

**The Effect of Inhibitors of Oxidative Phosphorylation  
on the *Drosophila* Heat Shock Response**

by

**Neil Anthony Winegarden**

**A thesis submitted in conformity with the requirements  
for the degree of Master of Science (MSc)  
Graduate Department of Zoology  
University of Toronto**

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# **The Effect of Inhibitors and Uncouplers of Oxidative Phosphorylation on the *Drosophila* Heat Shock Response.**

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M.Sc. Thesis

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## **Abstract:**

The heat shock response allows cells to cope, at the molecular level, with environmental and pathophysiological stresses. It is a highly conserved and universal response. Inhibitors of oxidative respiration are but one class of chemicals that can induce the heat shock response. In order to gain insight into the mechanisms and pathways involved in activation of the heat shock response, three different inhibitors of oxidative respiration: sodium salicylate, 2,4 dinitrophenol (DNP) and potassium cyanide (CN<sup>-</sup>) were examined in detail. It was found that all of these drugs dramatically reduced the level of intracellular ATP in *Drosophila* tissue culture cells, and that this decline in ATP correlated with the activation of the heat shock response as measured by HSF binding and heat shock gene induction. However, one of these agents, sodium salicylate, while inducing some aspects of the heat shock response did not induce heat shock gene transcription. It is suggested that decreased ATP levels can, at least in part, serve as a signal to the cell's stress response machinery, and that this may be one of the means by which certain inducers of the heat shock response act.

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Appendix, Figures A1 and A2 – These experiments were performed in their entirety by Ken Wong.

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## **Chapter 1**

### **General Introduction**

### **The Eukaryotic Heat Shock Response**



## A. Historical Perspectives

The heat shock response is a highly conserved mechanism by which organisms cope with cellular stress. The origin of such stress may either be environmental or pathophysiological in nature. This response was originally identified in *Drosophila* as a specific heat induced puffing pattern on the polytene chromosomes of a *Drosophila busckii* larvae. Although this response was originally characterized in *Drosophila*, it has now been identified in every organism studied to date indicating that it is a universal mechanism.

In 1962, Ferruccio Ritossa reported that if a *Drosophila busckii* larvae was subjected to elevated temperatures (heat shock, 30 °C) or to 1 mM 2,4 dinitrophenol, the polytene chromosomes found within its salivary glands showed a characteristic puffing pattern. Studies using the incorporation of tritiated cytidine indicated that during heat stress, these puffs were the sites of active transcription (Ritossa, 1962). It was later determined that the loci which displayed puffing activity in response to stress were the sites of a specific subset of genes. These genes encoded for the protein family known as the heat shock proteins (hsps<sup>1</sup>) (Tissierres, 1974; Scalenghe and Ritossa, 1977). The overall function of these proteins is to help repair damage caused within the cell by stress (Reviewed by Parsell and Lindquist, 1993, 1994). These early experiments lead to investigations of the mechanisms involved in controlling this response to stress, and to a characterization of the heat shock proteins.

## B. Heat Shock Proteins

Once expressed the heat shock proteins function to repair stress induced damage inside of the cell. Although some hsps are strictly stress inducible there are many constitutive

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<sup>1</sup> See the Appendix for a full list of abbreviations used.

members of the hsp families which function during normal physiological conditions and whose expression may or may not be enhanced during heat shock (Burel et al., 1992; Parsell and Lindquist, 1993; Parsell and Lindquist, 1994). These hsps are referred to as heat shock cognates or hscs. In general, hsps are molecular chaperones: their function being to prevent or reverse protein aggregation and denaturation. During normal cellular conditions hsps function to fold nascent polypeptides into their correct conformations or to assist in the unfolding and refolding of proteins which is necessary to facilitate protein transport across membranes. Two features indicate the relative importance of these proteins: 1) hsps are among the most highly conserved proteins known (hsp70 from bacteria and from humans share ~50% identity)(Parsell and Lindquist, 1994) and 2) during normal physiological conditions, hsps constitute 5-10% of the cell's total protein mass (Morimoto et al., 1994b). The fact that these proteins are so well conserved indicates that they perform essential functions which have been maintained throughout evolution. In addition, individual deletions of many of the heat shock proteins have been shown to be lethal (See Parsell and Lindquist, 1994 and references therein). Some of the heat shock proteins are developmentally regulated, and in fact, hsp70 is one of the first proteins expressed after fertilization (Burel et al., 1992). Heat shock proteins also function in thermotolerance. By subjecting cells or an organism to sub-lethal heat shock temperatures and allowing for recovery, organisms can then become thermotolerant to heat shock temperatures which would have otherwise been lethal (Reviewed in Parsell and Lindquist 1993,1994). This acquired thermotolerance is generally a result of an increased abundance of hsps prior to the lethal heat shock.

The heat shock protein family is divided into several subgroups. In eukaryotes, the family includes five major subgroups which are characterized on the basis of their molecular weights: hsp100, hsp90, hsp70, hsp60, and the small hsps. Not all organisms possess members of each subgroup however. In general, the absence of one subgroup is usually compensated for by increased expression of one or more of the other subgroups (See Parsell and Lindquist, 1993 and 1994 for reviews).

### *Hsp100*

The hsp100 family contains both constitutively expressed and heat inducible members (Parsell and Lindquist, 1994). The 100 kD hsp family has been found to be required for thermotolerance in yeast, particularly at higher temperatures (Parsell and Lindquist, 1994 for review) and also functions to provide tolerance to certain chemical toxins such as ethanol (Sanchez et al., 1992). Although hsp100 expression is induced by many different stress agents such as heat, ethanol, copper, cadmium and arsenite, it does not appear to function in tolerance to all of these (Sanchez et al., 1992). It is thought that the function of this family of proteins is to prevent protein aggregate formation and/or to reverse the aggregation process and may therefore only provide tolerance to conditions which promote such aggregation (Parsell and Lindquist, 1994). Although there are hsp100 members in yeast and mammals, it appears that there is no *Drosophila* hsp100 homologue (Parsell and Lindquist, 1993,1994). Hsp100 has two ATP binding domains (Parsell and Lindquist, 1994). In yeast these two domains are responsible for formation of a homo-oligomeric hsp104 complex, as well as for the ATPase function of hsp104 (Parsell and Lindquist, 1994).

### *Hsp90*

Hsp90 is present in cells prior to heat shock, and during heat shock it is strongly induced (Parsell and Lindquist 1993). There are members of the family which can be found in the nucleus, in the cytosol, and in higher eukaryotes, hsp90 can be found in the endoplasmic reticulum (Parsell and Lindquist, 1994). Studies in yeast have indicated that hsp90 is required at all temperatures (Parsell and Lindquist, 1994). This family contains proteins capable of interacting with and in many cases modulating the activity of a host of other proteins. Included in the list of proteins are kinases, such as casein kinase II (Miyata and Yahara, 1991), eIF-2 $\alpha$  kinase (Rose et al., 1989), and oncogenic tyrosine kinases (Brugge, 1986); steroid hormone receptors such as the estrogen, progesterone, androgen, dioxin, antherdiol and glucocorticoid receptors (Pratt et al., 1992; Smith and Toft, 1993; Brunt et al., 1990); cytoskeletal elements such as actin (Nishida et al., 1986) and tubulin (Redmond et al., 1989; Fostinis et al., 1992); and the regulatory protein calmodulin (Minami et al., 1993). Hsp90 shows specificity unlike the other hsps which have been shown to be promiscuous in their interactions (Parsell and Lindquist, 1994). Hsp90 possesses an ATP binding function and an autophosphorylating activity (Csermely and Kahn, 1991). In addition, hsp90 has a peptide stimulated ATPase activity (Nadeau et al., 1993). Hsp90 has been shown by mutation analysis to be required for stress tolerance in eukaryotic organisms (Parsell and Lindquist, 1993). The chaperone function of hsp90 has been demonstrated by its interaction with casein kinase II in which it was found hsp90 functions to prevent and dissolve aggregates of the

kinase (Miyata and Yahara, 1991). It is also possible that the modulating affect of hsp90 on other proteins may be necessary during stress (Parsell and Lindquist, 1994).

### *Hsp70*

Hsp70 is in most eukaryotes the most abundant hsp family (Craig and Gross, 1991; Parsell and Lindquist, 1994). There are many different members of the family, and many which are specific to a particular organelle or even to a particular tissue (Frydman and Hartl, 1994; Parsell and Lindquist, 1994). Hsp70 in the mitochondria, for example, is responsible for import of nuclear encoded proteins and then for the presentation of these proteins to another chaperone, hsp60 (Kang et al., 1990). There are heat inducible and constitutive members of the family (Burel et al., 1992; Parsell and Lindquist, 1994). All hsp70 members have a conserved ATP binding domain (Flaherty et al., 1990; Flaherty et al., 1994; McKay et al., 1994). Hsp70 members participate in protein folding, unfolding and disassembly in the cell during normal physiological conditions and during stress (See Gething and Sambrook, 1992; Frydman and Hartl, 1994 and Hightower et al., 1994 for review). Hsp70 interacts cotranslationally with nascent polypeptides (Beckmann et al., 1990). The constitutive members of the hsp70 family (also referred to as hsc70) are required for proper cell growth. Cells underexpressing hsc70 demonstrate temperature sensitive growth (Craig and Jacobsen, 1985). In yeast, hsp70 is required for survival during moderate heat shocks, but has little effect at high heat shock temperatures (Werner-Washburne et al., 1987). If, however, yeast cells contain a mutation in hsp104, then hsp70 is required for high temperature survival (Sanchez et al., 1993). In *Drosophila*, which does not possess an hsp100 member, hsp70 is the main protein involved in thermotolerance (Parsell and Lindquist, 1994). Although some of

the heat inducible hsp70 members are also present in the cell during non-shock conditions, the major heat inducible hsp70 in *Drosophila* is largely undetectable at control temperatures and is induced over 1000 fold upon heat shock (Velazquez et al., 1983). Hsp70 also seems to have a role in the proteolysis cycle of the cell, facilitating import of proteins into the lysosomes (Terlecky, 1994). Although there are hsp70 members present at low levels in the cell during normal physiological conditions, this level must be carefully controlled as overexpression of hsp70 can be detrimental to cell growth. It has been demonstrated that the chronic overexpression of hsp70 in non-stressed cells results in an inhibition of proliferative growth (Williams et al., 1993; Feder et al., 1992). The chaperone function of hsp70 is mediated by both ATP and K<sup>+</sup> ions. ATP seems to mediate the release of hsp70 bound peptides (Beckmann et al., 1990). However, it has been reported that ATP hydrolysis is not required for the release from hsp70, rather only the binding of ATP is required, and the presence of K<sup>+</sup> stimulates the peptide release (Palleros et al., 1993). There has also been suggestion that there may be an ATP independent chaperone function for hsp70 (Ciavarra et al., 1994). An additional member of the hsp70 family is the endoplasmic associated protein BiP. This protein is present in cells at all times. In yeast, BiP functions to aid in the translocation of proteins across the endoplasmic reticulum, and it is possible that it also plays a similar, but less crucial role in mammalian systems (Brodsky and Schekman, 1994).

### *Hsp60*

The hsp60 family was the first to be referred to as molecular chaperones, and the term chaperonins has been specifically reserved for this protein family (Ellis and Hemmingson, 1989). Hsp60 is the eukaryotic homologue of the well characterized GroEL protein from

prokaryotes (Burel et al., 1992). GroEL functions as a molecular chaperone by promoting the folding of newly translated proteins, and in eukaryotes, hsp60 functions in the chloroplasts and mitochondria to ensure proper folding of newly imported proteins (See Frydman and Hartl, 1994 for review). This is an ATP dependent process, and another protein (GroES in prokaryotes or hsp10 in eukaryotes) regulates this ATPase activity (Bochkareva et al., 1988; Ostermann et al., 1989; Parsell and Lindquist, 1994). Hsp60 is present in mitochondria and chloroplasts under normal physiological conditions and is responsible for the proper folding of imported proteins and for assembling multimeric structures (Cheng et al., 1989; Kang et al., 1990; Ostermann et al., 1989). Under conditions of elevated temperature there is an increased demand for the function of hsp60 in order to ensure the proper folding of proteins (Martin et al., 1992). Hsp60 expression is induced by conditions which cause protein denaturation within the cell and helps to ensure proper mitochondrial, and chloroplast function during stress (See Hightower, 1991 for review).

### *Small Hsps*

The small heat shock proteins belong to a family of several different proteins ranging in molecular size from (15 to 30 kD). The small hsps show a degree of structural similarity to the  $\alpha$ -crystallin proteins (Ingolia and Craig, 1982; Wistow, 1985; Arrigo and Landry, 1994).  $\alpha$ -crystallin itself has been shown to be heat inducible in its expression (Head et al., 1996). Despite showing similarity to  $\alpha$ -crystallin, members of the small hsps are less well conserved than the other hsp families (Arrigo and Landry, 1994). The number of members of this family varies dramatically amongst organisms with there being as few as one member in yeast and more than twenty members in plants (Arrigo and Landry, 1994). The small heat shock

proteins are developmentally as well as stress regulated (Bendena et al., 1991; Cheney and Shearn, 1983). These proteins are expressed during non-shock conditions and are strongly induced upon heat shock (Arrigo and Landry, 1994). It has also been demonstrated that the small hsps are involved in and in many cases are required for thermotolerance. In *Dictyostelium*, cells harboring mutations in the small hsps cannot acquire thermotolerance despite increased levels of the other hsps (Loomis and Wheeler, 1982). By selectively raising the abundance of the small hsps in *Drosophila* by the use of ecdysone, thermotolerance can be conferred without requiring a previous sub-lethal heat shock (Berger and Woodward, 1983). There is also indication that the small hsps function during exposure to other forms of toxic stress (Arrigo and Landry, 1994). One current theory as to the role of the small hsps in protecting cells during stress is that they protect the microfilament network from stress induced damage (Arrigo and Landry, 1994).

*Other heat induced proteins:*

In addition to the classic hsps, there are several other proteins which have been demonstrated to have heat induced expression. Among the list are  $\alpha$ -crystallin (Arrigo and Landry, 1994), calreticulin (Conway et al., 1995), peptidyl prolyl *cis-trans* isomerases (Sykes et al., 1993), and ubiquitin (Schlesinger, 1990). Of these, ubiquitin is one of the more interesting as it may work in concert with the protein repair mechanisms mediated by hsps. Upon stress polyubiquitin is increased in expression (Parsell and Lindquist, 1993). Ubiquitin is a protein that during both non-shock and stress conditions tags certain proteins for degradation (Schlesinger, 1990). These tagged proteins are then transported to the proteasome where they are degraded in an ATP dependent manner (Schlesinger, 1990).



There are three classes of enzyme which are involved in the tagging of proteins with ubiquitin: E1, E2 and E3 (Schlesinger, 1990), however it appears as though only the members of the E2 group (the conjugating enzymes) are heat inducible (Parsell and Lindquist, 1993). It is possible that the ubiquitin system triggers the degradation of proteins which are beyond repair by the other hsp's.

### **C. Regulation of the Eukaryotic Heat Shock Gene Expression**

#### Heat Shock Factor

In eukaryotes the expression of the heat shock proteins is controlled by an inducible *trans* acting transcriptional activator termed Heat Shock Factor (HSF) (reviewed by Morimoto et al., 1992; Lis and Wu, 1993; Morimoto, 1993; Morimoto et al., 1994a; Wu et al., 1994; Mager and DeKruiff, 1995; Wu, 1995). HSF preexists inside of cells in an inactive conformation, and is activated in response to stress (Zimarino and Wu, 1987). HSF controls the expression of the genes which encode for hsp's, activating their expression only when they are required. The HSF protein is essential for cell viability; not only at heat shock temperatures, but during normal cell growth as well (Jedlika et al., 1997; Sorger and Pelham, 1988).

#### *HSF Families*

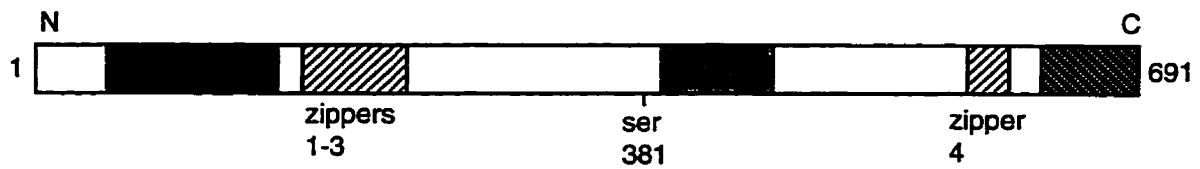
Although all eukaryotic organisms studied to date have been found to possess at least one HSF, some organisms have been found to possess a family of HSFs. In *Saccharomyces cerevisiae* (Sorger and Pelham, 1988; Wiederrecht et al., 1988), *K. lactis* (Jakobsen and Pelham, 1991), *Schizosaccharomyces pombe* (Gallo et al., 1993), *Drosophila* (Clos et al., 1990), and *Xenopus* (Stump et al., 1995), only one HSF has been identified. Mice (Sarge et





al., 1991) have been found to contain two distinct HSFs and three HSFs have been identified in humans (Rabindran et al., 1991; Schuetz et al., 1991; Nakai, 1997), tomato (Scharf et al., 1990), and chickens (Nakai and Morimoto, 1993). These various HSFs have been named numerically (i.e. HSF1, HSF2 and HSF3). In all cases, HSF1 is the stress activated member of the family (For Review see Morimoto et al., 1994a). HSF2 is not heat inducible (Sarge et al., 1993) but may be involved in regulating the developmental expression of hsp's and may have a role in spermatogenesis (Sarge et al., 1994). HSF2 has been found to be induced by hemin treatment in human erythroleukemia cells (Sistonen et al., 1992). For the remainder of this thesis the term HSF will refer to the stress inducible HSF1.

### *HSF Structure*

Although the heat shock response is in itself highly conserved amongst organisms, the HSFs which control the response show a fairly large degree of variation at the sequence level. There is, however, a functional conservation among HSFs. HSF has several important domains which contribute to its function and regulation (Figure 1). The DNA binding and oligomerization domains of HSF which are found in the amino terminal half of the protein show the most conservation amongst organisms (Clos et al., 1990; Wu, 1995). The DNA binding domain of HSF shows some sequence similarity to the recognition helix of bacterial  $\sigma$ -factors (Clos et al., 1990). Although sequence similarity to known DNA binding motifs is limited, the solution structures of *Kluyveromyces lactis* and *Drosophila* HSFs have revealed that the DNA binding domain of HSF resembles the helix-turn-helix DNA binding motif (Damberger et al., 1994; Vuister et al., 1994a; Vuister et al., 1994b and Wu, 1995 for review). HSF also contains a conserved oligomerization domain. Under conditions of normal

**Figure 1. The functional domains of the *Drosophila* heat shock factor.**



-  - DNA binding domain (amino acids (a.a.) 45-150)
-  - Hydrophobic repeats (leucine zippers)
  - zippers 1-3 are the oligomerization domain (a.a. 166-232)
  - zipper 4 is the suppressor of oligomerization and activation (a.a. 583-609)
-  - suppressor of activation (a.a. 392-462)
-  - activation domain (a.a. 629-691)

physiological growth, HSF exists as an inactive monomer, and it is not until the onset of stress that HSF becomes active, at which time it is converted to a homotrimeric conformation (Westwood and Wu, 1993). The oligomerization domain is comprised of a series of three hydrophobic heptad repeats known as leucine zippers (Wu, 1995), and the trimer is formed via a triple-stranded  $\alpha$ -helical coiled-coil (Peteranderl and Nelson, 1992). Among animals, there is an additional region of conservation in HSF which is found in the C-terminal portion of HSF (Wu, 1995). This region contains a fourth leucine zipper which acts as a repressor of oligomerization (Rabindran et al., 1993; Zuo et al., 1994). It has been suggested that this fourth leucine zipper is responsible for stabilizing the monomeric conformation of HSF by interacting with the first three leucine zippers found in the amino terminal half of the HSF molecule (Rabindran et al., 1993; Zuo et al., 1994). HSF also possesses another, less conserved domain which controls the transactivation functions of HSF. The activation domain is contained within the carboxy-terminal portion of the molecule, and in *Drosophila* it is located at the extreme C-terminus (Wisniewski et al., 1996). Although there is limited sequence similarity in this region among organisms, it has been demonstrated that the activation domain from human HSF can substitute for *Drosophila* HSF (Wisniewski et al., 1996). This would imply that although there is limited sequence conservation, there is a functional conservation to this domain. The activation domain of *Drosophila* HSF has been found to be rich in hydrophobic and acidic residues (Wisniewski et al., 1996). The activation domain of metazoan HSFs has only recently been examined in detail, and the mechanism by which this domain functions remains undetermined at this time.

### Heat Shock Element

Once HSF is activated it binds to a *cis* acting positive control element termed the Heat Shock Element (HSE). HSEs are found in the promoter regions of the heat shock genes and function to direct the action of HSF. The HSE was identified as a required sequence for the inducible transcription of the *Drosophila* hsp70 gene (Mirault et al., 1982, Pelham, 1982; Fernandes et al., 1994a). HSEs contain between three and six inverted repeats of the consensus sequence AGAAn (Amin et al., 1988; Fernandes et al., 1994a; Fernandes et al., 1994b; Pelham, 1982; Perisic et al., 1989; Xiao et al., 1991; Cunnif and Morgen, 1993; Kroeger and Morimoto, 1994). HSF shows equal affinity for both head to head and tail to tail arrangements of the five base pair blocks (Perisic et al., 1989). Heat shock promoters contain more than one HSE (Shuey and Parker, 1986). For the major heat shock protein genes of *Drosophila*, the promoters contain between three (for hsp22) and 6 HSEs (for hsps 23 and 26)(Simon and Lis, 1987). The distance of the HSEs from the transcriptional start site can vary, with increased distance having little effect on the ability of the element to regulate heat shock gene transcription (Simon and Lis, 1987).

In *Drosophila*, as with other organisms, heat shock causes a shift in the protein expression pattern. Within 20 minutes of a temperature up-shift to 36.5 °C *Drosophila* cells begin to synthesize only hsps; all other protein synthesis is halted (DiDomenico et al., 1982a). mRNAs from other genes are not degraded at higher temperatures, rather, they are stable during heat shock (DiDomenico et al., 1982a). This then indicates that heat shock mRNAs are preferentially translated during heat shock (DiDomenico et al., 1982a). It has been determined that there is a segment of the 5' untranslated region of the heat shock mRNAs

which is responsible for controlling the preferential translation of these messages (McGarry and Lindquist, 1985; Hultmark et al., 1986). It has also been reported that in certain cell lines, under specific disease conditions, there can be an uncoupling of heat shock gene transcription and hsp translation (Aquino et al., 1993), but this has been the only report of such an event.

In eukaryotes, the heat shock gene expression is mediated through the function of HSF. Activation of the heat shock gene expression is believed to be a multistep pathway which may involve some or all of the following 1) trimerization of HSF, 2) nuclear localization of HSF 3) binding to HSE, 3) hyperphosphorylation of HSF, and 4) stimulation of RNA pol II initiation/elongation by HSF (Figure 2).

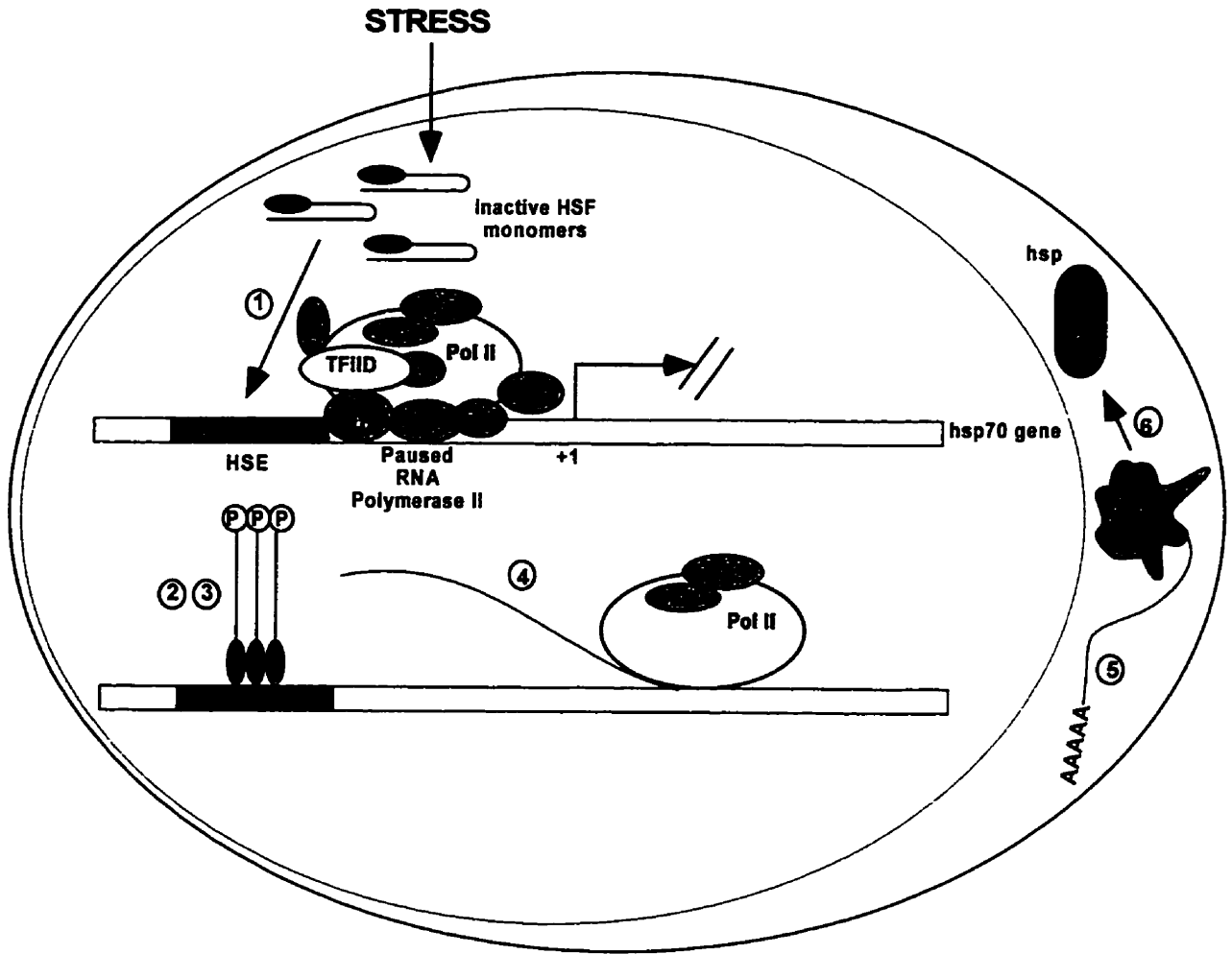
### Trimerization

During conditions of normal growth in metazoans and in the fission yeast *S. pombe*, HSF remains in an inactive configuration, that is, it is not bound to the HSE and upon heat shock (or during exposure to other forms of stress) HSF is activated to its DNA binding form (Zimarino and Wu, 1987; Kingston et al., 1987; Gallo et al., 1993). High affinity DNA binding is accompanied by oligomerization from a monomer to a trimer (Westwood et al., 1991; Sarge et al., 1993; Westwood and Wu, 1993). HSF itself preexists within cells (Zimarino and Wu, 1987; Kingston et al., 1987). In addition, any regulatory proteins must also be constitutively present as no protein synthesis is required for the activation or inactivation of HSF (Zimarino and Wu, 1987; Mosser et al., 1988; Zimarino et al., 1990). This situation of inducible DNA binding is somewhat different in the yeasts *S. cerevisiae* and *K. lactis* where HSF is constitutively bound to the HSEs (Sorger et al., 1987; Sorger and Pelham, 1988; Jakobsen and Pelham, 1991). Although HSF is always bound in these

**Figure 2. *Drosophila* heat shock gene expression.**

Stress is sensed by the cell leading to an activation of heat shock gene expression. This activation is a multistep process. (1) HSF becomes active and (2) forms trimers which bind to the heat shock element. During the activation of HSF, (3) it becomes hyperphosphorylated. Once bound to the HSE, a paused polymerase molecule is triggered into elongation and (4) the heat shock gene is transcribed. The mRNA is transported to the cytoplasm where (5) it is translated and (6) an hsp is formed to repair cellular damage caused by the stress condition.





organisms it has recently been shown that during heat shock there are additional, lower affinity HSEs which are bound by HSF (Giardina and Lis, 1995).

### Nuclear Localization

It has been demonstrated in *Drosophila* that HSF is always localized to the nucleus (Westwood et al., 1991). This localization may help to afford a rapid response to stress. At least some of the *S. cerevisiae* and *K. lactis* HSFs are nuclear at all times as they are constitutively bound to HSE. In addition, *Xenopus* HSF has also been found to be nuclear both before and after heat shock (Mercier et al., 1997). The mammalian situation has been a source of controversy. Some studies have suggested that during non-stress conditions, mammalian HSF is predominantly a cytoplasmic protein (Baler et al., 1993; Sarge et al., 1993) while others have demonstrated that HSF is a nuclear protein at all times (Wu et al., 1994; Kim et al., 1995; Martinez-Balbas et al., 1995; Mercier et al., manuscript in preparation). Those supporting the theory that unshocked mammalian HSF is cytoplasmic suggest that during normal growth conditions, HSF is found in the cytoplasm of cells and during heat shock, HSF is rapidly translocated into the nucleus (Baler et al., 1993; Sarge et al., 1993). This conclusion has been recently called into question and it has been found that in mammalian cells including HeLa, NIH-3T3 and Viro cell lines as well as in porcine cells, the majority of HSF is localized to the nucleus under all conditions (Wu et al., 1994; Kim et al., 1995; Martinez-Balbas et al., 1995; Mercier et al., manuscript in preparation). Determining the location of HSF continues to be an important goal as it will ultimately direct the focus of were to search for regulators of HSF activity.

### Binding to HSE

Once HSF is in the trimeric form it binds to HSEs with high affinity. Each one of the three HSF subunits interacts with one of the AGAAn repeats which make up an HSE (Wu, 1995; Perisic et al., 1989). Although HSF can bind to an HSE which is comprised of only two of the 5 bp repeats, three repeats are required for high affinity binding (Perisic et al., 1989). The binding of HSF to the HSE is cooperative on two separate levels. There is cooperation between the monomers of a trimer, as well as cooperation between trimers (Xiao et al., 1991). Although binding to the HSE is required for activation of heat shock gene transcription, it is not known whether it is sufficient and in fact there is evidence to suggest that it is not. Several cases have been reported where HSF becomes bound to the HSE but heat shock gene transcription is not activated (Giardina et al., 1995; Jurivich et al., 1995; Jurivich et al., 1992; Winegarden et al., 1996<sup>2</sup>).

### Hyperphosphorylation of HSF

During heat shock, HSF has been found to be hyperphosphorylated (Sorger and Pelham, 1988; Larson et al., 1988; Sarge et al., 1993; Winegarden et al., 1996). The actual timing of this event has not been conclusively determined and may occur before, after, or concomitantly with the acquisition of the trimeric conformation and DNA binding activity of HSF. Further, the role of HSF hyperphosphorylation has not been conclusively determined. It has been suggested that hyperphosphorylation may be required for acquisition of transcriptional competence for HSF (Sorger and Pelham, 1988; Xia and Voellmy, 1997). This theory seems to have been discounted, at least for mammalian cells as it was found that the amino acid analogue azetidine-2-carboxylic acid could induce heat shock gene activity without

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<sup>2</sup> The Data for Winegarden et al., 1996 is presented in chapter 2 and the appendix of this thesis.

detectably triggering the change in gel mobility normally associated with hyperphosphorylation of HSF (Sarge et al., 1993). It has also been demonstrated that HSF hyperphosphorylation is not required for HSF to obtain DNA binding activity in mammalian cells and in *Drosophila* (Sarge et al., 1993; Cotto et al., 1996; Winegarden et al., 1996). An alternate theory as to the role of HSF hyperphosphorylation suggests that it is required for the attenuation of the heat shock response and the inactivation of HSF (Hoj and Jakobsen, 1994; Chu et al., 1996). A recent report utilizing a reporter gene system suggests that hyperphosphorylation of HSF contributes to the ability of HSF to induce transcription and in addition stabilizes the interaction of HSF with the HSE, preventing dissociation from the promoter (Xia and Voellmy, 1997). It is also not yet known how HSF becomes hyperphosphorylated. It has not been determined whether HSF is hyperphosphorylated by a stress activated kinase or whether heat shock prevents the action of a phosphatase. Recently evidence has been presented which suggests that HSF is acted upon by a member of the mitogen activated protein kinases (MAPKs), specifically of the ERK-1 family (Chu et al., 1996).

#### Activation of Heat Shock Gene Transcription and Translation

The precise means by which HSF triggers the transcription of the heat shock genes has not yet been fully determined. It is assumed that HSF in some way interacts with the basal transcriptional machinery, however its target has not been identified. The promoters of most heat shock genes appear to have an RNA polymerase molecule already associated with them (Rougvie and Lis, 1988). This polymerase is transcriptionally engaged and is in a paused state

until heat shock (Rougvie and Lis, 1988). It is therefore assumed that the function of HSF is to release this paused polymerase into elongation.

### **Control of HSF Activation**

There has been a great deal of research focused on determining the means by which the activity of HSF is controlled. There is mounting evidence that the activities of HSF are separable. That is, HSF binding can be triggered without inducing the transcription of the heat shock genes (Jurivich et al., 1992; Giardina et al., 1995; Jurivich et al., 1995; Winegarden et al., 1996). In light of this, efforts to determine the means by which HSF activity is regulated must concentrate both on controlling HSF binding activity, as well as controlling transcriptional competency.

### **Regulation of HSF Binding Activity**

HSF appears to have a natural affinity for DNA when in its trimeric state. Therefore, it may hold true that regulating the oligomerization of HSF in turn regulates the DNA binding activity of HSF. Causing HSF to oligomerize by the addition of antibodies generated to the active form of HSF causes HSF binding to be activated (Zimarino and Wu, 1990). HSF appears to have a natural propensity to aggregate. When expressed in bacteria, *Drosophila* HSF forms higher oligomeric structures in the absence of stress (Clos et al., 1990; Rabindran et al., 1991). These oligomers are competent to bind DNA and to activate transcription from heat shock promoters *in vitro* (Clos et al., 1990). HSF, when expressed at low levels in *Xenopus* oocytes (Clos et al., 1990; Zuo et al., 1994), tissue culture cells (Rabindran et al., 1993; Clos et al., 1993), or reticulocyte lysates (Sarge et al., 1991) does not aggregate in the absence of stress, and exists in its inactive monomeric configuration. This implies that there is

some factor present in eukaryotic cells, and absent in bacteria, which is responsible for keeping HSF in its inactive configuration. One of the more attractive theories currently available is that one or more of the heat shock proteins or heat shock cognate proteins are responsible for maintaining HSF in its inactive conformation. This would imply that the heat shock response is self regulating.

Hsp70, or the 70 kD cognate, is one of the favoured candidates for fulfilling the role as a regulatory factor (For a full review see Craig and Gross, 1991). Briefly, this theory states that hsc70 is responsible for maintaining HSF in an inactive conformation through transient interactions. Heat, and perhaps other forms of stress, cause a denaturation of cellular proteins causing an increased need for the function of the hsc70 in the cell. When hsc70 leaves HSF unattended, HSF is able to take on its preferred trimeric conformation. Once in trimeric form, HSF can then bind to HSE. This theory also hypothesizes that during stress, HSF remains active until there is enough free hsc70/hsp70 present to relieve the cellular damage and to return HSF to its inactive form.

Support for this theory has come from a few different observations. The amount of hsp70 produced in a cell is directly related to the degree of stress (DiDomenico et al., 1982b; Mosser et al., 1993). That is, once the required amount of hsp70 for a particular stress is produced, the response is turned off. If the function of hsp70 is prevented in cells, for example by adding amino acid analogues, which will prevent the formation of functional hsps, there is an overexpression of the hsp70 gene, and a continual production of hsp70 (DiDomenico et al., 1982b). For each stress it appears there is a required level of functional hsps which needs to be reached before normal protein synthesis returns (DiDomenico et al.,

1982b). Overexpression of hsp70 in cells causes a decrease in detectable HSF binding activity as monitored by gel mobility shift assays (Price and Calderwood, 1992; Liu et al., 1993) and microinjection of hsc70 into *Xenopus* oocytes causes a decreased expression of a heat shock reporter gene during stress (Mifflin and Cohen, 1994b). Conversely, in yeast strains carrying mutations in two of the hsc70s encoded in this organism, hsps are expressed at high levels during normal growth temperatures (Craig and Jacobsen, 1985). Overexpression of hsps which is seen in cells carrying hsc70 mutations can be prevented by mutations in the HSEs of these cells (Boorstein and Craig, 1990). This has been cited as evidence of a link between hsc70 loss of function and HSF gain of function. Mutation of the HSEs presumably prevents HSF binding to the heat shock gene promoters. The fact that this loss of HSF function prevents the hsp overexpression under these conditions would indicate that the mutant hsc70s were unable to control the activity of HSF, allowing it to remain active in cells harboring such mutants. If hsp70 accumulation is prevented by the addition of RNA polymerase II inhibitors, HSF remains active for a longer duration during a subsequent heat shock (Price and Calderwood, 1992). Another piece of evidence which suggests an effect of hsp70 on the control of HSF activation comes from the observation that overexpression of hsp70 in mammalian cells prevents that heat inducible expression of a  $\beta$ -galactosidase reporter gene under the control of a heat shock promoter (Baler et al., 1996).

It is assumed that if hsp70/hsc70 is responsible for regulating the activity of HSF, these chaperone proteins would have to be able to bind to and interact with HSF. There has been a number of experiments that show that hsp70 is capable of binding HSF, however, the functional significance of this interaction is harder to determine. Anti-hsp70 antibodies have

been shown to supershift HSF-HSE complexes in gel mobility shift assays after prolonged heat shocks (Abravaya et al., 1992). In addition, hsp/hsc70 has been shown to coimmunoprecipitate with HSF from both heat shocked and control HeLa cells (Rabindran et al., 1994). It has also been suggested through western blotting analysis that hsp/hsc70 forms an unstable heterodimer with HSF during control conditions (Baler et al., 1996). Hsp90 has also been shown to interact with HSF (Nadeau et al., 1993) and it has been suggested that hsp70 and hsp90 may work together to regulate the activity of HSF (Mosser et al., 1993). It has been demonstrated that the addition of hsp70 to control extracts of HeLa cells can prevent HSF activation by detergents such as NP-40 (Abravaya et al., 1992). Moreover, overexpression of hsp70 has been shown to reduce the amount of HSF which is activated to bind HSE by both heat shock (Liu et al., 1993; Mosser et al., 1993) and by chemical treatment with arsenite or azetidine (Mosser et al., 1993). However, further experiments by another group has suggested that hsp overexpression cannot reduce the activation of HSF *in vivo* (Rabindran et al., 1994). In an attempt to explain this apparent controversy, a third group has suggested that although overexpression of hsps may not reduce the amount of HSF activated to bind HSE, it may be sufficient to delay the onset of HSF binding activity (Baler et al., 1996).

Although there is some debate as to whether hsp70 or other hsps can control the binding activity of HSF, there is a growing body of evidence, and a fairly strong consensus that hsps can accelerate both the attenuation of HSF binding and the inactivation of HSF during recovery. Cell lines overexpressing hsp70 show more rapid attenuation of HSF binding (Mosser et al., 1993). Elevated expression of all of the hsps accelerates the loss of HSF



binding activity during recovery in rat cells, and accelerates the attenuation of HSF binding in *Drosophila* cells (Rabindran et al., 1994). Whether hsp's can modulate the activity of HSF or not is still in question, however at this time, a more attractive, or better founded model has yet to be presented.

Although there may be some negatively acting factor which keeps HSF in an inactive state, at least part of the role of stabilizing HSF's monomeric configuration is intrinsic to the HSF molecule itself. As previously mentioned, a leucine zipper in the C-terminal portion of the HSF molecule interacts with the other three leucine zippers of the oligomerization domain thereby both suppressing HSF trimerization and stabilizing the monomer (Rabindran et al., 1993; Zuo et al., 1994). It has also been suggested that HSF itself can directly "sense" an increase in temperature as it has been shown that the DNA binding activity of unshocked purified HSF can be activated *in vitro* by heat shock (Goodson and Sarge, 1995, Larson et al., 1995). Therefore, part of the role of maintaining HSF in an inactive monomeric conformation may be a result of an intrinsic stability of the monomeric molecule itself. This stability may be overcome by increased temperature. This does not rule out a role for some other factor which assists in enhancing the HSF monomer's stability. Under the physiological conditions of the cell, the intrinsic stability may not be as high as it is when HSF is in pure form. If hsp70 is involved in stabilizing the monomeric form, the inherent stability of the HSF monomer may be enough to allow the interaction with hsp70 to be a transient one, but may be limiting enough that if left unchecked, the monomers in the cell will unfold, and trimers will form. It is also apparent that the regulation of HSF activity may not be a simple mechanism. In an attempt to understand the means by which HSF activity is regulated, human cells were transfected with

*Drosophila* HSF, and vice versa, to see at what temperature, the exogenous factor would be activated (Clos et al., 1993). It was thought that if the activation temperature is intrinsic to the factor itself, activation would occur at the native temperature for that HSF. On the other hand, if activation temperature is determined by the cell, the newly introduced HSF would act similarly to the endogenous HSF in that cell. Unfortunately, no clear answer was provided. *Drosophila* HSF expressed in HeLa is constitutively active at the normal growth temperature for HeLa (37 °C) (Clos et al., 1993), indicating that the activation temperature is determined by the HSF molecule. However, human HSF expressed in *Drosophila* takes on the activation temperature of *Drosophila* HSF indicating that activation temperature is controlled by the cell (Clos et al., 1993). It appears from this report that activation of HSF is a complex function regulated both by the factor itself and by conditions inside of the cell.

#### Regulating HSF Transcriptional Competency

The activation domains of HSF from yeast (Nieto-Sotelo et al., 1990), *Drosophila* (Wisniewski et al., 1996), mouse (Shi et al., 1995) and human (Green et al., 1995; Zuo et al., 1995) have been mapped to the C-terminal portion of the molecule. Recently, it has also been demonstrated for mammalian HSE, that there exists another region of the molecule which maps to the region between the DNA binding domain and the oligomerization domains, (and may include portions of each of these domains) which is responsible for negatively regulating the activity of the transactivation domain of HSF (Green et al., 1995, Shi et al., 1995; Zuo et al., 1995). It is thought that perhaps the molecule is folded on itself masking the activation domain, and that heat causes an unfolding of the structure causing the transactivation domain to be exposed. As has been previously mentioned, a role of hyperphosphorylation in the

control of transactivation has also been considered, however no definitive answer has been presented. It has also been suggested that there may be requirements for factors other than HSF in order to stimulate the activity of heat shock genes. One group has suggested that there is an additional constitutive HSE binding factor which is required for full activation of the heat shock response (Liu et al., 1993).

#### **D. Inactivation of the Heat Shock Response**

Inactivation of the heat shock gene expression, like its activation, involves multiple steps including 1) loss of HSF binding, 2) termination of heat shock mRNA translation and 3) degradation of hsp mRNAs. There are two conditions under which some of these events happen. The response may be attenuated during a continual heat shock or stress and the response is also inactivated during recovery from heat shock. During recovery from heat shock or an extended continual heat shock, HSF has been shown to lose its DNA binding activity (Abravaya et al., 1991; Mosser et al., 1993; Zimarino and Wu, 1987). The puffing of heat shock loci also regresses during recovery from heat shock (Ashburner, 1970; Winegarden et al., 1996) and during attenuation under conditions of a constant heat shock (Han et al., 1985). Transcription from the heat shock genes is stopped, as would be expected after the release of HSF from the HSE (Abravaya et al., 1991). During recovery from heat shock, there is a decrease in the translation of hsps and the translation of the normal cellular proteins returns to normal (DiDomenico et al., 1982a). The loss of hsp expression appears to be mostly due to a selective degradation of the heat shock mRNAs (DiDomenico et al., 1982a; Petersen and Lindquist, 1988).

## **E. Inducers of the Heat Shock Response**

### **The “Stress Signal”**

One important goal in understanding the molecular biology of the heat shock response is to identify the means by which stress is sensed in the cell. There are numerous different inducers which activate the heat shock response (See Ashburner and Bonner, 1979 and Nover, 1991 and references therein). In an attempt to understand how such a diverse group of inducers can all stimulate activation of the same response, many researchers have attempted to identify a “universal” signal. It was originally thought that there may be some molecule, intermediate other than HSF, and/or signalling pathway through which all forms of stress activate the heat shock response. However, evidence is accumulating that there may be a few different signals which induce the heat shock response, and that different classes of inducers may activate the response via different means.

### **Classes of Inducers**

Although the classic inducer of the heat shock response is elevated temperature, there are many other inducers which have been identified. Table 1 outlines the inducers which have been identified for *Drosophila*. These different inducers can be grouped into several different classes: oxidative respiration inhibitors/oxidizing agents, anion transporters, potassium ionophores, transition series metals, amino acid analogues, inhibitors of gene expression, inhibitors of topoisomerase II, steroid hormones, membrane destabilisers, teratogens, mutagens, carcinogens and miscellaneous other chemicals. As can be seen from Table 1, there are several different aspects of the heat shock response which are assayed for activation by the different chemical inducers. Originally inducers of the response were identified by determining

Table 1. Inducers of the *Drosophila* Heat Shock Response

Inducing Agent	Chromosome Puffing	Activity Induced By Agent		
		HSF Binding	HS Gene Transcription	Hsp Translation
<b>Drugs affecting oxidative reparation and oxydizing agents</b>				
Amytal (0.3 mM)	20			
Anoxia and recovery	1, 19, 25			19
Antimycin A (saturated)	20			
Arsenate (10 mM)				
Arsenite (10-100 $\mu$ M)	19, 20			19,32
Azide (3 mM)	20, 25			
Cyanide (1-10 mM)		35	35	
2,4 Dinitrophenol (0.1-1 mM)	13, 19, 20, 23, 24	35	35	19
2-Heptyl-4-hydroxyquinoline- <i>N</i> -oxide	20			
H <sub>2</sub> O <sub>2</sub> (0.05 - 1 mM)	10			
Hydroxylamine (10 mM)	27			
Menadione (1 mM)	20			
Methylene blue (10 mM)	20			
Oligomycin + Potassium cyanide	20			
Rotenone (saturated)	20			
Salicylate (3-30 mM)	24, 25, 34	34		
<b>Anion transporters</b>				
4,4'-Diisothiocyanostilbene 2,2' disulfonate (100 $\mu$ M) (DIDS)				28
4-Acetamido 4'-isothiocyanostilbene 2,2' disulfonate (100 $\mu$ M)				28
Flufenamic acid (100 $\mu$ M)				28
Mefanamic acid (100 $\mu$ M)				28
Niflumic acid (100 $\mu$ M)				28
<b>K<sup>+</sup>-ionophores</b>				
Dinactin (0.1-1 $\mu$ M)	23			
Trinactin	4			
Valinomycin (10 $\mu$ M)	23			
<b>Transition series metals</b>				
Cd <sup>2+</sup> (10-100 $\mu$ M)				11
<b>Amino Acid Analogs</b>				
Azetidine-2-carboxylic acid (5-50 mM)	36	36	12	12
Canavanine (0.1-1 mM)			12	12, 33
s-aminoethylcysteine				33
$\beta$ -Hydroxyleucine			12	12, 33
<b>Inhibitors of Gene Expression</b>				
Chloramphenicol (1 mM)	3			3 (during recovery)
<b>Drugs affecting membrane structure</b>				
Ethanol (2-6%)				31
Digitonin, saponins, Sarkosyl, Triton X-100, Nonidet P-40	22		22	
<b>Steroid hormones<sup>†</sup></b>				
Dexamethasone (0.1 mM, during glucose deprivation)				7

<i>Inducing Agent</i>	<i>Chromosome Puffing</i>	<i>Activity Induced By Agent</i>		
		<i>HSF Binding</i>	<i>HS Gene Transcription</i>	<i>Hsp Translation</i>
Diethylstilbestrol (10 $\mu$ M)				7
Ecdysterone (1 $\mu$ M)				9, 16, 17
Hydrocortisone				8
Methyltestosterone (10 $\mu$ M)				7
<b>Teratogens, carcinogens, mutagens</b>				
Coumarin (0.1-1 mM)				7
Diphenylhydantoin (0.1-1mM)				7
Pentobarbital (0.1-1 mM)				7
Tolbutamide (0.1-1 mM)				7
5-Azacytidine (30 mM)				7
Thalidomide (1 mM)				7
<b>Miscellaneous Inducers</b>				
Abnormal proteins				14, 15, 18
Ammonium chloride	27			
Ether				
Dicoumarol (1 mM)	25			
Uridine (10 mM)	27			
Vitamin B-6	19			19

#### References:

- Ashburner, 1970
- Baretino et al., 1982
- Behnel, 1982
- Behnel and Seydewitz, 1980
- Bournias-Vardiabasis and Buzin, 1986
- Bultmann, 1986
- Buzin and Bournias-Vardiabasis, 1984
- Caggese et al., 1983
- Cheney and Shearn, 1983
- Compton and McCarthy, 1978
- Courgeon et al., 1984
- DiDomenico et al., 1982a
- Ellgaard, 1972
- Hiromi and Hotta, 1985
- Hiromi et al., 1986
- Ireland and Berger, 1982
- Ireland et al., 1982
- Karlik et al., 1987
- Koninkx, 1976
- Leenders and Berendes, 1972
- Love et al., 1986
- Myohara and Okada, 1988
- Rensing, 1973
- Ritossa, 1962
- Ritossa, 1964
- Rowe et al., 1986
- Scalenghe and Ritossa, 1977
- Sherwood et al., 1989
- Singh and Gupta, 1985
- Strand and McDonald, 1985
- Tanguay, 1983
- Vincent and Tanguay, 1982
- Westwood and Steinhardt, 1989
- Winegarden et al., 1996
- Winegarden, N.A. Unpublished Results
- Stevens, M. L. Unpublished Results

† Steroid hormones are probably not true inducers of the heat shock response. These chemicals induce the expression of the small heat shock proteins only, and are thought to do so through the function of separate hormone response elements in the promoter of these genes.

which agents could cause the heat shock puffing pattern described by Ritossa in 1962. Once other aspects of the heat shock response were identified, such as hsp synthesis and heat shock gene expression, there were other means by which inducers could be identified.

### *Denatured proteins*

Elevated temperatures, and several chemical inducers have been suggested to activate the heat shock response via the accumulation of a denatured protein pool (Westwood and Steinhardt, 1989; Mifflin and Cohen 1994a,b). Support for this theory comes from the observation that denatured proteins are capable of inducing the response in the absence of any other forms of stress. Microinjection of denatured proteins into *Xenopus* oocytes causes the activated transcription from a reporter gene which is under the control of a heat shock promoter (Ananthan et al., 1986; Mifflin and Cohen, 1994a). Expression of mutant actin genes in *Drosophila* flight muscle triggers the constitutive expression of hsps (Hiromi et al., 1986). It has been demonstrated that the trigger cannot be any denatured protein, and that it is only those with the propensity to aggregate into larger complexes or precipitates which are effective (Mifflin and Cohen, 1994a). Further evidence for a role of denatured proteins comes from the fact that chemicals which can function to stabilize protein conformation such as glycerol or D<sub>2</sub>O can prevent the activation by heat (Edington et al., 1989), and by agents such as urea, or detergents such as NP40 and Triton X-100 (Mosser et al., 1990). This theory of denatured proteins as a signal for activating the heat shock response agrees well with the thought that the heat shock response is auto-regulated. Denatured proteins would serve as new substrates for hsp70 activity, thus causing HSF to be activated. A further link has been provided by the observation that only those denatured proteins which were found to activate

the heat shock response were bound by hsc70 (Mifflin and Cohen, 1994b). Denatured proteins which had no effect on the heat shock response were not bound hsc70. There is currently a fairly strong consensus that denatured proteins can induce the response, however, it is not known if this is the means by which all inducers trigger the response.

#### *Changes in pH and Ca<sup>2+</sup> concentration*

Another theory that has received some attention is that inducers of the heat shock response cause changes in intracellular pH (pH<sub>i</sub>) and/or intracellular calcium (Ca<sup>2+</sup><sub>i</sub>) concentration. Indeed in both yeast and *Drosophila*, DNP (Weitzel et al., 1985) and heat shock (Drummond et al., 1986; Weitzel et al., 1985) decrease pH<sub>i</sub>. Aspirin, in humans (Flescher et al., 1995) and heat shock in *Drosophila* have been shown to increase Ca<sup>2+</sup><sub>i</sub> concentrations (Drummond et al., 1986). Despite this however, there are discrepancies as to whether these conditions can trigger activation of the heat shock response. While it has been reported that decreased pH or increased Ca<sup>2+</sup> can trigger HSF activation *in vitro* (Mosser et al., 1990), and low pH<sub>i</sub> can cause hsp70 synthesis *in vivo* (Nishimura, 1989), it has also been reported that neither condition is sufficient nor are they required for activation of the heat shock response in *Drosophila* (Drummond et al., 1986). This theory may still have some foundation however as both conditions are known to affect protein conformation and may contribute to activation through denatured proteins as explained above (Mosser et al., 1990).

#### *Reactive Oxygen Intermediates*

A third theory is that certain inducers cause a build up of reactive oxygen intermediates in the cell, and that these intermediates somehow activate the heat shock response. Agents such as menadione which can cause elevated levels of reactive oxygen



species in the cell can activate HSF binding in mammalian cells (Bruce et al. 1993). The major piece of evidence supporting this theory however is that  $H_2O_2$  can rapidly activate the binding of HSF to HSE (Bruce et al., 1993; Jacquier-Sarlin and Polla, 1996; Becker et al., 1990) and induces the heat shock puffing pattern (Compton and McCarthy, 1978).  $H_2O_2$  induced activation of HSF is so rapid that it has led to speculation that the effect of  $H_2O_2$  on HSF may be direct (Becker et al., 1990). It was suggested that if this action is direct on HSF, then  $H_2O_2$  may be the final signal involved in activating HSF. It should be noted that many of the agents which produce reactive oxygen intermediates in the cell such as menadione and methylene blue also interfere with the production of ATP via oxidative respiration (see next section).

#### **F. Induction of the Heat Shock Response by Inhibition of Oxidative Respiration**

Many of the conditions which lead to a reduction of the ATP inside of cells cause the activation of the heat shock response. Both lack of an essential metabolite such as glucose or oxygen, and exposure to chemicals which inhibit respirative metabolism can cause dramatic reductions in intracellular ATP. It is thought that the heat shock response is activated to protect the cells under such conditions. It has in fact been demonstrated that an overexpression of hsp70 can protect mammalian cells against injury induced by metabolic stress (Williams et al., 1993).

##### Effect of Glucose or Oxygen Deprivation

By depriving cells of glucose or of oxygen, the intracellular ATP ( $ATP_i$ ) levels can be dramatically reduced. The effects of such a deprivation on the heat shock response has been studied in some detail. In cultured myogenic cells, the removal of glucose from the media

causes a 50% decrease in the amount of ATP<sub>i</sub>, but has no observable effect on the heat shock response (Benjamin et al., 1992). The effect of oxygen deprivation is different. Reduction (hypoxia) or elimination (ischemia/anoxia) of oxygen both lower ATP<sub>i</sub>. The activation of HSF binding is induced during the reduction of oxygen (Giaccia et al., 1992). In this case, the binding of HSF to the HSE does not, however, result in the induced expression of the hsp genes (Giaccia et al., 1992). Ischemic/hypoxic conditions do lead to increased expression of hsps but it is not until during recovery that hsp expression is induced (Giaccia et al., 1992; Van Why et al., 1994). In addition to this induced expression, recovery from ischemic conditions causes induced puffing of the *Drosophila* polytene chromosomes at the heat shock loci (Ritossa, 1964) as well as hsp synthesis (Drummond and Steinhardt, 1987). As can be seen, ischemia/hypoxia induces the entire heat shock response, but the onset all aspects of the response are not coincident with the actual stress condition: it is only the initial stage, the binding of HSF to HSE, that is triggered, during reduced oxygen. The reason for this has not yet been determined.

#### Effects of ATP Depleting Agents

One of the major sources of energy for cells is the respirative metabolism pathway. This pathway is thought to use the free energy derived from electron transport in order to pump protons from the mitochondrial matrix into the intermembrane space (Hatefi, 1985 for review). This causes a proton gradient to be formed which stores a large amount of electrochemical potential energy. This energy is then utilized by a specialized enzyme named F<sub>0</sub>F<sub>1</sub>-ATPase (Hatefi, 1985; Vignais and Lunardi, 1985). The inner mitochondrial membrane is impermeable to protons and therefore the only way that the protons can be returned to the

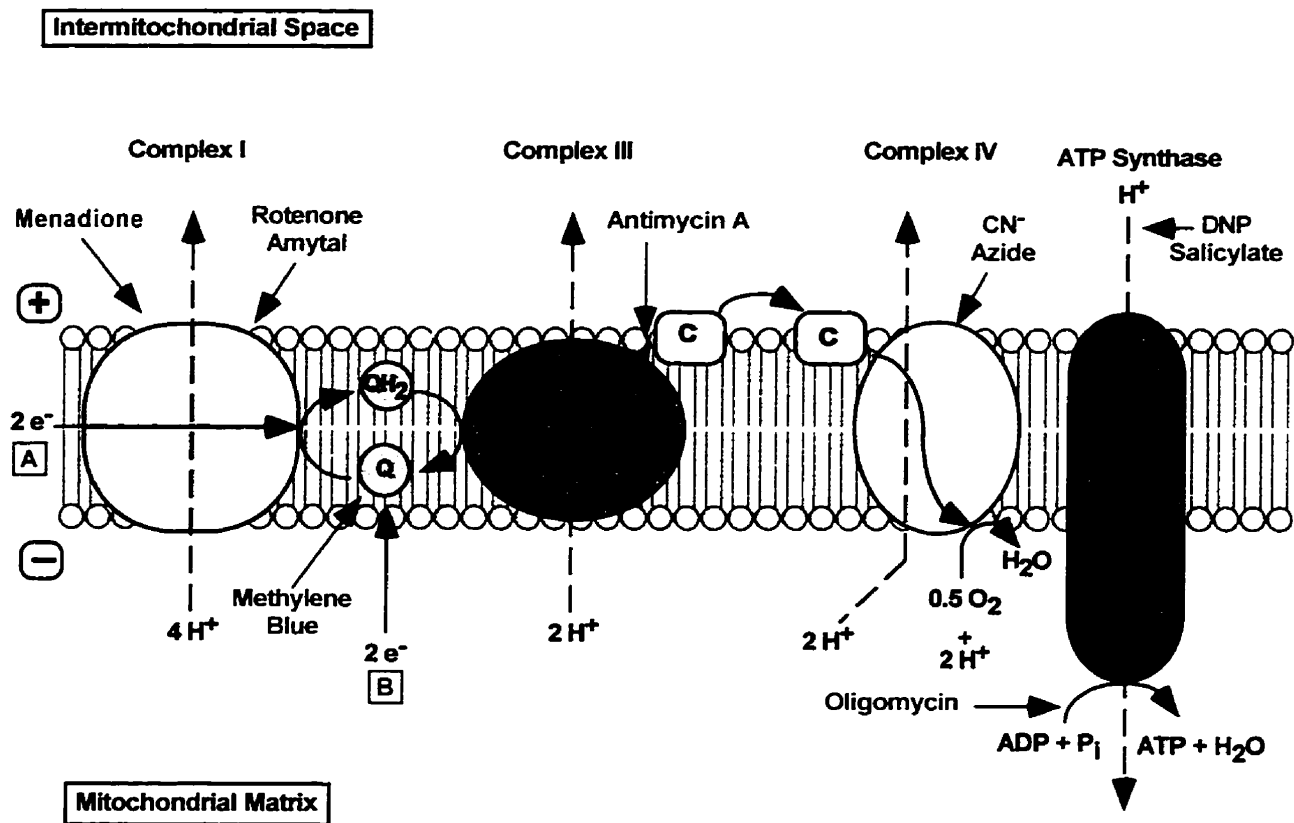
matrix is through the ATPase. The ATPase (or ATP synthase) is thought to use the energy stored in the proton gradient to drive the production of ATP from ADP. This entire process is referred to as oxidative phosphorylation. Due to the many steps involved in this process, there are many points at which the production of ATP can be interrupted (Figure 3). Agents such as rotenone, antimycin A and cyanide are termed respiratory inhibitors and function by interrupting electron transfer (Hatefi, 1985; Leenders and Berendes, 1972). Other agents such as menadione and methylene blue act as electron acceptors and interfere with initiation of the electron transport through the electron transport chain (Leenders and Berendes, 1972). Chemicals such as 2,4 dinitrophenol and salicylate uncouple electron transport from oxidative phosphorylation (Brody, 1956; Leenders and Berendes, 1972). This function is performed by shuttling protons back across the mitochondrial membrane, bypassing the ATP synthase.

Each of these types of agents have been previously studied for their effects on the heat shock response. Originally, the discovery that such agents could induce the heat shock response lead to the suggestion that lowered ATP levels may be a trigger to initiate the response. Further investigation, using other ATP depleting agents caused some in the field to discount this theory (see references below). There has also been controversy as to which agents are capable of inducing the response.

Inhibitors of complex I function include drugs such as amytal and rotenone. It has been demonstrated that both of these agents are capable of inducing heat shock puffs on the polytene chromosomes of *Drosophila* larvae (Leenders and Berendes, 1972) and rotenone induces the DNA binding activity of mammalian HSF (Benjamin et al., 1992). In addition, if rotenone is added to mammalian cells in glucose free media, hsp70 and hsp90 expression is

**Figure 3. Points of action of drugs affecting the mitochondrial electron transport chain.**

The mitochondrial electron transport chain is composed of five different complexes (I, III, IV, and V are shown here). Complex II transfers electrons from an alternate source, succinate. Drugs affecting the production of ATP are indicated in red.



**A** - electrons transferred from NADH

**B** - electrons transferred from succinate via Complex II

induced (Gabai and Kabakov, 1994). Antimycin A and 2-*n*-heptyl-4-hydroxy-quinoline-*N*-oxide (HOQNO) inhibit electron flow through complex III of the electron transport chain. Once again, both of these agents trigger polytene chromosome puffing at the heat shock loci (Leenders and Berendes, 1972). Antimycin A has also been shown to induce the production of hsps in chick embryos (Landry et al., 1985). It appears from this data that inhibitors of either of these two complexes will trigger the activation of the heat shock response.

The situation is more complex however when examining inhibitors of complex IV. The two classic inhibitors of this complex are azide and cyanide (CN<sup>-</sup>). Azide has been demonstrated to be effective in inducing the heat shock puffs of both *Drosophila* (Leenders and Berendes, 1972; Ritossa, 1964) and *Sarcophaga bullata* (Bultmann, 1986) polytene chromosomes. Cyanide by itself (0.005 to 10 mM) is not capable of inducing the heat shock puffing pattern (Behnel and Rensing, 1975; Leenders and Berendes, 1972). If cyanide is used in conjunction with oligomycin, however, (at a concentration of oligomycin which is ineffective by itself, see below), the heat shock puffing pattern is observed (Leenders and Berendes, 1972).

When other aspects of the heat shock response are assayed, the situation becomes even more unclear. In *S. cerevisiae* concentrations of 10 mM or greater cyanide cause the expression of hsps (Weitzel et al., 1987). In mammalian cells, however, cyanide has been reported to be unable to induce hsp expression (Burdon, 1986). Interestingly, if cyanide is used in conjunction with 2-deoxyglucose, both hsp70 mRNA and protein are found at elevated levels (Iwaki et al., 1993). The fact that CN<sup>-</sup>, on its own, did not induce puffing in *Drosophila* or hsps in mammalian cells was key in the discreditation of the theory that

lowered ATP levels could serve as a trigger for the heat shock response (Leenders and Berendes, 1972; Behnel and Rensing, 1975; Burdon, 1986).

The fifth complex in the electron transport chain is the  $F_0F_1$  ATPase. Although there are several inhibitors of this enzyme, only one has been studied in any detail in relation to the heat shock response. Oligomycin B prevents the phosphorylation of ADP to ATP by the ATP synthase. Originally it was reported that oligomycin (when added as a saturated solution) did not induce the puffing of the polytene chromosomes of a *Drosophila* larvae (Leenders and Berendes, 1972). A later report however showed a concentration dependence of the action of oligomycin. While  $10^{-6}$  M oligomycin was ineffective in inducing puffing,  $10^{-5}$  to  $10^{-4}$  M solutions did induce puffing of the polytene chromosomes (Behnel and Rensing, 1975). Interestingly, this concentration dependence parallels the effectiveness of oligomycin in lowering intracellular ATP levels, with the lower concentration having no effect on ATP levels, and the higher concentrations lowering intracellular ATP (Behnel and Rensing, 1975).

A final group of chemicals affecting a cell's ability to produce ATP are the uncouplers. There are a large list of chemicals which are capable of functioning as uncouplers, and several have been examined for their effect on the heat shock response. Among the chemicals studied are arsenate, carbonylcyanide phenylhydrazone (CCP), carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 2,4 dinitrophenol (DNP) and the negative enantiomer of gossypol ((-)-gossypol). Each of these chemicals have been shown to have at least some effect on the heat shock response. Arsenate induces hsp synthesis in both *Drosophila* (Bournais-Vardiabasis et al., 1990) and mammalian cells (Kim et al., 1983). CCP (Haveman et al., 1986), CCCP (Gabai and Kabakov, 1993) and (-)-gossypol (Benz et al., 1990; Benz et

al., 1991) induce hsp expression in mammalian cells. DNP is perhaps the best characterized of the uncouplers, in terms of the effect on the stress response. DNP was the first chemical inducer of the heat shock response identified, inducing the same puffing pattern in *Drosophila* chromosomes as heat (Ritossa, 1962; Koninkx, 1976; Leenders and Berendes, 1972). It has also been shown that DNP is a potent inducer of HSF binding in *Drosophila* tissue culture cells (Zimarino and Wu, 1987). When examining the expression of hsps, however, there has been some controversy. Similar to what is seen with CN, DNP has been shown to induce the expression of the stress proteins in *S. cerevisiae* (Weitzel et al., 1985; Weitzel et al., 1987). DNP can also induce hsp expression in *Drosophila* (Koninkx, 1976). In mammalian cells, it was originally reported that DNP cannot induce hsp synthesis (Burdon, 1986; Haveman et al., 1986). Further examination has indicated that expression of hsps can be induced in mammalian cells by DNP, but the amount of expression seems to be variable, ranging from only a moderate induction to a rather robust induction of the hsps (Edbladh et al., 1994; Wiegant et al., 1994).

#### ATP Decrease as a Stress Signal

Decreases in ATP levels have been implicated in signaling stress in the cell almost from the time of the original discovery of the response. However, it has been difficult to unambiguously determine whether decreases in ATP do cause activation of the heat shock response. The fact that certain ATP depleting conditions do not induce puffing of *Drosophila* polytene chromosomes, or expression of hsps in certain cell lines has led to a dismissal of this theory by some researchers. The problem may lie in the fact that many agents which affect ATP levels, when used at the concentrations in which they exhibit their maximal affect on



respiration, have no effect on inducing hsp (Lanks, 1986). One possible explanation is that once ATP levels have been decreased past a certain point, cells might lack sufficient energy to elicit a response to the stress condition.

### ***G. Sodium Salicylate - A Drug With Pleotropic Effects***

Although the salicylates are administered for a wide variety of reasons, the means by which they exert the effects that are attributed to them is not well understood. Salicylate, and its close relative, acetylsalicylic acid (aspirin) perform numerous functions within the cell (Table 2). These functions range from modulating the activity of several different proteins and enzymes within the cell, to altering the levels of certain ions or metabolites. Unless otherwise specified, the following description of salicylate's effects were determined in mammalian systems.

#### **Effects on Protein Function and Gene Transcription**

The effects of the salicylates on protein function is complex. In some cases it appears as though the action is direct. Such is the case where aspirin inhibits the activity of cyclooxygenase inside of the cell. Aspirin carries out this function by transferring its acetyl group to cyclooxygenase, thus inactivating it (Roth and Majerus, 1975). In other cases it appears that there may be an indirect function of the salicylates on protein activity. It is possible that it is one of the other functions of salicylates, such as affecting ion balances, which may in turn cause an effect on the activity of many cellular proteins and enzymes.

Salicylate has been demonstrated to have both positive and negative effects on the transcriptional activity of a number of genes. In plants, salicylate can accumulate in disease ridden organisms and is believed to be involved in the induction of pathogenesis related genes

Table 2. The effects of salicylate and acetylsalicylic acid (Aspirin)

<i>Function Affected</i>	<i>Effect</i>	<i>Effective Concentration (mM)</i>		<i>Reference</i>
		<i>Salicylate</i>	<i>Aspirin</i>	
<b>Effects on Protein/Enzyme Activity</b>				
Mitochondrial ATPase	Stimulates	2.5-5		2
	Inhibits	16	13	3
Neutrophil activation/aggregation	Inhibits	2-3	2-3	1
NF- $\kappa$ B activation	Inhibits	2-20		10
NF- $\kappa$ B mediated transcription	Inhibits	2-20		10
I- $\kappa$ B degradation	Inhibits	20		10
<b>Effects on Metabolism</b>				
Ca <sup>2+</sup> uptake	Inhibits	0.15-3		1, 17
Ca <sup>2+</sup> release from mitochondria	Stimulates	0.3		17
cAMP concentrations	Stimulates	3	3	1
Inorganic Phosphate Uptake	Inhibits	2	2	2
Inorganic Phosphate Levels	Increases	90		15
O <sub>2</sub> Consumption	Stimulates	2	2	2
Intracellular ATP	Decreases	3-10	10-20	5, 12, 14
Glutathione Depletion	Stimulates	6 <sup>a</sup>	6 <sup>a</sup>	9
Phosphocreatine Levels	Decreases	1-20		13, 14
Glucose Consumption	Stimulates	10-60		15
Lactate Production	Stimulates	10-60		15
Glycolysis	Inhibits	90		15
Fatty Acid Oxidation	Inhibits	1-3 <sup>a</sup>	1-3 <sup>a</sup>	1, 4
Lipid Peroxidation	Stimulates	2 <sup>a</sup>		6
ATP, ADP, AMP concentration	Decreases	90		15
<b>Other Effects</b>				
Oxidative Phosphorylation	Uncouples	1-2		2, 7
Membrane Conductance	Increases	1	10	8
Mitochondrial Swelling	Stimulates	2-30	2	7, 16
Metal Binding (Fe, Cu, Co, Mn)	Binds	2 <sup>a</sup>		6, 18
Respiration	Stimulates then Inhibits	0.9-1.5 <sup>a</sup>	0.2-0.5;10-20	11, 12, 14

**References:**

- Abramson et al., 1985
- Brody, 1956
- Chatterjee and Stefanovich, 1976
- Deschamps et al., 1991
- Glader, 1976
- Gunther and Höllriegel, 1989
- Gutknecht, 1990
- Gutknecht, 1992
- Kaplowitz et al., 1980
- Kopp and Gosh, 1994
- Martens and Lee, 1984
- Millhorn et al., 1982
- Olson et al., 1992
- Spenny and Bhowan, 1977
- Worathumrong and Grimes, 1975
- You, 1983
- Yoshida et al., 1992
- Dawson, et al., 1993

<sup>a</sup> These values are approximate and are calculated under the assumption that 1 kg of living tissues contains approximately 90 % water.

(Delaney et al., 1994). In contrast, other genes expressed during leaf wounding apparently are inhibited by salicylate (Li et al., 1992).

In human cells, salicylate has been shown to inhibit nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation and transcription of genes regulated by this transcription factor (Kopp and Ghosh, 1994). It appears as though the effect of salicylate on NF- $\kappa$ B activation is mediated through a prevention of the degradation of the inhibitor of NF- $\kappa$ B function, I- $\kappa$ B (Kopp and Ghosh, 1994). Since I- $\kappa$ B is not degraded in the cells, NF- $\kappa$ B remains associated with the inhibitory polypeptide, and remains localized to the cytosol of the cell (Kopp and Ghosh, 1994). Salicylate can also increase the expression of genes in mammalian cells. Aspirin and salicylate both cause elevated levels of cytochrome P450 mRNA (Damme et al., 1996). In certain cases, salicylate can either enhance or suppress the activity of a certain gene depending on the conditions of the cell. Nitric oxide synthase (iNOS) is stimulated in its expression in murine macrophage cells during stimulation by either lipopolysaccharide (LPS) or interferon-gamma (IFN- $\gamma$ ) (Kepka-Lenhart et al., 1996). Interestingly, both aspirin and salicylate inhibit the expression of iNOS mRNA in macrophage cells which have been stimulated by LPS, but they both enhance expression of iNOS mRNA in cells stimulated with IFN- $\gamma$  (Kepka-Lenhart et al., 1996).

#### Effects of Salicylate on Cellular Physiology and Metabolism

Salicylate has many different effects on the metabolism and physiology of cells. These effects are manifest in salicylate's ability to change intracellular concentrations of certain ions and molecules as well as modify certain metabolic processes within the cell.

It has been demonstrated that salicylate and aspirin can change the intracellular concentrations of molecules which may be involved in cell signaling events. Two such molecules are calcium and cyclic AMP (cAMP). It has been shown that salicylate affects intracellular calcium levels (Abramson et al., 1985; Yoshida et al., 1992). Salicylate causes overall increases in the amount of cytosolic calcium within cells. This effect appears to be solely the result of the release of intracellular stores of calcium, such as those from the mitochondria (Yoshida et al., 1992), as salicylate prevents extracellular calcium uptake (Abramson et al., 1985; Yoshida et al., 1992). Both aspirin and salicylate have also been demonstrated to stimulate an increase of cAMP within cells (Abramson et al., 1985). This stimulated increase of cAMP has been implicated in some of the other functions of salicylate/aspirin such as the aggregation and activation of neutrophils (Abramson et al., 1985).

The concentrations of other ions in the cell are also affected. One of the first functions attributed to salicylate, and indeed the one originally suspected to be involved in the activation of the heat shock response by this drug, was the ability of this drug to chelate certain ions in the cell. Salicylate binds manganese (Mn), copper (Cu), iron (Fe), cobalt (Co) and nickel (Ni) tightly (Dawson et al., 1993). In addition, there is an effect on the rate of potassium ( $K^+$ ) efflux. Salicylate causes increases in the unidirectional efflux of  $K^+$  out of cells (Shennan, 1992). Related to the effects on metabolism, salicylate also affects the balance of intracellular inorganic phosphate. Specifically, there is an inhibition of inorganic phosphate uptake (Brody, 1956) but at high concentrations (90 mM), salicylate causes increases in intracellular inorganic

phosphate, primarily through removal of phosphates from high energy species such as ATP, ADP and AMP (Worathumrong and Grimes, 1975).

It has long been known that salicylate can affect cellular metabolism, causing increased oxygen and glucose consumption (Brody, 1956; Terada et al., 1990; Worathumrong and Grimes, 1975). However, this drug prevents cells from generating the energy required for cellular processes. Salicylate has been demonstrated to be an uncoupler of oxidative phosphorylation thus preventing the production of ATP (Brody, 1956). At high concentrations (10-90 mM), salicylate also inhibits glycolysis and causes decreases in ADP and AMP levels (Worathumrong and Grimes, 1975). There is also evidence for the inhibition of the uptake of inorganic phosphate into cells (Brody, 1956). Gluconeogenesis, the process by which glucose is synthesized from non-carbohydrate sources is also inhibited by salicylate (Rognstad, 1991; Terada et al., 1990). Other effects of salicylate on metabolism include inhibition of ketogenesis (Terada et al., 1990), proteoglycan synthesis (Hugenberg et al., 1993), inhibition of fatty acid oxidation (Abramson, 1985; Deschamps et al., 1991; Rognstad, 1991), stimulation of lactate production (Worathumrong and Grimes, 1975) and of lipid peroxidation (Gunther and Höllriegl, 1989) and decreases in the levels of phosphocreatine (Olson et al., 1992; Spenny and Bhowan, 1977) and glutathione (Kaplowitz et al., 1980). Overall these effects of salicylate compromise cellular metabolism and lead to decreases in cellular energy.

## **H. Specific Aims of Thesis**

This thesis examines three different chemical agents which cause decreases in intracellular ATP: sodium salicylate, 2,4-dinitrophenol, and potassium cyanide. Initially the

goal of the research presented here was to examine several different inducers of the *Drosophila* heat shock response in an effort to determine whether there is a universal signal for the stress response.

The second chapter involves an examination the effect of sodium salicylate on the heat shock response in *Drosophila*. This particular inducer was chosen because in recent years there has been conflicting reports on how and to what degree salicylate induced the heat shock response in yeast and human cells. Salicylate had always been considered an inducer of the *Drosophila* heat shock response as it was found to induce the puffing of the heat shock loci on polytene chromosomes (Ritossa, 1963). Later studies in yeast and human systems indicated that salicylate was in fact not an inducer of heat shock gene expression. We chose to examine the effect of salicylate in *Drosophila* in order to determine whether this drug was inducing the heat shock response. We found that although salicylate induced puffing of the heat shock gene loci and binding of HSF to the polytene chromosomes, and to a synthetic HSE oligonucleotide, no transcription of the heat shock genes could be detected.

An additional goal was to attempt to identify how salicylate was capable of inducing certain aspects of the heat shock response. Salicylate was reported to induce HSF binding in both yeast and human cells. Each of the groups examining these systems formulated hypotheses as to how salicylate might activate the heat shock response. The authors examining salicylate in yeast suggested that activation of the heat shock response might be a result of changes in intracellular pH (Giardina and Lis, 1995). Alternatively, it was suggested that in human cells, salicylate might contribute to increases in denatured proteins within the cell thus activating the heat shock response in much the same way that elevated temperatures

are thought to (Jurivich et al., 1995). Neither investigation provided strong evidence supporting their theories, however. We then attempted to determine what the signal might be for activation of the *Drosophila* heat shock response during salicylate exposure. During this investigation it was found that activation of heat shock factor binding was coincident with a decrease in the levels of intracellular ATP. It was therefore hypothesised that this may be the means by which salicylate might activate the heat shock response.

Chapter three contains experiments which were performed in order to test this theory, and to determine if the observed partial heat shock response was a common feature during metabolic stress. These experiments involve the use of two additional inducers: DNP and  $\text{CN}^-$  which are both potent depletors of intracellular ATP.

## **Chapter 2**

### **Sodium Salicylate and the *Drosophila* Heat Shock Response**



## A. Introduction

One of the most widely administered family of drugs is the anti-inflammatory salicylates. Although many aspects of their action have been characterized, such as their effect on prostaglandin synthesis (Vane, 1971), a total understanding of their pharmacological mechanism has not been developed.

Over thirty years ago Ritossa reported that heat (30°C), 1 mM 2,4-dinitrophenol and 10 mM sodium salicylate induced the puffing of a specific set of loci in the polytene chromosomes of *Drosophila* salivary gland cells (Ritossa, 1962; Ritossa, 1963). These “heat shock” puffs have always been assumed to be the site of active transcription of the heat shock genes because <sup>3</sup>H-uridine is incorporated at these sites (Ritossa, 1964; Ashburner and Bonner, 1979) and heat shock gene cDNAs hybridize to RNA found at the puff sites (Livak et al., 1978). Furthermore, the addition of RNA synthesis inhibitors blocks the induction of the heat shock puffs (for review see Ashburner and Bonner, 1979). Since the discovery of the heat shock puffs, the heat shock response has been described in every organism examined and a multitude of different “stress” inducers have been identified (for review see Nover, 1991). In eukaryotic cells, the transcriptional response to stress is mediated through a transcription factor known as heat shock factor (HSF) (for reviews see Mager and DeKruiff, 1995; Morimoto et al., 1994a; Wu et al., 1994; Wu, 1995). In metazoans, stress induces a transformation of HSF from a monomer (for HSF1) to a trimer; and it is this latter form which binds to a specific DNA sequence known as the HSE (for review see Fernandes et al., 1994a). Binding of HSF to the HSE is presumed to be required for heat shock gene transcription but it is not clear if it is sufficient.

In *Drosophila* tissue culture cells (Schneider Line 2, SL2), heat, 2,4-dinitrophenol, and salicylate treatments have been shown to induce the high affinity binding of HSF *in vivo* (Zimarino and Wu, 1987). Experiments using human cells have indicated that salicylate is capable of inducing both HSF binding and heat shock gene transcription at certain concentrations, while other concentrations induce HSF binding only (Jurivich et al., 1992; Jurivich et al., 1995). The situation is somewhat different in yeast where sodium salicylate stimulates HSF binding, but transcription of heat shock genes does not occur. Surprisingly, addition of salicylate prevents the induction of heat shock genes by heat in this organism (Giardina and Lis, 1995).

In this study, it is shown that sodium salicylate (3-30 mM) induces activation of HSF binding activity in SL2 tissue culture cells. It has also been shown that HSF binding and chromosomal puffing are induced in *Drosophila* salivary glands treated to 10 mM salicylate, and to a lesser extent to 3 mM salicylate (Winegarden et al., 1996; Figures A1 and A2, Appendix). No puffing was observed in salivary glands exposed to 30 mM salicylate. Surprisingly however, salicylate treatments (3-30 mM) do not induce transcription of the hsp70 heat shock genes in salivary glands nor in SL2 cells as assayed by primer extension analysis. These results suggest that chromosomal puffing and gene transcription are likely separable events. In addition, and similar to what is seen in yeast, salicylate (30 mM) prevents heat induced transcription of the heat shock genes.

It is not known how salicylate activates HSF binding but models have been proposed which indicate that the lowering of intracellular pH (Giardina and Lis, 1995) or the accumulation of newly synthesised aberrant proteins (Jurivich et al., 1992; Jurivich et al.,

1995) might be involved in the activation process. Here it is shown that, in *Drosophila* cells, salicylate dramatically inhibits cellular ATP levels and prevents the HSF hyperphosphorylation normally seen with heat induced activation of HSF. It is proposed that sodium salicylate interferes with oxidative respiration resulting in decreased cellular ATP. A model as to how lowered ATP levels might activate HSF binding and prevent transcription of the heat shock genes is discussed.

## **B. Experimental Procedures**

### Tissue Culture

*Drosophila* Schneider line 2 (SL2) cells were used for all experiments involving tissue culture cells. Cells were obtained from the laboratory of Dr. Carl Wu (NCI, NIH Bethesda MD).

### *Heat Inactivation of Fetal Bovine Serum*

Fetal bovine serum (FBS; HyClone) was heat inactivated prior to use in tissue culture media. The heat inactivation protocol was provided and adapted from HyClone Laboratories Inc. Frozen sera was thawed 14 h in a 21 °C incubator. An empty serum bottle was used as a temperature control. This bottle was filled with 500 ml of water, and a thermometer was placed into this bottle such that the tip was suspended in the water, and did not touch the sides or the bottom of the bottle. This bottle and the bottle of sera were placed in a temperature controlled circulating water bath (Neslab) preheated to 56 °C. Bottles were swirled every 10 minutes to ensure proper mixing of the contents. Once the contents of the bottle reached 56 °C, the bottles were incubated 30 minutes. At the end of the 30 minute incubation, the bottle of sera was incubated in an ice water bath for an additional 20 minutes. The sera was then aliquoted into sterile glass bottles (100 ml volumes) which were either frozen or added to the tissue culture media.

*Tissue Culture for Electrophoretic mobility shift assays (Figure 4), Primer extensions (Figure 6) ATP assays (Figure 8) and Immunoprecipitation (Figure 9)*

Cells were grown in Shields and Sang M3 Insect Media (Quality Biological, Gaithersburg, MD) supplemented with 10% heat inactivated FBS (HyClone) and 20 µg/ml

gentamycin solution (Sigma). Cells were grown at 21-23 °C in T-75 tissue culture flasks (for suspension cells - Starstedt) at a volume of 15-20 ml. Prior to all experiments, cells at a concentration of  $1-1.4 \times 10^7$  cells/ml were transferred to 50 ml polypropylene tubes at a volume of 2.5-6.5 ml. Cells were aerated in these tubes by shaking at 175 rpm, 21 °C in a New Brunswick model shaking incubator, for 4 or more hours prior to the experiment. For maintenance of the culture, cells were split 1:3 every three days.

#### *Tissue Culture for RNA stability assay (Figure 7)*

Cells were grown in HyClone CCM3 serum free media for insect cells (HyClone) with 20 µg/ml gentamycin solution (Sigma). Cells were grown at 21-23 °C in T-75 tissue culture flasks (for suspension cells - Starstedt) at a volume of 15 ml. Prior to all experiments, cells at a concentration of  $5-8 \times 10^6$  cells/ml were transferred to 50 ml polypropylene tubes at a volume of 3-5 ml. Cells were aerated in these tubes by shaking at 200 rpm, 21 °C, for 6 or more hours prior to the experiment. For maintenance of the culture, cells were split 1:5 every three days.

#### Cell Treatments

Controls (21 °C) were prepared by taking cells which had not been subjected to any stress agents, but had undergone the aeration described above. All other cell treatments were also performed after aeration. Heat shocks (36.5 °C) were performed by incubating the cells in 50 ml polypropylene tubes (for volumes of 2-6 ml) or in standard microfuge tubes (for 1 ml samples) in a temperature controlled circulating water bath (Neslab). Cells were gently swirled every five minutes during heat shock to ensure constant suspension. Salicylate was added to cells from a 1 M Na-salicylate stock prepared in a physiological *Drosophila* saline

(45 mM K-glutamate, 45 mM Na-glutamate, 8.7 mM MgSO<sub>4</sub>, 5.0 mM Bis-Tris, 6.8 mM CaCl<sub>2</sub>·H<sub>2</sub>O, 12 g/L glucose, pH 6.9), to the concentrations indicated in each experiment. 30 mM salicylate plus heat shock experiments were performed by incubating cells in 30 mM salicylate for 5 min at 21 °C and then transferring the cells to 36.5 °C for the remaining time interval. For experiments studying the effects of protein synthesis inhibition, cycloheximide was added to cells at a concentration of 118 μM 30 min prior to further cell treatments. This concentration of cycloheximide has previously been reported to prevent 98% of protein synthesis in *Drosophila* cells (Zimarino and Wu, 1987; Zimarino et al., 1990). Cells were continually shaken at 175 rpm during this period.

#### Salivary Gland Treatments

Salivary glands were dissected from wandering late third instar “Oregon R” *Drosophila melanogaster* larvae in a drop of modified TB1 buffer (15 mM HEPES, pH 6.8, 80 mM KCl, 16 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% polyethylene glycol 6000)(Bonner, 1981; Myohara and Okada, 1987). Glands were dissected and transferred to a depression slide containing 200 μl modified TB1. Depression slides were kept in humidified chambers. Glands were first allowed to incubate at room temperature for 1 h prior to further treatment. After the one hour period, glands were subjected to either heat shock or chemical treatment. For heat shock, glands were transferred to 1.5 ml microfuge tubes containing 50 μl of TB1, and were incubated in a temperature controlled circulating waterbath (Neslab) at 36.5 °C for the time indicated in each experiment. For salicylate treatments, the appropriate volume of a 1 M sodium salicylate stock (prepared in buffered physiological saline - see above) was added to the glands. After treatment, glands were pelleted at 10,000 g at 4 °C for 5 minutes.

Supernatants were aspirated off, and pellets were either used for RNA extraction immediately or were stored at -72 °C until further use.

#### Protein Extracts for Mobility Shift Assays

Cells were treated to the conditions described in each experiment. 1 ml aliquots of cells were transferred to 1.5 ml microfuge tubes, and pelleted at 7000 rpm, at 4 °C, for 2 min in a Beckman microfuge. Supernatants were aspirated off, and cell pellets were immediately frozen under liquid nitrogen and either stored at -72 °C or processed immediately as follows: pellets were thawed by resuspension in 5 pellet volumes of lysis buffer (10 mM HEPES pH 7.9, 0.4 M NaCl, 0.1 mM EGTA, 5.0% glycerol, 0.5 mM DTT and 0.5 mM PMSF) and then centrifuged at 10,000 g for 10 min at 4 °C. Supernatants were then transferred to a new 1.5 ml tube, frozen under liquid nitrogen and stored at -72 °C or were used immediately in electrophoretic mobility shift assays (EMSA).

#### Electrophoretic Mobility Shift Assays (EMSA)

An HSE consensus sequence (HSE3; 5'-GGG CGT CAT AGA ATA TTC TCG AAT TCT GGG TCA GG-3') was annealed to a shorter complimentary oligonucleotide (5'-CC TGA CCC AGA ATT CGA G-3') and the overhang was filled in using the Klenow fragment of DNA polymerase with 0.167 mM of each of dATP, dTTP and dGTP, 50 µCi of <sup>32</sup>P-α-dCTP (3000 Ci/mmol; New England Nuclear/Amersham) and 2.5 units of Klenow (New England Biolabs) used in the labelling reaction. Unincorporated nucleotides were removed using a gel filtration (Sephadex G25 superfine, Pharmacia) spin column.

### *Spin Columns*

A Sephadex G-25 slurry was prepared by incubating 5 g of Sephadex G-25 in 50 ml TE (10 mM Tris; 1 mM EDTA; pH 8.0) at room temperature for 12 hours. The buffer remaining unabsorbed was aspirated off. The beads were washed once with 1 volume (of the swollen beads) of TE and then 1 volume of TE was added to the swollen beads producing a 1:1 slurry of Sephadex G-25.

1 ml of the 1:1 slurry (well mixed) was pipetted into a 1.0 ml BioRad spin column. The solvent was allowed to run through the column for 10 minutes, and then the tip of the column was inserted into a 1.5 ml microfuge tube from which the lid had been removed. This 1.5 ml tube and the spin column were placed into a 15 ml polypropylene tube and was spun at 3000 rpm for 5 minutes in a swinging bucket centrifuge. The TE collected in the microfuge tube was decanted and another 1 ml of slurry was added to the column. This was once again centrifuged at 3000 rpm for 5 minutes. The microfuge tube was removed and replaced with a new clean tube to collect the eluate during purification of the labelled probe. The labelling reaction mixture was added to the top of the column and the column was spun at 3000 rpm for 5 minutes.

To calculate incorporation, the amount of activity (in counts per minute - CPM) remaining in the column and the amount collected in the microfuge tube was determined by geiger counter, and approximate incorporation was determined as:

$$\%incorporation = \frac{CPM_{microfuge\_tube}}{CPM_{column} + CPM_{microfuge\_tube}}$$



### *Gel Shifts (EMSAs)*

Gel shift assays were performed essentially as described in (Zimarino and Wu, 1987). 4  $\mu$ l of *Drosophila* SL2 cell extract was mixed with 6.1  $\mu$ l of reaction mix containing: 4  $\mu$ l ddH<sub>2</sub>O, 1  $\mu$ l 10X buffer mix (100 mM HEPES pH 7.9, 500 mM NaCl, 30% glycerol w/v), 1  $\mu$ l 10X BSA/nucleotide mix (0.5 mg/ml *E. coli* DNA, 0.2 mg/ml poly d(N)<sub>6</sub>, 2 mg/ml yeast tRNA, 20 mg/ml BSA) and 0.1  $\mu$ l <sup>32</sup>P labelled HSE3 (0.01 pmole). The binding reaction was allowed to proceed for 10 min on ice (0 °C) and then 2  $\mu$ l of 6X loading dye (0.25% Bromophenol Blue, 0.25% xylene cyanol, 30% glycerol (w/v), 3X TBE) was added. Samples were electrophoresed in a 1% agarose gel (Seakem ME) for 1.25 h at 82 V in 0.5X TBE buffer. Gels were blotted and dried onto Whatman DE81 paper underlaid with Gel Blot paper (GB003 - Schneider and Schuel) and exposed to preflashed Kodak XRP-1 film with a Cronex Lightning Plus Intensifying Screen (Du Pont) at -72°C. The salicylate EMSA was performed four times, all with very similar results. The cycloheximide EMSA was performed three times producing the same pattern each time. In each case a representative gel is shown in the results.

### RNA Preparation

#### *Preparation of RNA from SL2 cells for primer extension (Figure 6):*

For each preparation,  $5 \times 10^7$  cells were used. After treatment, cells were pelleted, and washed once in 1 volume *Drosophila* saline. RNA extractions were performed using the guanidinium/CsCl method for total RNA extraction essentially as described in Ausubel et al., 1995. 3.5 ml of 4 M guanidinium solution, pH 5.5 (4 M guanidinium isothiocyanate; 20 mM sodium acetate, pH 5.2; 0.5 % sarkosyl all prepared in DEPC H<sub>2</sub>O) was added to the cell

pellet and was repeatedly drawn through a 20 ga needle until viscosity was no longer detectible. 1.5 ml of 5.7 M CsCl (in DEPC H<sub>2</sub>O) was aliquoted into a 13.51 mm autoclaved polyallomer ultracentrifuge tube (Nalgene). The 3.5 ml of lysate was layered on top of the CsCl cushion in order to create a step gradient. The gradients were centrifuged 20 hours in a Beckman SW 50.1 rotor at 150,000 g at 18 °C. Supernatants were removed by Pasteur pipette, and the tubes were inverted to drain remaining liquid (10 minutes). Pellets were resuspended by repeat pipetting in 360 µl TES (10 mM Tris•HCl, pH 7.4; 5 mM EDTA, 1 % SDS). Resuspended solutions were allowed to sit 10 minutes at 21 °C. Solutions were then transferred to RNase free 1.5 ml microfuge tubes (DiaMed). 40 µl of 3 M sodium acetate, pH 5.2 and 1 ml 100 % ethanol was then added to the solution. RNA was precipitated 30 minutes in a 95 % ethanol bath at -72 °C. RNA was pelleted by centrifuging 10 minutes at 10,000 g. The supernatant was aspirated off, and pellets were dried 10 minutes. Pellets were then resuspended in 360 µl of DEPC H<sub>2</sub>O and were reprecipitated as above. Supernatants were again aspirated off and pellets were dried 20 minutes. Pellets were dissolved in 200 µl DEPC H<sub>2</sub>O and quantified via A<sub>260</sub> and A<sub>280</sub> readings. RNA was stored at -72 °C until further use.

*Preparation of RNA from SL2 cells (Figure 7) and Salivary Glands (Figure 5) for Primer Extension*

For SL2 cells,  $1 \times 10^7$  cells were used for each sample preparation. For salivary glands, total RNA was extracted from 20 pairs of salivary glands for each treatment studied. RNA was prepared using the RNeasy RNA extraction kit (Qiagen) following the instructions supplied. RNA was quantified by spectrophotometry (A<sub>260</sub> and A<sub>280</sub>). For 20 pairs of glands,

or  $10^7$  cells, the following volumes of reagent were used: 350  $\mu$ l Lysis Buffer RLT; 350  $\mu$ l 70% ethanol; 700  $\mu$ l wash buffer RW1; 2 X 500  $\mu$ l wash buffer RPE and 30  $\mu$ l DEPC H<sub>2</sub>O. RNA samples were stored at -72 °C until use in primer extension analysis.

### Primer Extension Assays

#### *Primer Extension of RNA from Salicylate Treated SL2 cells (Figure 6) or Salivary Glands (Figure 5)*

For SL2 cells, 5 or 10  $\mu$ g of total RNA was lyophilized and resuspended in 18.5  $\mu$ l final volume of buffer containing 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 20 units/ $\mu$ l RNAGuard (Pharmacia) and 0.1 picomoles <sup>32</sup>P end-labelled hsp 70 oligomer (5'-CCC AGA TCG ATT CCA ATA GCA GGC-3') and/or H2B oligomer (5'-GCC TTT CCA CTA GTT TTC GGA GGC-3'). For salivary glands, 1 or 2  $\mu$ g of total RNA was lyophilized and resuspended in 9.25  $\mu$ l of the above buffer and 0.05 picomoles of each oligomer was added. Oligomers were end labelled using 30  $\mu$ Ci  $\gamma$ -<sup>32</sup>P-ATP (3000 Ci/mmol, New England Nuclear/Amersham) and T4 polynucleotide kinase (New England Biolabs) according to (Ausubel et al., 1995). 10  $\mu$ l reactions were used for labelling including: oligomers (10 pmol), 1  $\mu$ l T4 buffer (from a 10X stock - New England Biologicals),  $\gamma$ -<sup>32</sup>P-ATP (30  $\mu$ Ci), 10 U T4 polynucleotide kinase, and DEPC H<sub>2</sub>O to bring the volume to 10  $\mu$ l. Reactions were incubated for 60 minutes at 37 °C. Reactions were stopped by the addition of 2  $\mu$ l 0.5 M EDTA, 1  $\mu$ l tRNA (10  $\mu$ g/ $\mu$ l) and 38  $\mu$ l TE, and heat inactivating the enzyme at 65 °C for 5 minutes. Labelling reactions were followed by removal of unincorporated ATP with a Sephadex G-25 (Pharmacia) spin column. The reaction was heated to 85 °C for 5 min and then transferred to 42 °C and incubated overnight (14-18 h).

The next morning dNTPs were added to a final concentration of 2 mM and then 100 units of reverse transcriptase (Superscript II; Gibco/BRL) were added to give a final total volume of 20  $\mu$ l (or 50 units reverse transcriptase and a final volume of 10  $\mu$ l for salivary gland RNA). The reaction was then incubated at 42 °C for a further 60 min. Primer extension reactions were then ethanol precipitated with 3  $\mu$ l 2 M Na-acetate and 50  $\mu$ l 100 % ethanol, washed with 70 % ethanol and dried. Primer extension products were resuspended in 10  $\mu$ l of formamide loading dye (Stop solution, United States Biochemical Sequenase Rapidwell DNA Sequencing Kit), denatured by heating to 100 °C, and electrophoresed on an 8% PAGE/7M urea gel in 1x TBE which had been pre-run for 30 min at 200 V. Gels were run until the bromophenol blue marker had run 2/3 of the length of the gel. The gel was dried and autoradiographed using Kodak XAR5 film at -72 °C with a Lighting Plus (Du Pont) intensifying screen. The molecular sizes of the hsp70 and H2B primer extension products were originally determined on large gels (35 x 40 cm) and determined to be 275 bp (for hsp70) and 78 bp (for H2B) which was in good agreement with the 270 bp and 70 bp sizes estimated from the sequences of the genes. The experiment dealing with the salivary gland RNA was repeated one other time with a similar result. The other primer extension assays were performed three times each. In all cases the results were very similar and representative gels have been chosen for the figures.

*Primer Extension of RNA to Test RNA Stability - Revised Protocol (Figure 7)*

For each sample, 5  $\mu$ g of total RNA was lyophilized and resuspended in 12.5  $\mu$ l of a solution composed of 1.2X 1st strand buffer (Gibco BRL); 12 mM DTT (Gibco BRL); and 0.1 pmol of primer (see above protocol). Samples were heated at 85 °C for 5 minutes and

then at 42 °C for one hour. To each tube 2.5 µl of the a second mixture containing 100 U of reverse transcriptase (Superscript II RT; Gibco BRL); 20 U of RNA guard (Pharmacia) and 10 mM dNTPs (10 mM dATP; 10 mM dCTP; 10 mM dGTP and 10 mM dTTP; Boehringer Mannheim) was added. Tubes were then incubated at 42 °C for 60 minutes. To each tube, 3 µl 2M sodium acetate (pH 5.2) and 50 µl 100% ethanol was added. Samples were precipitated overnight at -72 °C. Ethanol was then removed and samples were dried 60 minutes under vacuum. Samples were then resuspended in 10 µl of formamide loading dye (0.05 % bromophenol blue, 0.05 % xylene cyanol, 20 mM EDTA all dissolved in 100% formamide) and boiled 2 minutes to denature. The samples were electrophoresed on an 6% PAGE/7M urea gel in 1x TBE which had been pre-run for 30 min at 200 V. Gels were run 45 minutes at 200 V. The gel was dried and autoradiographed using preflashed Kodak XRP-1 film at -72 °C with a Lighting Plus (Du Pont) intensifying screen. This experiment was repeated three times with similar results. A representative gel was chosen for the figure.

#### Densitometry of Autoradiograms

All autoradiograms which were to be analysed by densitometry were prepared on Kodak XRP-1 film which had been preflashed with an Amersham Sensitize flash unit to obtain a linear range of exposure. Autoradiograms were digitized using an Agfa Arcus II scanner (settings were: transparency, 300 ppi, grayscale). Densitometric analysis was performed using the profile analyst feature of the Molecular Analyst software program v.2.1 (Bio-Rad). For the EMSA assays, HSF binding activity was expressed as a percent of the heat shock (36.5 °C) cell sample. For the primer extension assays, the hsp70 signal intensities were first normalized using the H2B signals. These normalized values were then used to

compare signal intensities between different treatments and the results were expressed as the ratio (fold) of hsp70 message relative to the control (21 °C) level. In some instances no hsp70 signal was detectable under control conditions and thus relative amounts could not be calculated.

### ATP Assays

Intracellular ATP was measured using Boehringer Mannheim's Constant Light Source Kit II. Cell extracts were prepared as per the instructions included with the kit. 110 µl of cell suspension (approximately  $1 \times 10^6$  cells) were added to 1000 µl of boiling 100 mM Tris pH 7.75, 4 mM EDTA solution. Samples were boiled for 2 min and then centrifuged for 3 min at  $10,000 \times g$  and 4 °C. Supernatants were transferred to a clean microfuge tube and kept at 4 °C or stored frozen at -20 °C. For each reading, 100 µl of the extract was added to 100 µl of the luciferase reagent provided in the kit. Readings were taken using a scintillation counter (Wallac model 1409) over 10 or 20 s intervals with the windows wide open. All conditions were performed in triplicate. ATP levels were determined with the aid of a standard curve prepared using pure ATP over a  $10^{-5}$  to  $10^{-9}$  M concentration range. The ATP assay was performed three times, each time producing similar results. The results from one experiment was chosen for the figure.

### Protein Labelling and Immunoprecipitation

100 µCi of  $^{32}\text{P}$ -*o*-phosphate (New England Nuclear or Amersham) was added to 1 ml of cells at  $1.0 \times 10^7$  cells/ml in phosphate-free M3 media. Immediately after addition of phosphate, salicylate was added to the cells, or cells were heat shocked by submersion in a 36.5 °C water bath. After the appropriate time interval, cells were pelleted by centrifugation

at 10,000 x g for 30 s. Supernatants were removed, and 10 volumes TETN400 buffer was added (25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5), 400 mM NaCl, 1% Triton X-100 v/v) (Firestone and Winguth, 1990). Pellets were immediately lysed and resuspended by drawing through a 28.5 gauge needle. Samples were stored at -72 °C until processed by immunoprecipitation. Immunoprecipitations were performed essentially as described in (Firestone and Winguth, 1990) with a only few modifications. Cell extracts were made using a buffer with increased salt concentration (400 mM NaCl) and thus in order to maintain a 250 mM NaCl concentration, an equal volume of TETN100 (5 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5), 100 mM NaCl, 1% Triton X-100 v/v) rather than TETN250 was added to each extract for binding reactions to give a final 250 mM NaCl concentration. One  $\mu$ l of anti-*Drosophila* HSF antisera (943) (Westwood et al., 1990) was added to each 100  $\mu$ l binding reaction and the entire sample was immunoprecipitated and loaded onto a 8% PAGE gel. This experiment was performed twice, and a representative gel is shown in the figure.

## C. Results

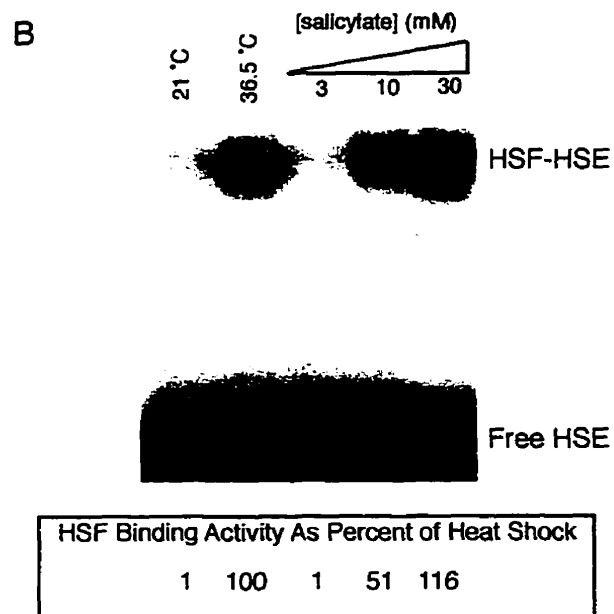
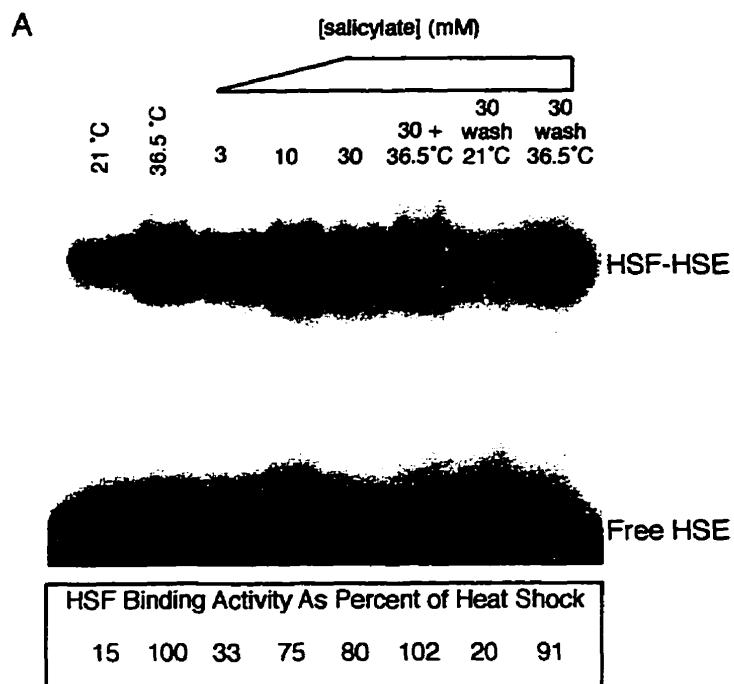
### *Salicylate Induces the Binding of HSF to HSE in Drosophila Tissue Culture Cells-*

In SL2 cells, HSF binding activity to an HSE was found to be induced by salicylate in a dose dependent manner (Figure 4A). Binding of HSF was activated within 20 min at 3, 10 and 30 mM concentrations of salicylate. 3 mM salicylate had only minor effects on HSF binding activity, whereas 10 mM and 30 mM salicylate strongly induced binding of HSF to the HSE oligonucleotide. The HSF binding pattern for heat and salicylate treated cells at 60 min was similar to the 20 min pattern (data not shown). In order to test the effect of salicylate under conditions of heat shock, cells were heat shocked (36.5 °C) in the presence of 30 mM salicylate. Under these conditions, the amount of HSF binding activity was similar to heat shock and visibly stronger than 30 mM salicylate alone, implying that salicylate at 30 mM does not fully induce the available HSF pool (Figure 4A). To determine if the effect of salicylate is reversible, cells were exposed to 30 mM salicylate for 20 min, washed once in physiological saline and resuspended in salicylate-free media. Cells were incubated in the absence of salicylate for 40 min, and after this period of recovery, the salicylate induced DNA binding of HSF was reversed (Figure 4A). The level of HSF binding in cells treated in such a manner was reduced to levels comparable to a normal 21 °C control. If cells treated in a similar fashion were heat shocked after resuspension in salicylate-free media, a typical heat shock induced level of HSF binding was observed (Figure 4A). Heat shock was capable of triggering HSF binding in the presence of the protein synthesis inhibitor cycloheximide; and cycloheximide by itself did not induce HSF binding within the 50 minute time interval of the experiment (Figure 4B). In an attempt to determine whether protein synthesis is required for salicylate to induce the binding of HSF, salicylate was added to cells



**Figure 4. Sodium salicylate activates the binding of HSF to HSEs in *Drosophila* SL2 cells in the absence of protein synthesis.**

*A*, *Drosophila* tissue culture cells were exposed to the conditions indicated for a period of 20 minutes and HSF binding activity was analyzed by electrophoretic mobility shift assays. A radiolabeled HSE consensus sequence was used for a probe. *B*, the experiment was performed as described in *A* with the exception that prior to each condition, cells were incubated with 118  $\mu\text{M}$  cycloheximide for 30 minutes in order to prevent protein synthesis (Zimarino et al., 1990a). Binding reactions were run out on 1 % agarose gels in 0.5 X TBE.



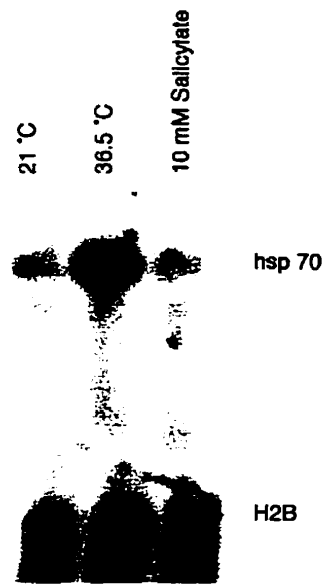
pre-treated with and in the presence of cycloheximide. Cycloheximide treatment modestly affected HSF binding induced by 10 or 30 mM salicylate treatments (Figure 4B). HSF binding in the control (21.5 °C) sample and in the 3 mM salicylate treated cells appeared to be inhibited by cycloheximide as both had noticeably lower HSF binding activity in comparison to the non-cycloheximide treated sample. The ability of cycloheximide to inhibit induction of HSF binding has previously been observed in *Drosophila* for control and submaximal heat shock temperatures (Zimarino and Wu, 1987; Zimarino et al., 1990). These results suggest that protein synthesis is not a prerequisite for induction of HSF binding activity by higher concentrations of salicylate (10 and 30 mM) but may play a role in the induction of HSF binding activation at lower salicylate concentrations (3 mM). This finding differs somewhat from one reported in mammalian cells where cycloheximide treatment prevented induction of HSF binding by salicylate at all of the concentrations tested (Jurivich et al., 1995).

*Salicylate Fails to Induce the Transcription of Hsp70 in Drosophila Salivary Glands and SL2 cells-*

In an attempt to correlate the induced puffing of the 87C locus of the polytene chromosomes (Winegarden et al., 1996; also see appendix) with transcription of the hsp70 gene, primer extension analysis was performed to assay the levels of hsp70 message present in the salivary glands (Figure 5). Little detectable message was present at the control temperature (21 °C). Upon heat shock (36.5 °C for 20 min) the amount of hsp70 message detected greatly increased (Figure 5) and this correlated with large puffs at 87C and 87A (Figure A1B in appendix). A 10 mM salicylate treatment for 1 h induces maximal puffing at the hsp70 loci (Figure A1G; appendix), but no hsp70 message could be detected by primer

**Figure 5. Sodium salicylate does not activate hsp70 gene transcription in *Drosophila* salivary glands.**

RNA extracts were prepared from *Drosophila* salivary glands which were incubated for 2 hours in TB1 buffer at 21 °C, incubated 1.5 hours at 21 °C in TB1 and then heat shocked for 36.5 °C for 20 minutes, or incubated 1 hour at 21 °C in TB1 then for 1 hour in 10 mM sodium salicylate. Heat shock and salicylate-treated salivary glands show maximal puffs at the 87C locus at these time points. Hsp70 and histone H2B gene transcripts in these RNA samples were identified by primer extension analysis and extension products were separated on 8%PAGE/7M Urea gels. Densitometry was performed with the aid of the Molecular Analyst program (Bio-Rad)(See Experimental Procedures). Hsp70 message signals were first normalized by comparing them to the H2B message signals. After normalization, the ratio of hsp70 message in comparison to the level present in the control (21 °C) sample was calculated and is shown below the autoradiogram.



Relative Amount of Hsp70 Message as Compared to Control

1.0 50 1.2

extension analysis (Figure 5). This result was surprising given that heat induced puffing at the heat shock gene loci has previously been correlated with heat shock gene transcription (see Discussion). This result also suggests that chromosomal puffing and transcription of the heat shock gene loci can be uncoupled.

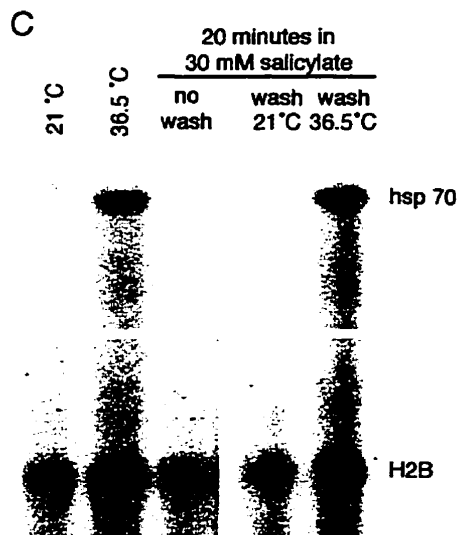
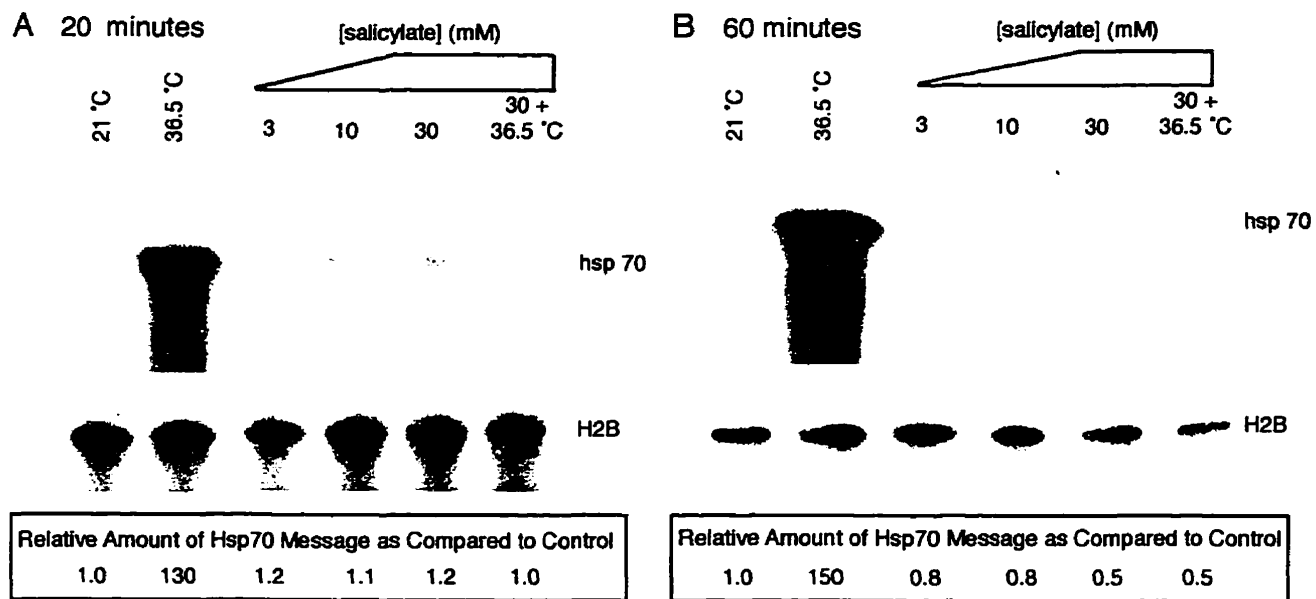
To ascertain the effects of heat and salicylate treatments on heat shock gene expression in SL2 cells, hsp70 mRNA levels were also monitored by primer extension analysis. During heat shock (36.5 °C) the amount of hsp70 transcripts increased approximately 130-150 fold with large amounts of hsp70 extension products seen at 20 min (Figure 6A) and at 60 min (Figure 6B). None of the concentrations of salicylate used (3-30 mM) were capable of inducing hsp70 expression within the 60 min interval. Furthermore, 30 mM salicylate prevented heat induced expression of the hsp70 genes. To determine if heat shock messages would be expressed during recovery from salicylate treatments, primer extensions were performed on RNA isolated from cells which were washed and allowed to recover. A 40 minute recovery did not cause an increase in hsp70 transcripts. However, after a recovery period, heat shock inducibility of hsp70 gene expression was restored (Figure 6C).

#### *Salicylate does not Affect hsp70 mRNA Stability-*

The above primer extension results give an indication of steady state hsp70 message levels in cells and do not rule out the possibility that hsp70 messages might be induced but are extremely unstable in salicylate treated cells. Methods can be used to detect newly initiated transcripts (e.g. nuclear run-on analysis), however, most of these methods require a subsequent *in vitro* step utilising excess ATP, and because salicylate affects ATP levels (see

**Figure 6. Salicylate fails to induce hsp70 gene transcription in SL2 cells and inhibits heat-induced hsp70 transcription.**

Total RNA extracts were prepared from *Drosophila* SL2 cells after exposure to the noted conditions for 20 minutes (A) or 60 minutes (B). The amounts of hsp70 and H2B gene transcripts were determined by primer extension analysis as described in the legend to Figure 5. C, the experiment was performed as described above using extracts from cells which were treated for 20 minutes with 30 mM salicylate, washed in saline, and allowed to recover 20 minutes at 21 °C. Cells were then either kept at 21 °C or heat shocked at 36.5 °C for an additional 20 minutes. Densitometry was performed as described in the legend of figure 5. No values are given for C, as the signals present in the control (21 °C) sample were not strong enough to be detected, and thus ratios to this value could not be calculated.





next section), these types of analyses are less likely to give an accurate picture of hsp gene expression in salicylate treated cells.

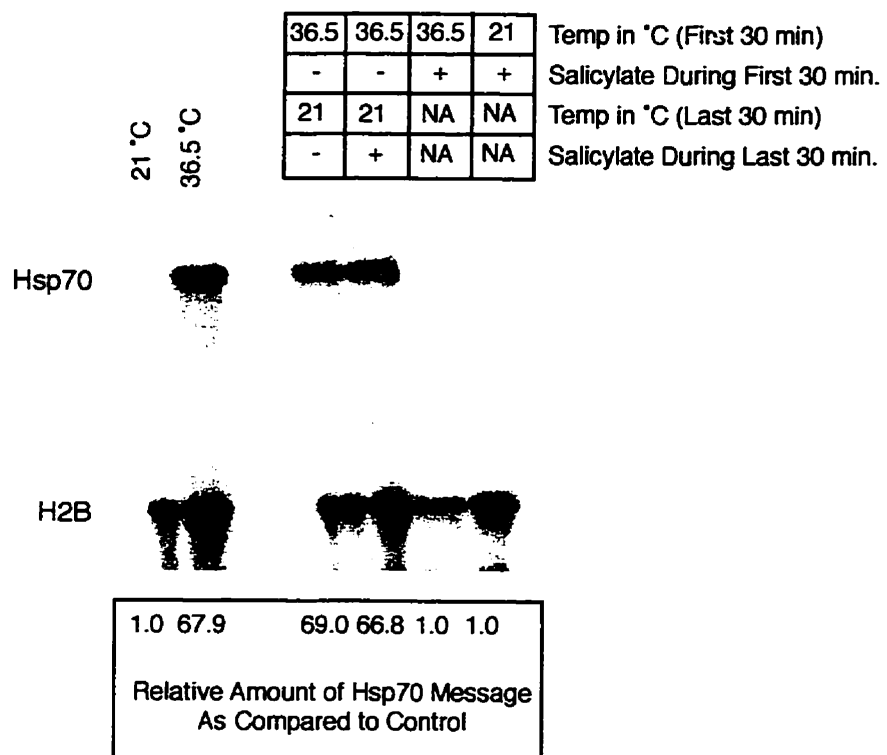
In an effort to distinguish between lack of expression of the hsp70 gene and increased heat shock message instability the following experiment was performed. Two sets of cells were heat shocked at 36.5 °C for 30 minutes to elevate the amount of hsp70 message to a detectable level. After heat shock, one set of cells was allowed to recover at 21 °C for 30 minutes. To the other sample, salicylate was added to a final concentration of 30 mM, and the cells were incubated for 30 minutes at 21 °C. Primer extension analysis was performed on RNA prepared from these cells to determine the effect of salicylate on the elevated hsp70 mRNA pool (Figure 7). There was no apparent effect of salicylate on the hsp70 mRNA pool, as both the salicylate treated and the control samples showed equal levels of hsp70 message. This result implies that salicylate does not cause instability in the heat shock messages. Therefore, the lack of hsp70 message in salicylate treated cells is probably due to the lack of hsp70 gene transcription. Further, in the case of salicylate plus heat shock experiments, salicylate must in fact be inhibitory of heat induced heat shock gene transcription.

#### *Salicylate Causes a Rapid Decrease in Intracellular ATP Levels in Drosophila SL2 Cells-*

While investigating the induction of HSF binding activity by a number of different inducers of the heat shock response, we noticed that induction caused by sodium salicylate was at least temporally very similar to that seen by induction by 2,4-dinitrophenol and other inhibitors of oxidative respiration (see Chapter 2 of this text). This prompted us to examine what effect sodium salicylate might have on ATP production inside of *Drosophila* cells. Under normal physiological conditions, the amount of intracellular ATP in *Drosophila* SL2

**Figure 7. Salicylate does not affect hsp70 message stability.**

Lanes 1 and 2 contain a 21 °C control, and a 30 minute heat shock (36.5 °C) respectively. Experimental samples were performed as follows: cells were heat shocked at 36.5 °C for 30 minutes to elevate the level of hsp70 transcript to detectable levels. Cells were then allowed to recover at 21 °C for 30 minutes either in the presence of (+) or absence of (-) 30 mM salicylate. The last two lanes show that salicylate (30 mM) prevents heat induced transcription of hsp70 and that 30 mM salicylate does not induce hsp70 gene expression. Total RNA was extracted from the SL2 cells and hsp70 transcripts were assayed via primer extension analysis (See Experimental Procedures). Desitometry was performed as described in the figure legend for figure 5 and in the Experimental Procedures section. NA, indicates that the condition noted is not applicable to that particular experiment as no recovery step was performed.



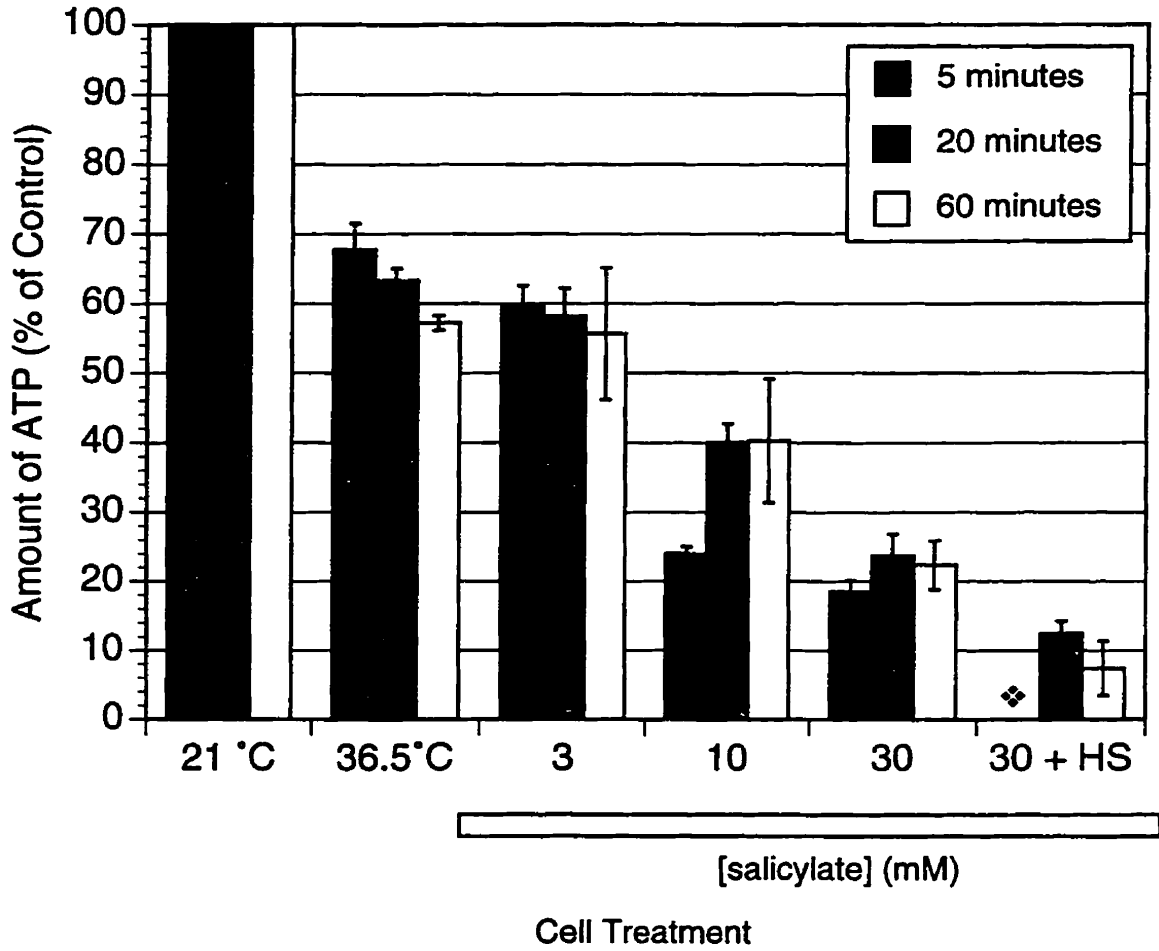
cells was found to remain fairly constant (results not shown). Heat shock reduced intracellular ATP levels by approximately 35 % (Figure 8). Salicylate treatment had a rapid and dramatic effect on the level of ATP within the cells. 3 mM salicylate dropped ATP levels slightly below that seen in heat shock (i.e. a 40–45 % reduction) and higher concentrations of salicylate decreased this level even further. 10 mM salicylate decreased ATP levels by more than 60%, and 30 mM salicylate by more than 75 %. The majority of this effect was seen within 5 min of the addition of salicylate. Heat shocking cells in the presence of 30 mM salicylate had the greatest effect on intracellular ATP where levels dropped by more than 85 % when compared to untreated cells.

*Salicylate does not Induce the Hyperphosphorylation of HSF-*

Heat shock has been reported to induce the hyperphosphorylation of HSF in yeast and human cells (Sorger et al., 1987; Larson et al., 1988) and it has been suggested that this modification could play a role in HSF activation and/or inactivation (see General Introduction (Chapter 1) and Discussion). The phosphorylation of *Drosophila* HSF (dHSF) in response to heat and salicylate treatments was assayed by immunoprecipitation of HSF from SL2 cells transiently labelled with  $^{32}\text{P}$ -orthophosphate. Heat shock induced the hyperphosphorylation of dHSF (Figure 9). Salicylate treatments, however, did not induce this hyperphosphorylation of HSF. In fact, salicylate treatments decreased the amount of HSF phosphorylation normally seen in untreated cells (Figure 9, compare 21 °C to salicylate treatments). At 10 and 30 mM salicylate, no phosphorylation of HSF is detected at all. Surprisingly, 30 mM salicylate also prevented the heat induced hyperphosphorylation of HSF.

**Figure 8. Salicylate rapidly decreases intracellular ATP levels in a dose-dependent manner.**

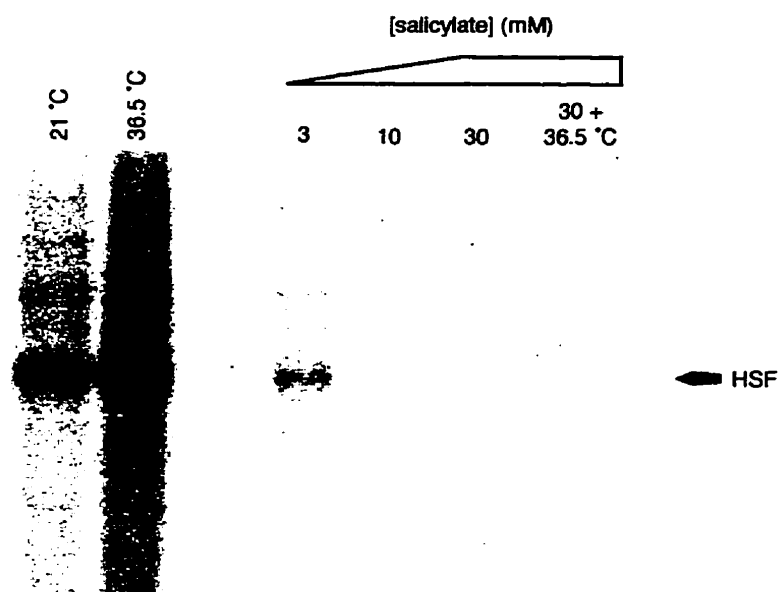
*Drosophila* SL2 cells were subjected to each of the treatments indicated. Cells were lysed and proteins denatured by boiling in 100 mM Tris (pH 7.75), 4 mM EDTA solution for 2 minutes. ATP levels were determined with the use of a luciferase based assay (Constant Light Source Kit II – Boehringer Mannheim) and were detected and quantified with a scintillation counter. Values are expressed as percent of control (21 °C), which was approximately  $2.7 \times 10^{-4}$  M ATP/ $10^5$  cells.



◆ Treatment not tested

**Figure 9. Salicylate inhibits the phosphorylation of *Drosophila* HSF.**

HSF was immunoprecipitated from extracts of *Drosophila* SL2 cells which were treated to the conditions indicated for 20 minutes, in the presence of  $^{32}\text{P}$   $\alpha$ -phosphate. Immunoprecipitates were separated on 8% SDS PAGE gels and autoradiograms were made. The band representing HSF is indicated.





#### D. Discussion

Sodium salicylate has long been known to be an inducer of heat shock puffs in *Drosophila* salivary glands (Ritossa, 1962; Ritossa, 1963). These puffs have been assumed to be the sites of heat shock gene transcription and, for heat induced puffing, this has been demonstrated to be true (for review see Ashburner and Bonner, 1979). More recent studies in human cells (Jurivich et al., 1992; Jurivich et al., 1995) and in *S. cerevisiae* (Giardina and Lis, 1995) have suggested that sodium salicylate can induce certain but not all aspects of the heat shock response. That is, salicylate induces HSF binding at the promoters of heat shock genes, as demonstrated by *in vivo* footprinting. However, salicylate's ability to activate heat shock gene transcription appears to be dependent on the concentration of salicylate used in human cells (Jurivich et al., 1992) and does not occur at all in yeast (Giardina and Lis, 1995). These observations prompted a reexamination of the salicylate-induced heat shock response in *Drosophila* using both salivary glands from third instar larvae and Schneider line 2 (SL2) cells.

The results of this study show that salicylate (3-30 mM) induces HSF binding activity in *Drosophila* tissue culture cells. It should be noted that this binding is as judged by a gel mobility shift assay, and thus is an *in vitro* assay in that the HSF has been removed from the cells and allowed to bind to an artificial HSE. The binding data is supported by HSF binding experiments which examine HSF localisation on *Drosophila* polytene chromosomes. This type of assay is *in vivo* in nature as the HSF is fixed to the HSEs prior to visualization. Although, not performed in this investigation it is well noted that if higher resolution *in vivo* analysis of HSF binding were required, *in vivo* footprinting assays could be employed.

The induced binding is rapid and strong for the higher concentrations of salicylate used (10 and 30 mM). We have also demonstrated that sodium salicylate causes a binding pattern of HSF to *Drosophila* polytene chromosomes which is very similar to that induced by heat shock (Winegarden et al., 1996; Figures A1 and A2 in appendix). This indicates that in terms of HSF binding activity, sodium salicylate and heat shock are similar inducers.

Salicylate reduces ATP concentrations in *Drosophila* SL2 cells in a dose dependent manner. Heat shock also decreases intracellular ATP but not to the same extent as the salicylate concentrations used in this experiment. Three mM salicylate decreases ATP to levels slightly below the level induced by heat shock (40 % versus 35 % inhibition) and weakly activates HSF binding. Higher concentrations of salicylate dramatically affect cellular ATP; 10 mM salicylate decreases ATP by up to 60 % and 30 mM by up to 75 % of control levels. Salicylate has previously been reported to decrease intracellular ATP in rat astrocytes (Olson et al., 1992). Salicylate has also been identified as an uncoupler of oxidative phosphorylation (Brody, 1956). Most of the observed inhibitory effect on ATP levels in *Drosophila* SL2 cells is exhibited within 5 min of the addition of salicylate. This time course of ATP inhibition correlates well with the activation of HSF binding by salicylate indicating that decreases in intracellular ATP may be an important factor in the activation of HSF binding by this drug.

Numerous inducers of heat shock puffs in *Drosophila* have been shown to be inhibitors of oxidative respiration (see Ashburner and Bonner, 1979; Leenders and Berendes, 1972; Rensing, 1973; Leenders et al, 1974; Behnel and Rensing, 1975). The concept that decreased cellular ATP levels could play an important role in the activation of heat shock puffs in *Drosophila* was suggested in a number of early studies (Ritossa, 1964; Leenders and

Berendes, 1972; Ashburner, 1970). There was good evidence which showed that heat and certain inhibitors of oxidative respiration indeed did lower intracellular ATP levels and induced puffing at the heat shock gene loci (Leenders et al., 1974; Behnel and Rensing, 1975). However, the authors of these studies concluded that decreased cellular ATP was not the causative agent of induction chiefly based on experiments in which certain inhibitors or combinations of inhibitors lowered cellular ATP but did not induce puffing. Our results show that high concentrations of oxidative respiration inhibitors (e.g. 30 mM salicylate) induce HSF binding with no or minimal puffing, suggesting that such inhibitors can activate the heat shock response to varying degrees (Winegarden et al., 1996; Figures A1, A2 in appendix). We propose that in *Drosophila*, all agents which lower intracellular ATP will likely activate HSF binding.

We have demonstrated that sodium salicylate at 3 and 10 mM concentrations induce puffing of the *Drosophila* heat shock loci on polytene chromosomes. Surprisingly, however sodium salicylate does not appear to induce heat shock gene transcription even at the concentrations which clearly induced heat shock puffs. The effect of salicylate on *Drosophila* hsp70 gene expression was originally investigated in SL2 cells and it was found that there was no induced expression of the hsp70 gene at any of the concentrations used. But, because puffing was strongly induced by 10 mM sodium salicylate in salivary glands, we originally interpreted the results to signify that there may be a difference in the responses between salivary glands and tissue culture cells to this drug. To clarify this, expression of hsp70 in salivary glands was also investigated, and it was found that salicylate does not induce the transcription of the hsp70 gene, in spite of the fact that large puffs were observed at 87A and

87C, the sites of the *hsp70* genes. This was a surprising result given that previous studies had demonstrated that radiolabelled RNA precursors are incorporated into heat induced heat shock puffs and that inhibitors of RNA synthesis such as actinomycin D and  $\alpha$ -amanitin block the formation of the heat shock puffs (Ritossa, 1964; Berendes, 1968; and see Ashburner and Bonner, 1979 for a review). We have interpreted this result to signify that for heat shock genes, puffing of the gene(s) does not necessarily mean the gene is actively transcribing, and therefore that puffing can be uncoupled from transcription.

What does puffing represent? Studies on the intermolt puff found at 68C, the site of three salivary gland secretion (*sgs*) protein or glue genes, have led Meyerowitz and coworkers to question whether puffs actually signify transcription. The insertion of the three 68C glue genes, *Sgs-3*, *Sgs-7* and *Sgs-8*, into the germline of *Drosophila* does produce a new puff at the insertion site (Meyerowitz et al., 1987). Likewise, new heat inducible puffs have been reported by Lis and colleagues in *Drosophila* having either a *hsp70* gene with its promoter or a *hsp70* gene promoter fused to the *lacZ* gene inserted into their germline (Lis et al., 1983; Simon et al., 1985). However, transformants that contain only the *Sgs-3* gene do not have puffs at the insertion sites even though the inserted *Sgs-3* gene is transcribed at high levels (Crosby and Meyerowitz, 1986). Furthermore, the reverse has also been demonstrated in the *l(1)npr-1* mutant, that is, no glue gene mRNA is detected in this mutant even though the 68C locus is prominently puffed (Crowley et al., 1984). Thus, Meyerowitz and coworkers have observed situations where highly transcribed genes do not produce puffs as well as puffs at sites which are not making mRNA. For salicylate induced puffs, the reduction in ATP levels caused by salicylate might explain why puffing is observed but not transcription. Many of the

enzymatic activities of RNA polymerase II holoenzyme, particularly those associated with the MO15, ERCC2, and ERCC3 subunits of TFIIF (Seroz et al., 1995) have been shown to require ATP. ATP is also required as a substrate for the synthesis of the RNA molecule. Perhaps salicylate induced puffs represent a localised decondensation of heat shock gene loci brought about by HSF binding and early transcription events such as formation of the transcription initiation complex and promoter clearance. However, the large size of the puffs induced by 10 mM salicylate would suggest that the decondensation of the polytene chromosome is likely occurring over a fairly large area (i.e. not just the promoter region). HSF binding alone does not appear to cause prominent puffs because 30 mM salicylate induced HSF binding but very small puffs or no puffs were observed. Further experiments will have to be performed to determine what puffs represent.

How does salicylate induce the high affinity binding of HSF to the HSE? It has been suggested that salicylate may be inducing HSF binding by generating abnormal proteins within the cell by a yet to be described mechanism (Jurivich et al., 1995). It has also been suggested that salicylate primarily affects newly synthesised proteins because the addition of cycloheximide prevented salicylate induced HSF binding in human cells (Jurivich et al., 1995). In this study, we have found that in *Drosophila* cells, cycloheximide addition inhibited the induction of HSF binding by 3 mM salicylate but did not appear to inhibit the induction of HSF binding by 10 or 30 mM salicylate. Abnormal proteins have been proposed to be a “universal” intracellular stress inducer and are believed to be generated by a number of the agents which trigger the heat shock gene expression and heat shock protein synthesis (see Nover, 1991; Ananthan et al., 1986; Mifflin and Cohen, 1994a; Mifflin and Cohen, 1994b).

The newly synthesized heat shock proteins are then thought to go on to perform a number of functions including disaggregating, refolding and degrading denatured or abnormal proteins. Heat shock proteins (e.g. hsp/hsc70) might also be directly responsible for maintaining HSF in its inactive configuration and/or converting the active DNA binding form of HSF back to its inactive form (see Morimoto et al., 1994a; Wu et al., 1994). Thus, the generation of abnormal proteins could substantially increase the number of substrates for hsc/hsp70 and therefore, other substrates such as HSF may not be attended to, and in the case of HSF, “aggregate” to its active trimeric configuration.

Could reduced cellular ATP activate HSF binding? The hsp70 family of heat shock proteins are ATPases which require hydrolysable ATP to release bound peptides (Frydman and Hartl, 1994). Thus, if hsp70 activity is required for maintenance of inactive HSF, any treatment which serves to substantially decrease ATP could result in the conversion of HSF to its high affinity DNA binding form. Alternatively, decreases in cellular ATP and the subsequent inhibition of heat shock proteins could lead to an accumulation of misfolded and partially folded proteins within the cell. An increase in abnormal proteins has been reported to occur in ATP depleted cells (Nguyen and Bensaude, 1994).

Why is there no transcription of the hsp 70 gene in *Drosophila* salivary gland and SL2 cells even though HSF is binding to the HSEs? At present this question cannot fully be answered, but the results of this study suggest a number of possible explanations. In SL2 cells, ATP levels are reduced at all the concentrations tested (3-30 mM). The large reduction in ATP seen at 10 and 30 mM salicylate might account for the prevention of heat shock gene synthesis for the reasons described above, even when cells are heat shocked. It would be

difficult to invoke this hypothesis at 3 mM salicylate given that ATP levels with this treatment are only reduced to approximately the same level as seen by a heat shock treatment. However, it should be noted that 3 mM salicylate only weakly induced HSF binding activity and, therefore, a large increase in heat shock gene synthesis might not be expected. Another possibility is that salicylate activated HSF is physically different from heat activated HSF. All concentrations of salicylate used in this study reduced the amount of HSF that was phosphorylated and/or the number of phosphate groups per HSF molecule. In human cells, salicylate also appears to alter HSF phosphorylation (Jurivich et al., 1995). We observed that 30 mM salicylate prevented heat induced heat shock gene transcription and the hyperphosphorylation of HSF which normally accompanies heat shock. These results indicate that HSF hyperphosphorylation is not required for the activation of HSF binding activity *in vivo*. A similar observation has been noted in human cells for certain stress agents (Sarge et al., 1993). It has been proposed that hyperphosphorylation of HSF is a prerequisite for the transcriptional activation of heat shock genes in yeast and in human cells (Sorger et al., 1987; Larson et al., 1988; Sorger and Pelham, 1988). If this hypothesis is true, the fact that salicylate treatments do not result in the hyperphosphorylation of HSF would explain why no transcription of the heat shock genes occurs in SL2 cells. However, the role of HSF hyperphosphorylation in the transcriptional activation of heat shock genes has been called into question, and in yeast, it appears that hyperphosphorylation of HSF is likely not required for the transcriptional activation of heat shock genes but probably is involved in the deactivation of HSF from its active state (Hoj and Jakobsen, 1994).

Additional studies with other inhibitors of ATP production should give us insight as to whether salicylate's effects on the induction of HSF binding activity, the induction heat shock puffs, and the inhibition of heat shock gene transcription and HSF hyperphosphorylation can be ascribed to decreased ATP levels alone.



## **Chapter 3**

### **Activation of the *Drosophila* Heat Shock Response by 2,4 Dinitrophenol and Potassium Cyanide**

## A. Introduction

Initial studies investigating the effects of sodium salicylate on the *Drosophila* heat shock response indicated that decreased ATP levels may be a trigger for activation of some aspects of the heat shock or stress response. At the same time it was determined that sodium salicylate only induced a partial heat shock response, in that HSF binding was activated, but HSF hyperphosphorylation and heat shock gene transcription were not induced. In an attempt to help determine whether a reduction in ATP levels always induces the same aspects of HSF activity, two other inhibitors of oxidative respiration were examined, the uncoupler 2,4 dinitrophenol (DNP) and the electron transport chain inhibitor potassium cyanide (CN<sup>-</sup>).

### *Modes of Action of DNP and CN<sup>-</sup>*

Both DNP and CN<sup>-</sup> have been widely examined, and their functions and modes of action have been clearly identified. DNP is a classical inhibitor of oxidative phosphorylation. It functions to shuttle protons across the mitochondrial membrane and thus dissipates the proton gradient established by the electron transport chain (Brody, 1956). Because of this action, there is no electrochemical potential energy with which the mitochondria can drive the generation of ATP. Salicylate is believed to have a similar mode of action (see Figure 3 in Chapter 1). CN<sup>-</sup> is an inhibitor of electron transport in mitochondria (Leenders and Berendes, 1972). CN<sup>-</sup> associates with complex IV, the terminal member of the electron transport chain and prevents it from being oxidised. This causes a back-up in the electron flow and prevents further pumping of protons into the intermembrane space of the mitochondria (See Figure 3 in Chapter 1).

### *DNP and CN and the Heat Shock Response*

Both DNP and CN have been studied in relation to activation of the heat shock response. DNP has been demonstrated to be effective in inducing all aspects of the heat shock response. DNP induces the heat shock puffing pattern in *Drosophila* polytene chromosomes (Koninkx, 1976; Ritossa, 1962), the binding of HSF to HSE (Zimarino and Wu, 1987). DNP induced transcription and translation of hsp70 has also been reported in *Drosophila* (Koninkx, 1976), yeast (Weitzel et al., 1985; Weitzel et al., 1987) and mammals (Edbladh et al., 1994; Wiegant et al., 1994), indicating that the inhibition of oxidative respiration may produce different effects on the heat shock response than salicylate. The effect of CN the heat shock response has been less clear. Originally it was reported that CN was not an inducer of the heat shock response as it fails to induce the heat shock puffing pattern in *Drosophila* (Behnel and Rensing, 1975; Leenders and Berendes, 1972). This discovery had also lead to the conclusion that decreased ATP levels are not sufficient for inducing the stress response in *Drosophila*.

In light of the initial discoveries in *Drosophila* which showed that CN is ineffective in inducing the heat shock puffing pattern, further examination of this chemical on the *Drosophila* heat shock response has been limited. Salicylate induced strong puffing at the hsp70 loci without causing an increase in hsp70 message in the salivary gland of *Drosophila* (Winegarden et al., 1996). The reverse has also been witnessed in which transformants that contain the 68C glue gene, *Sgs-3* do not have puffs at the insertion sites even though the inserted *Sgs-3* gene is transcribed at high levels (Crosby and Meyerowitz, 1986). We believe that the original conclusion that CN is not an inducer of the heat shock response may be

erroneous and here we re-examine the effects of  $\text{CN}^-$  to determine if it is capable of inducing part of or the entire heat shock response in *Drosophila*. We have also examined the effects of DNP for comparison.

It was found that both these inducers did indeed cause rapid decreases in intracellular ATP, and that this drop in ATP was coincident with the onset of the activation of HSF binding to the HSE. Surprisingly, both DNP and  $\text{CN}^-$  were found to induce the transcription of the *hsp70* gene in *Drosophila* tissue culture cells, albeit at a level which is noticeably lower than that induced by heat shock. In addition, it was found that at least for DNP, the hyperphosphorylation of HSF is not a requirement for the activation of *hsp70* gene expression. Overall, further evidence has been provided that the stress response in *Drosophila* can be induced as a result of decreased intracellular ATP.

## **B. Experimental Procedures**

### Tissue Culture

*Drosophila* Schneider line 2 (SL2) cells (obtain from the laboratory of Dr. Carl Wu at NCI, NIH Bethesda, MD) were grown in HyClone CCM3 serum free media for insect cells (HyClone) with 20 µg/ml gentamycin solution (Sigma). Cells were grown at 21-23 °C in T-75 tissue culture flasks (for suspension cells - Starstedt) at a volume of 15 ml. Prior to all experiments, cells at a concentration of  $5-8 \times 10^6$  cells/ml were transferred to 50 ml polypropylene tubes at a volume of 3-5 ml. Cells were aerated in these tubes by shaking at 200 rpm, 21 °C, for 6 or more hours prior to the experiment. For maintenance of the culture, cells were split 1:5 every three days.

### Cell Treatments

21 °C controls were prepared by taking cells which had not been subjected to any stress agents, but had undergone the aeration described above. All other cell treatments were also performed after aeration. 36.5 °C heat shocks were performed by incubating the cells in 50 ml polypropylene tubes (for volumes of 2-6 ml) or in standard microfuge tubes (for 1 ml samples) in a temperature controlled circulating water bath (Neslab). Cells were gently swirled every five minutes during heat shock to ensure constant suspension. 2,4 dinitrophenol was added to cells from a 10 mM dinitrophenol stock prepared in a physiological *Drosophila* saline (45 mM K-glutamate, 45 mM Na-glutamate, 8.7 mM MgSO<sub>4</sub>, 5.0 mM Bis-Tris, 6.8 mM CaCl<sub>2</sub>·H<sub>2</sub>O, 12 g/L glucose, pH 6.9), to the concentrations indicated in each experiment. Cyanide was added to cells from a 100 mM stock prepared in the same physiological *Drosophila* saline.

### Protein Extracts for Mobility Shift Assays

Protein Extracts were prepared as described in the Experimental Procedures section of Chapter 2.

### Electrophoretic Mobility Shift Assays (EMSA)

EMSA was performed as described in the Experimental Procedures section of Chapter 2. The DNP EMSA assay was repeated three times with very similar results. The CN<sup>-</sup> assay was repeated five times. Although there was variation with the 3.0 mM condition, 1.0 mM CN<sup>-</sup> always produced results similar to what is seen in the figure included. 3.0 mM CN<sup>-</sup> always produced binding to a level lower than what was detected during exposure to 1.0 mM CN<sup>-</sup>, however the degree of binding did vary to some degree. The autoradiogram is a representative example and reflects the most common result seen.

### RNA Preparation

One x 10<sup>7</sup> cells were used for each sample preparation. RNA was prepared using the RNeasy RNA extraction kit (Qiagen) following the instructions supplied. (See also the experimental procedures section of Chapter 2). RNA was quantified by spectrophotometry (A<sub>260</sub> and A<sub>280</sub>). RNA samples were stored at -72 °C until use in primer extension analysis.

### Primer Extension Assays

Primer extensions were performed as described in the Experimental Procedures section of Chapter 2 under the heading ‘Primer Extension of RNA to Test RNA Stability - Revised Protocol’. The DNP experiment was repeated three times with very similar results. The CN<sup>-</sup> assay was performed twice, also with similar results.

### Densitometry of Autoradiograms

Refer to the Experimental Procedures section of Chapter 2 for this protocol.

### ATP Assays

ATP assays were performed as described in the Experimental Procedures section of Chapter 2.

### Protein Labelling and Immunoprecipitation

Cells grown at a concentration of  $8 \times 10^8$  cells/ml in CCM3 media were pelleted, washed twice in physiological saline, and resuspended in phosphate free Sheilds and Sang M3 media at a concentration of  $1 \times 10^7$  cells/ml.  $^{32}\text{P}$ -*o*-phosphate (New England Nuclear/Amersham) was aliquoted into 1.5 ml microfuge tubes in 100  $\mu\text{Ci}$  amounts. One ml of cells were then added to each tube. Immediately after addition to phosphate, DNP was added to the cells, or cells were heat shocked by submersion in a 36.5 °C water bath. After the appropriate time interval, cells were pelleted by centrifugation at 10,000 x g for 30 s. Supernatants were removed, and 10 volumes TETN400 buffer was added (25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5), 400 mM NaCl, 1% Triton X-100 v/v supplemented with the following phosphatase inhibitors: 0.1 mM Na-vanadate, 1 mM benzimide, 10 mM NaF, 10 mM  $\beta$ -glycerol phosphate, 1 mM  $\text{K}_2\text{PO}_4$ , and 1 mM  $\text{KH}_2\text{PO}_4$ ) (Firestone and Winguth, 1990). Pellets were immediately lysed and resuspended by drawing through a 28.5 gauge needle. Samples were stored at -72 °C until processed by immunoprecipitation. Immunoprecipitations were performed essentially as described in (Firestone and Winguth, 1990) with a only few modifications. Cell extracts were made using a buffer with increased salt concentration (400 mM NaCl) and thus in order to maintain a 250 mM NaCl

concentration, an equal volume of TETN100 (5 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 7.5), 100 mM NaCl, 1% Triton X-100 v/v) rather than TETN250 was added to each extract for binding reactions to give a final 250 mM NaCl concentration. One  $\mu$ l of anti-*Drosophila* HSF antisera (943) (Westwood et al., 1991) was added to each 100  $\mu$ l binding reaction and the entire sample was immunoprecipitated and loaded and separated via SDS-PAGE on 8% polyacrylamide gels. The labelling was performed in duplicate, and each time produced similar results.



## C. Results

### *2,4-Dinitrophenol Induced HSF DNA Binding Activity in SL2 Tissue Culture Cells -*

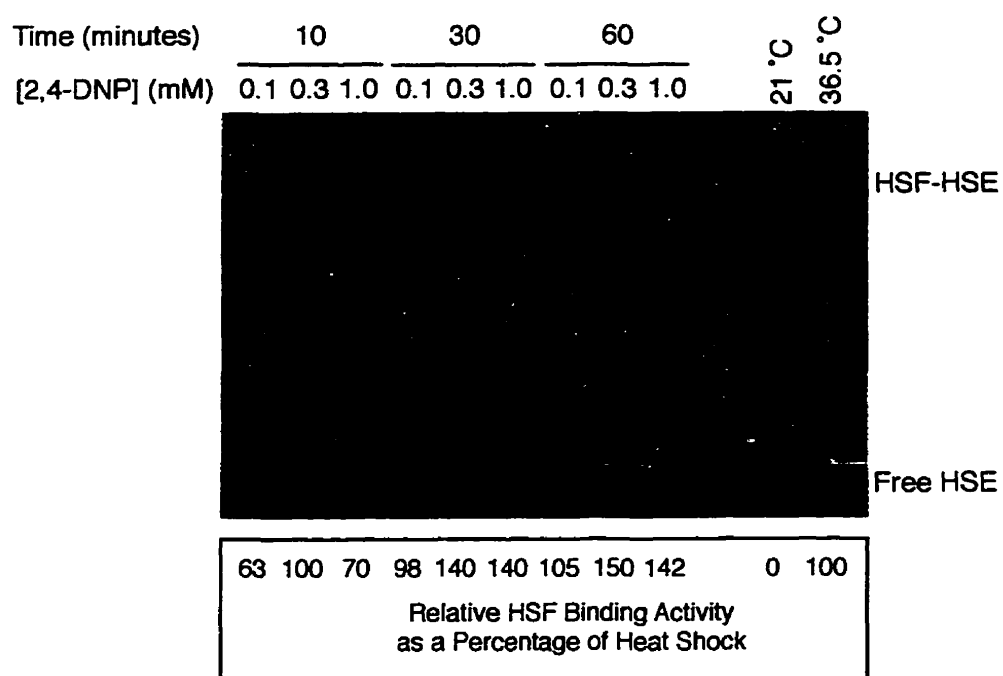
2,4-dinitrophenol (DNP) rapidly and strongly induced the DNA binding activity of HSF in *Drosophila* tissue culture cells over the concentration range of 0.1 to 1.0 mM (Figure 10). HSF binding to a synthetic HSE was activated within 10 minutes of exposure to DNP at each of the three concentrations tested (0.1, 0.3 and 1.0 mM). With 0.1 mM DNP, HSF binding slightly increased with time. Both 0.3 and 1.0 mM DNP provided noticeably higher levels of HSF binding than 0.1 mM DNP and increased binding with time, with 0.3 mM DNP inducing more HSF binding than 1.0 mM. At 30 and 60 minutes, both 0.3 and 1.0 mM DNP caused more HSF binding than a 20 minute heat shock. In addition, the mobility of the HSF bound HSE in the gel decreased with time and this effect was most pronounced at the 0.3 mM and 1.0 mM DNP concentrations. By 60 minutes, the HSF binding activity had similar mobilities as a 20 minute heat shock. This change in mobility could reflect changes in HSF's conformation or shape but probably does not reflect increased HSF phosphorylation (see Figure 16). HSF binding activity did not increase with 1.0 mM DNP, and thus the two lower concentrations were used for the subsequent analysis of the DNP induced heat shock response.

### *Cyanide Induced Rapid Binding of HSF to the HSE in Drosophila Tissue Culture Cells -*

CN<sup>-</sup> also induced HSF binding activity in *Drosophila* tissue culture cells (Figure 11). Binding activity was detectable after 5 minutes of exposure to 1.0 mM. 3.0 mM only appeared to induce minimal HSF binding activity. Throughout the entire 60 minute time course of the experiment, 1.0 mM CN<sup>-</sup> continued to be a potent inducer of HSF binding

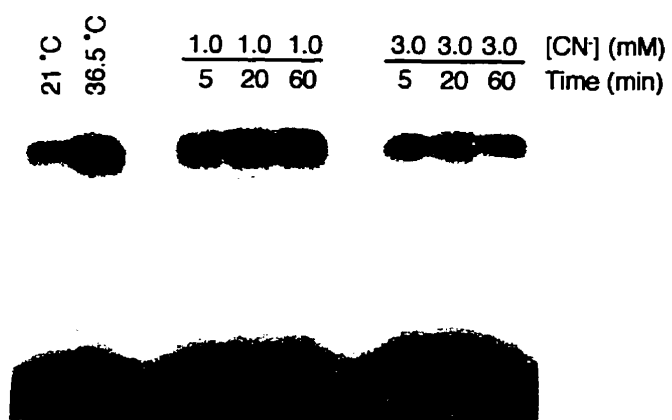
**Figure 10. DNP induces the HSF DNA binding activity.**

*Drosophila* SL2 cells were treated to the conditions noted, and protein extracts were made (see Experimental Procedures). EMSAs were performed to test for the binding activity of HSF using a radiolabeled synthetic HSE as a probe. The 36.5 °C heat shock was for 30 minutes. Binding reactions were separated on 1 % agarose gels in 0.5 X TBE. Densitometry values given are expressed as a percentage of heat induced binding.



**Figure 11. HSF binding activity is induced by potassium cyanide.**

Cells were treated to the conditions noted and EMSAs were performed on the protein extracts as described in the legend for Figure 10.



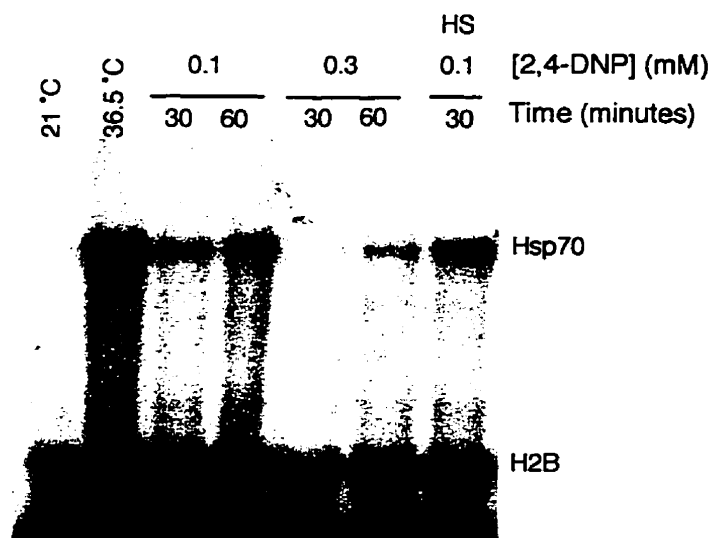
activity, and 3.0 mM continued to only show a moderate effect. CN<sup>-</sup> at 0.3 mM was also investigated for its ability to induce HSF binding activity but the results obtained with this concentration were highly variable, with the majority of results indicating that this concentration is incapable of inducing HSF binding activity.

*DNP Induces hsp70 Expression in a Concentration Dependent Manner -*

Primer extension analysis was utilized to examine the ability of DNP to induce the expression of the hsp70 in *Drosophila* (Figure 12). After 30 minutes, 0.1 mM DNP induced hsp70, but to a level which was lower than that induced by a 30 minute heat shock. The level of binding increased over the 60 minute time period but remained at a level which was lower than a 30 minute heat shock. DNP at 0.3 mM was less effective in inducing hsp70 expression. No signal is detectable at the 30 minute interval, and only a minor signal is detected at 60 minutes of exposure. The decreased H2B signal in the 0.3 mM 30 minute lane indicated that this lane was misloaded. A longer exposure (3 times the length of time) was used to see if the hsp70 signal could be detected but no signal is observed in this lane (data not shown). The effect of a combined heat shock with 0.1 mM DNP was also investigated. It can be seen that the combination of these two conditions did induce hsp70 gene expression, however, this level was lower than what was seen with a 30 minute heat shock. But, it also appears that this combination induced a higher level of expression than what is seen with a 0.1 mM DNP treatment alone. At this time, the effect of 0.3 mM DNP and heat shock has not been investigated.

**Figure 12. DNP induces hsp70 gene transcription in *Drosophila*.**

Total RNA was extracted from *Drosophila* SL2 cells which had been treated to the conditions indicated. Hsp70 and histone H2B transcript levels were determined via primer extension analysis (See Experimental Procedures). Densitometry was performed as described in the legend of figure 5 and in the Experimental Procedures section.



0	100	30	50	0	13	50
Hsp70 Message as A Percentage of Heat Shock (36.5 °C) Level						



### *CN Induced hsp70 Gene Expression -*

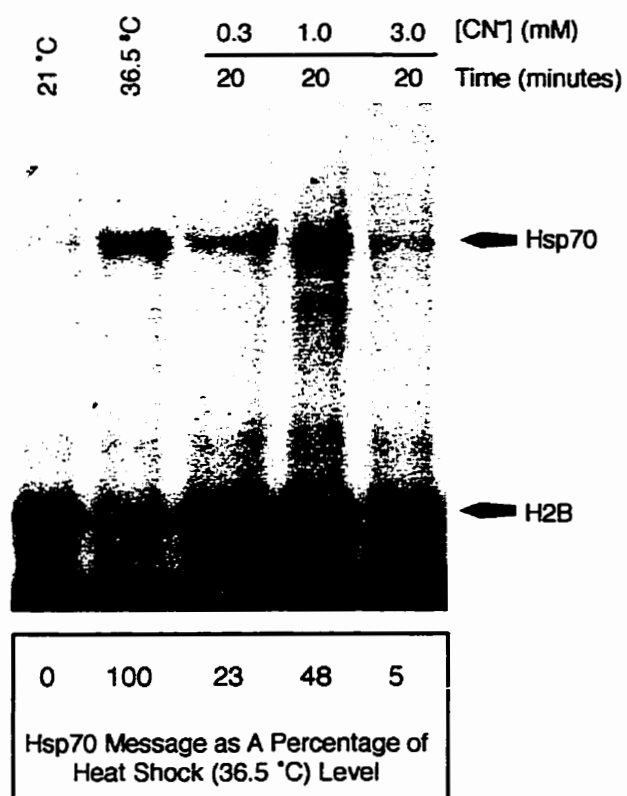
CN also induced the expression of the hsp70 gene in *Drosophila* (Figure 13). Three different concentrations of CN : 0.3 mM, 1.0 mM and 3.0 mM were examined for their ability to induce hsp70 message after a 20 minute exposure. This time point was chosen because the mobility shift analysis indicated that 20 minutes appeared to be the point of maximal HSF binding. Each of these three concentrations induced a moderate amount of hsp70 message with 3.0 mM producing the least amount of message and 1.0 mM producing the most.

### *DNP Caused a Concentration Dependent Decrease in Intracellular ATP Levels in Drosophila SL2 Cells-*

DNP is a known uncoupler of oxidative respiration and for this reason the effect of this drug on intracellular ATP was examined. As had been previously observed (see Chapter 2), ATP levels remained relatively constant during normal physiological conditions. Heat shock reduced intracellular ATP levels by approximately 31%, a level comparable to that seen in the previous examination of salicylate's effect on ATP (compare Figures 8 and 14). Exposure to DNP caused a rapid and dramatic decrease in intracellular ATP (Figure 14). At 0.1 mM, DNP caused a reduction in ATP concentration by approximately 43% of normal levels. At 0.3 mM, DNP decreased ATP levels by more than 85%. The majority of the effect of DNP was observed within the first 20 minutes of exposure. At 60 minutes, 0.1 mM DNP further lowered ATP concentrations to 40% of normal (ie a 60% decrease), and 0.3 mM DNP had no further effect.

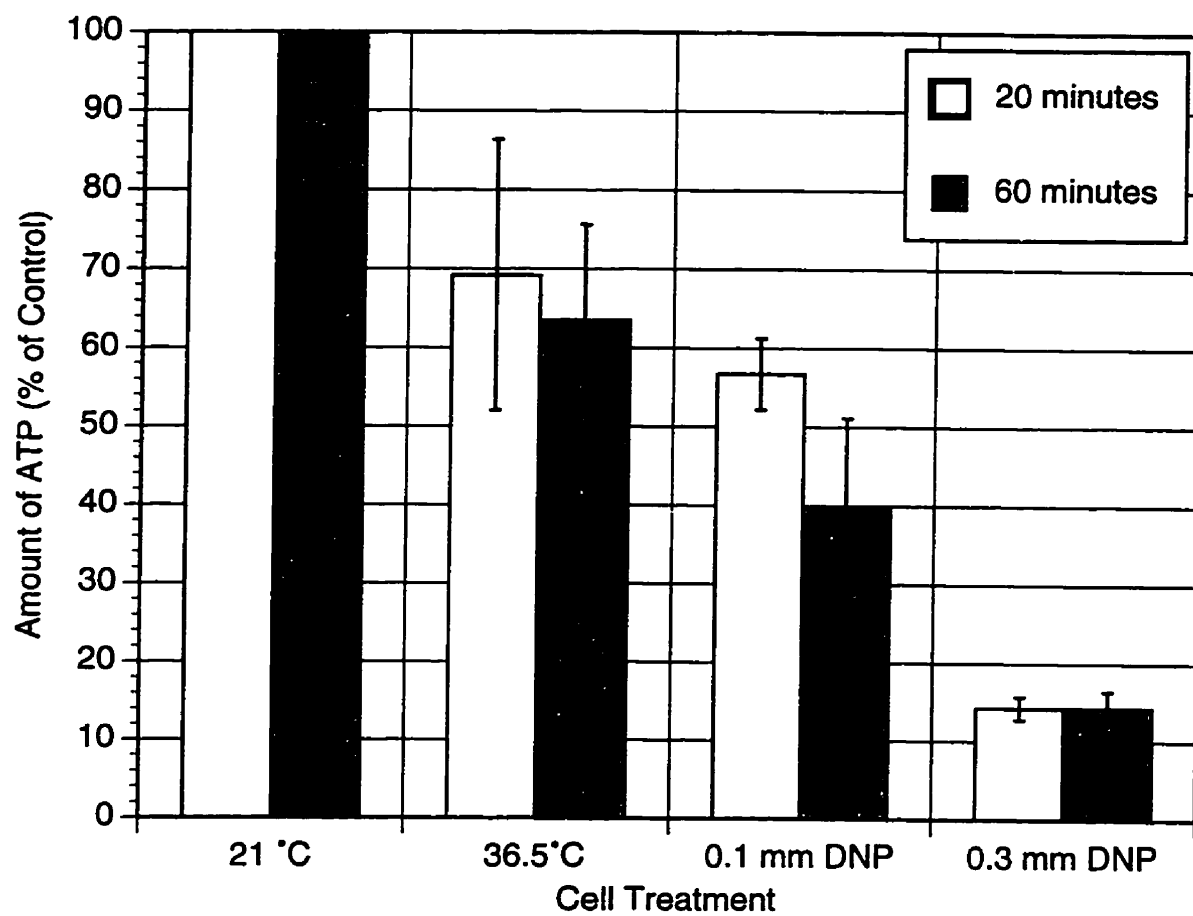
**Figure 13. CN<sup>-</sup> induces hsp70 gene transcription in *Drosophila*.**

Total RNA was extracted from *Drosophila* SL2 cells which had been treated to the conditions indicated. Hsp70 and histone H2B transcript levels were determined via primer extension analysis (See Experimental Procedures). Densitometry was performed as described in the legend of figure 5 and in the Experimental Procedures section.



**Figure 14. DNP reduces intracellular ATP in a dose-dependent manner.**

*Drosophila* SL2 cells were subjected to each of the treatments indicated. Cells were lysed and proteins denatured by boiling in 100 mM Tris (pH 7.75), 4 mM EDTA solution for 2 minutes. ATP levels were determined with the use of a luciferase based assay (Constant Light Source Kit II – Boehringer Mannheim) and were detected and quantified with a scintillation counter. ATP is expressed as a percentage of the control (21 °C) values which were approximately  $2.4 \times 10^{-4}$  mM ATP/ $10^5$  cells.



*CN Rapidly Decreased Intracellular ATP -*

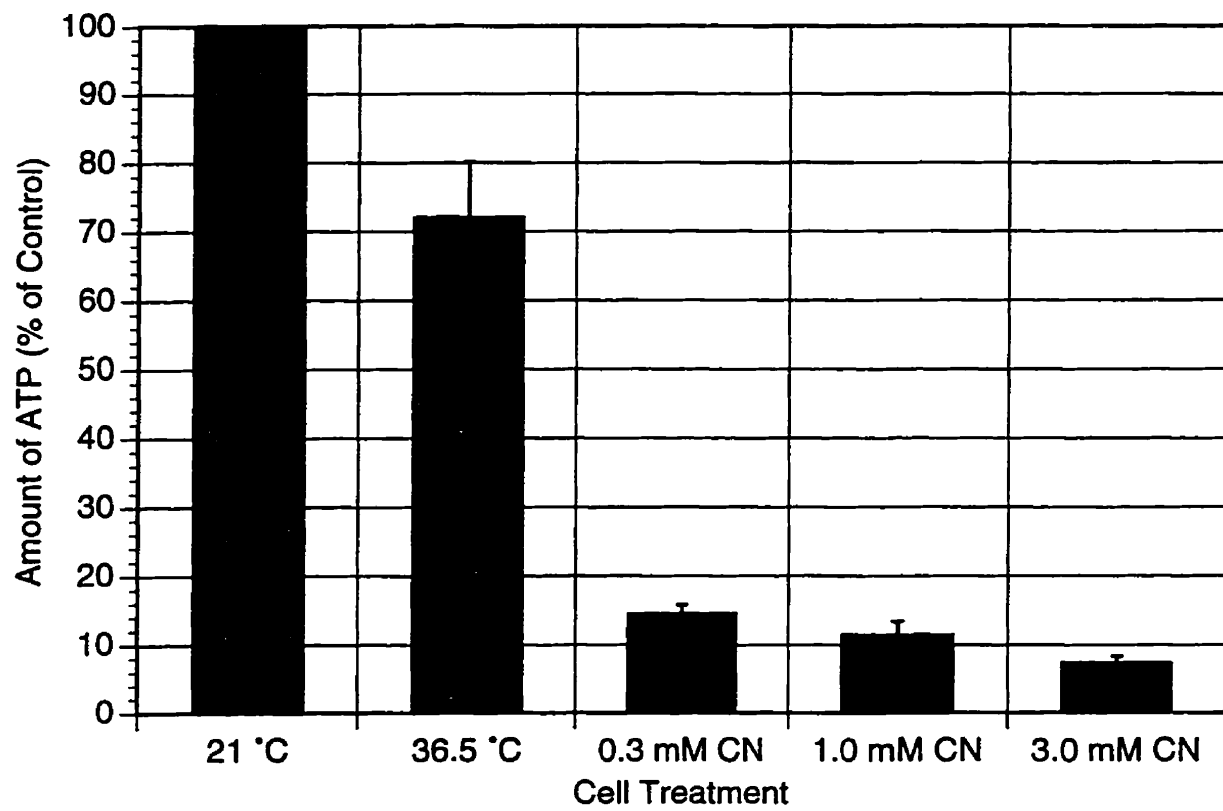
CN<sup>-</sup> also caused a dramatic decrease in the levels of intracellular ATP in *Drosophila* tissue culture cells (Figure 15). After 20 minutes 0.3 mM CN<sup>-</sup> had decreased the intracellular ATP levels by 85%. The higher concentrations of CN<sup>-</sup> had an even greater effect with 1.0 mM CN<sup>-</sup> decreasing ATP by 89% and 3.0 mM by 93%.

*DNP failed to induce HSF hyperphosphorylation -*

The effect of DNP on HSF hyperphosphorylation was examined using immunoprecipitation of transiently labelled *Drosophila* SL2 cells (Figure 16). Heat shock induced the HSF hyperphosphorylation that is normally observed. DNP however did not induce the hyperphosphorylation of HSF and the overall level of HSF phosphorylation appears to be less than that which is observed during normal physiological conditions (i.e. 21 °C).

**Figure 15. CN<sup>-</sup> strongly reduces intracellular ATP in *Drosophila* SL2 cells.**

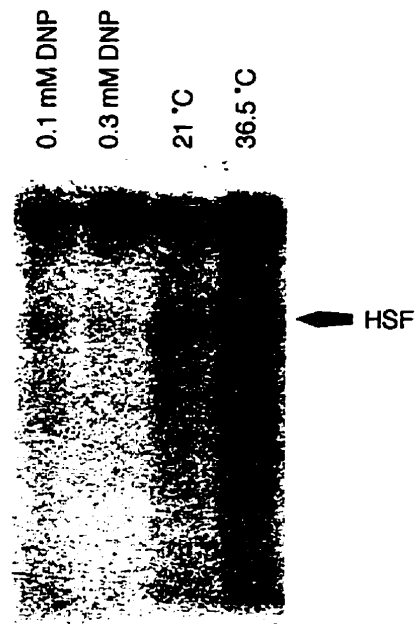
*Drosophila* SL2 cells were subjected to each of the treatments indicated. Cells were lysed and proteins denatured by boiling in 100 mM Tris (pH 7.75), 4 mM EDTA solution for 2 minutes. ATP levels were determined with the use of a luciferase based assay (Constant Light Source Kit II – Boehringer Mannheim) and were detected and quantified with a scintillation counter. All measurements were taken after a 20 minute exposure to the condition noted. ATP is expressed as a percentage of the control (21 °C) value which was approximately  $2.5 \times 10^{-4}$  mM ATP/ $10^5$  cells.





**Figure 16. DNP fails to induce the hyperphosphorylation of HSF.**

HSF was immunoprecipitated from extracts of *Drosophila* SL2 cells which were treated to the conditions indicated for 20 minutes, in the presence of  $^{32}\text{P}$   $\alpha$ -phosphate. Immunoprecipitates were separated on 8% SDS PAGE gels and autoradiograms were made. The band representing HSF is indicated.



#### D. Discussion

DNP was one of the first chemicals identified as an inducer of the heat shock response inducing the same puffing pattern as heat on the polytene chromosomes of *Drosophila* (Ritossa, 1962; Koninkx, 1976; Leenders and Berendes, 1972). It has also been demonstrated to induce the binding of HSF to the HSE in *Drosophila* (Zimarino and Wu, 1987), and the expression of hsps in yeast (Weitzel et al., 1985; Weitzel et al., 1987) and *Drosophila* (Koninkx, 1976). Some discrepancy seemed apparent when it was reported that DNP could not induce the expression of hsps in mammalian cells (Burdon, 1987), however, this controversy has seemingly been explained with the report that DNP can cause hsp synthesis in mammalian cells, but that this expression is dosage dependent (Edbladh et al., 1994; Wiegant et al., 1994). The effect of cyanide on the heat shock response has been even less clear. Several researchers have demonstrated that  $\text{CN}^-$  (0.005 to 10 mM) is incapable of inducing the heat shock puffing pattern of *Drosophila* polytene chromosomes (Behnel and Rensing, 1975; Leenders and Berendes, 1972). It has also been reported that  $\text{CN}^-$  cannot induce the synthesis of hsps in human cells (Burdon, 1987). In contrast,  $\text{CN}^-$  has been shown to be capable of inducing hsps in *S. cerevisiae* (Weitzel et al., 1987). The complexity in the effect of  $\text{CN}^-$  on the heat shock response is demonstrated by two separate phenomena. 1) Although  $\text{CN}^-$  cannot induce the puffing of *Drosophila* polytene chromosomes, addition of oligomycin, an agent that further lowers ATP levels through the inhibition of ATP synthase caused the heat shock puffing pattern to occur (Leenders and Berendes, 1972). 2) Similar to the experiment with oligomycin, exposure of cells to  $\text{CN}^-$  in a media containing 2-deoxyglucose caused expression of hsp70 mRNA and protein in mammalian cells (Iwaki et al., 1993). It

appears that actions which further reduce ATP levels can bring about activation of the heat shock response in  $\text{CN}^-$  treated cells.

In this study, DNP and  $\text{CN}^-$  were utilized to determine whether decreased ATP levels could induce part of, or the entire heat shock response. This was done in order to lend support to the theory that salicylate could be activating the heat shock response in *Drosophila* via decreased intracellular ATP. In addition, an attempt was made to determine if the effect seen with salicylate, in which only part of the heat shock response was activated, is common to ATP depleting agents.

DNP (0.1 to 1.0 mM) was found to strongly induce HSF binding activity in *Drosophila* SL2 tissue culture cells. The degree of activated binding was found to be dosage and time dependent. Interestingly, the intermediate concentration used 0.3 mM DNP, appeared to be the strongest inducer of HSF binding activity. Similarly,  $\text{CN}^-$  (0.3 to 3.0 mM) induced the binding of HSF, however, only the lower concentration used (1.0 mM) was effective in inducing this binding. The higher concentration of  $\text{CN}^-$  used (3.0 mM) had reduced HSF binding activity. Although further testing will be required, preliminary investigation has indicated that a possible explanation for this decrease is that there is an increase in cell mortality during exposure to 3.0 mM  $\text{CN}^-$ . Trypan Blue viability assays might be performed to establish whether this hypothesis is valid.

Both of these agents also lowered intracellular ATP as expected, with  $\text{CN}^-$  having the greatest effect. 0.1 mM DNP reduced ATP to approximately 57% of pretreatment values after 20 minutes and to 40% after 60 minutes. 0.3 mM DNP had a much more pronounced effect reducing ATP to 14 % after 20 minutes and remaining unchanged upto 60 minutes.

CN<sup>-</sup> was a potent ATP depleting agent. 0.3 mM CN<sup>-</sup> reduced ATP to 14 % and 1.0 mM reduced it to 11 % after only 20 minutes. Similar to what is seen with salicylate, both DNP and CN<sup>-</sup> reduce intracellular ATP on a time scale which is consistent with the induction of HSF binding activity in *Drosophila* tissue culture cells.

Puffing experiments in *Drosophila* have indicated that DNP can induce the transcription of the heat shock genes, but that CN<sup>-</sup> is ineffective in the induction of stress related puffs. Due to the previous results with salicylate, we believe that puffing is not always an appropriate measure of transcriptional activity of the heat shock genes. Therefore, it was important to analyse the expression of hsp genes by directly measuring transcript levels. Since DNP induced hsp synthesis in *Drosophila* had been previously been reported, it was expected that we would detect some transcription for DNP, but CN<sup>-</sup> like salicylate was expected to be ineffective as an inducer of hsp70 gene transcription. We observed that DNP, and suprisingly, CN<sup>-</sup> are capable of inducing the expression of hsp70 in *Drosophila* tissue culture cells. The maximal level of hsp70 expression was observed with 0.1 mM DNP at 60 minutes (50% of a standard 30 minute heat shock). Interestingly, 0.3 mM DNP was less effective in the induction of hsp70 gene expression. At 30 minutes, no expression of hsp70 is seen with this concentration of DNP. After 60 minutes, a minor amount of expression is detectable (13% of a standard 30 minute heat shock for the figure shown). Similar results were obtained with CN<sup>-</sup>. That is, the intermediate concentration of CN<sup>-</sup> used (1.0 mM) was most effective in inducing hsp70 gene expression. After 20 minutes, 1.0 mM CN<sup>-</sup> had induced expression of hsp70 to a level which is approximately 50% of what is seen with a 20 minute heat shock.

Both the higher and the lower concentrations of  $\text{CN}^-$  used (3.0 mM and 0.3 mM) were less effective inducers of hsp70 expression at this time point.

What causes decreased expression with higher concentrations of DNP and  $\text{CN}^-$ ? For  $\text{CN}^-$ , it is possible to invoke the same hypothesis as was used to explain the decreased HSF binding activity with higher concentrations of  $\text{CN}^-$ . As previously mentioned, preliminary experiments indicated that 3.0 mM  $\text{CN}^-$  causes increased cell mortality when compared to 1.0 mM  $\text{CN}^-$ . Initial experiments have indicated that after 20 minutes, 3.0 mM  $\text{CN}^-$  caused approximately 70% mortality which can be compared to 1.0 mM  $\text{CN}^-$  which caused only 20% mortality. It is harder to explain the effect of higher concentrations of DNP as during preliminary tests, all concentrations of DNP tested (from 0.1 to 1.0 mM) had similar levels of viability (90-95%) when tested at 20 minutes of exposure. As mentioned however, more tests must be done to be able to attribute statistical significance to these figures. Why then is the level of hsp70 expression lower at 0.3 mM than it is at 0.1 mM? This phenomenon is even more difficult to reconcile in the light of the fact that 0.3 mM DNP causes a much higher level of HSF binding than does the lower concentration of 0.1 mM. In some respects what is seen with 0.3 mM DNP is reminiscent of the situation observed with salicylate. Salicylate induced strong binding of HSF and yet did not activate hsp70 gene expression. It is possible that 0.3 mM DNP lowers ATP levels below that which the cell requires to drive such processes as gene transcription. Further analysis may be required to determine the point at which DNP fails to induce hsp70 gene transcription in order to fully understand the effect of this drug on the heat shock response.

One of the theories that came out of the investigation of salicylate and the heat shock response was that salicylate's failure to activate the transcription of the hsp70 gene may be linked to the fact that salicylate inhibits the hyperphosphorylation of HSF. It was then decided that HSF hyperphosphorylation would be analysed during exposure to DNP. If the theory that salicylate does not induce hsp70 gene expression due to a lack of HSF phosphorylation were to hold true, DNP would have to induce HSF hyperphosphorylation as it was capable of inducing hsp70 gene expression. What was observed was unexpected. DNP failed to induce HSF hyperphosphorylation and similar to what is observed with salicylate, the level of phosphorylation on HSF during DNP exposure appears lower than what is seen during normal physiological conditions. This result also indicates that hyperphosphorylation of HSF is not required in *Drosophila* to induce the expression of the hsp70 gene. Further experiments with CN<sup>-</sup> and other inducers of the heat shock response (e.g. amino acid analogues) should be performed in order to confirm this finding.

Despite the fact that there still remain questions which need to be answered, it is apparent that these two agents, DNP and CN<sup>-</sup> are both capable of inducing both HSF binding and heat shock gene transcription. For CN<sup>-</sup> this is an unexpected result as it has previously been reported that this drug is incapable of inducing the heat shock response in *Drosophila*. This study lends further evidence that puffing analysis is not sufficient for determining inducers of the heat shock response. The results further support our theory that any condition which lowers intracellular ATP will at the minimum induce the binding of HSF to HSE. It has also been demonstrated that in some instances this effect may be sufficient to initiate the expression of the heat shock genes in *Drosophila*. Further investigation is required to

determine whether the rest of the response is activated by these two agents. In particular, is hsp expression induced in *Drosophila* during exposure to DNP and CN<sup>-</sup> and can exposure to these agents induce thermotolerance in this organism?



## **Chapter 4**

### **General Discussion**

#### **The Effect of Inhibitors and Uncouplers of Oxidative Respiration on the *Drosophila* Heat Shock Response**

## A. Discussion

The heat shock response is a universal and conserved mechanism employed by cells to prevent and reverse damage which is induced by stress. In eukaryotes the response is mediated by a specific *trans* activator termed heat shock factor (HSF). Activation of the heat shock response is a multistep process involving activation of the HSF and subsequent expression of the heat shock genes and synthesis of heat shock proteins (hsps). One of the most actively examined aspects of the heat shock response is the signal(s) which activates this cell's defense mechanisms during stress. Many researchers have attempted to identify a universal inducer for the heat shock response, however, it is now becoming clear that there may be more than one means by which the stress response may be activated. In order to further elucidate the mechanisms involved in activation of the heat shock response, it is important to study the induction of the response by numerous different inducers.

This particular study began as an investigation of the *Drosophila* heat shock response during exposure to sodium salicylate (hereafter referred to as salicylate). During the course of this investigation it was determined that exposure of *Drosophila* tissue culture cells to salicylate caused the activation of HSF binding activity, as judged by gel mobility shift assay, and puffing of the heat shock loci on polytene chromosomes (see Appendix), but failed to induce the transcription of the hsp70 gene, or the hyperphosphorylation of HSF. It was also discovered that salicylate caused dramatic decreases in the levels of intracellular ATP. This lead to the theory that salicylate may function to induce the heat shock response through such a reduction of ATP. Further analysis was deemed necessary, and as such, two additional

agents capable of reducing intracellular ATP were employed to study the effects of decreased intracellular ATP on the *Drosophila* heat shock response.

### ***Does Reduced Intracellular ATP Activate the Heat Shock Response?***

There has been a great deal of interest focused on determining the effect of metabolic inhibitors on the heat shock response. During the 1970s many researchers were attempting to determine how many kinds of agents could act as inducers of the heat shock response. Their methods were limited and induction of the heat shock response was assayed mainly by looking at the induction of the heat shock puffing pattern on *Drosophila* polytene chromosomes, the method originally described by Ritossa (1962). In fact, the first chemical inducers of the stress response were uncouplers of oxidative respiration, DNP and salicylate (Ritossa, 1962).

Two investigations in particular focused on examining metabolic inhibitors for their ability to induce this stress related puffing pattern in *Drosophila*. In 1972, Leenders and Berendes published a report which examined numerous metabolic inhibitors. This study utilized drugs which acted at each of the individual points of the electron transport chain and oxidative phosphorylation process. These researchers were lead to the conclusion, however, that decreased ATP levels are not sufficient to activate the heat shock response. This conclusion was the result of observations that certain agents which deplete cells of ATP, in particular potassium cyanide and oligomycin, were unable to induce the puffing of *Drosophila* polytene chromosomes. A later study involved similar experiments, and although at this time, oligomycin was found to induce the puffing of the polytene chromosomes, cyanide remained ineffective in the induction of the stress related puff sites (Behnel and Rensing, 1975). These authors were also lead to the same conclusion, that ATP reductions were insufficient to act as

a signal for the activation of the heat shock response. These reasons for the dismissal of the theory are no longer sufficient. As we have demonstrated, puffing of the *Drosophila* chromosomes does not necessarily represent transcriptional activity (Winegarden et al., 1996).

In this thesis it has also been demonstrated, with cyanide that the lack of puffing (shown previously by other researchers) does not signify the lack of gene expression (Compare Figure 13 and Leenders and Berendes, 1972; Behnel and Rensing, 1975). For this reason, it is possible that despite the lack of puffing observed in *Drosophila* during exposure to cyanide and certain other chemicals, the heat shock response can still be activated at some other level. Also, inducers like cyanide may only be inducing a partial response (e.g. only HSF binding). In yeast, it has already been shown that cyanide, when used at high concentrations (10 mM) can induce the expression of hsp's (Weitzel et al., 1987).

Many chemicals which are capable of lowering the levels of intracellular ATP have also been demonstrated to be inducers of the heat shock response. The data presented in this thesis has shown that three agents which decrease ATP levels induce at least some aspects of the heat shock response in *Drosophila*. But, not all aspects of the heat shock response are activated. Salicylate apparently only induces the binding of HSF and the puffing of the heat shock loci. DNP induces HSF binding, chromosome puffing, and hsp70 gene transcription (hsp synthesis was not assayed). DNP, however, failed to induce the hyperphosphorylation of HSF that normally accompanies a heat shock. Cyanide induced the HSF binding activity and transcription of hsp genes (at 0.3 to 3.0 mM). Others have shown that cyanide fails to induce the heat shock puffing pattern (over a concentration range of 0.05 to 10 mM)(Leenders and Berendes, 1972; Behnel and Rensing, 1975). Interestingly, and perhaps more telling, is the

fact that the effects on the heat shock response induced by each of these chemicals are induced on a time scale that is similar, and consistent with the rate at which these agents decrease ATP inside of the cell.

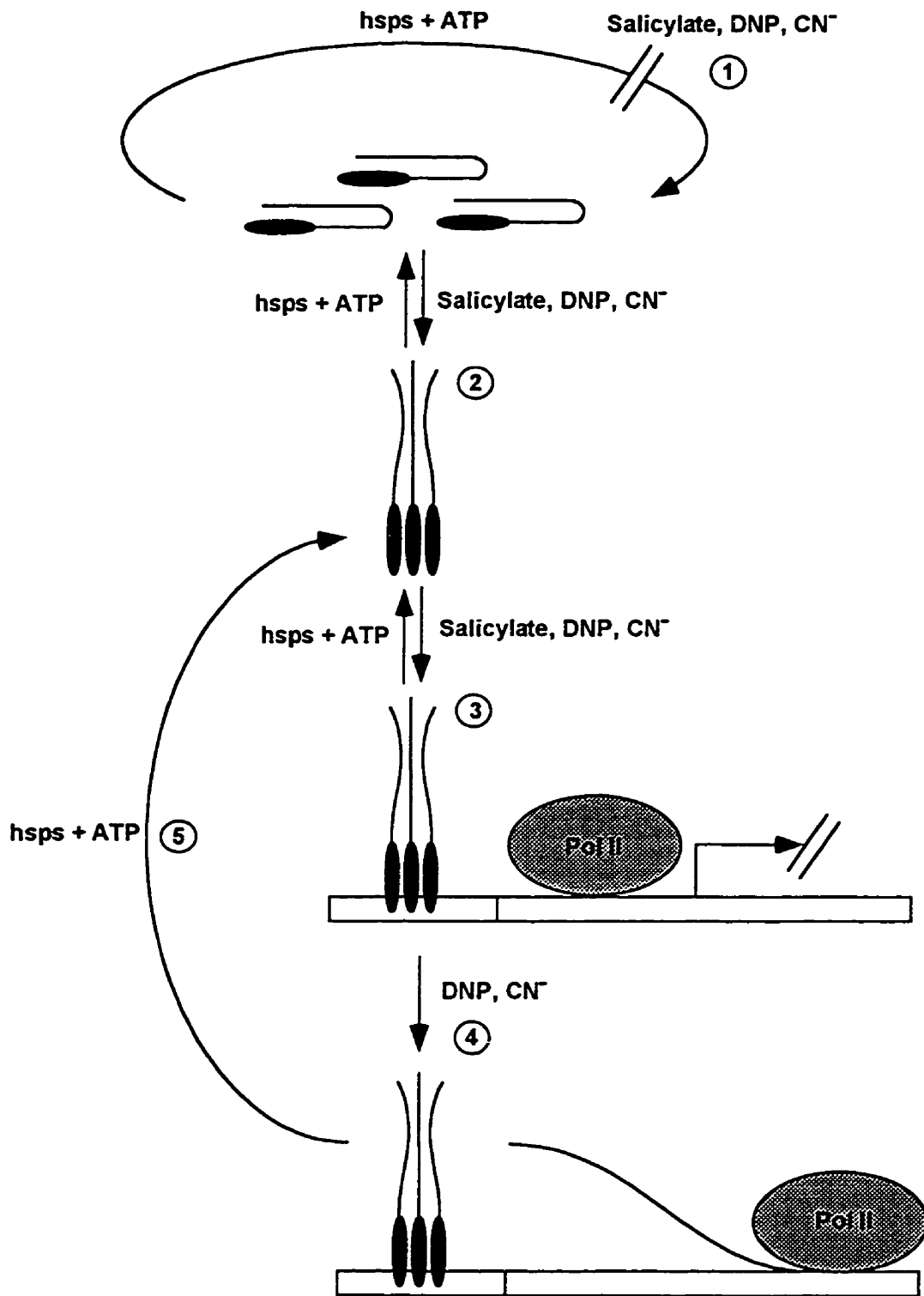
### ***How Might Reduced ATP Induce the Heat Shock Response?***

In order to formulate a hypothesis as to how decreases in intracellular ATP might cause the heat shock response to be activated, it is important to examine the current theories as to how the heat shock response is regulated. One prevailing theory is that the heat shock gene transcription is auto-regulated. This theory stipulates that HSF is maintained in an inactive conformation through the action of constitutively present hsps. Therefore, it is thought that stress is recognized in cells when the interaction of HSF with these heat shock proteins is interrupted. During heat shock it is thought that elevated temperatures cause a denatured protein pool to be generated. This causes increased demand in the cell for hsps. The constitutively present hsps are then required elsewhere in the cell and HSF is left unchecked causing it to take on an active conformation.

Can a similar hypothesis be put forward for reduced cellular ATP? In chapter one of this thesis it was suggested that reduced ATP might also act to prevent the interaction of HSF with the hsps. Several of the hsps, and hsp70 in particular, require ATP in order to carry out their cellular functions. In light of this, it is possible that reduced ATP might prevent hsp function and thus HSF could not be maintained in its inactive conformation. This would at least explain how HSF binding comes about during ATP decreases (See Figure 17 for model). An alternative and related explanation is also possible. It has been reported (Nguyen and Bensaude, 1994) that decreased levels of intracellular ATP cause severe protein denaturation

**Figure 17. A proposed model for HSF activation during ATP depletion.**

(1) HSF is maintained in an inactive monomeric configuration via the action of hsp(s) and ATP. Salicylate, DNP and  $\text{CN}^-$  might interrupt this action by reducing intracellular ATP and consequently inhibit the activity of the ATP dependent hsps. This would result in the trimerization of HSF (2). HSF is proposed to be returned to its inactive monomeric state via hsps and ATP, but during exposure to inhibitors of oxidative respiration, this action is inhibited. (3) After trimerization, HSF binds to the HSEs in the promoters of the heat shock genes. Once again, HSF might be removed from HSEs via the action of hsps and ATP. Salicylate, DNP and  $\text{CN}^-$  would prevent this. During salicylate exposure, this appears to be the final step in the activation of the response. DNP and  $\text{CN}^-$  initiate a further step of the response, allowing HSF to activate the expression of the heat shock genes (4). During attenuation of the response, hsps and ATP might remove HSF from the HSE (5) thus terminating transcription from the heat shock genes. It is not known at this time whether or not this aspect of the heat shock response takes place during DNP or  $\text{CN}^-$  exposure.



in the cell. In particular, it seems that proteins of the cytoskeleton are especially sensitive to such ATP decreases. How this denaturation comes about is not clear, and it is conceivable that this is also a result of a loss of hsp function inside of the cell. This protein denaturation would then induce the heat shock response in much the same way that heat shock is thought to.

Further indication that HSF activation is a result of loss of hsp function comes from investigation of the attenuation of the heat shock response. During a constant heat shock, HSF binding has been shown to attenuate (Abravaya et al., 1991). It is thought that this effect is due to the newly synthesized hsps which interact with HSF thus inactivating it. During exposure to salicylate however there is no attenuation of the response (K. Wong, and T. Westwood, unpublished results), possibly due to the lack of hsp synthesis/function in the cell. HSF binding and chromosome puffing are maintained throughout exposure to salicylate. To test whether this lack of attenuation is due to the lack of hsps, further experiments with CN<sup>-</sup> and DNP could be performed. It is expected that these inducers will cause hsps to be synthesised as they induce expression of the hsp70 gene. If the lack of attenuation of HSF binding during salicylate exposure is a result of the lack of hsps in the cell, then there may be an attenuation of HSF binding in cells treated with CN<sup>-</sup> and DNP. If, however, the theory of reduced ATP preventing hsp function is correct, it would be expected that regardless of *de novo* hsp70 synthesis, no attenuation of the response should be observed as these newly synthesised chaperones will continue to lack the ATP required for their function.



### ***Why is Only a Partial Heat Shock Response Induced?***

It is interesting that each of these inducers only activate parts of the heat shock response. As each of the three inducers induces a different subset of the parts of the heat shock response, they may have to be looked at individually before a common denominator can be identified.

#### **Salicylate**

Previously it was suggested that salicylate may not induce transcription of the heat shock genes in spite of the fact that HSF binding activity is induced because of the decreased ATP levels in the cell. We had suggested that since certain members of the basal transcriptional machinery, particularly the MO15, ERCC2, and ERCC3 subunits of TFIIF require ATP for their function (Seroz et al., 1995), the reduced ATP levels may prevent transcription from occurring. We also suggested that the lack of transcription may have been due to the lack of HSF hyperphosphorylation during salicylate exposure. These explanations had appeared to be valid at the time, and would also have explained salicylate's ability to inhibit heat induced transcription of the heat shock genes. Our further experiments, however, would indicate that these hypotheses are no longer valid. First, the theory that reduced ATP prevented transcription of the heat shock genes can no longer be used to explain what is happening with salicylate. This is evident from the observation that despite the fact that both DNP and CN<sup>-</sup> dramatically lower ATP levels, even more so than with salicylate, hsp70 gene transcription was observed (see Figures 12 and 13). This would imply that there is sufficient ATP present in the cells during exposure to salicylate to drive the transcription process. Second, our alternate explanation that lack of hsp70 transcription was due to the lack of HSF

being hyperphosphorylated would also appear to be invalid. As was seen with DNP, HSF was not hyperphosphorylated and yet transcription of the hsp70 gene was observed (see Figures 12 and 16). Thus, it appears that the hyperphosphorylation of HSF is not required for hsp70 gene expression. This is contradictory, however, to a recent publication which has demonstrated that the lack of hsp70 gene expression seen during salicylate exposure could be overcome by measures which caused HSF to become hyperphosphorylated. Addition of the phosphatase inhibitor calyculin A caused HSF hyperphosphorylation in the presence of salicylate (Xia and Voellmy, 1997). When cells were pretreated with calyculin A prior to salicylate exposure, salicylate gained the ability to strongly induce expression from a heat shock reporter gene (Xia and Voellmy, 1997). These contradictions might be explained in that these authors utilized an artificial assay system, in a human cell line. Specifically, it was the expression of a transfected chloramphenicol acetyl transferase reporter gene under the control of a heat shock promoter which was assayed. It is not possible to ensure that there are no differences between such a system and a *Drosophila in situ* system.

How then can we explain the fact that salicylate induces HSF binding but not transcription of the heat shock protein genes? It is quite possible that this question will not be fully answered until the actual means by which HSF activates transcription is understood. It has been demonstrated previously, that salicylate has numerous inhibitory functions inside of the cell (refer to Table 2). It is possible that the inhibitory effect of salicylate on the heat shock response may be related to one of its many other functions. Once it is understood how HSF and the basal transcriptional machinery interact, it may be possible to determine if salicylate in some way interrupts this interaction. Salicylate can modify protein functions in

the cell and may interfere with HSF itself or with one or more of the members of the heat shock response. It is also still not known the means by which HSF becomes fully activated. Salicylate could conceivably interfere with this activation process preventing HSF from taking on a fully active conformation.

### DNP

DNP appears to fully induce the heat shock response with the exception of the hyperphosphorylation of HSF. Although puffing and protein synthesis were not examined in this thesis, both have previously been reported to be induced by DNP in *Drosophila* (Koninkx, 1976). It is apparent from the data obtained with DNP that heat shock gene transcription can occur during conditions of reduced intracellular ATP. In addition, the DNP data also suggests that hyperphosphorylation of HSF is not a prerequisite for heat shock gene transcription (Figures 12 and 16). There are a few possible explanations for this. If the hyperphosphorylation that accompanies heat shock is due to an activated kinase, it is possible that this kinase is strictly heat inducible. It is also possible that the hyperphosphorylation of HSF is due to the inactivation of a phosphatase. Once again, if this is the situation, there may be a heat labile phosphatase which is unaffected by inducers other than heat. Finally, decreases in intracellular ATP may cause a shift in the equilibrium between phosphatase and kinase activities. Lowered ATP levels would reduce the amount of ATP available to kinases, and thus may lower the amount of phosphorylation of HSF.

We conclude that HSF hyperphosphorylation is not an absolute prerequisite for transcription of hsp70, at least not when HSF is induced by DNP. It is not clear, however, whether or not hyperphosphorylation plays any role in the activation of hsp70 gene

expression. It is possible, for example, that the hyperphosphorylation of HSF is required for heat induced transcription of the heat shock genes but not during DNP induced stress, or that maximal induction of the heat shock gene requires the hyperphosphorylation of HSF.

The hypothesis that hyperphosphorylation of HSF might enhance expression of the hsp70 gene is supported by some recent experiments. Hyperphosphorylation of HSF has been demonstrated to prolong the time period for which HSF remains bound to HSE (Xia and Voellmy, 1997). In this thesis, DNP was shown to induce hsp70 gene expression, but at a level that was considerably lower (50%) than what is seen with heat shock induced expression (Figure 13). If DNP is preventing HSF hyperphosphorylation, it may not allow maximal expression of this gene. This might also explain the fact that during a combined heat shock and DNP exposure, the level of hsp70 gene expression is lower than that obtained for a heat shock alone.

#### CN<sup>-</sup>

Cyanide was found to induce HSF binding and hsp70 gene transcription. Polytene chromosome puffing, HSF hyperphosphorylation and hsp synthesis were not examined. It has previously been demonstrated that CN<sup>-</sup> over a wide range of concentrations (0.05 to 10 mM) is not capable of inducing polytene chromosome puffing (Behnel and Rensing, 1975; Leenders and Berendes, 1972). Why there is no puffing of the chromosomes is unclear. This does however underscore the fact that chromosomal puffing cannot be used as an indication of transcription for the heat shock genes. It will also be interesting to determine if CN<sup>-</sup> does in fact induce the synthesis of hsps in *Drosophila*. It may be possible that puffing requires a certain minimal amount of ATP to be present. We previously observed that high

concentrations of salicylate (30 mM) which decreased levels of ATP more than intermediate concentrations (10 mM) also produced smaller heat shock puffs (Winegarden et al., 1996). This hypothesis might be addressed by examining much lower concentrations of CN<sup>-</sup>, that reduce ATP to a level comparable to salicylate or DNP.

It is clear that there may not be one single explanation as to why these inducers only activate parts of the heat shock response. It is likely the case that each of these agents operate under slightly different conditions as they each operate through unique means. Although they have a common effect of reducing intracellular ATP, the differences in the means by which they do so might account for the differences in the stress response elicited during exposure to these agents. Also, each of these agents might be affecting other processes in the cell that in some way contributes to the activation of the heat shock response.

#### *Is Reduced ATP a Signal to Induce the Heat Shock Response?*

It is unlikely that reduced ATP is “the” common signal for induction of the stress response. Heat shock does reduce ATP levels but only to a level which is similar to 3 mM salicylate. As was seen with this concentration of salicylate, there is very little activation of the heat shock response. If heat shock was to activate the response via reduced ATP, 3 mM salicylate should also activate the response to a level similar to heat shock (unless salicylate is inhibiting other cellular process as well). We cannot rule out the possibility, though, that heat induced reductions in ATP levels does contribute to the activation of HSF, however, it is more likely that different classes of inducers work via different means. While metabolic inhibitors may function to activate the stress response via reduced ATP levels, other inducers such as heat, ethanol and amino acid analogues are likely to exert their effect through formation of a

denatured protein pool. Indeed, reduced ATP levels may also be creating “abnormal” proteins by interfering with the ATP dependent chaperones. While although it is unlikely that all inducers function to activate the heat shock response via decreases in intracellular ATP, it is conceivable that a number of inducers do function by this action. There is an indication that the activation of the heat shock response (as judged by HSF binding activity measurements) is temporally related to the decrease in intracellular ATP caused by the three agents examined in this investigation.

### **B. Future Directions**

As was previously mentioned, it is possible to formulate a theory as to how reduced ATP might activate the heat shock response. However, whether reduced ATP is sufficient to activate the response is not conclusively known at this time. Briefly, our theory extends on the already popular model that HSF activity is regulated by one or more of the heat shock gene products (see Figure 17). This theory states that constitutively active hscs/hsp maintain HSF in its inactive conformation through transient interactions until the onset of stress. At this point, the hscs/hsp are required elsewhere in the cell to repair denatured and aggregated proteins, and thus HSF is left to take on an active conformation. Our extension of the theory attempts to account for the possible activation of HSF via lowered intracellular ATP. We suggest that because hsp/hsc function is ATP dependent, the loss of ATP may prevent these chaperone molecules from maintaining HSF in its inactive form.

How might this theory be tested further? Unfortunately this is not a simple problem to examine. In order to test the theory, an exogenous source of ATP could be provided. In doing so it could be determined whether the onset of the response during exposure to one of the ATP depleting drugs could be delayed or prevented. Unfortunately, ATP will not cross

biological membranes in an intact form (ATP is metabolized and then transported across the membrane), so it is not possible to add exogenous ATP to cells during exposure to one of the agents which decrease intracellular ATP. Another method of delivering ATP to cells in an exogenous form would be to using cell permeablising agents. Once again a problem exists in that agents which permeabilise cell membranes, which would in turn allow for passage of ATP into cells, will also likely activate the heat shock response themselves, thus this method cannot be employed. Additionally, it may be possible to examine the response *in vitro*. Certain inducers of the heat shock response are capable of activating HSF binding *in vitro* (Mosser et al., 1990). If one of the three agents examined were capable of inducing binding *in vitro*, exogenous ATP could be added to a lysate to test for a delayed activation of HSF. It is unlikely, however, that these agents will function *in vitro* as they are functioning via an inhibition of mitochondrial function which is likely to be compromised in a cell lysate. Microinjection of ATP into cells is a further possibility, however, the act of microinjection itself may cause undue stress on the cells and cause activation of the stress response. Finally, ATP levels may be increased in cells prior to adding one of the chemical agents. By adding a precursor to ATP production such as phosphocreatine, intracellular ATP might be elevated.

The answer may rely on analysis of the recovery of these cells from stress. As has been demonstrated with salicylate, this response is reversible. Salicylate can be washed out of cells, and HSF binding is attenuated. Recovery profiles for both HSF binding and ATP levels may indicate if recovery of these two events is also temporally linked. Although this will not conclusively prove a role of ATP reduction, it will strengthen the argument that it is a possible signal for activation of the heat shock response.

Further evidence might come by studying how HSF is attenuated (i.e. converted from the trimer to the monomer). By purifying an activity which converts trimeric HSF to monomeric HSF and identifying its components one would either lend support to, or discount the involvement of hsp's in the regulation of HSF. The role of ATP during this process could also be examined.

This study has indicated that it may be possible to identify a common signal for specific classes of inducers. It also suggests that it is unlikely that there is one "universal" signal. In order to fully understand the activation and control of the heat shock gene expression, it will be necessary to fully analyse several of these inducer classes. In addition, there are many important questions to be answered regarding how HSF activates transcription of the heat shock genes. The proteins involved in HSF activation will probably have to be identified in order to fully understand the events required to activate HSF to its fully transcriptionally competent form.



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## **APPENDICES**

## APPENDIX 1

**Abbreviations used:**

**ADP** - adenosine diphosphate  
**AMP** - adenosine monophosphate  
**ATP** - adenosine triphosphate  
**BSA** - bovine serum albumin  
**cAMP** - cyclic adenosine monophosphate  
**cDNA** - complementary DNA  
**CN** - cyanide  
**CPM** - Counts per minute  
**C-terminal** - carboxy terminal  
**DEPC** - Diethylpyrocarbonate  
**DNP** - 2,4 dinitrophenol  
**DTT** - dithiothreitol  
**EDTA** - ethylenediamine tetra-acetic acid  
**EGTA** - ethyleneglycol-bis(B-aminoethyl ether) N,N,N',N' tetraacetic acid  
**EMSA** - electrophoretic mobility shift assay  
**FBS** - fetal bovine serum  
**H2B** - histone 2B  
**HEPES** - N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid  
**HOQNO** - 2-*n*-heptyl-4-hydroxy-quinoline-*N*-oxide  
**hscs** - heat shock cognates  
**HSE** - Heat Shock Element  
**HSF** - Heat Shock Factor  
**hsps** - heat shock proteins  
**I- $\kappa$ B** - Inhibitor of  $\kappa$ B  
**iNOS** - inducible nitric oxide synthase  
**K** - potassium  
**MAPK** - Mitogen Activated Protein Kinase  
**mRNA** - messenger RNA  
**Na** - sodium  
**NF- $\kappa$ B** - Nuclear factor  $\kappa$ B  
**N-terminal** - amino terminal  
**PAGE** - polyacrylamide gel electrophoresis  
**PMSF** - phenylmethylsulfonyl fluoride  
**ppi** - pixels per inch  
**SDS** - Sodium dodecylsulfate  
**SL2** - Schnieder Line 2  
**TB1** - transcription buffer 1  
**TBE** - Tris-borate-EDTA  
**TE** - Tris-EDTA  
**TES** - Tris-EDTA-SDS

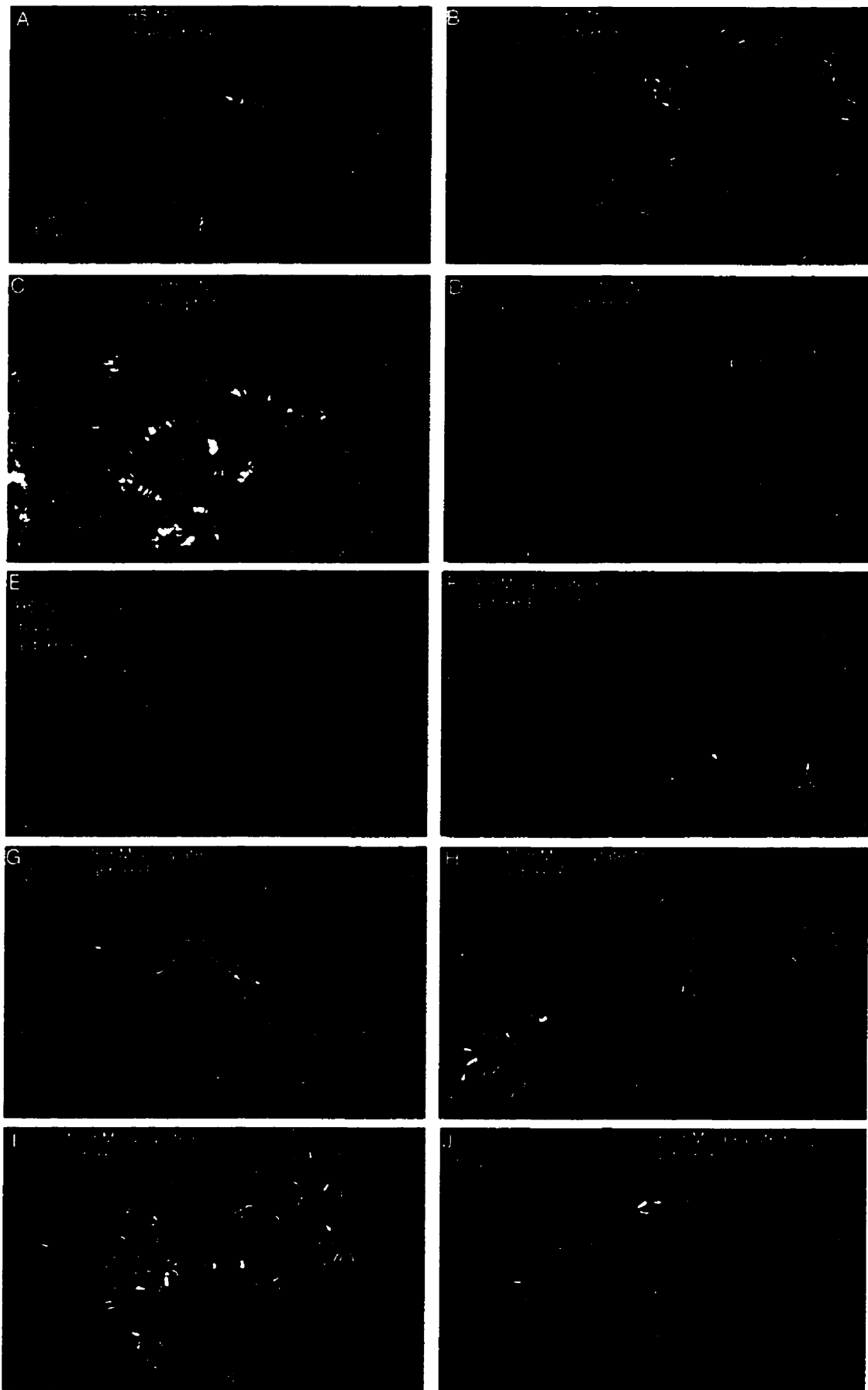
## **APPENDIX 2**

**Figures From Winegarden et al., 1996**

**All Experiments and Figures in this Appendix are the Work of Ken Wong  
and have been included with his permission.**

**Figure A1. Sodium salicylate induces HSF binding and heat shock puffs in the polytene chromosomes of *Drosophila* third instar salivary glands.**

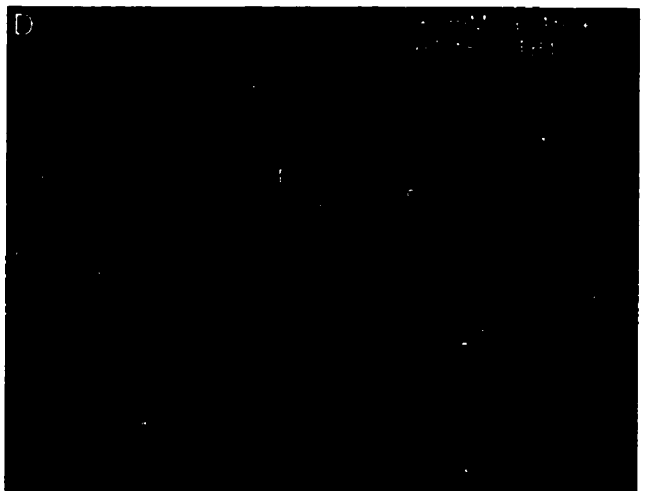
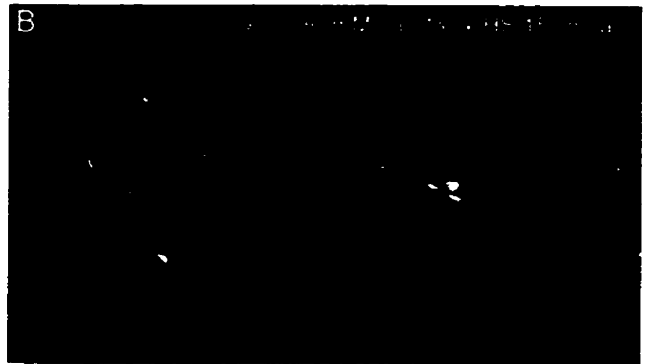
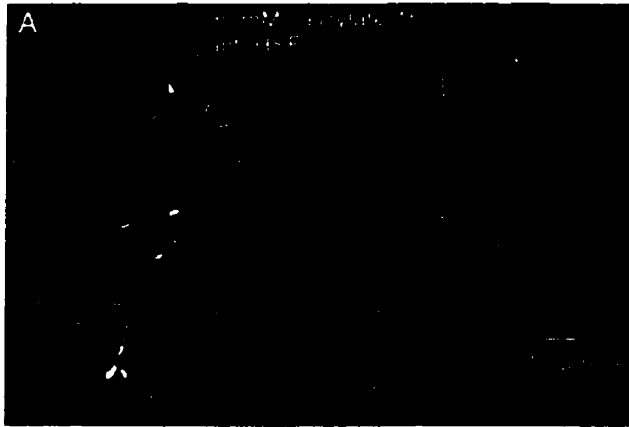
Salivary glands from *D. melanogaster* third instar larvae were dissected in TB1 buffer and kept in organ culture for a minimum of 60 minutes prior to stress treatment. Chromosomal squashes were prepared from control glands (21 °C, C and D), heat-shocked glands (15 minutes, 36.5 °C, A and B), or glands treated with 3, 10, 30 mM sodium salicylate (E-J). The duration of exposure to salicylate is indicated. Chromosomes were stained for DNA using bisbenzimidazole (Hoescht) (A and C) and for HSF using a rabbit anti-HSF primary antisera (943) followed by a goat anti-rabbit fluorescein isothiocyanate labeled secondary antibody (B, D-I). 87C is the location of three copies of the hsp70 gene. The bar represents 10 μm.



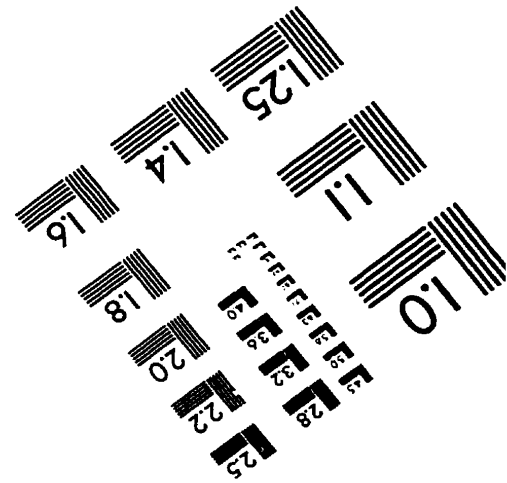
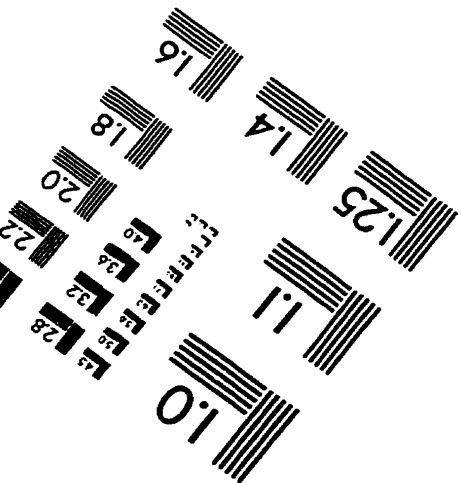
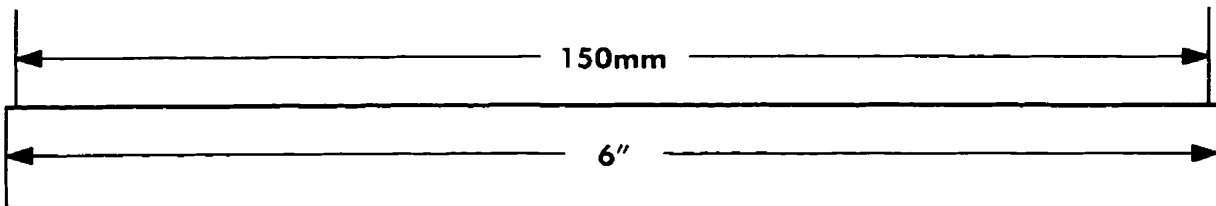
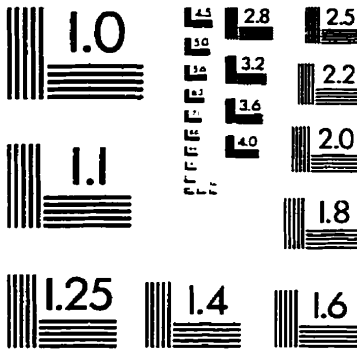
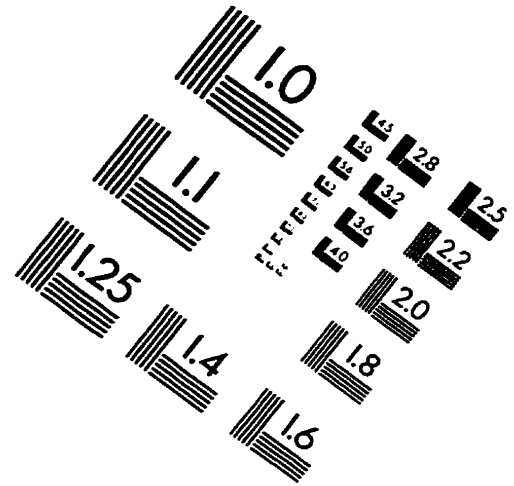
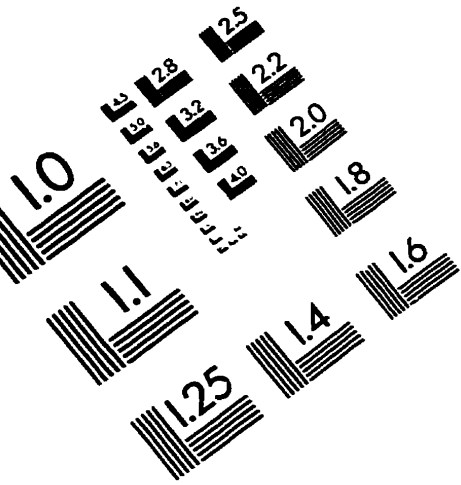


**Figure A2. 30 mM sodium salicylate prevents induction of the heat shock puffs by heat.**

Salivary glands were dissected as described in Figure A1 and chromosomal squashes prepared from glands treated with 30 mM salicylate (A), 30 mM salicylate plus a heat shock (15 min, 36.5 °C) in the presence of salicylate (B), 30 mM salicylate followed by a heat shock in the absence of salicylate (C), and 30 mM salicylate followed by an incubation in normal buffer at the control temperature (D). HSF was stained as described in Figure A1. The *bar* represents 10 μm.



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