Novel structure of heat shock messenger ribonucleoproteins in *Drosophila* Kc cells

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Poly(A)$^+$ polyribosomal mRNA is normally complexed with a specific set of proteins forming ribonucleoprotein particles, termed polyribosomal mRNPs. We have found that in *Drosophila* during the heat shock response, their structure is profoundly altered as shown by their greatly decreased density in Cs_2SO_4 gradients. RNA-protein cross-linking, conducted in vivo, revealed that polyribosomal mRNA is associated with only two polypeptides of 70 and 38 kDa; while those from cells raised at 25°C revealed a more complex protein pattern. The 38 kDa protein is located on the poly(A) sequences.

Drosophila *Polyribosomal mRNP* *Heat shock messenger* *Poly(A)-protein* *UV cross-linking*

1. INTRODUCTION

Drosophila cells respond to mild heat treatment by activating a small set of specific genes, called heat shock genes. After heat shock, most of the genes normally active appear to be repressed. Heat shock is characterized by rapid synthesis of a small number of proteins, the so-called heat shock proteins [1,2]. There is evidence that new messenger RNAs coding for the heat shock proteins are transcribed, processed and transported into the cytoplasm to be translated [3–6].

At all steps in gene expression, mRNA is intimately associated with various proteins as messenger ribonucleoprotein particles (mRNPs) [7–9]. Normally, these mRNPs contain protein and RNA in a mass ratio of about 4:1, as estimated from their buoyant density in Cs_2SO_4 of 1.34 g/cm^3 [10–12]. Recently though, Mayrand and Pederson [13] have found that pre-messenger RNA synthesized during the heat shock response in *Drosophila* cells forms a profoundly altered nuclear ribonucleoprotein structure with a density of 1.58 g/cm^3, which is equivalent to 10% proteins and 90% RNA.

We will show that upon heat shock, translatable mRNPs in the cytoplasm have a similar buoyant density of 1.55 g/cm^3 in a Cs_2SO_4 density gradient, which means that it also has a protein-deficient structure. It is, therefore, of interest to know the protein constituents of this particle. UV irradiation which leads to photochemical formation of covalent crosslinks between mRNA and its proteins was conducted in vivo [14–18]. Here, we report that during heat shock *Drosophila* polyribosomal mRNA is associated with 2 polypeptides of 70 and 38 kDa only, while those from cells raised at 25°C revealed a more complex pattern.

2. MATERIALS AND METHODS

2.1. *Cell culture and radioisotopic labeling*

Drosophila Kc cells [19], adapted to serum free growth, were grown at 25°C in spinner cultures at approx. 2×10^6 cells per ml. To pulse-label RNA,

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exponentially growing cells were concentrated 5-fold and incubated with [^{3}H]uridine for 20 min at 25°C (4 μCi/ml). 5-Fluoruridine was added to 5 μg/ml to inhibit rRNA synthesis [20,21].

2.2. *Heat shock, in vivo UV crosslinking and cell fractionation*

For heat shock, the cell suspension was transferred to a 37°C water bath, as in [13]. Both the 25 and 37°C cell suspensions were thereafter chilled rapidly with ice-cold buffered saline (140 mM NaCl, 10 mM K-phosphate, pH 7.1), pelleted by centrifugation (1000 × g, 10 min, 2°C), and washed twice with the same buffer. A suspension of 10^8 cells/ml was irradiated with 254 nm UV light at 3700 μW/cm^2 with stirring in stainless-steel petri dishes at 2°C for 15 min [11,16]. The irradiated cells were centrifuged (1000 × g, 10 min, 2°C) and resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 10% sucrose, 4 mM dithioerythritol, 0.2% Triton X-100). Phenylmethylsulfonyl fluoride (PMSF, Serva) was added to a final concentration of 0.5 mM. The cells were disrupted in a Dounce homogenizer by 15 strokes of the smooth pestle. Nuclei, mitochondria and unbroken cells were removed from the cytosol fraction by 2 sucessive centrifugations (3000 × g, 10 min; 16000 × g, 10 min, 2°C). The supernatant was layered on 15–45% (w/w) sucrose gradients in 10 mM Tris-HCl, pH 7.6, 10 mM NaCl, 2 mM $MgSO_4$, and centrifuged in a Beckman SW-28 rotor (27000 rpm, 3.5 h, 4°C). Finally, fractions of 1.2 ml were collected from the gradients after passage through the flow cell of a recording spectrophotometer. Aliquots of 200 μl were precipitated with trichloroacetic acid and analyzed in a scintillation counter. Fractions containing particles larger than disomes were pooled, and ultimately sedimented through 4-ml cushions of 2 M sucrose in a Beckman Ti-60 rotor run at 36000 rpm for 17 h at 4°C [15].

2.3. *Oligo(dT)-cellulose affinity chromatography*

The polyribosomal pellet was resuspended in SDS-containing buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% SDS). It was then made 0.5 M in NaCl and heated at 60°C for 3 min. After cooling to room temperature the sample was chromatographed on an oligo(dT)-cellulose column, previously equilibrated with binding buffer (10 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 14 mM mercaptoethanol, 0.5% SDS) [15]. Poly(A)$^+$ polyribosomal mRNPs were released with 5 ml elution buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% SDS), rechromatographed, eluted, precipitated with ethanol and then pelleted by centrifugation at 16000 × g for 60 min at 0°C. The pellet was dissolved in digestion buffer (62.5 mM Tris-HCl, pH 6.5, 10% glycerol, 14 mM mercaptoethanol, 0.5% sarkosyl) and treated with ribonuclease A. Poly(A)-protein complex was then isolated from the digested material by another affinity chromatography on an oligo(dT)-cellulose column as described above. Eluted fractions were again precipitated with ethanol, pelleted, dissolved in digestion buffer and digested with 10 units ribonuclease T_2 for 60 min at 37°C. The samples were then made 2% in SDS and electrophoresed in 12.5% polyacrylamide slab gels (PAGE) as described [15,22].

3. RESULTS

3.1. *Characterization of* Drosophila *mRNPs isolated under culture condition and after heat shock*

One expects to find most of the translatable mRNA bound to ribosomes; thus polyribosomes were isolated from 37°C heat-shocked and 25°C control cultures by means of sucrose gradient centrifugation (fig.1). The A_{254} profile clearly shows that upon heat shock the polyribosomes were significantly reduced, while the 80 S monoribosomes were increased. But the radioactivity profile indicated that a significant amount of mRNA was still present in the polyribosomal fraction (i.e. 9% of the total ^{3}H-labeled acid precipitable counts vs 22% in control experiments). Since cells grown at elevated temperature preferentially synthesize heat shock proteins [3–6], the polyribosomal region (fraction 1–16, fig.1b) should be enriched in heat shock mRNAs.

Previous investigations have demonstrated that a set of major mRNP-proteins remains associated with mRNA throughout the translation cycle [23–25]. In a first step we have used Cs_2SO_4 density gradient centrifugation to analyze the density of the [^{3}H]uridine pulse-labeled mRNP of the polyribosomal region from cells raised at 25°C and after a temperature shift to 37°C. To ensure that

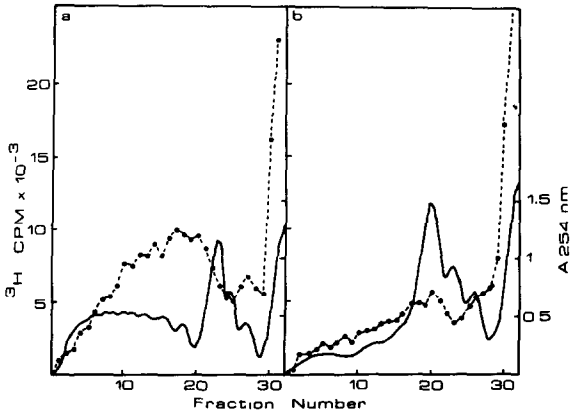

Fig.1. Sedimentation profile of *Drosophila* cytoplasmic supernatant in sucrose gradients. *Drosophila* Kc cells were labeled with [^{3}H]uridine for 20 min in the presence of 5 μg/ml of 5-fluoruridine. A cytoplasmic supernatant was prepared and layered on 15–45% (w/w) sucrose gradients and centrifuged as described in section 2. Fractions of 1.2 ml were collected after passage through the flow cell of a recording spectrophotometer to monitor the distribution of A_{254}. 200-μl aliquots of each fraction were analyzed for trichloroacetic acid precipitable radioactivity. Identical amounts of ^{3}H radioactivity were loaded on the 2 gradients. (a) From cells raised at 25°C, (b) from cells under heat shock at 37°C. (——) A_{254}, (– – –) ^{3}H radioactivity.

the isolated mRNPs were preserved as they existed in the living cell, cells were irradiated with UV light in vivo prior to fractionation to crosslink the mRNA to its associated proteins [14–18]. This treatment allowed a rigorous purification procedure to prevent adventitious protein association.

As shown in fig.2a, heat-shocked cells contain 2 distinct types of mRNPs with densities of 1.34 and 1.55 g/cm^3. Control cells (25°C) possess only one principal buoyant density peak of 1.34 g/cm^3 (fig.2b), while protein-free mRNA has a density of 1.64 g/cm^3 (fig.2c).

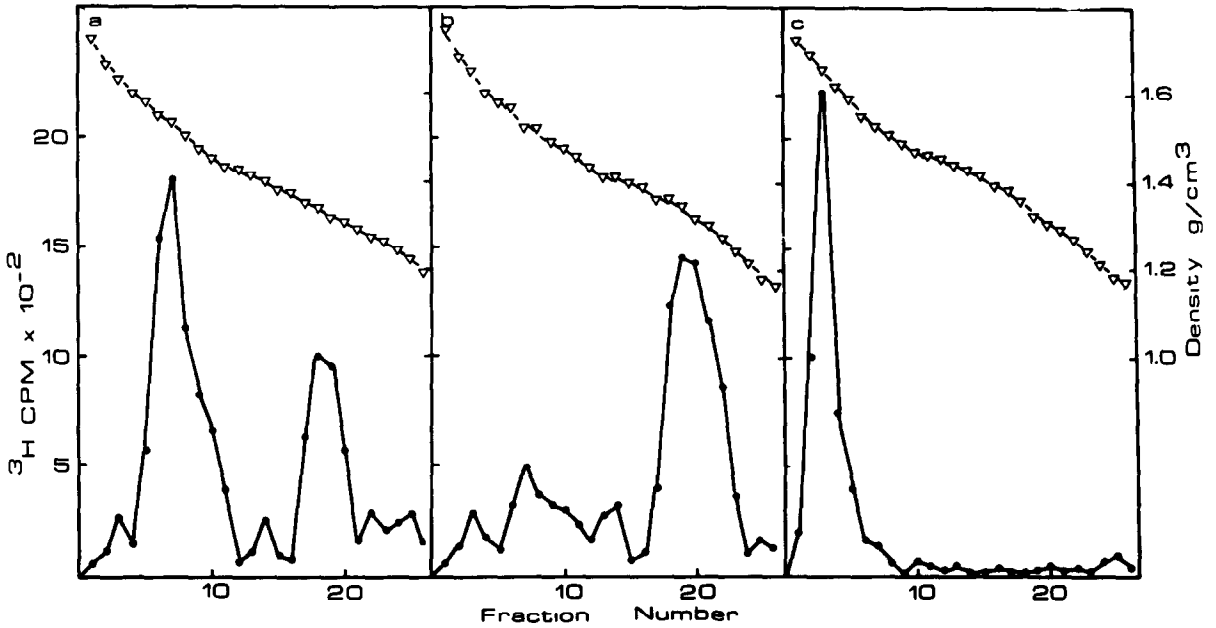

Fig.2. Cs_2SO_4 banding of polyribosomal mRNPs from control and heat-shocked cells. Cells were labeled with [^{3}H]uridine for 20 min and UV irradiated in vivo. Polyribosomes resuspended in 10 mM Tris-HCl, pH 7.6, 10 mM KCl, 0.01% Triton X-100, 15% (v/v) DMSO, were layered onto 15–30% (w/w) Cs_2SO_4-DMSO preformed gradients in the same buffer and centrifuged in a Beckman SW-56 rotor at 40000 rpm for 20 h at 20°C. 150-μl fractions were collected. The density of each fraction was determined by weighing 20-μl aliquots. Trichloroacetic acid precipitable ^{3}H radioactivity was measured from 100-μl samples. (a) From cells raised at 37°C, (b) from cells raised at 25°C, (c) [^{3}H]uridine-labeled mRNA as control.

3.2. *Proteins associated with poly(A)$^+$ mRNA from cells raised at 25 °C and during heat shock at 37 °C*

The Cs_2SO_4 density gradient analysis showed that upon heat shock a 'denser' type of mRNP was assembled preferentially; this difference in density was most likely due to differences in the protein composition of the mRNPs. Poly(A)$^+$ mRNPs of in vivo irradiated cells were separated from the polyribosomes by affinity chromatography on oligo(dT)-cellulose in the presence of SDS and high salt to prevent any unspecific adsorption of proteins to mRNP or to the matrix. Poly(A)$^+$ mRNPs were eluted, digested with RNases A, T_1 and T_2, and then analyzed by SDS-PAGE [15]. Fig.3 (lane C) shows the proteins associated with poly(A)$^+$ mRNPs from heat-shocked cells where mainly 2 polypeptides of 70 and 38 kDa were found. When RNase treatment was omitted, no proteins entered the gel, indicating that they were crosslinked to mRNA.

Control mRNPs from cells raised at 25°C revealed a more complex protein pattern. Fig.4 (lane A) shows proteins of 110, 75, 70, 60, 55, 42 and 38 kDa as well as some minor bands (bands of the 3 RNases are also seen).

It is already well established that in mammalian cells a specific protein of 75 kDa interacts with the

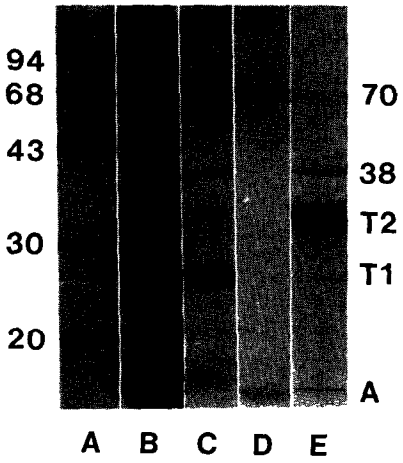

Fig.3. Proteins which could be crosslinked to poly(A)$^+$ mRNA from cells under heat shock. Poly(A)$^+$ mRNPs were obtained through affinity chromatography on oligo(dT)-cellulose as described in section 2. The mRNP-proteins were analyzed in SDS-PAGE after digestion with RNases A, T_1, and T_2. The gels were stained with Coomassie brillant blue. Molecular masses of the proteins are given in kDa. Lanes: (A) Marker proteins: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor. (B) Polyribosomes from heat-shocked cells digested with RNases A, T_1 and T_2; before chromatography through oligo(dT)-cellulose. (C) Proteins crosslinked to poly(A)$^+$ polyribosomal mRNA from cells under heat shock, digested with RNases A, T_1, and T_2. (D) Proteins crosslinked to mRNA sequences other than poly(A) after digestion with RNase A in high salt and rechromatography on oligo(dT)-cellulose; non-rebound fraction. (E) Oligo(dT)-cellulose bound fraction of the proteins crosslinked to poly(A) after digestion with RNase T_2.

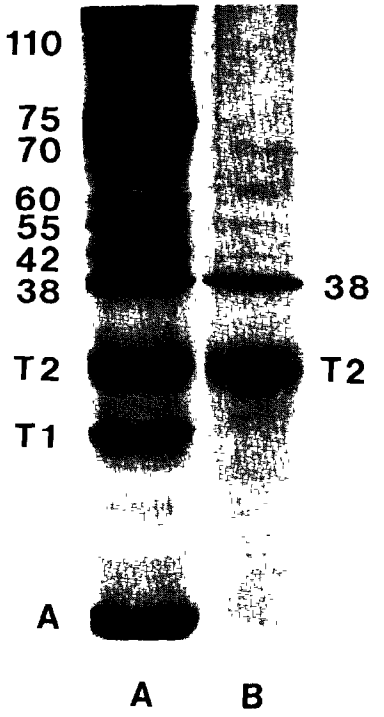

Fig.4. Proteins crosslinked to poly(A)$^+$ mRNA from cells raised at 25°C. Poly(A)$^+$ mRNPs were purified through affinity chromatography on oligo(dT)-cellulose as described in section 2. The proteins in the various fractions were analyzed in SDS-PAGE after digesting with RNases A, T_1 and T_2. The gels were stained with Coomassie brillant blue. Molecular masses of the proteins are given in kDa. Lanes: (A) Proteins crosslinked to poly(A)$^+$ mRNA digested with RNases A, T_1, and T_2. (B) Proteins crosslinked to the poly(A). Poly(A)$^+$ mRNPs were digested with RNases A and T_1 in high salt. The poly(A)-protein complexes were rechromatographed through oligo(dT)-cellulose and digested with RNase T_2.

poly(A) sequences of polyribosomal mRNA [15, 26–31]. To determine whether this was also true for the approx. 70 kDa protein in *Drosophila* cells, oligo(dT)-purified mRNPs were digested with RNase A in high salt. Under these conditions poly(A) sequences are preserved, while other phosphodiester bonds are hydrolyzed [15]. A poly(A)-protein complex was isolated by rechromatography of the digested mRNPs on oligo(dT)-cellulose. Treatment with RNase T_2 to hydrolyze poly(A) [32] surprisingly liberated a single protein of 38 kDa, as detected by SDS-PAGE analysis (fig.3, lane E, and fig.4, lane B). When RNase T_2 digestion was omitted, no such band was obtained, indicating that the 38 kDa protein-poly(A) complex had not entered the gel. On the other hand the 70 kDa protein was present in the fraction not binding to oligo(dT)-cellulose (fig.3, lane D); we conclude that it is associated with mRNA sequences other than poly(A).

Finally, it is interesting to mention that mRNPs obtained from monoribosomes and the 40 S region of heat-shocked cells shared density and protein composition with the polyribosomal mRNP found in the 25°C control cells (not shown).

4. DISCUSSION

We have combined UV crosslinking in vivo and Cs_2SO_4 density gradient centrifugation to elucidate the structure of *Drosophila* poly(A)$^+$ polyribosomal mRNPs under heat shock and normal culture conditions. We show that these mRNPs under heat shock form a protein-deficient structure, which is composed of 2 proteins of 38 and 70 kDa only. Whereas mammalian cells possess a poly(A)-protein of 75 kDa [15,26–31], the 70 kDa protein is associated with sequences other than poly(A). *Drosophila* poly(A) sequences are asociated with a protein of 38 kDa. Poly(A)-proteins of similar molecular mass have also been found in other lower organism like *Artemia salina* [33] and the slime mold *Dictyostelium* [34]; and Jacobson, A. personal communication). It would be interesting to know to what extent these 2 proteins are genetically related due to their similar specific affinity for poly(A).

The scarcity of proteins in heat shock mRNPs may mean that under stress, messengers work with a minimum of helper proteins for emergency translation. Our results are in every respect analogous to the findings of Mayrand and Pederson [13]. They isolated protein deficient pre-mRNPs from *Drosophila* nuclei during heat shock. According to their interpretation, transcripts of genes which lack intervening sequences enter a fast pathway that requires merely capping and polyadenylation before transport to the cytoplasm. This hypothesis could certainly be broadened, that upon reaching the cytoplasm these mRNAs are immediately translated and with no resting phase as free mRNPs. This 'minor' pathway employs only a small number of RNA-binding proteins, i.e. those associated with poly(A) sequences and caps. This could explain why we always find a small amount of mRNPs banding at 1.55 g/cm^3 in Cs_2SO_4 density gradients as shown in fig.2b. In retrospect such a small peak between 1.55 and 1.63 g/cm^3 has often been observed in normal cells [13,35,36].

Recently, Kloetzel and Bautz [37], working on proteins associated with *Drosophila* hnRNA, reported that heat shock proteins also occur on cytoplasmic mRNA, while others claimed that they are absent from cytoplasmic mRNP fractions [38]. We have not been able to prove whether the messenger associated 38 kDa or the 70 kDa proteins are genuine heat shock proteins. The availability of monoclonal antibody against the heat shock 70 kDa protein, as recently published by La Thangue [39], will certainly solve the problem.

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