% $NaDodSO_4$. This corresponds to 1–3 ng of poly(A)/aliquot in cytoplasmic extracts and 0.5–1.0 ng/aliquot for the nucleus. [^{3}H]Poly(U) (5000 cpm, 7.0×10^5 cpm/μg) was added and the samples were incubated at 43 °C for 15 min. They were put on ice immediately, and 2 mL of ice-cold 2 × SSC was added. Pancreatic ribonuclease was added to 20 μg/mL, and the samples were incubated for 15 min on ice. RNase-resistant, Cl_3CCOOH-precipitable radioactivity was collected on either Millipore HAWP or Whatman GF-C filters and counted in a toluene-based scintillation fluid. Each time point was assayed in triplicate, and each experiment was repeated at least twice. Schneider's cells contain 12.8 ± 0.3 fg of cytoplasmic poly(A)/cell and 2.1 ± 1.24 fg of poly(A)/cell in the nucleus with our assays. Thus, 85% of steady-state poly(A) is cytoplasmic (see Results). In typical [^{3}H]poly(U) assays, 0.5–1.0 ng of nuclear poly(A) was used or 4×10^5 cell equiv. This number of cells would contain 0.2 μg of DNA (Lengyel et al., 1975). When we assayed 1 μg of *Drosophila* DNA, there was the equivalent of ≤0.06 ng of [^{3}H]poly-(U)-hybridizable material. DNA does not therefore contribute substantially to the nuclear poly(A) determinations or to the results obtained following heat shock.

Sizing of Steady-State Poly(A). Nuclear and cytoplasmic fractions were extracted with phenol and chloroform–isoamyl alcohol (24:1) and then precipitated with 2 volumes of ethanol at −20 °C overnight. Samples were resuspended in 0.25 mL of 2 × SSC and digested with 20 μg/mL pancreatic ribonuclease and 20 units/mL T_1 ribonuclease for 30 min at 37 °C. After the addition of 3 μL of DEPC, 0.2 mL of water, and 20 μg/mL carrier RNA, digested material was precipitated with 2 volumes of ethanol. Samples containing 25 ng of poly(A) were resuspended in 25 μL of electrophoresis buffer (0.04 M Tris, pH 7.4, 2 mM EDTA, 0.2% $NaDodSO_4$, 10% glycerol, and 0.001% bromophenol blue) and electrophoresed on 10% polyacrylamide gels (Weinberg et al., 1967) until the marker dye was 1 cm from the bottom. Gels were sliced into 2-mm slices and placed in 200 μL of $NaDodSO_4$ buffer. Poly(A) was eluted overnight at 37 °C with shaking and assayed by using [^{3}H]poly(U) as described above.

Materials. Cyclohexamide, actinomycin D, puromycin, chloramphenicol, pancreatic ribonuclease, and T_1 ribonuclease were purchased from Sigma Chemical Co., St. Louis, MO. [^{3}H]uridine (37.9 Ci/mM) and [^{3}H]leucine (47 Ci/mM) were purchased from New England Nuclear.

Results

Steady-State Levels of Poly(A) at 25 and 37 °C. Changes in mRNA half-lives may be one way that cells control gene expression. Destabilization of preexisting mRNA may clear the way for new mRNA translation. Spradling et al. (1977) noted an 80% decrease in cytoplasmic poly(A) during the first 30–60 min following 37 °C heat shock in *Drosphila* Schneider's line 2 cells. When we measured poly(A) levels by hybridizing cytoplasmic or nuclear RNA with [^{3}H]poly(U) and observed the amount of radioactivity remaining acid precipitable, we obtained the results shown in Figure 1.

At 25 °C cytoplasmic and nuclear steady-state levels of poly(A) remain essentially constant for the 2 h of observation. At 37 °C, however, both nuclear and cytoplasmic levels change dramatically. The amount of cytoplasmic poly(A) decreases 50% in the first 30 min at 37 °C. Cytoplasmic poly(A) continues to decay during the next 90 min at 37 °C but at a much slower rate. On the other hand, nuclear poly(A) *increases* 3-fold in the first 60–90 min following transfer to 37 °C. A slight decrease from the 3-fold nuclear poly(A) maximum increase is seen at 2 h (Figure 1).

To confirm our observations, we repeated these experiments using Echalier's KC_0 cell line with the same results (not shown). There is a rapid decay of cytoplasmic poly(A) at 37 °C of 50–70% followed by a much slower decay. This would argue against poly(A) degradation representing an artifact found only in Schneider's cells.

We determined the amounts of cytoplasmic rRNA species contaminating our nuclear preparations at 25 °C and at 60 and 120 min into heat shock. No significant differences in the amounts of rRNA in the nuclear preparations were observed (Table II). We therefore conclude that the rapid

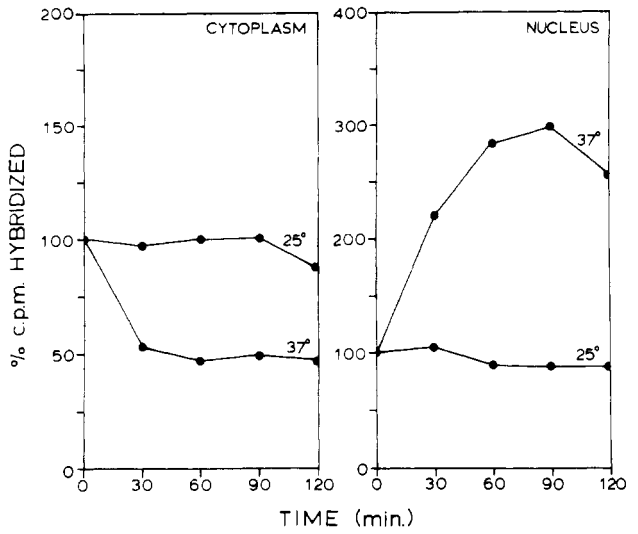

FIGURE 1: Effect of heat shock on cytoplasmic and nuclear poly(A) levels. 100-mL cultures of *Drosophila* cells (2×10^6–4×10^6 cells/mL) were placed at either 25 or 37 °C. Aliquots were removed at intervals, separated into nuclear and cytoplasmic fractions, and assayed for poly(A) by using [^{3}H]poly(U) hybridization. For all of the figures, the 100% values seen at 0 min correspond to 2–4 ng of poly(A) for the cytoplasm and 0.5–1.0 ng for nuclear poly(A) per aliquot. The [^{3}H]poly(U) probe had a specific activity of 700 cpm/ng.

Table II: Nuclear Contamination with 19S and 26S rRNA

min at 37 °C	% rRNA in nuclear fraction[a]
0	8.4
60	7.0
120	7.0

[a] Calculated from 19S plus 26S rRNA in nuclear vs. cytoplasmic fractions. rRNA was fractionated on 15–30% sucrose gradients in an SW41 rotor at 25 000 rpm, for 12 h at 22 °C. Gradients were scanned on ISCO flow cell at 254 nm, and area under peaks was determined by weight.

changes in poly(A) levels during heat shock are not merely a reflection of increased adherence of cytoplasmic material to nuclear pellets after treatment of the cells at 37 °C.

Spradling et al. (1977) used cells concetrated 5-fold prior to heat shock and reported an 80% decrease in cytoplasmic poly(A) during the first 30–60 min. When we use concentrated cells, we find a similar decrease in poly(A) (Figure 2). The more rapid decay seen in concentrated cells is due either to a pH change from CO_2 loss as volume decreases or to more rapid temperature equilibration.

Effect on pH on Poly(A) Levels at 25 and 37 °C. To determine if pH affected poly(A) levels, we concentrated cells 4–5-fold and added Hepes buffer (12.4 mM final concentrations, pH 6.8) to half the culture. Cells were allowed to recover for 30 min at 25 °C and then transferred to 25 or 37 °C. Equal aliquots of cells were removed at 30-min intervals, fractionated into nuclei and cytoplasm, and assayed for poly(A). The results (Figure 2) demonstrate that extracellular pH affects both nuclear and cytoplasmic poly(A) metabolism in concentrated cell cultures even at 25 °C. Without buffer, 75% of the cytoplasmic poly(A) at 25 °C decays by 90 min and the remaining 25% appears relatively stable. Poly(A) levels do not change appreciably at 25 °C for concentrated cells with buffer (Figure 2) and nonconcentrated cells (Figure 1). The biphasic curve suggests that two populations of poly(A) exist in the cytoplasm, one decaying rapidly and one being relatively stable.

At 37 °C in cells without additional buffering, cytoplasmic poly(A) decays twice as fast as it does in buffered cells.

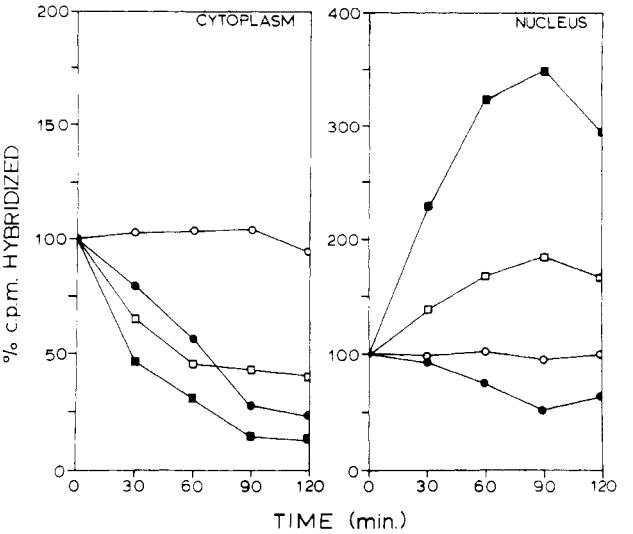

FIGURE 2: Effect of concentrating cells and buffer on nuclear and cytoplasmic poly(A) levels during heat shock. 100-mL cultures of *Drosophila* cells (2×10^6–4×10^6 cells/mL) were concentrated 5-fold in media containing or lacking 12.5 mM Hepes, pH 6.8, and allowed to recover for 30 min at 25 °C. They were then transferred to 37 or 25 °C. Aliquots were removed at indicated intervals, separated into nuclei and cytoplasm, and assayed for poly(A) by using [^{3}H]-poly(U). (●) 25 °C, without buffer; (○) 25 °C, with buffer; (■) 37 °C, without buffer; (□) 37 °C, with buffer.

Without buffer, the slow component continues to decay, while in buffered cells, this component is stabilized.

Nuclear poly(A) in nonbuffered concentrated cells decays slowly at 25 °C to 75% of its original value by 90 min. In contrast, nuclear poly(A) remains constant for at least 90 min in nonconcentrated (Figure 1) or concentrated cells maintained in buffered media (Figure 2). At 37 °C in concentrated cells without buffer, nuclear poly(A) increases 3.6-fold then drops slightly. When pH is maintained, the poly(A) levels increase only 2-fold. In nonconcentrated cells, a 3-fold increase in nuclear poly(A) occurs after 90 min. Therefore, external pH can affect steady-state levels of both nuclear and cytoplasmic poly(A).

Kinetics of Poly(A) Decay at 37 °C. Figures 1 and 2 indicate that there are at least two components in the cytoplasmic poly(A) population in *Drosophila* cells at 37 °C, a rapidly decaying component (component I) and a more stable one (component II). For determination of half-lives of these components, cells were placed at 37 °C and at 10-min intervals an aliquot was passed through a funnel containing frozen crushed ISB to rapidly cool the cells. Because the rate of labeling of mRNA after heat shock is ≤10% of the rate at 25 °C (Spradling et al. 1977), de novo RNA synthesis probably does not represent a substantial fraction of total poly(A) at early times in the experiments. For estimation of the half-life of poly(A) at 25 °C, 10 μg/mL actinomycin D was added (Table II).

Analysis of poly(A) levels demonstrated a biphasic curve at 37 °C (Figure 3a). Component I decays rapidly with an apparent half-life of 26 min and constitutes 46% of the poly(A) in the cytoplasm. Component II appears stable and constitutes 54% of the poly(A) in the cytoplasm. When we correct the decay curve for component I by subtracting the contribution of component II, we obtain a half-life of 10 min (Figure 3b).

At 25 °C it was necessary to inhibit RNA synthesis with actinomycin D. Although the drug may affect the results, it nevertheless allows us to estimate the stability of poly(A) in cells grown in 25 °C. Figure 3a shows that at 25 °C there is a single kinetic component of poly(A) which decays with

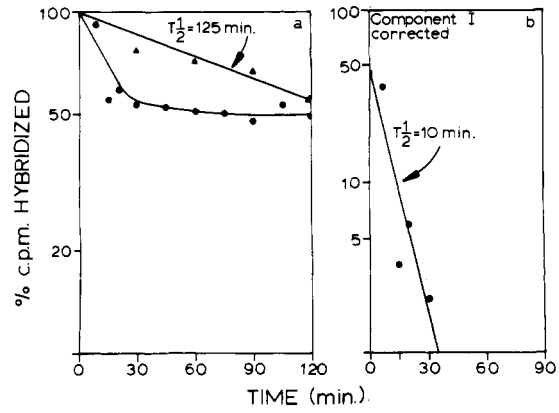

FIGURE 3: Kinetics of decay of cytoplasmic poly(A). (a) 100-mL cultures of *Drosophila* cells (2×10^6–4×10^6 cells/mL) were placed at 37 °C. Aliquots removed at 10-min intervals were separated into nuclei and cytoplasm, and cytoplasmic fractions were assayed for poly(A) content by using [^{3}H]poly(U). For determination of the stability of 25 °C poly(A), actinomycin D (10 μg/mL) was added at time 0 and aliquots were taken every 30 min. (▲) 25 °C, with actinomycin D; (●) 37 °C, without actinomycin D.

a half-life of 125 min. This is slightly faster than the estimated half-life of poly(A) in HeLa cells (3.5–4 h) when actinomycin D is used (Sheiness et al., 1975). Thus, component I at 37 °C decays 12 times faster than poly(A) at 25 °C.

Poly(A) Degradation Does Not Require RNA or Protein Synthesis. Several studies have shown that new RNAs are synthesized in heat-shocked cells (Spradling et al., 1977; Mirault et al., 1978; Moran et al., 1978). To determine if new RNA synthesis was necessary for the rapid drop in cytoplasmic polynA) at 37 °C, we assayed poly(A) levels in cells pretreated with actinomycin D. As shown in Figure 4a,b at 37 °C, the decrease in cytoplasmic poly(A) was the same in the presence or absence of actinomycin D for the first 60 min, indicating that new RNA synthesis is not necessary for the initial decrease of poly(A). After 60 min in cells minus actinomycin D, the poly(A) levels remain constant whereas in pretreated cells poly(A) levels continue to decline. After 120 min this results in a 15–20% difference in poly(A) levels between the treated and untreated cultures. Since the cytoplasmic poly(A) levels are identical at 30 min in cells in the presence and absence of actinomycin D at 37 °C, we conclude that rapid degradation of component I does not require new RNA synthesis. Comparison of the 37 °C curves with and without actinomycin D shows that the increase in nuclear poly(A) is somewhat reduced with the drug indicating a portion of the increase may reflect de novo RNA synthesis (see Discussion).

Six to nine new polypeptides are synthesized during heat shock (Lewis et al., 1975; Koninkx, 1976). To determine if new protein synthesis is necessary for the rapid decrease in poly(A), we followed the fate of poly(A) in the presence of several inhibitors of protein synthesis. None of the inhibitors used affected the rapidly decaying poly(A) component I. The results in Figure 4c,d show that with cyclohexamide at 37 °C, cytoplasmic poly(A) decreases rapidly in the first 30 min just as it does without the drug, indicating protein synthesis is not a requirement for poly(A) degradation. At 25 °C, cyclohexamide has only a slight effect on cytoplasmic poly(A) levels. Poly(A) component II is apparently destabilized by cyclohexamide treatment at 37 °C. Cyclohexamide has little effect on nuclear poly(A) levels in cells incubated at 25 or 37 °C, indicating that the increase in nuclear poly(A) does not require protein synthesis.

Puromycin and chloramphenicol also did not block the rapid 37 °C induced decay of component I poly(A) (data not

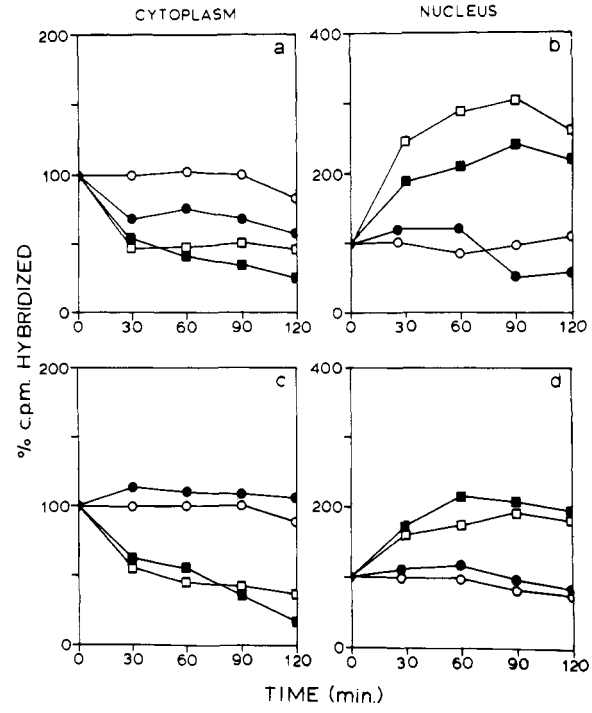

FIGURE 4: RNA synthesis and protein synthesis are not necessary for heat-shock effects. (a and b) 100-mL cultures of *Drosophila* cells were incubated for 20 min with 10 μg/mL actinomycin D and then placed at either 25 or 37 °C. Aliquots were removed at 30-min intervals, separated into nuclei and cytoplasm, and assayed for poly(A) content by using [^{3}H]poly(U). (c and d) 100-mL cultures were concentrated 5-fold and allowed to recover for 30 min at 25 °C in medium containing 12.5 mM Hepes, pH 6.8. Cyclohexamide was added (20 μg/mL) and the cells were incubated an additional 20 min. Cultures were transferred to 25 or 37 °C, and aliquots were removed at 30-min intervals, fractionated into nuclei and cytoplasm, and assayed for poly(A) content by using [^{3}H]poly(U). (□) 37 °C, without inhibitor; (○) 25 °C, without inhibitor; (■) 37 °C, with inhibitor; (●) 25 °C, with inhibitor.

shown). However, as shown in Table I, neither drug was an adequate inhibitor of protein synthesis at 37 °C.

Effect of Temperature. Several of the heat-shock puffs show a characteristic temperature of induction. In addition, with increasing temperature, the size of the puffs increases (Lewis et al., 1975; Ashburner & Bonner, 1979). To determine whether the alterations in poly(A) metabolism exhibit a similar behavior, we placed 100-mL cultures of cells at various temperatures. Equal size aliquots were removed at 30-min intervals, separated into nuclei and cytoplasm, and assayed for poly(A) content. As shown in Figure 5a, marked effects of temperature on cytoplasmic poly(A) content are first visible at 37 °C. As the temperature is increased, we see faster rates of poly(A) decay. At 39 °C, component I decays faster and constitutes more of the total cytoplasmic poly(A) than at 37 °C. Component II is no longer stable and decays at 39 °C. These effects are more pronounced at 41 °C where most of the cytoplasmic poly(A) has decayed by 60 min.

We see a similar effect of temperature for nuclear poly(A) (Figure 5b). The results demonstrate both a temperature threshold for the changes in poly(A) content and increased severity of response as the temperature is raised. In figure 5c, the poly(A) levels at 60 min have been plotted vs. temperature to illustrate the effect. Interpolation between the points in Figure 5c indicates cytoplasmic poly(A) degradation and nuclear poly(A) accumulation are both induced at 34 °C.

Size of Nuclear and Cytoplasmic Poly(A) during Heat Shock. At least two models are consistent with the loss of 50% of cytoplasmic poly(A) during heat shock. In model 1, every

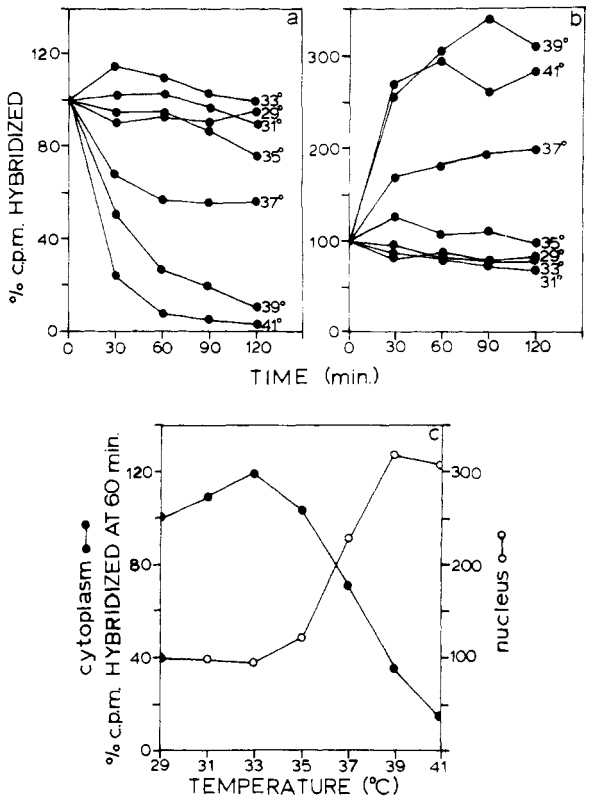

FIGURE 5: Effect of varying temperatures on cytoplasmic and nuclear poly(A) levels. 100-mL cultures were placed at the indicated temperatures, and aliquots were removed every 30 min. Samples were fractionated into nuclei and cytoplasm and assayed for poly(A) content by using [^{3}H]poly(U). (a) Cytoplasm; (b) nucleus; (c) 60-min poly(A) levels replotted vs. temperature to demonstrate the induction threshold temperature more clearly.

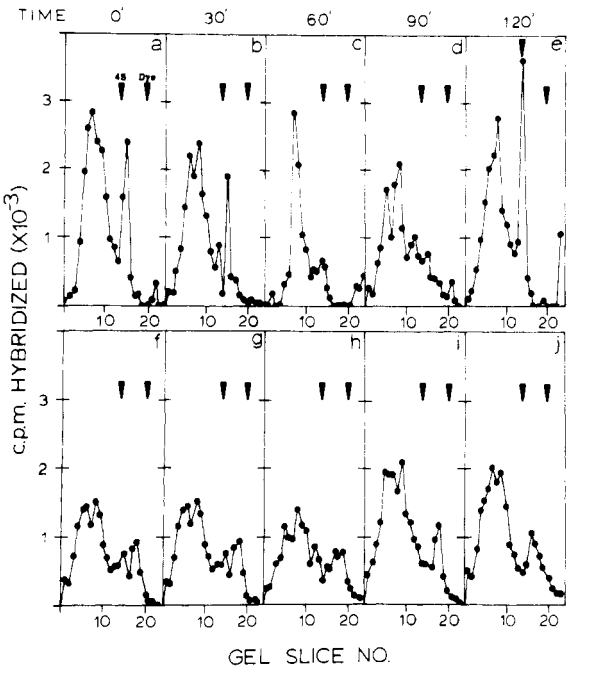

FIGURE 6: Sizing of nuclear and cytoplasmic poly(A) during heat shock. 100-mL cultures of cells (2×10^6–4×10^6 cells/mL) were placed at 37 °C. Aliquots were removed at 30-min intervals and fractionated into nuclei and cytoplasm. Samples were extracted with phenol and chloroform and digested with T_1 and pancreatic nucleases, and resistant material was electrophoresed on 6-cm 10% polyacrylamide gels. Poly(A) was eluted, and amounts in each fraction were determined by [^{3}H]poly(U) hybridization. 25 ng of poly(A) was loaded on each gel. Data have been corrected for differential recovery from the gels. (Panel a) 0-min cytoplasm; (panel b) 30-min cytoplasm; (panel c) 60-min cytoplasm; (panel d) 90-min cytoplasm; (panel e) 120-min cytoplasm; (panel f) 0-min nucleus; (panel g) 30-min nucleus; (panel h) 60-min nucleus; (panel i) 90-min nucleus; (panel j) 120-min nucleus.

preexisting mRNA in the cytoplasm loses half its length of poly(A), and therefore an overall decrease in poly(A) size would be predicted. In the second model, half the mRNAs lose all of the poly(A) tail while the other portion of mRNA retains a full length poly(A). Model 2 results in no change in overall poly(A) size, just a decrease in the total amount per cell.

To determine which of these models is correct, we have studied the size of bulk poly(A) sequences at various times following heat shock. Following digestion with T_1 and pancreatic ribonucleases, nuclease-resistant RNA was electrophoresed on polyacrylamide gels (Weinberg et al., 1967). Poly(A) was eluted from gel slices and hybridized to [^{3}H]-poly(U).

Before heat shock begins (Figure 6a), we detect two cytoplasmic poly(A) size classes. The first species, designated poly(A)$_\alpha$, is broad and heterogeneous but ~150 nucleotides in length with a peak at slice 8. The second species, designated poly(A)$_\beta$, migrates just ahead of a 4S marker and is roughly 28 nucleotides in length (Wilt, 1977; Burness et al., 1975). Poly(A)$_\alpha$ is of a size which corresponds to polysomal cytoplasmic mRNA and poly(A)$_\beta$ probably corresponds to mitochondrial poly(A) on the basis of its size (Hirsch et al., 1974; Hirsch & Penman, 1973; Brawerman & Diez, 1975). Poly(A)$_\beta$ is ~23% of total cytoplasmic steady-state poly(A) at 25 °C. During heat shock (see Figure 6b–d) the average size of poly(A)$_\alpha$ remains approximately the same, always peaking at slice 8. Poly(A)$_\beta$ decreases as a percentage of total cytoplasmic poly(A) during heat shock until 120 min, but the length is relatively constant at all times. The relative amounts of poly(A)$_\beta$ during heat shock are quantitated in Table III. We

Table III: Degradation of Poly(A)$_\beta$ during Heat Shock

min at 37 °C	% of material in poly(A)$_\beta$[a]
0	22.2
30	13.7
60	12.9
90	11.4
120	22.6

[a] Determined in two ways: (1) summation of cpm hybridized in fractions 13–16 and dividing by total cpm hybridized; (2) measurement of the area under the curves with a Numonics digital tracer and dividing the area under fractions 13–16 by the total area.

see that poly(A)$_\beta$ drops from 22.5% of total at time zero to 11–14% of total cytoplasmic poly(A) during heat shock by 90 min. A preferential decrease of poly(A)$_\beta$ would not therefore account for the overall 50% drop in cytoplasmic polyA) during heat shock. Because we detect no gross size changes in either poly(A) component, we conclude that the decrease in poly(A) content during heat shock does not reflect removal of 50% of each mRNAs poly(A) but complete degradation of some poly(A)'s.

Similarly, accumulation of poly(A) in the nucleus during heat shock could result from (1) synthesis of new RNAs with poly(A) attached, (2) addition of adenosine residues to preexisting polyadenylated RNA sequences, or (3) random polyadenylation of any primer. To distinguish between poly(A) lengthening and the other hypotheses, we have sized nuclear poly(A). We find two peaks of poly(A) in steady-state nuclear poly(A) (Figure 6f). The larger size material migrates more slowly than peak α cytoplasmic poly(A) indicating it has

a slightly larger size. It is clear from the size profiles during heat shock that the size distribution remains essentially unchanged. We see no evidence of a size increase in nuclear poly(A) eliminating model 2, lengthening of preexisting poly(A). Five to ten adenylate residues could be added but would not account for the 2–3-fold increase in nuclear poly(A). The small poly(A) peak in the nucleus may represent the oligo(A) seen by Nakazato et al. (1973, 1974) in HeLa cells.

Discussion

We have shown that upon transfer of *Drosphila* cells from 25 to 37 °C, there are dramatic alterations in the metabolism of preexisting poly(A). In the nucleus, poly(A) accumulates for the first 60–90 min following transfer, peaking at levels 2 or 3 times higher than at 25 °C. Nuclear poly(A) is not increasing beyond normal length. It seems unlikely that the increase is due entirely to synthesis of heat shock specific RNA. Spradling et al. (1977) showed that the rate of [^{3}H]uridine incorporation during heat shock is ≤10% of the normal rate. Our studies with actinomycin D (Figure 4) show that accumulation of nuclear poly(A) is decreased only 20–30% when RNA synthesis is inhibited. We have also measured the rate of [^{3}H]uridine incorporation in the nucleus during heat shock and find that it is decreased by 50% after 60 min at 37 °C (unpublished results). These results make it unlikely that new RNA synthesis alone can account for the rapid increase in nuclear poly(A). The results are consistent with poly(A) being added nonspecifically to preexisting nonadenylated RNA molecules in the nucleus. Continued synthesis of poly(A) in the absence of RNA synthesis has been observed in HeLa cells (Darnell et al., 1971) but not to the extent seen during heat shock. Alternatively, nuclear poly(A) may accumulate because of an increased transit time for newly adenylated molecules during heat shock.

Preexisting poly(A) in the cytoplasm decays when the temperature is raised to 37 °C. Kinetic studies show that there are two apparent components designated I and II. Component I decays with first-order kinetics and has a half-life of 10 min. It constitutes 46% of the poly(A) in the cytoplasm. We have not attempted to measure the half-life of component II. Any accumulation with time of heat-shock mRNA would alter the half-life of long-lived components. We estimate 33% of the total poly(A) after 120 min at 37 °C results from new RNA synthesis (Figure 4a,b), indicating that accumulation of new RNA contributes substantially to total cytoplasmic poly(A) at later times during heat shock. By measuring the size of cytoplasmic poly(A), we have shown that degradation is apparently complete for component I. Component II appears to retain its poly(A) relatively intact.

The half-life of 10 min calculated for component I differs from reported RNA lifetimes in insect cells. Lengyel & Penman (1977) found a component of RNA with a half-life of 60 min in the cell line used in these studies. A component with a half-life of 72 min has been described in *Aedes albopticus* cells (Spradling et al., 1975). These measurements were made with RNA selected on oligo(dT)–cellulose; therefore, we can directly compare these half-lives with that of component I. At 37 °C there is a poly(A) population which decays 6–7 times faster than the most rapidly turning over poly(A) population at 25 °C thus far observed.

Inhibitors of RNA synthesis or protein synthesis do not prevent the rapid changes in poly(A) levels in nucleus or cytoplasm (component I). However, the inhibitors do alter the apparent stability of component II. Alterations in component II RNA half-life by the drugs or contributions of newly synthesized heat-shock RNA to the pool have not been distinguished. Neither RNA nor protein synthesis is required for the extremely rapid changes in poly(A), indicating that the responsible factor(s) exist prior to the heat shock and are rapidly activated by temperature changes.

It is tempting to speculate that poly(A) binding proteins, many of which have been described (Mazur & Schweiger, 1978; Kwan & Brawerman, 1972; Morel et al., 1973), may play a role in the heat-induced rapid decrease in poly(A) content. In vitro studies have shown that purified poly(A) polymerase (EC 2.7 7.19) can degrade poly(A) when the ATP concentration is lowered (Abraham & Jacob, 1978), a condition that is known to exist in heat-shocked cells (Leenders et al., 1974).

Rapid degradation of cytoplasmic poly(A) may serve to signal the cell that the message is not longer needed. The message may then be destroyed or sequestered for later use. Sequestering might explain how cells can recover from a 2-h heat shock (unpublished results), despite degradation of 50–80% of the poly(A). Deadenylated message can survive in the cell and may be utilized (Fromson & Verma, 1976; Gedamu, & Dixon, 1976; Levenson & Marcu, 1976; Kaufman et al., 1977; Ruderman & Pardue, 1977). It is also known that nonadenylated message can by adenylated and used under certain conditions (Slater & Slater, 1974; Dolecki et al., 1977). The fate of the rest of the molecule must be determined in order to determine if mRNA is degraded or sequestered. These studies are in progress.

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1,25-Dihydroxyvitamin D_3 Stimulated Increase of 7,8-Didehydrocholesterol Levels in Rat Skin†

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ABSTRACT: A convenient, accurate assay was developed for determining skin cholesta-5,7-dien-3β-ol (7,8-didehydrocholesterol) concentrations. Ultraviolet spectrophotometry provided quantitation of the sterol from rat skins following saponification and chromatography on Lipidex and high-performance liquid chromatography. Correction for recoveries was accomplished by using 7,8-didehydro[3α-^{3}H]cholesterol as an internal standard. Chronic dosing of vitamin D-deficient rats with 1,25-dihydroxyvitamin D_3 caused a 4-fold increase in skin 7-dehydrocholesterol content. This rise was not the result of changes in food consumption, body weight, or plasma calcium. Cholesterol concentrations were not significantly elevated although some of the other nonsaponifiable lipid components found in the high-performance liquid chromatogram appeared to be increased by the treatment. These results suggest that the vitamin D hormone 1,25-$(OH)_2D_3$ may exert a positive feedback regulation on the production of vitamin D_3 in skin.

Windaus and his co-workers (Windaus et al., 1935) synthesized cholesta-5,7-dien-3β-ol (7,8-didehydrocholesterol; Δ5,7) under the premise that it was the natural provitamin for vitamin D in animal tissues. Vitamin D_3 was identified in ultraviolet (UV) irradiation mixtures of synthetic Δ5,7 (Windaus et al., 1936). This group then spectrophotometrically detected Δ5,7 in several animal tissues, finding the highest concentrations in skin samples. They then isolated and identified Δ5,7 from pig skin (Windaus & Bock, 1937). This work led to the long held assumption that vitamin D_3 is the natural antirachitic substance produced in the skin of animals by irradiation with UV light. This idea was supported recently by the isolation and identification of vitamin D_3 (Holick et al., 1977; Esvelt et al., 1978) and its obligatory precursor, previtamin D_3 (Holick et al., 1979) from UV irradiated rat skins.

The skin is a highly active sterol biosynthesizing tissue which, unlike most tissues, accumulates significant quantities of sterol intermediates, including Δ5,7 (Kandutsch, 1964; DeLuca, 1971). In the skin, where the unsaturation at C-24 is reduced early in sterol biosynthesis (Clayton et al., 1963), Δ5,7 serves as a direct precursor of cholesterol. Δ5,7 is formed in an irreversible oxygen-dependent reaction from Δ7-cholestenol (Dempsey et al., 1964), and Δ5,7 can be converted to cholesterol in an irreversible nicotinamide adenine dinucleotide phosphate (NADP) dependent reaction (Frantz et al., 1964). The low level of Δ5,7 in other tissues (Dempsey et al., 1964;

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0006-2960/80/0419-6158$01.00/0