Heat shock response in *Drosophila*

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Abstract. Major alterations in genetic activity have been observed in every organism after exposure to abnormally high temperatures. This phenomenon, called the heat shock response, was discovered in the fruit fly *Drosophila*. Studies with this organism led to the discovery of the heat shock proteins, whose genes were among the first eukaryotic genes to be cloned. Several of the most important aspects of the regulation of the heat shock response and of the functions of the heat shock proteins have been unraveled in *Drosophila*.
Key words. Drosophila; heat shock; stress; heat shock protein; gene regulation.

Introduction

Nearly thirty years ago, in the December 1962 issue of this journal, a short article appeared by the Italian geneticist Ferrucio Ritossa, entitled: "A New Puffing Pattern Induced by Temperature Shock and DNP in *Drosophila*"[75]. Ritossa had discovered that a small temperature elevation, as well as treatment with particular chemicals, produced a strikingly new puffing pattern on the giant salivary gland chromosomes: a small number of new puffs appeared, while most of the many puffs present before the heat shock (hs) regressed, and often disappeared altogether. The work of Beermann[7] had suggested that puffs were the sites of active loci, and the observations of Ritossa therefore indicated that mild hs as well as stresses induced by some chemicals led to profound alterations of gene expression. It is known today that a small number of specific genes, the so-called heat shock genes, located at the sites of the hs-induced puffs, are vigorously activated under those conditions, while the expression of many of the genes active in the flies before the stress is precluded. It seemed an ideal situation in which to study gene regulation in eukaryotes. This was the start of a new field of research aimed at understanding why and how hs and stress of other origins so dramatically, although transiently, modify the normal course of gene expression. Following the cloning of the hs genes of *Drosophila* at the end of the seventies, it became feasible to investigate the role of particular DNA sequences of the promoter (heat shock element, HSE) able to activate the transcription of the hs genes when a protein, the heat shock factor (HSF), became bound to it. It is now known that the products of the activated genes, the heat shock proteins (hsps), already present in small amounts in many cells in the absence of stress, rapidly increase under stress, and in some instances reach fairly large concentrations.

Work done in the 1930s had shown that a strong hs induced non-heritable defects of development, called phenocopies, which mimic the phenotype of homeotic mutations[26]. Phenocopies have been extensively studied in *Drosophila*[24, 56], and more recently in other organisms including mammals[94]. They can be interpreted as the consequence of alteration of the expression of genes involved in specific steps of development[56, 57].

When *Drosophila* are exposed to severe hs (about 40 °C), the majority of the animals die. If, however, just prior to the severe stress, they undergo a mild shock (around 33 °C) sufficient to activate the synthesis of hsps without disturbing normal protein synthesis, many animals survive and do not show any phenocopies[58]. This phenomenon is called thermotolerance or thermoprotection. It is thought that the hsps made during the mild hs protect the organism from lethal stress. Recent works suggest that several hsps are implicated in this protection (reviewed in Hightower[30] and Morimoto et al.[59]).

Following the early work on *Drosophila*, a similar heat shock response was observed in all organisms examined, from archaebacteria to man, showing that the response is universal. Remarkable homologies of nucleotide sequences were found, not only between hs genes of very distant eukaryotes, but also between hs genes from prokaryotes and eukaryotes: the hs genes appear to be among the most conserved genes.

Historical background

In the papers of Ritossa[75–78] several important features of the heat shock response were reported. The induction of the new puffing pattern, whether by hs, 2,4-dinitrophenol, sodium salicylate, sodium azide, dicumarol or the release from anoxia, always included the same puffs, suggesting that a small number of specific genes were involved. Experiments with radioactive markers (cytidine, uridine) showed that the newly-induced puffs were the sites of intense transcription. Cycloheximide had no effect on the induction of the hs puffs, pointing out that protein synthesis was probably not required. Identical puffs were induced at different stages of development. They were also induced in the midgut and in the hindgut by hs, release from anoxia, and dinitrophenol, indicating that the response was not limited to salivary glands and

hence was not tissue specific. Excised salivary glands treated at high temperature, or with other inducers, showed the typical hs puff pattern. These experiments suggested that the response takes place at the cellular level. When shocked larvae (30 min at 30 °C) were placed again at 25 °C, the new puffs disappeared after about 1 h. When the larvae were maintained at 30–32 °C, the new puffs regressed after about 3 h and the non-heat shock puffs partially reappeared at about the same time. These observations showed that the response is transient. This work was extended to *Drosophila melanogaster*, where nine hs puffs were identified[6].

Ritossa's papers raised several questions which were answered mainly in the seventies. First it was shown that heat shock resulted in the rapid increase of synthesis of about seven new protein species as seen on polyacrylamide gels, while the synthesis of most of the many different proteins made before the shock was inhibited, sometimes drastically. This was seen in all the different tissues which were examined: salivary glands, brain, Malpighian tubes and wing imaginal discs. The puff at locus 87C showed consistently stronger RNA labeling than any other puff and the major band on protein gels, known today as that of hsp70, showed much stronger labeling than any other band. It was therefore postulated that the protein in this major band was coded for by mRNA made at locus 87C[91]. Work done in subsequent years clearly demonstrated that mRNAs made at heat shock puff sites were translated into hsps[43, 50, 51, 53, 88]

Heat shock genes

The hs genes from *Drosophila* were among the first eukaryotic genes to be cloned and to have their organization established[15, 16, 33, 46, 81, 93]. Nucleotide sequences revealed homologous regions with dyad symmetry upstream of the heat shock genes, and it was proposed that they served for their thermal activation[28, 34, 38].

In brief, the major hsp genes of *Drosophila* consist of the hsp70 gene family, the hsp83 gene and the small hsp gene family. Besides the hsp70 genes present in several copies, and the single copy hsp68 homologous gene, the *Drosophila* genome bears seven genes strongly homologous to hsp70, called cognates, which are constitutively expressed[45]. The existence of a family of hsp70 genes, variously regulated, seems to be the general picture emerging in higher eukaryotes[59]. Hsp83, the largest major hsp of *Drosophila*, is encoded by a single gene[33]. It is expressed constitutively and moderately activated by hs[61]. The small hsp gene family includes the genes encoding hsp22, 23, 26 and 27. They share homologies among themselves and with mammalian α-crystallin genes[36]. The major hsp genes of *Drosophila* are devoid of introns.The only exception is the hsp83 gene, which has one small intron preceding the start of the coding region[29, 34]. Since splicing is inhibited by hs (see below), this absence of intron probably results from selective pressure to ensure that hsp genes are correctly expressed during hs.

Heat shock elements and heat shock factors

Upstream of all hs genes of eukaryotes, in the promoter region, there are multiple copies of short sequences called heat shock elements (HSE), necessary for stress-induced transcriptional activation of these genes. The first functional investigation of the regulatory region of an hs gene took place with the hsp70 gene of *Drosophila*. Promoter deletions transfected into monkey COS cells were examined for heat-induced transcription[54, 66]. Pelham showed that sequences between nucleotides −47 and −66 upstream of the hsp70 gene are needed for heat-induced transcription, and he deduced a 14 bp consensus with dyad symmetry, CnnGAAnnTTCnnG, the heat shock element, upstream of the TATA box[66]. Later experiments with germ line transformants of *Drosophila*[13, 19, 83], or in *Drosophila* cells[1], showed that a single HSE produces at most one or a few percent of the level of thermal induction seen with wild type hsp70 genes. An additional HSE is necessary to reach full activation. With the hsp23 and 26 genes studied in germ line transformants, at least three HSEs scattered over several hundred bp upstream of these genes are required for full hs expression[63].

Recently the importance of particular nucleotides flanking two consensus motives upstream of the hsp70 gene was investigated[2, 99]. The results showed that nucleotides immediately flanking each of the two HSEs play a positive role in the rate of transcriptional activation of the hs gene. The basic unit is now seen as 5 bp, nGAAn or its complement nTCCn, and an HSE is made of at least three units (for instance nGAAnnTTCnnGAAn). Regulatory regions of hs genes are made of multiple short arrays, or of single long array, of 5 bp units. The number of 5 bp units is variable. For example, in the hsp83 gene there are eight contiguous 5 bp units in the regulatory region. Each unit is a site of interaction with the heat shock factor HSF[69, 101].

Studies of protein-DNA interactions in nuclei of *Drosophila* tissue culture cells[97] and in vitro transcription assays prepared from these cells[62] each identified a protein, the heat shock factor (HSF), which binds with high affinity to HSEs after hs. It is a rare protein that has been purified and cloned from several species (see ref. in Westwood et al.[95]), including yeast[86, 96] and *Drosophila*[12, 98]. Active HSF appears to act as trimers or hexamers[12, 69, 87, 95]. In yeast, HSF is always bound to promoters of hsp genes, and is activated after hs by phosphorylation[85, 86]. In higher eukaryotes, in a first step during hs, the HSF molecules from the preexisting pool become capable of binding to the 5 bp units of HSEs, and in a second step phosphorylation of the HSF would create a complex with high transcriptional efficiency (see for details Morimoto et al.[59], and Sorger and

Pelham[86]). How HSF is modified to bind to HSE, and how transcription is activated, remains to be seen. It has been suggested that HSF is not only involved in the activation of hsp genes, but possibly also in the repression of transcription of normal genes following hs[95].

Regulation of the heat shock response

The overall regulation of the hs response, in particular its repression during prolonged stress or after return to a normal temperature, is still poorly understood. The first manifestations of the hs response, which involve the binding of HSF to HSE, can be detected within seconds after an elevation of temperature[105]. The question is therefore what cellular structure acts as a sensor of abnormal temperature or other physiological perturbations. Denatured or improperly folded polypeptides have been proposed as the inducer of the hs response (see ref. in Hightower[30]). *Drosophila* offers a striking example of induction of hsps in absence of elevated temperatures: in mutants that produce abnormal actins, an hs response is found in some flight muscles which express most strongly the mutated proteins[31].

When cells are returned to low temperatures after hs, all the cellular activities disrupted by the stress start to be restored. The speed of this recovery depends on the severity of the stress. Observed at the level of protein synthesis, the recovery is characterized by a gradual increase of translation of normal mRNAs (both those present before hs and the newly transcribed ones) and a gradual decrease of translation of hsp transcripts[17]. This decrease is asynchronous, hsp70 being repressed before hsp83[18]. The arrest of hsp70 production is essentially due to degradation of its mRNA, whose half-life is at least 20-fold higher at 37 °C than at 25 °C[72]. The instability of hsp70 messages at normal temperatures appears to be due to their 3′ untranslated region[73]. Finally, there is evidence that the down-regulation of hsps synthesis during the recovery period is regulated by the amount of hsps produced[17]. Hsp70 has been suggested to play a major role in this regulation[104].

Other effects of heat shock in Drosophila

Heat shock was found to alter the nucleolar morphology[82]. This alteration is probably the result of the transient inhibition of synthesis and processing of rRNAs[20]. Changes in the post-translational modifications of histones, ubiquitination[42], phosphorylation[25], ADP-ribosylation[60], acetylation and methylation[3], have been observed. In particular, the deacetylation of all four core histones, H2A, H2B, H3 and H4[3], may cause alterations in chromatin structure and transcriptional activity[27].

The processing of mRNAs, particularly their termination and splicing, is also strongly inhibited by hs. Inhibition of splicing occurring before any cleavage at the 5′ splice site of introns was first observed in *Drosophila*[102,103], and later in many other organisms (see ref. in Yost et al.[104]). Termination of transcription and/or 3′ end processing of many RNAs is also altered during strong hs. This leads for example to the accumulation of a 5S RNA precursor longer by 15 nucleotides[79] or to hs mRNAs with 3′ extensions exceeding 1 kb[8,64].

Several cytoplasmic activities are also affected by hs, especially protein synthesis[44,53,89,91]. On the one hand, translation of most pre-existing mRNAs is blocked by hs, and polysomes dissociate. On the other hand, new polysomes are assembled with the hsp mRNAs[50]. Interestingly, the pre-existing transcripts are not degraded, but simply remain, apparently unmodified, in the cytoplasm[41,89]. The preferential translation of hsp mRNAs appears to be due to sequences present in their long untranslated leaders, especially a conserved sequence at their 5′ end[35,40,49]. The absence of strong secondary structures in the leader sequences of hsp transcripts might also play a role.

Cell-free lysates prepared from stressed cells have been shown to discriminate between "normal" and hsp messages, suggesting that the preferential translation of hsp mRNAs is not solely due to their 5′ sequences but also to a stable, although reversible, modification of the translational machinery[41,80,89]. Recent studies indicate that hs inactivates a factor involved in the recognition of the cap structure of mRNAs[47]. The observation that the hsp70 message does not seem to need the presence of cap binding protein to be translated (J. Sierra, cited in Yost et al.[104]) might explain its preferential translation at elevated temperature.

Another reason for the rapid disappearance of normal polysomes might be the profound alterations of the cytoskeleton, such as the collapse of intermediate filaments and the disintegration of actin filaments, induced by hyperthermia[9,55].

Functions of the heat shock proteins

Many of the disturbances described above occur only in cells submitted to strong hs. They are not observed when the cells are first made thermotolerant by exposure to a mild hs before the strong stress. Since thermotolerant cells have synthesized hsps during the first stress, these observations provide circumstantial evidence for a function of hsps in the protection of vital cellular activities such as RNA processing[102,104] and translation[58,71]. The view emerging today is that the protection against adverse effects of hs is conferred by several of the different hsps, if not by all of them[30,59].

In the search for the functions of proteins, obvious questions concern their localization in the cell and their interactions with other molecules and particular cellular structures. With regard to hsps, these questions were investigated with *Drosophila* tissue culture cells and salivary glands. Analysis was done by cellular fractionation, with the appropriate controls to ensure that the observed

localization did not result from artifacts of the fractionation procedures[5]. During hs, hsp83 is mainly cytoplasmic, whereas the other hsps are to a great extent in the nucleus and return to the cytoplasm after the stress. The result for hsp70 was later confirmed by immunofluorescence with monoclonal antibodies[92]. Moreover, the possibility of concentration of hsp70 within nucleoli was suggested[5], and this was later confirmed by immunofluorescence[67].

When *Drosophila* hsp70 was constitutively expressed in mouse L cells transfected with a plasmid overproducing hsp70, the nucleolar lesions induced by hs[82] were more rapidly repaired during the recovery period[67]. The hypothesis put forward was that hsp70 binds to damaged ribosome precursors and catalyzes their ordered reassembly. Pelham proposed that during hs, proteins become denatured, exposing hydrophobic regions which interact to form insoluble aggregates; hsp70 would bind to hydrophobic surfaces and promote the dissolution of the aggregates and the refolding of the denatured polypeptides[68]. This concept found support when members of the hsp70 family were shown to act as molecular chaperones in the translocation of proteins across membranes of the endoplasmic reticulum and of mitochondria[21,30,59] (Burel et al., in this issue).

The role of hsp83 in stressed *Drosophila* cells has not been worked out, but this protein, like its mammalian homolog, may be part of intracellular transport mechanisms or of the regulation of steroid hormone receptors. The receptor of the molting hormone ecdysone is a candidate target for hsp83. Similarly to hsp70, the small hsps move to the nucleus during hs[4,5]. These proteins display a sequence homology to α-crystallins[36] which probably accounts for their tendency to form high molecular weight oligomers[4]. The function of the small hsps is unknown. It has recently been reported that a related, heat-inducible, 25 kDa turkey protein acts as an inhibitor of actin polymerization[55]. Some small hsps could be implicated in the organization or in the protection of the cytoskeleton.

Expression of heat shock proteins in the absence of stress

As briefly mentioned earlier, most hsps or their homologs are expressed in the absence of stress, often in a tissue-specific manner and at particular stages of development. To begin to understand their role at normal temperatures, it is important to describe precisely where and when they are expressed. The presence of large amounts of hsp83 at normal temperatures was revealed by protein labeling[44,53] and mRNA studies[48,61,106]. Immunoprecipitation showed the presence of hsp83 in several of the *Drosophila* tissues that were tested: salivary glands and brains of larvae and prepupae, and thoracic epithelium of young pupae[11]. In the ovary, hsp83 is present in the maturing germ cells, but not in the somatic follicle cells[106].

The hsp70 genes appear to be almost completely inactive in the absence of stress, but seven other members of the hsp70 family are strongly expressed at normal temperatures[45]. One of these genes, hsc 70-4, encodes a polypeptide whose abundance during *Drosophila* development seems always to be higher than that reached by hsp70 at maximal thermal induction, especially in cells active in endocytosis[70].

The small hsps are independently expressed at several developmental stages[48,84,106]. The developmental expression of two of these genes has been characterized in detail. At the mRNA level, transcription of hsp26 was found in several tissues, including the central nervous system and the germ cells[22]. Hsp27 was studied using an antibody and found to have a developmental profile quite similar, but not identical, to that of hsp26[65]. The activation of the small hsp genes at the beginning of the pupal stage seems to be regulated in part by the molting hormone ecdysone[37,90]. There is good evidence that the DNA sequences necessary for the regulation of hsp genes during development are different from those involved in hs induction[14,22,23,32,52,74,100].

A special case of induction of some of the small hsps deserves to be mentioned. Several drugs that act as teratogens in mammals have been shown to induce the synthesis of hsp22 and hsp23 in *Drosophila* embryonic cells. The level of activation of the two proteins apparently correlates with the inhibition of differentiation of these embryonic cells[10]. Whether this observation will be relevant for the understanding of the function of the small hsps remains to be seen.

Conclusion

The discovery of the heat shock response in *Drosophila* thirty years ago has led to numerous studies which have shown the universality of the stress response and the involvement of heat shock proteins in critical cellular processes. Heat-inducible promoters are now frequently used in developmental biology, for instance to analyze the effect of ectopic expression of a gene. We expect that new exciting results will continue to arise from the study of the heat shock response. For example, studies of the chromatin structure of hs genes have recently led to the discovery of DNA sequences called "boundaries" which separate higher order chromosomal domains involved in long-range gene regulation[39].

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Mammalian heat shock protein families. Expression and functions

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Abstract. When prokaryotic or eukaryotic cells are submitted to a transient rise in temperature or to other proteotoxic treatments, the synthesis of a set of proteins called the heat shock proteins (hsp) is induced. The structure of these proteins has been highly conserved during evolution.
The signal leading to the transcriptional activation of the corresponding genes is the accumulation of denatured and/or aggregated proteins inside the cells after stressful treatment. The expression of a subset of hsp is also induced during early embryogenesis and many differentiation processes.
Two different functions have been ascribed to hsp:
– a molecular chaperone function: chaperones mediate the folding, assembly or translocation across the intracellular membranes of other polypeptides, and
– a role in protein degradation: some of the essential components of the cytoplasmic ubiquitin-dependent degradative pathway are hsp.
These functions of hsp are essential in every living cell. They are required for repairing the damage resulting from stress.
Key words. Heat shock proteins (hsp); chaperones; protein degradation; ubiquitin.

Introduction

The first contribution of this volume has already dealt with the discovery and general description of the cellular heat shock response in *Drosophila*. Two books[46, 51] and a recent meeting review[30] give up-to-date information on heat shock proteins (hsp) and heat shock gene expression.
The following contribution will focus on the heat shock response in mammalian, mainly mouse cells. Our aim is to draw attention to the following, less well-known points:
– Heat shock protein synthesis is only one phase of a general adaptive response towards heat shock and similar stresses.
– Heat shock proteins are members of large families of proteins which have similar functions, but different expressions.
– Heat shock protein synthesis can be regulated in a specific way, independently of any stress treatment, in different physiological conditions, for instance during gametogenesis or early development.
– Heat shock proteins and related proteins fulfil essential functions in the normal cell.
– The same functions are required in stressed cells.

The mammalian heat shock response

When a mouse cell is submitted to proteotoxic treatments, such as a transient rise in temperature (transition from 37 °C to 42–43 °C for 15 min), or to chemical stresses such as addition of sodium arsenite, ethanol, heavy metals or amino acid analogs, it is possible to distinguish three successive phases in the cellular response to these aggressive treatments:
– The first phase, immediately following the beginning of the stress treatment, corresponds to an alteration phase. There is a general decrease in gene transcription and mRNA translation. There are also changes in cell morphology and modifications of chromatin and of the cytoskeleton. Some enzymatic activities are decreased or lost, probably due to the denaturation of the corresponding proteins by the stress treatment. However, other enzymes, such as some protein kinases, are activated during this first phase. The activation of these protein kinases does not require protein synthesis. These protein kinases might fulfil an adaptive function. For instance, activation of eiF2 kinase might be partly responsible for the decrease in protein synthesis observed after stress[14]. This decrease would prevent the synthesis of incorrectly folded proteins during stress treatment. Recently an-