HeLa cell messenger RNAs of widely differing sizes encode certain heat shock proteins in the M_r region of 72 000–74 000

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Cloned cDNA sequences coding for specific 72 000–74 000 M_r HeLa cell heat shock proteins (HSPs) have been used as probes to examine cytoplasmic and nuclear RNA of heat shocked HeLa cells. The mRNAs for these HSPs vary widely in size. Whilst the β-, δ- and ε-HSPs are encoded in mRNAs of 1.9 kb, the γ-HSP arises from an mRNA three times that size. A search for putative nuclear precursors to these mRNAs revealed high M_r candidates at 15.8 kb which accumulate 1–2 h after the initial heat shock, thus supporting the notion that transcriptional control is important in the initial heat shock response.

HeLa cell *Heat shock response* *Heat shock protein* *Messenger RNA* *Pre-messenger RNA*

1. INTRODUCTION

We have reported that a brief treatment of HeLa cells at a temperature of 45°C for 10 min followed by a development period of 2 h at 37°C leads to the increased synthesis of three groups of heat shock proteins (HSPs) in the M_r regions of 100 000, 72 000–74 000 and 37 000 [1]. Experiments with actinomycin D suggested that some control of this response may be exercised at the transcriptional level [1].

In vitro translation of cytoplasmic poly(A)$^+$ or poly(A)$^-$ RNA isolated from such heat-shocked cells, followed by two-dimensional gel electrophoretic analyses of the translation products, showed that while the HSPs in the 100 000 M_r and 37 000 M_r groups are encoded by single mRNA species, the 72 000–74 000 M_r group of HSPs (designated α, α^1, β, γ, δ, ε and ζ) are coded for by seven mRNAs which can be found in both polyadenylated and non-polyadenylated forms [2]. We now show that the mRNAs for the β, δ and ε HSPs are in the size range expected of mRNA coding for polypeptides of 72 000–74 000 M_r, but the mRNA for the γ-HSP is approximately three times its expected size.

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2. MATERIALS AND METHODS

2.1. *Isolation of cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNA from heat-shocked HeLa cells*

Cytoplasmic poly(A)$^+$ RNA and poly(A)$^-$ were isolated 2 h after a 10 min heat shock at 45°C as described in [2].

2.2. *Sucrose gradient analysis of poly(A)$^+$ RNA*

Sucrose gradient centrifugation of poly(A)$^+$ RNA was carried out according to the procedure in [3]. Cytoplasmic poly(A)$^+$ RNA (0.3 mg) from heat-shocked cells was dissolved in 10 mM Hepes (pH 7.5), 1 mM EDTA, heated at 65°C for 10 min, cooled rapidly on ice, and immediately layered on a 5–20% linear sucrose density gradient in the same buffer. The gradients were centrifuged in a Beckman SW 40 rotor at 35 000 rev./min for 16 h at 4°C. Eighteen fractions were collected and each fraction was precipitated with ethanol two times prior to translation in a rabbit reticulocyte protein synthesising system using [^{32}S]methionine as radioactive label. After electrophoresis through

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8.75% polyacrylamide gel the products were detected by fluorography as described in [2].

2.3. *Isolation of nuclear RNA from control and heat-shocked HeLa cells*

Nuclei were isolated from control and heat shocked HeLa cells as described in [2] and nuclear RNA was isolated according to the method in [4] with modifications. Pelleted nuclei were suspended in 10 vol. (w/v) of 8 M guanidine–HCl, 20 mM sodium acetate (pH 5), 0.7 M 2-mercaptoethanol at 0°C and passed twelve times through a 16 mm gauge needle to shear high M_r/DNA weight. This solution was carefully layered on a 1.5 ml 5.7 M CsCl, 1 mM EDTA (pH 7.0) and centrifuged for 48 h at 35 000 rev./min in a Beckman SW 40 rotor at 18°C. After the centrifugation the RNA was found precipitated at the bottom of the tube, whilst proteins remained at the top and DNA at the interphase. DNA and proteins were removed by aspiration and the RNA dissolved in a solution of 10 mM Tris–HCl (pH 7.0), 1 mM EDTA and re-precipitated overnight with 2.5 vol. ethanol, 0.3 ml sodium acetate (pH 5.0) at –20°C.

2.4. *Gel electrophoresis of RNA*

RNA was separated on 1% agarose gels (20 cm long by 3 mm thick) with methyl mercury (II) hydroxide (Ventron, Lancaster Synthesis, England) as denaturing agent [5]. The agarose was boiled in electrophoresis buffer (50 mM boric acid, 5 mM sodium borate, 10 mM Na_2SO_4, 1 mM EDTA (pH 8.2)), cooled to 60°C, and methyl mercury hydroxide was added to a concentration of 5 mM. The gel was then poured in a well-ventilated hood. The RNA was loaded in a two-fold dilution of electrophoresis buffer containing 10% glycerol, 0.1% (w/v) bromophenol blue and 0.1% (w/v) Xylene cyanol FF. Electrophoresis was performed for 1 h at 20 mA and 6 h at 60 mA in electrophoresis buffer. After electrophoresis the gel was prepared for transfer to diazobenzyloxymethyl cellulose (DBM)-paper as described in [6]. HeLa cell 28 S, 18 S and 4 S RNAs and single-stranded *Hin*dIII restriction fragments of λ bacteriophage DNA were used as size markers. It should be emphasised that due to the lack of proper RNA standards at the high M_r size range, the sizes of the 'Northern' bands relative to DNA standards are only a rough estimation.

2.5. *Transfer and hybridisation of the RNA*

The RNA was partially cleaved by alkali and transferred to DBM-paper in 200 mM sodium acetate (pH 4.0) as described in [6]. To prepare hybridisation probes, HeLa cell heat shock cDNA plasmids were nick-translated to a spec. act. of 3×10^6 cpm/μg according to the method of Roop et al. [7]. Pre-hybridisation and hybridisation conditions were as described in [6] except that the hybridisation buffer contained 10% (w/v) dextran sulphate (Pharmacia Fine Chemicals, London) and in addition to sonicated denatured calf thymus DNA as carrier, 50 μg/ml poly(C) was added to block non-specific hybridisation by the poly(dG · dC) of the cDNA plasmids. Washing of the filters after hybridisation was carried out according to the method in [6].

2.6. *Construction and characterisation of cDNA recombinant plasmids*

Construction and characterisation of the two recombinant plasmids (pHS2 and pHS6) containing HeLa cell heat shock polypeptide mRNA sequences is described in [8].

3. RESULTS

Cytoplasmic poly(A)$^+$ RNA isolated from HeLa cells incubated at 37°C for 2 h after a 10 min heat shock at 45°C was fractionated on 5–20% sucrose gradients. Eighteen fractions were collected and the RNA from each fraction was isolated and translated in a rabbit reticulocyte cell-free protein synthesising system using [^{35}S]methionine as label and fluorography to detect the products after gel electrophoresis. From fig.1 it can be seen that the poly(A)$^+$ RNA fractions coding for the 72 000–74 000 M_r group of HeLa HSPs sedimented rather broadly in fractions 1–10 (~ between 28 S and 18 S). Although previous studies [2] indicated that this group of HSPs were also coded for by poly(A)$^-$ mRNAs, a similar separation profile was obtained when poly(A)$^-$ RNA rather than poly(A)$^+$ RNA was used (results not shown). Since the 72 000–74 000 M_r group comprises several polypeptides coded apparently by seven poly(A)$^+$ or poly(A)$^-$ mRNA species [2], it is possible that this surprisingly broad sedimentation profile may be a consequence of secondary structures adopted by these RNAs

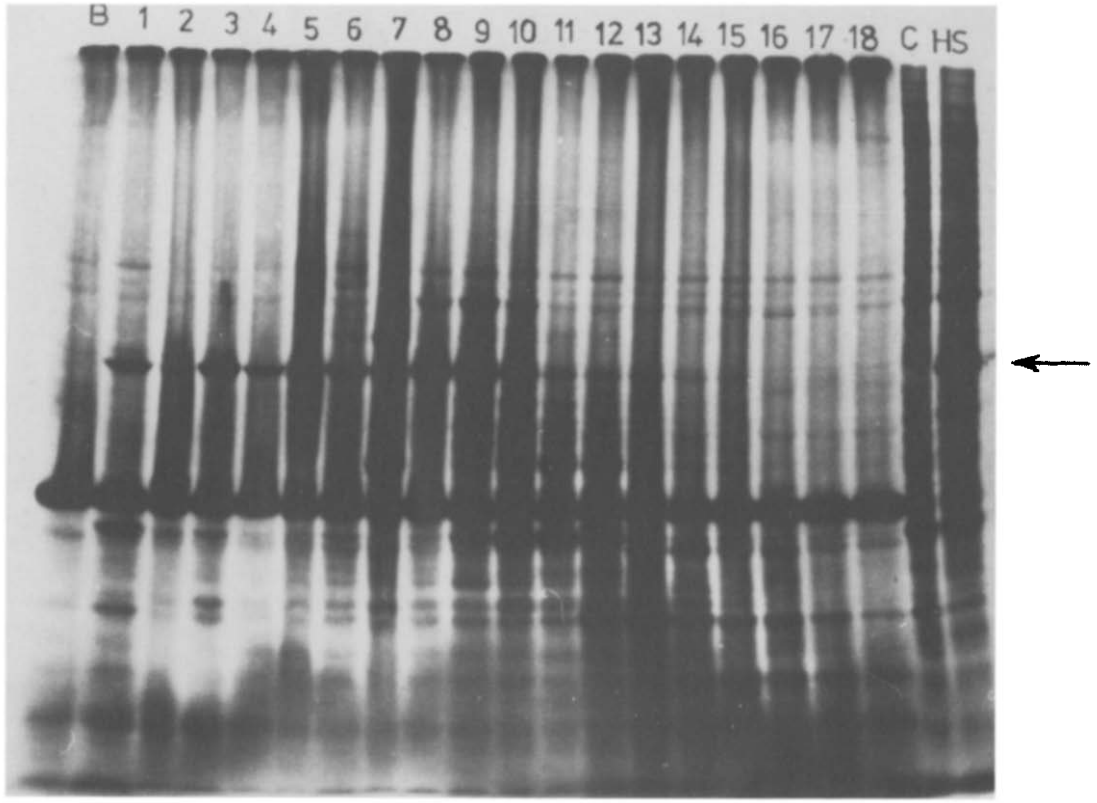

Fig.1. Fluorogram of [^{32}S]methionine labelled polypeptides synthesised in vitro from cytoplasmic poly(A)$^+$ RNA of heat-shocked HeLa cells following fractionation on sucrose gradients. Total cytoplasmic poly(A)$^+$ RNA was fractionated on 5–20% (w/v) sucrose density gradients [3]. 1 μg RNA from each fraction was translated in a rabbit reticulocyte cell-free translation system and the [^{35}S]methionine labelled products were analysed by one dimensional electrophoresis through 8.75% sodium dodecyl sulphate–polyacrylamide gels. The numbers corresponding to the gradient fractions are noted above the individual gel lanes (fractions are numbered from the bottom of the gradient). Lane B contains proteins made by the reticulocyte lysate in the absence of added exogeneous RNA. Lanes C and HS contain proteins labelled by incubation of intact control and heat-shocked cells with [^{32}S]methionine (see [1]). The arrow indicates the position of the 72 000–74 000 M_r heat shock proteins.

during centrifugation in non-denaturing gradients. Alternatively, this unusual sedimentation profile may result from different sizes of mRNAs coding for the individual HSPs of the 72 000–74 000 M_r group. To investigate these possibilities, cytoplasmic RNA from heat-shocked cells was subjected to agarose gel electrophoresis in the presence of methyl mercury hydroxide as denaturing agent and the RNA transferred to DBM-paper for hybridisation with specific ^{32}P-labelled cDNA probes. We have recently reported the construction of two recombinant plasmids containing cDNA sequences. One of these, pHS2 contains sequences encoding the γ-HSP of the 72 000–74 000 M_r group and the other pHS6 contains a cDNA sequence that cross-hybridises with RNAs for the β, δ and ε-HSPs of the same group [8]. These two plasmids were used in 'Northern' blot hybridisation experiments to determine the sizes of some of the mRNAs for the 72 000–74 000 M_r group of HSPs. Fig.2 (slot a) shows that plasmids pHS6 hybridises to cytoplasmic poly(A)$^+$ RNA of size

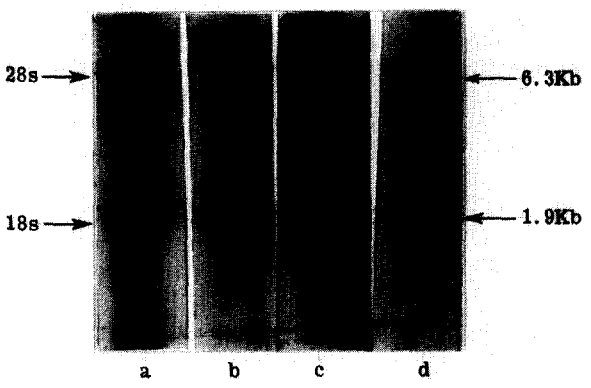

Fig.2. Electrophoretic analysis of cytoplasmic RNA sequences coding for the major 72 000–74 000 M_r group of HeLa cell heat shock proteins. Cytoplasmic poly(A)$^+$ RNA (slots a and c) and poly(A)$^-$ RNA (slots b and d) (5 μg) were separated on 1% agarose gels containing 5 mM methyl mercury hydroxide, transferred to DBM-paper and hybridised separately with ^{32}-P-labelled pHS6 (slots a and b) or pHS2 (slots c and d). The mobility of the rRNA markers was determined by ethidium bromide staining.

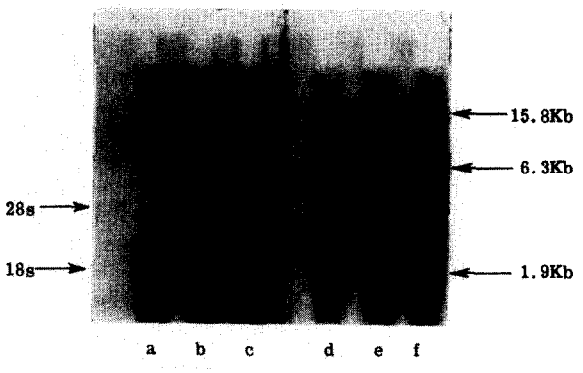

Fig.3. Electrophoretic analysis of HeLa cell nuclear RNA containing HSP mRNA sequences from HeLa cells. Nuclear RNAs from control and heat-shocked cells were separated on agarose gels containing methyl mercury hydroxide as denaturing agent. The nuclear RNAs were then transferred to DBM-paper and hybridised with nick-translated HeLa cells heat shock cDNA plasmids. The autoradiogram shows the hybridisation pattern with plasmids pHS6 (slots a–c) and pHS2 (slots d–f). Slots a,d: nuclear RNA from control HeLa cells. Slots b, e: nuclear RNA isolated 1 h after heat shock. Slots c, f: nuclear RNA isolated 2 h after heat shock.

1.9 kb but pHS2 hybridises to poly(A)$^+$ RNA with a size of 6.3 kb (fig.2 slot c). Since a similar hybridisation pattern was observed for poly(A)$^-$ RNA from heat-shocked cells (fig.2 slots b and d), it appears that the presence or absence of a poly(A) tail does not explain the differences in size of the mRNAs coding for the β, δ, ε and γ-HSPs. Whilst 1.9 kb is the range expected of an mRNA coding for a protein of 72 000–74 000 M_r (assuming the average M_r of an amino acid residue to be 120), an mRNA at 6.3 kb is clearly unusual. It should be pointed out that no sequence homology exists between the intragenic fragments of plasmids pHS2 and pHS6 as determined by filter hybridisation (results not shown) and when these plasmids are bound separately to DBM-paper and hybridised to cytoplasmic RNA from heat-shocked cells, the RNA that specifically hybridises to each of the plasmids after elution and translation in vitro produced a single protein band at 72 000–74 000 M_r on sodium dodecyl sulphate–polyacrylamide gels [8]. These proteins can be further separated on two-dimensional gels to give the γ-HSP on the one hand and the β, δ and ε-HSPs on the other hand [8].

The differing sizes of the mRNAs coding for the 72 000–74 000 M_r group of polypeptides may of course reflect differences at the level of nuclear RNA. Total RNA was isolated from nuclei of control cells and cells harvested 1 h and 2 h at 37°C after the heat treatment at 45°C for 10 min. These RNAs were electrophoresed through agarose gels containing methyl mercury hydroxide, transferred to DBM-paper and hybridised separately with each of the two cDNA plasmids. The results in fig.3 show that nuclear RNAs homologous to both probes and greater in size than the corresponding cytoplasmic mRNAs can be detected in cells after heat shock. The high level of hybridisation at 2 h corresponds to the time of maximal heat shock protein synthesis [1], and supports the notion that control of heat shock protein induction is exercised at the transcriptional level and that possible nuclear mRNA precursors accumulate at that time.

Another notable feature is that irrespective of the two cDNA probes used, the largest nuclear RNA transcripts detected were ~ 15.8 kb. Whether these represent the primary transcripts of HSP genes remains to be determined. When pHS6 is used as probe whilst there are complementary sequences smaller than the readily detectable 15.8 kb component (fig.3 lane c), a 1.9 kb species corresponding to the cytoplasmic mRNA is also easily detected. With pHS2 the 15.8 kb species is slightly less conspicuous but a 6.3 kb species corresponding to the relevant cytoplasmic mRNA is evident (fig.3 lane f). However, it is also very clear that a considerable amount of nuclear RNA hybridises to both the cDNA probes but smaller in size than the corresponding mRNAs (fig.3 lanes b, c, e and f). These could represent heat shock protein messages at various stages of splicing or prematurely terminated transcripts. Use of suitable genomic sequences as hybridisation probes could help clarify these points as well as the overall complexities of the nuclear processing events involved.

4. DISCUSSION

The large size of the mRNA for the γ-heat shock polypeptide is surprising. This of course could be due to the presence of unusually long 3′ and/or 5′-untranslated regions. In the case of *Drosophila* heat shock protein mRNAs there is nothing unusual about the 3′-untranslated regions but the 5′-untranslated regions range from 111 to 253 nucleotides [9]. This led to a suggestion that such untranslated regions may be important at the DNA level for the binding of protein involved in modulating expression of *Drosophila* heat shock genes [9]. This may also be true for the human γ-polypeptide. Another mRNA which is around three times the size expected is that for the small t 'transformation antigen' coded for by SV40 DNA [10]. In this case the 3′-untranslated region accounts for the bulk of the excess mRNA length.

On the other hand in chick embryo fibroblasts there is a small heat shock protein (p22) which is nevertheless coded for by a large mRNA. However it has been shown by immunoprecipitation studies that this particular protein is synthesised initially in a high M_r precursor form of about twice the size of the mature protein [3]. Although there is no similar heat shock protein in human cells, the possibility that the γ-polypeptide, unlike the other proteins of the 72 000–74 000 M_r group, actually arises from a larger precursor cannot be ruled out. However our in vivo labelling and in vitro mRNA translation data make it seem unlikely [1,2]. Although the 6.3 kb messenger may even be polycistronic, a final explanation for the different sizes of mRNA for the 72 000–74 000 polypeptides may well lie in the complexities of nuclear processing events.

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