Polypeptides encoded by polyadenylated and non-polyadenylated messenger RNAs from normal and heat shocked HeLa cells

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SUMMARY

There is an increased synthesis of proteins in the molecular weight region of 100,000-72,000-74,000 and 37,000 two hours after treatment of HeLa cells for 10 min at 45°C. In vitro translation, using a rabbit reticulocyte cell-free protein synthesising system, of HeLa cell cytoplasmic RNA shows that the prominent 72,000-74,000 M₀ heat shock protein band comprises seven polypeptide species (namely adfçf&cc) and these polypeptides are directly encoded by both polyadenylated and non-polyadenylated mRNA.

INTRODUCTION

We have recently reported that increased synthesis of three sets of proteins in the 100,000, 72,000-74,000 and 37,000 M₀ regions occurs maximally two hours after cultured HeLa cells had been subjected to heat treatment at 45°C for 5-10 min (1). The increased synthesis of these particular proteins is blocked by actinomycin D but not by cycloheximide or sodium fluoride (1) and has led to the suggestion that some control of this response may be exercised at the transcriptional level.

In Drosophila cells there is evidence that heat treatment induces 'puffs' at limited number of genetic loci on the polytene chromosomes of salivary glands (2, 3). Moreover, it has been shown that RNA is transcribed in large amounts at the sites of the induced 'puffs' (4-6) and that some of these RNAs are preferentially translated into heat shock polypeptides (7, 8). Most of the cytoplasmic RNA complementary to the Drosophila melanogaster heat shock 'puff' 93D is non-polyadenylated (poly(A)⁻) (9), whilst all of the other heat shock 'puffs' hybridise strongly with polyadenylated (poly(A)⁺) cytoplasmic RNA (10-12). In addition the RNA coding for the major heat shock protein, hsp 70 is found in both poly (A)⁺ and poly (A)⁻ cytoplasmic RNA classes.

In this report we show that the major HeLa cell heat shock proteins
at 72,000–74,000 M comprises seven polypeptide species which we have
designated αβγδεζηξ and these polypeptides are directly encoded by both
polyadenylated and non-polyadenylated mRNAs.

MATERIALS AND METHODS

HeLa cell culture and heat shock procedure

HeLa cells were grown in culture as monolayers in Eagle's minimal
essential medium (Glasgow modification) supplemented with 10% (v/v)
calf serum (1). 2 x 10⁷ cells were seeded and grown for 2-3 days
in rotating Winchester bottles. Heat shock treatment was carried
out by immersion of the bottles in a waterbath at 45°C for 10 min and
thereafter returning the culture to the normal growth temperature of
37°C for 2 hr.

Isolation of HeLa cell cytoplasmic RNA

The cell monolayers were washed twice with BSS (balanced salt solution)
(1), scraped off the glass in BSS with a rubber scraper, collected by
centrifugation at 600 g, and washed twice with further portions of BSS.
The cells were then swollen for 5 min in hypotonic buffer (0.01 M NaCl,
1.5 mM MgCl₂, 0.01 M Tris-HCl pH 7.4) at a concentration of 5 x 10⁵ cells/
ml and disrupted by 10 strokes of a Dounce hand homogeniser. Nuclei were
collected by centrifugation at 600 g for 10 min, and the resulting
supernatant fluid made 0.5% sodium dodecylsulphate, 10 mM EDTA and the
cytoplasmic RNA extracted using the phenol-chloroform method as described
by Penman (13).

Separation of poly (A)⁺ and poly (A)⁻ RNAs

Cytoplasmic RNA, precipitated by ethanol (13) was collected by centrifug-
ation at 10,000 g, dissolved in binding buffer (0.2% N-Lauroyl sarcosine,
0.4 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl pH 7.4), heated at 70°C for 5 min,
cooled rapidly on ice and fractionated on a poly (U)-Sepharose column at
room temperature. The poly (U)-Sepharose was obtained from Pharmacia
(Great Britain) Ltd., and 1 ml columns were prepared according to their
instructions. The unbound material was eluted with 10 vol binding buffer,
whilst bound material, poly (A)⁺ RNA was eluted with 90% formamide, 0.5%
N-lauroyl sarcosine, 0.01 M EDTA, 0.1 M tris-HCl pH 7.4, 0.2 M NaCl and
precipitated overnight with 2 vol ethanol at -20°C.

The original unbound material was then applied to a second poly (U)-
Sepharose column, eluted with 10 vol binding buffer and applied to a
third poly (U)-Sepharose column. After elution with 10 vol binding buffer,
the solution was made 0.2 M NaCl and the poly (A) RNA precipitated with 2 vol ethanol overnight at -20°C.

**In vitro** translation of RNA

Both cytoplasmic poly (A) RNA was translated **in vitro** in an mRNA dependent rabbit reticulocyte cell free protein synthesising system (obtained from New England Nuclear, Manheim). This was based on the procedure used by Pelham and Jackson (15) and the actual translation was carried out as outlined by the manufacturers. Each 25 µl assay contained approximately 40 µCi [³⁵S]methionine (specific activity 967.5 Ci/mmol).

**One dimensional electrophoretic analysis of in vitro translation products**

The assay mixtures containing the products of translation as described above were mixed with 1 vol 2% (w/v) sodium dodecyl sulphate, 3% (v/v) 2-mercaptoethanol, 50 mM tris-HCl pH 6.8, heated at 100°C for 3 min, cooled on ice then applied to a 8.75% (w/v) polyacrylamide slab gel overlaid with a 3% (w/v) polyacrylamide stacking gel and electrophoresed as described previously (1).

**Two dimensional electrophoretic gel analysis of in vitro translation products**

The assay mixtures containing the product of translation as described above were mixed with 1 vol 9.5 M urea, 2% (w/v) NP-40, 2% ampholines pH range 3.5-10 (LKB Ltd.), 5% (v/v) 2-mercaptoethanol. First dimension isoelectric focussing and second dimension electrophoresis were carried out as previously described (1).

**Fluorography**

Detection of [³⁵S] labelled polypeptides was carried out using fluorography as previously described (1).

**RESULTS**

Cytoplasmic polyadenylated (poly(A)) RNA was isolated from HeLa cells 2 hr after being subjected to heat shock (10 min at 45°C). When this was translated in a rabbit reticulocyte cell free protein synthesising system and the products analysed by one dimensional polyacrylamide gel electrophoresis the pattern of polypeptides made closely resembles that directed by poly(A) RNA from control cells. However two protein bands were prominent amongst the translation products of cytoplasmic poly(A) RNA from heat shocked cells (Figure 1, lanes 2 and 3). The particular bands have electrophoretic mobilities on dodecyl sulphate-polyacrylamide gels that are similar to the proteins that become labelled
Fluorogram of \([^{35}S]\) methionine labelled polypeptides resulting from the translation of total cytoplasmic poly(A)\(^{+}\) and poly(A)\(^{-}\)RNA from control and heat shocked HeLa cells.

Total cytoplasmic poly(A)\(^{+}\) and poly(A)\(^{-}\) RNAs were extracted from HeLa cells cultured at 37\(^{\circ}\)C and cells heat shocked at 45\(^{\circ}\)C for 10 min followed by 2 hr at 37\(^{\circ}\)C. 1 \(\mu\)g of poly(A)\(^{+}\) and 5 \(\mu\)g poly(A)\(^{-}\)RNA from control and heat shocked cells were translated in a rabbit reticulocyte cell-free system using \([^{35}S]\) methionine as radioactive label. Incubations were carried out for 60 min at 37\(^{\circ}\)C and the \([^{35}S]\) labelled polypeptides analysed by dodecyl sulphate polyacrylamide gel electrophoresis and detected by fluorography.

Lane 1, no RNA added to translation system; Lane 2, poly(A)\(^{+}\)RNA from control cells added; Lane 3, poly(A)\(^{-}\)RNA from heat shocked cells added; Lane 4, poly(A)\(^{-}\)RNA from control cells; Lane 5, poly(A)\(^{-}\)RNA from heat shocked cells; Lane 6, proteins labelled \textit{in vivo} by incubating intact HeLa cells with \([^{35}S]\) methionine for 1 hr; Lane 7, proteins labelled \textit{in vivo} by incubating heat shocked cells with \([^{35}S]\) methionine for 1 hr. Arrows indicate the migration of proteins at \(M_{r}=100,000\) and \(M_{r}=72,000-74,000\) respectively.
at 100,000 M\textsubscript{r} and 72,000-74,000-M\textsubscript{r} which appear in intact HeLa cells when they are held at 45\degree C for 10 min and exposed to \[^{35}\text{S} \text{methionine} \] after return to 37\degree C (ref. 1 and Figure 1, lane 7). These heat shock proteins are not made in large amounts in control cells cultured at 37\degree C, nor were they translated in large amounts in vitro from the poly(A)\(^{\dagger}\) RNAs from normal HeLa cells cultured at 37\degree C (Figure 1, lanes 2 and 6). It should be pointed out that the translation products also include two other prominent bands (Figure 1, lane 1) possibly residual globin synthesis and the "Endo" protein described by Morch and Benicourt (14). This "Endo" protein arises from the post-translational enzymatic addition of \[^{35}\text{S} \text{methionine} \] to a protein already present in the reticulocyte lysate (14).

Comparison of the electrophoretic mobilities of the translation products of cytoplasmic poly(A)\(^{\dagger}\) RNA from control and heat shocked cells showed that similar to the situation with cytoplasmic poly(A)\(^{\dagger}\) RNA most of the protein products made from the cytoplasmic poly(A)\(^{\dagger}\) RNA from heat shocked cells appear to be present in the products translated from the cytoplasmic poly(A)\(^{\dagger}\) RNA from control cells except for an enrichment of a protein band in the 72,000-74,000-M\textsubscript{r} region (Figure 1, lanes 4 and 5). It is of course possible that this particular protein arises from small amounts of poly(A)\(^{\dagger}\) RNA contaminating the poly(A)\(^{\dagger}\) RNA. However it should be pointed out that the preparation of the cytoplasmic poly(A)\(^{\dagger}\) RNA involved three successive cycles of poly(U)-Sepharose chromatography. At each chromatographic step a small proportion of the poly(A)\(^{\dagger}\) RNA was retained on the columns. When the 'bound' and 'unbound' RNA at each cycle of the affinity chromatography was translated in vitro, it was found that whilst the 'unbound poly(A)\(^{\dagger}\) RNA' did not change with respect to its ability to code for the 72,000-74,000-M\textsubscript{r} heat shock protein the 'bound' RNA had no significant translational activity. This 'bound' RNA could be ribosomal RNA retained on the column by virtue of its secondary and tertiary structures. On the other hand these procedures do not exclude the possibility that some poly(A)\(^{\dagger}\) RNA comprises species with extremely short poly A tails which would not permit their retention by poly(U)-Sepharose.

A previous report from this laboratory has shown by two dimensional electrophoretic analysis that the 100,000 M\textsubscript{r} protein band from intact heat shocked HeLa cells is made up of at least two polypeptide species, whilst the 72,000-74,000 M\textsubscript{r} comprises at least seven polypeptides. In order to investigate whether all these heat shock polypeptides result
directly from mRNA translation, a comparison of the in vitro translation products of cytoplasmic poly(A)\(^+\) and poly(A)\(^-\) RNA was made using the two-dimensional electrophoretic separation system of O’Farrell (16). This procedure revealed a number of interesting features not observed in one dimensional gel electrophoresis.

Some polypeptide spots in the fluorogram of the translation products of poly (A)\(^+\) RNA from control cells were not observed in the pattern of translation products of poly(A)\(^-\) RNA from heat shocked cells (in Figure 2a such polypeptides are indicated by arrows). Secondly, whereas in the area of the 72,000-74,000 \(M_r\) heat shock proteins seven polypeptide spots \(a,a',\beta,\gamma,\delta,\epsilon\) and \(\zeta\) are encoded by the poly(A)\(^+\) RNA from heat shocked cells only three \(\gamma,\epsilon\) and \(\zeta\) seem to be coded by poly(A)\(^-\) RNA from control cells. Thirdly only one strong polypeptide spot was found in the position expected of the 100,000 dalton heat shock proteins after translation of cytoplasmic poly(A)\(^+\) RNA from heat shocked cells (Figure 2b). This polypeptide spot was also formed but with slightly reduced intensity in the translation products of the poly(A)\(^+\) RNA from control cells (Figure 2a). This is perhaps not too surprising as the in vivo increase in synthesis of this group of proteins after heat shock was found to be only 18% (1).

Fourthly, although it is not always easy to see the polypeptides migrating at 37,000 \(M_r\), two dimensional analysis of products from the heat shocked cell poly(A)\(^+\) RNA translation show a single spot in this region which is not present in the translation products from control cell poly(A)\(^+\) RNA.

Figure 2
Fluorogram of the two-dimensional electrophoretic separation of \[^{35}S\]methionine labelled in vitro translation products of cytoplasmic poly(A)\(^+\) RNA from control and heat shocked HeLa cells.

Total cytoplasmic poly(A)\(^+\) RNA was translated in vitro using a rabbit reticulocyte cell free translation system and the translation products were analysed by two-dimensional electrophoretic gels. (a) Total cytoplasmic poly(A)\(^+\) RNA (1 \(\mu\)g) from control cells; (b) total cytoplasmic poly(A)\(^+\)RNA (1 \(\mu\)g) from cells heat shocked at 45\(^\circ\) for 10 min. and then returned to 37\(^\circ\) for 2 hr.

The brackets indicate the spots of the multiple forms of actin. \(a, a', \beta, \gamma, \delta, \epsilon, \) and \(\zeta\) refer to the proteins of the 72,000-74,000 \(M_r\).

100 \(K\) indicates the 100,000 \(M_r\) HeLa heat shock protein. Similarly 37 \(K\) is likely to be the 37,000 \(M_r\) HeLa heat shock protein.

The arrows in Figure 2a point to the proteins which do not appear amongst the translation products of the poly(A)\(^+\) RNA from heat shock cells (see Figure 2b for comparison).
This polypeptide may be the 37,000 $M_r$ heat shock protein. Similarly, a number of polypeptide spots are made by poly(A)$^-$$^+$RNA from control cells but which are not encoded in the poly(A)$^-$$^-$RNA from heat shocked cells. Some of these 'missing' polypeptides are indicated by arrows in Figure 3a and those that are exclusively poly(A)$^-$$^+$ translation products are both arrowed and numbered (see Figure 3a). On the other hand similar to the situation with poly(A)$^-$$^-$RNA, only three proteins of the 72,000-74,000 $M_r$ (a, b, and c) are translated from cytoplasmic poly(A)$^-$$^+$RNA from control cells, whilst all seven (a, a', b, y, d, e, and c) were encoded by cytoplasmic poly(A)$^-$$^+$RNA from heat shocked cells (Figures 3a and 3b). A faint 100,000 dalton protein spot was also found in the translation products of poly(A)$^-$$^+$RNA from heat shocked cells (Figure 3b) as well as a polypeptide migrating in a position expected of the 37,000 dalton heat shock protein. It appears thus that despite possible differences in relative abundances both the cytoplasmic poly(A)$^+$ and poly(A)$^-$$^-$mRNA from heat shocked cells code for the same overall variety of heat shock proteins at 72,000-74,000 $M_r$ (and possibly in the molecular weight region of 37,000 and 100,000 as well).

Figure 3

Fluorogram of the two-dimensional electrophoretic separation of [$^{35}$S]methionine labelled in vitro translation products of cytoplasmic poly(A)$^-$$^+$RNA from control and heat shocked HeLa cells.

Total cytoplasmic poly(A)$^-$$^+$RNA was translated in vitro using a rabbit reticulocyte cell free translation system and the translation products were analysed by two dimensional electrophoretic gels. (a) Total cytoplasmic poly(A)$^-$$^+$RNA (5 pg) from control cells; (b) total cytoplasmic poly(A)$^-$$^+$RNA (5 pg) from cells heat shocked at 45°C for 10 min. and then returned to 37°C for 2 hr.

The brackets indicate the spots of the multiple forms of actin $a$, $a'$, $b$, $y$, $d$, $e$ and $c$ refer to the proteins of the 72,000-74,000 $M_r$. 100 K indicates the 100,000 $M_r$ HeLa shock protein. Similarly 37 K is likely to be the 37,000 $M_r$ HeLa shock protein.

The arrows in Figure 3a point to the proteins which do not appear amongst the translation products of the poly(A)$^-$$^+$RNA from heat shock cells (see Figure 3b for comparison). The arrows with numbers refer in Figure 3a not only to this type of translation product but in particular to those coded for exclusively by poly(A)$^-$$^+$RNA rather than poly(A)$^-$$^-$RNA (see Figure 2a for comparison).
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DISCUSSION

A number of mRNAs have now been shown to exist in both poly(A)$^+$ and poly(A)$^-$ forms (17). The structural protein actin is synthesised by both poly(A)$^+$ and poly(A)$^-$ mRNA from Friend cells (18), HeLa cells (19), and other cell types (20-23). Heat shock of HeLa cells seems to have little effect on the translational activity of both poly(A)$^+$ and poly(A)$^-$ mRNAs for the multiple forms of actin (see bracketed area of Figures 2 and 3). Histones (24), myosin heavy chain (25), albumin (26), and the Drosophila hsp 70 (9, 27) are a few of the increasing list of proteins that have been shown to be encoded by both poly(A)$^+$ and poly(A)$^-$ mRNAs.

The reasons for the existence of mRNA in both poly(A)$^+$ and poly(A)$^-$ forms is not clear. It is possible that their production results from different nuclear processing pathways (28) or they may be simply derived from poly(A)$^+$ mRNAs in a process where the length of the poly(A) segment decreases with mRNA age (29). In Drosophila there are data indicating the gradual loss of the 3'-poly(A) tail from the mRNA for the hsp 70 protein (27).

A surprising observation was that whilst a number of proteins arise from the translation in vitro of mRNAs from control cells, these did not appear amongst the translation products of the mRNAs isolated from heat shocked cells. Previous in vivo experiments in which proteins in intact control and heat shocked HeLa cells were labelled with $^{35}$S methionine showed no such translational 'shut-off' in the synthesis of specific proteins after heat shock. It may be that certain mRNAs from heat shocked cells are particularly susceptible to degradation during the isolation procedures used or are simply not translated efficiently in the rabbit reticulocyte cell free system. A suggestion is that certain mRNAs from heat shocked cells have unusual requirements for ribosomes and initiation factors etc. which are not provided by a heterologous translation system (30). In this connection it should be noted that the preferential translation of Drosophila heat shock mRNA observed in vivo can only be reproduced in vitro by using homologous cell free protein synthesising systems prepared by lysing heat shocked Drosophila cells (27, 30).

There has been considerable speculation regarding the function of heat shock proteins. From studies of the phenomenon in chick embryo fibroblasts, Kelley and Schlesinger (31) made the suggestion that they might be membrane proteins involved in hexose metabolism or transport. Our observations that the size and characteristics of the in vitro...
translation products from mRNAs of heat shocked HeLa cells correspond well with those of the HeLa cell heat shock proteins found in vivo (1), suggest that the heat shock proteins at least in HeLa cells are likely to be direct translation products. Membrane proteins might be expected to arise after translation as precursors with extra amino acid sequences to facilitate their insertion into cell membranes (32). In addition studies with the antibiotic tunicamycin on intact cells confirm that the heat shock proteins are not extensively glycosylated (M. McMahon, unpublished results). Other types of post-translational modifications may explain the occurrence in vitro of one rather than the two polypeptide spots expected at 100,000 Mₐ or 37,000 Mₐ regions from the earlier in vivo studies with heat shocked HeLa cells (1).

REFERENCES