Abnormal Proteins Serve as Eukaryotic Stress Signals and Trigger the Activation of Heat Shock Genes

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Heat shock protein (hsp) genes, a group of ubiquitous genes, are activated by various metabolic stresses. The suggestion that denaturation of intracellular proteins may be produced by the metabolic stresses and then signal the activation of the hsp genes was examined by co-injection of purified proteins and hsp genes into frog oocytes. Activation of hsp genes was observed if the proteins were denatured prior to injection but not if they were introduced in their native form. Furthermore, the activation of hsp genes by abnormal proteins and by heat shock appears to occur by a common mechanism. A model for the transcriptional regulation of the genes is based on competition for degradation between abnormal intracellular proteins and a labile regulatory factor.

THE EXPOSURE OF CELLS TO TEMperatures above the ones to which they are normally adapted (heat shock) activates a small set of genes in *Drosophila* (*1*). The expression products of these so-called heat shock protein (hsp) genes have been identified (*2*) and the genes have been isolated (*3*). Recent work shows the existence of related genes and expressed protein products in mammalian, plant, and even bacterial cells (*3*); some of these genes have also been cloned. The *Drosophila* and human hsp genes are transcribed correctly and in a heat-regulated fashion in various eukaryotic cells, as shown by gene transfer studies (*4–8*).

Hsp genes are activated not only by heat but also by seemingly unrelated stimuli (Table 1). Many treatments that make the intracellular environment less physiological appear to prompt cells to activate hsp genes. For this reason the genes have also been referred to as stress-induced genes, and the entire phenomenon as stress response. Circumstantial evidence for a role of this response in protecting cells from damage in adverse situations has been presented (*9*). The wide spectrum of situations that leads to the activation of these genes suggests that the system regulating their expression may be as generalized as the ones controlling, for example, the bacterial SOS response, the bacterial general amino acid regulation, or catabolite repression.

It is assumed that a single mechanism controls the activity of the hsp genes, and that all the different inducing agents or treatments ultimately affect the concentration of a common signal molecule. Obviously, the identification of such a signal is crucial for understanding the mechanism of hsp gene regulation. Most of the conditions that induce hsp genes are known or thought (*10, 11*) to cause denaturation of preexisting or of newly made intracellular proteins (Table 1). This observation led to our efforts to demonstrate directly that accumulation of abnormal proteins of any kind signals the activation of hsp genes in eukaryotic cells. In addition, Goff and Goldberg (*10*) recently demonstrated that production of large amounts of abnormal proteins activates transcription of heat shock proteins in *Escherichia coli*.

Table 1. Agents or treatments that activate hsp genes.

Inducing agent or treatment	Proposed effects	References
Group I		
Ethanol	Translation errors	(*25*)
Amino acid analogs, puromycin	Abnormal proteins	(*26*)
Group II		
Heat shock	Increased unfolding of proteins	(*3, 27*)
Various heavy metals, copper-chelating agents, arsenite, iodoacetamide, *p*-chloromercuribenzoate	Binding to sulfhydryl groups, conformational changes in proteins	(*28*)
Return from anoxia, hydrogen peroxide, superoxide ions and other free radicals	Oxygen toxicity, free radical fragmentation of proteins	(*29*)
Ammonium chloride	Inhibition of proteolysis	(*30, 31*)
Amytal, antimycin, azide, dinitrophenol, rotenone, heptyl-hydroxy-quinoline N-oxide, ionophores	Inhibition of oxidative phosphorylation, changes in redox state, covalent modifications of proteins	(*32*)
Hydroxylamine	Cleavage of asparagine-glycine bonds in proteins	(*31, 33*)

Xenopus laevis oocytes were used because materials can be readily introduced into these large cells by microinjection. *Drosophila* hsp genes, and even a human hsp gene, were previously shown to be transcribed correctly and in a heat-regulated fashion in *Xenopus* oocytes (*6–8*) [for reasons that have been discussed elsewhere (*12*), the activities of the endogenous hsp genes cannot be measured accurately in the later stages of oocyte development]. It was also shown that the heat-induced production of β-galactosidase by hsp-β-galactosidase hybrid gene constructs provides a convenient and sensitive assay for the transcriptional regulation of these hsp genes (*8*). This assay is used here.

Coinjection of purified bovine β-lactoglobulin or bovine serum albumin (BSA) does not result in the activation of the *Drosophila* hsp70-β-galactosidase hybrid gene 622C (Fig. 1). When the same two proteins, however, are first denatured by reductive carboxymethylation and then injected at the same concentration as the respective native proteins, each causes the activation of the coinjected hsp hybrid gene. This basic experiment, which has been repeated seven times with different batches of oocytes, clearly demonstrates that proteins with no known direct relation to the heat shock regulation system, and no physiological role in the cell system used, stimulate the expression of an hsp hybrid gene when denatured but not in their native conformation. The level of hsp gene expression in oocytes injected with denatured proteins is about one order of magnitude lower than that reached after a 90-minute heat shock at 36.5°C. We assume this to mean that the amount of intracellular protein that is denatured by heat shock is larger than the effective amount of denatured proteins that could be delivered by microinjection in these experiments. Similar results were obtained from experiments in which DNA was injected first, and the proteins several hours later.

The possibility that only proteins whose conformations had been altered dramatically by chemical modification are capable of inducing hsp genes has been ruled out by the following experiment. Components of

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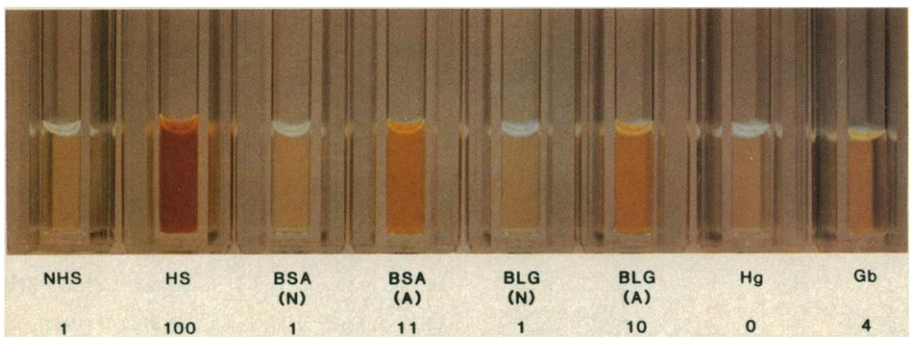

Fig. 1. Measurements of the expression of a *D. melanogaster* hsp70-β-galactosidase hybrid gene in microinjected *Xenopus* oocytes by the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Oocytes were prepared and nuclear injections were carried out as described (*7*). Batches of 20 oocytes were injected with 10 ng per oocyte of plasmid 622C DNA (Fig. 2) and, where indicated, with 85 ng per oocyte of denatured (A) (*23*) or native (N) bovine β-lactoglobulin (BLG), bovine serum albumin (BSA), human globin (Gb), or hemoglobin (Hg). One of the two batches that had received only DNA (HS, NHS) was subjected to a 90-minute heat treatment at 36.5°C (HS). All other samples were kept at 21°C. After overnight incubation at 21°C, extracts were prepared, β-galactosidase activities were determined (*8*), and the reaction mixtures were photographed (after transfer into plastic cuvettes). Relative activities, calculated from absorbance measurements at 420 and 550 nm, are indicated below.

multimeric proteins appear to be folded less tightly in their monomeric form than when they form part of proper multimeric structures, as evidenced by their increased sensitivity to proteolytic digestion (*13*); that is, they appear to be partially denatured proteins and are recognized as such by the cellular proteolytic system. We have prepared globin monomers by extraction of the heme group from human hemoglobin (type IV, Sigma). As expected, globin but not hemoglobin stimulates hsp gene expression (Fig. 1).

The above experiments appear to demonstrate that any abnormal protein triggers the activation of a *Drosophila* hsp gene, and a similar experiment with a human hsp gene construct shows that our findings are not restricted to any particular hsp gene (Fig. 2). These experiments do not indicate, however, whether heat shock or any of the other inducing conditions activate hsp genes through the accumulation of abnormal proteins or by other mechanisms. To obtain evidence that heat shock and abnormal proteins activate hsp genes by a common mechanism, we determined the location of the signals in the promoter of a *D. melanogaster* hsp70 gene that are involved in the regulation of the gene by the two stimuli. The regulated expression of a number of hsp hybrid genes with promoter segments of different lengths was examined (Fig. 2). Constructs with 194-, 88-, and 67-bp–long promoter regions are highly active in heat-shocked oocytes; a gene with only 50 bp of promoter sequence, however, is less active by about an order of magnitude than the genes with longer promoter segments. Analogous results were obtained for the abnormal protein–induced expression of the mutant genes. Thus, a short region located 50 to 67 bp upstream from the capping site of the hsp70 gene contains essential information for the regulation of the gene by both heat shock and abnormal proteins (the sequence element responsible for heat shock regulation has been mapped between −48 and −62 in previous studies (*5, 6*). This result strongly suggests that a common regulatory sequence is being used for the activation of the hsp70 gene by heat shock and abnormal proteins; that is, the two activation mechanisms include a common step.

That different purified abnormal proteins are capable of inducing the expression of hsp genes links the regulation of these genes to the catabolism of proteins. This finding, and the earlier findings that hsp gene activation occurs in the absence of protein synthesis (*14*), and that the genes are regulated in a positive fashion by the binding of a heat shock gene-specific factor (*15*) to defined sequences such as the above −48 to −62 element in their promoters, lead us to propose a model for the regulation of hsp genes. A somewhat different model has been suggested recently by others (*16*).

We propose that the heat shock gene-specific factor is present predominantly in an inactive form (1 in Fig. 3) in noninduced cells. Some of this factor may be converted at any time into an active form (2 in Fig. 3) by an unknown modification step. The active factor is suggested to be labile in noninduced cells; it is rapidly inactivated proteolytically and, therefore, does not accumulate. However, when cells undergo a heat shock, or are subjected to other treatments that induce hsp genes, a fraction of the intracellular proteins (3 in Fig. 3) is denatured. Such denatured proteins (4 in Fig. 3) are preferentially degraded and can be expected to compete effectively with the active form of the factor for the limiting component of the intracellular proteolytic system; this limitation may be at the level of ubiquitination or at a later step. As a result of this competition, the active factor can accumu-

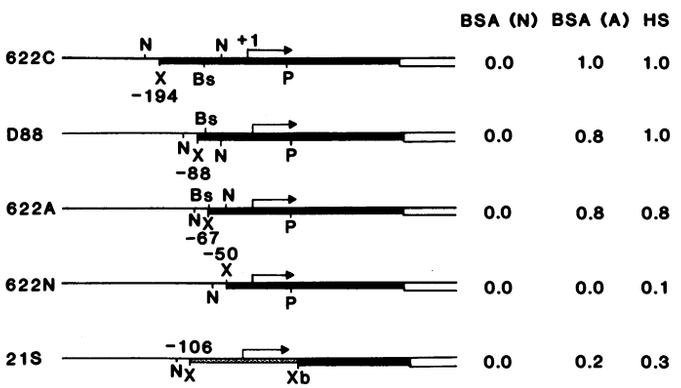

Fig. 2. Expression of different hsp70-β-galactosidase hybrid genes in frog oocytes coinjected with denatured BSA (A), native BSA (N), or following heat treatment (HS; see Fig. 1 for experimental details). Assays of β-galactosidase were carried out as in Fig. 1. The values in columns A and N are relative to the activity in oocytes coinjected with abnormal BSA and 622C DNA, and those in HS relative to that in 622C-injected, heat-treated oocytes. Readings on reaction mixtures containing extracts from hybrid gene–injected, non-heat-treated oocytes were used to estimate the background of the assay and were subtracted from all experimental values. All hsp-β-galactosidase constructs (*24*) have been inserted in the same orientation, between the Xma III and Bam HI sites of vector pSVOd. Maps of relevant segments of the hybrid genes used are shown. 622C, D88, 622A, and 622N are *D. melanogaster* hsp70-β-galactosidase hybrid genes containing 194, 88, 67, and 50 bp, respectively, of hsp70 promoter sequence. 21S is an analogous human hsp70 hybrid gene with 106 bp of human hsp70 promoter sequence. Dark areas are *D. melanogaster* and stippled areas human hsp70 gene promoter and RNA leader sequences; blank areas represent *E. coli* β-galactosidase gene sequences and thin lines pSVOd vector sequences. +1 refers to start of transcription sites; the transcriptional orientation is shown by arrows. −194, −88, and so forth indicate the lengths in base pairs of promoter segments. Bs: Bss HII, N: Nru I, P: Pst I, X: Xho I, Xb: Xba I.

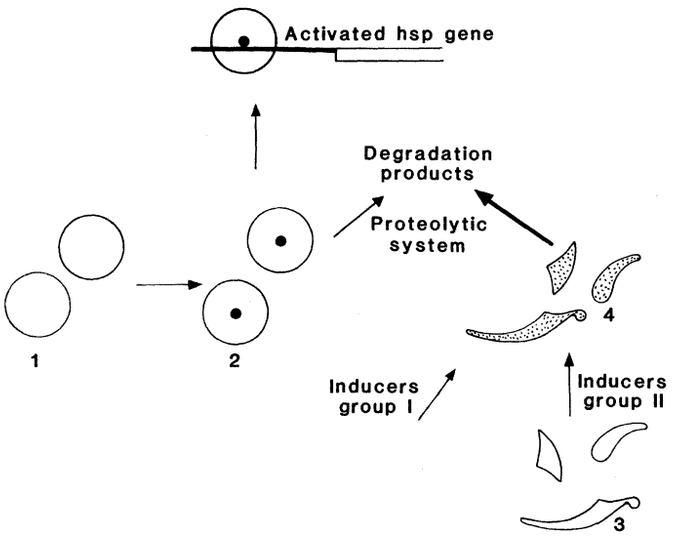

Fig. 3. Model of hsp gene regulation discussed in the text. Groups I and II inducers are listed in Table 1.

late, leading to the activation of the hsp genes.

The model predicts that amounts of abnormal proteins that are capable of mediating hsp gene activation should reduce significantly the rates of degradation of other short-lived proteins. As a test we examined in frog oocytes the effects of denatured and native BSA (injected in quantities similar to those used in the above expression experiments) on the degradation to acid-soluble material of a small amount of coinjected, tritiated transferrin. Indeed, with coinjected native BSA about 35 percent of the radioactivity in transferrin was converted to acid-soluble form during a 60-minute incubation of the oocytes, whereas with denatured BSA only about 25 percent of the radioactivity was solubilized.

Several isolated observations are consistent with and support the above model of hsp gene regulation. A previously isolated mouse cell line, ts85, is temperature-sensitive in ubiquitin-protein conjugation (thought to target proteins for degradation in eukaryotic cells) and in the degradation of short-lived (abnormal) proteins; the same mutant also produces hsps at elevated rates (*17*). Hsps are produced constitutively in indirect flight muscles of certain *Drosophila* flies that produce mutant forms of actin III (*18*). Furthermore, some normally short-lived polypeptides were stabilized in heat-shocked cells (*19*). Examination of hsp gene expression in *Drosophila* cells exposed to different temperatures has shown that at temperatures below 37°C, activation of the genes is transient (*20*). Thus, the heat shock system is self-regulating. According to the above model, the rate-limiting step in the deactivation of hsp genes is the degradation of large quantities of abnormal proteins. Increased synthesis of critical components of the degradative system during and after periods of active expression of hsp genes would accelerate this deactivation process. It would therefore not be totally unexpected if components of the proteolytic system themselves were encoded by hsp genes. Indeed, mammalian ubiquitin has recently been shown to be the product of a heat shock–induced gene (*21*).

Finally, we would like to point out the similarity of the above model for hsp gene regulation in eukaryotes with the one emerging from studies of the *E. coli* system. When these cells are forced to overproduce a single protein that is unfolded and rapidly degraded (such as human tissue plasminogen activator or human serum albumin), expression of the bacterial hsp genes rises (*10*) and the capacity of the bacteria to degrade abnormal proteins increases (*22*). These findings strongly suggest that the bacterial hsp genes are regulated by a mechanism that is very similar to the one operating in eukaryotic cells. Furthermore, that one of the bacterial heat shock genes encodes the major intracellular protease, protease La (*22*), parallels the finding that ubiquitin is a eukaryotic heat shock protein. We are intrigued by this unprecedented evolutionary conservation of a mechanism of gene regulation.

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23. Twenty milligrams of BSA (fraction V, Sigma) or β-lactoglobulin (United States Biochemical Co.) were incubated for 4 hours at 37°C in 8*M* urea, 0.4*M* buffered NaCl (*p*H 8), and 10 m*M* dithiothreitol. Iodoacetic acid was added to a final concentration of 0.25 m*M* and the mixture was incubated in the dark for 20 minutes at room temperature. The reaction was terminated by the addition of 0.1*M* 2-mercaptoethanol. The carboxymethylated proteins were purified by gel filtration through Sephadex G-50, followed by extensive dialysis in the cold against water, and finally against injection buffer [10 m*M* tris-HCl (*p*H 7.5) and 68 m*M* NaCl]. The proteins were subsequently concentrated in an Amicon Centricon-10 microconcentrator.
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