STUDIES ON AN INDUCIBLE GENE SYSTEM:
THE HEAT SHOCK RESPONSE IN TROUT CELLS

by

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B.Sc., The University of British Columbia, 1979

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Department of Biochemistry

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June 1984

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Date June 19, 1984
Abstract

The heat shock phenomenon has been characterized in cultured fibroblasts of the rainbow trout, *Salmo gairdnerii*. The response was elicited by one of two methods: temperature elevation or sodium arsenite exposure. The stress situations resulted in the rapid expression of a set of novel polypeptides, the heat shock polypeptides (hsp), normally absent in trout cells. At least six hsp have been identified and molecular weights assigned; these are referred to as hsp30, hsp32, hsp42, hsp62, hsp70, and hsp87. Translational control on pre-existing mRNAs was observed in cells under prolonged arsenite exposure. The heat shock response is a reversible process in trout cells.

Two cDNAs, THS70.7 and THS70.14, encoding partial information for two distinct species of trout hsp70 were isolated and characterized. These sequences are identical at 73.3% of the nucleotide positions in their regions of overlap, and their degree of sequence conservation at the polypeptide level is 88.1%. The two derived trout hsp70 polypeptide sequences show extensive homology with amino acid sequences for hsp70 from *Drosophila* and yeast. Southern blot analysis of trout testis DNA reveals a small number of bands hybridizing to the hsp70 genes in this species. The trout hsp70 cDNA sequences cross-hybridize with restriction fragments in genomic DNA from HeLa cells, bovine liver, nematodes, and *Drosophila*. 
Northern blot analysis of RNA from arsenite-induced RTG-2 cells (the trout cell line), using the trout hsp70 cDNAs as probes, reveals the presence of three hsp70 mRNA species. Both heat shock and sodium arsenite result in rapid synthesis of trout hsp70 mRNA. Similarly, the repression of hsp70 mRNA is very rapid, especially during recovery from a temperature stress.

An artifact of cDNA cloning was identified, i.e. an IS-element (named T31) was isolated and characterized as originating from a trout cDNA library. However, further analysis proved T31 to be a prokaryotic mobile element that had inserted itself into pBR322 during the preparation of the cDNA library.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bisacrylamide</td>
<td>N,N'-methylene bisacrylamide</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAA</td>
<td>cas-amino acids</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>chloroform</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DAP</td>
<td>dianiminopimelic acid</td>
</tr>
<tr>
<td>ddNTP</td>
<td>dideoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethylsulphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetate, disodium salt</td>
</tr>
<tr>
<td>Gu•HCl</td>
<td>guanadninum chloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HZ</td>
<td>hydrazine</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthiogalactoside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>Kd</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>mA</td>
<td>milliamperes</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NC</td>
<td>nitrocellulose</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>RF</td>
<td>replicative form</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPC</td>
<td>reverse phase chromatography</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N',-tetramethylethylene diamine</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>W</td>
<td>watts</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
</tbody>
</table>
Acknowledgements

First and foremost, I would like to acknowledge the support of my supervisor, Dr. Peter Candido, whose patience and invaluable discussions were greatly appreciated. Second, the assistance of many colleagues played a crucial role in the development of this thesis; my thanks to Jane Baker, Balwant Bhullar, Jeff Hewitt, Chris Kreis, Anne Rose, Colin Hay, Elizabeth Burgess, Roland Russnak, Rob Kay, and in particular Don Jones (without his help, many parts of this thesis would not have been possible). Third, I thank the members of the Biochemistry Department for creating an exciting environment in which to work and play. Last but not least, I thank Ms. Debbie Bunyak for typing this thesis.
DEDICATION

to

my parents

for waiting so long
1.1 The Heat Shock Response: General Characteristics

The heat shock response provides an excellent system for the study of the processes which accompany rapid gene induction in eukaryotic cells. When organisms are subjected to a heat-shock, transcription of most genes is suppressed and the expression of a novel set of proteins is enhanced (reviewed in 1-3). These induced proteins are termed the heat-shock polypeptides (hsp5). A variety of agents also elicit the same response, suggesting that the heat shock response is probably a reaction to metabolic stress rather than to temperature per se. However, since the response of cells to different stress stimuli is so similar, the term "heat shock response" is used to describe the general phenomenon. Similarly, "heat-shock polypeptides" is used to describe the proteins induced by these different agents.

In addition to the rapid induction and transcriptional control, the heat shock response is characterized by the presence of a translational control mechanism. Finally, the heat shock response is characterized by its highly conserved nature. The phenomenon is present in all organisms studied and homology at the molecular level is observed.

1.2 Historical Perspective

The discovery of the heat shock response dates back to 1962, when F. Ritossa first observed the changes in puffing patterns of the polytene chromosomes of Drosophila busckii upon temperature elevation (4). This
discovery was followed up by reports characterizing the puffing activity induced by heat-shock (5-8). Thus, for almost ten years after its discovery, most of the data on the heat shock response came from cytological studies on the polytene chromosomes of Drosophila salivary glands. Due to the limitations of the available techniques, studies of the induction mechanism or of the function of the heat shock response were not attempted.

In 1974, Tissières et al. (9) reported the initial results from studies on the molecular effects of the heat shock response. Dramatic changes in protein synthesis were correlated with the puffing activity of polytene chromosomes from heat-shocked Drosophila. These changes included heat-shock induced synthesis of a novel set of polypeptides and repressed synthesis of the normal complement of proteins. Soon after, heat-shock induced puffs were shown to be sites of active genes responsible for the production of hsp mRNA (10-13). The cloning of DNA from these puff sites was soon to follow and the first heat-shock induced sequences to be cloned were the αβ-repetitive units of the 87C1 locus (14, 15). As it turned out, the αβ units did not code for any of the known hsp genes. Genes coding for the hsp genes were soon cloned and analyzed (16-19).

It was 1978 before studies on the heat-shock phenomenon in organisms other than Drosophila began in earnest. Since then, the occurrence of the heat shock response in a wide variety of organisms has been reported (reviewed in ref. 1, also see Table II).

1.3 Mechanisms of Induction

As the name implies, the heat shock response is induced by exposure of
cells to slightly elevated temperatures. However, the response is not limited to temperature elevation; a growing list of alternative stimuli are being discovered (see Table I). Due to the variety of inducing agents and the rapid response of cells to these perturbations, the existence of a common cellular target is likely.

### TABLE I. Inducers of the Heat Shock Response

<table>
<thead>
<tr>
<th>Agent</th>
<th>Organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylated nucleotides</td>
<td>Salmonella typhimurium</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>21</td>
</tr>
<tr>
<td>Amino acid analogs</td>
<td>Mammalian cells</td>
<td>20</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Drosophila</td>
<td>5</td>
</tr>
<tr>
<td>Cold</td>
<td>Tetrahymena</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Rana cultured cells</td>
<td>23</td>
</tr>
<tr>
<td>Chelating agents</td>
<td>Chick embryo cells</td>
<td>24-26</td>
</tr>
<tr>
<td>Deciliation</td>
<td>Tetrahymena</td>
<td>27</td>
</tr>
<tr>
<td>Ecdysterone</td>
<td>Drosophila</td>
<td>28</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Chinese hamster cells</td>
<td>29</td>
</tr>
<tr>
<td>Heat</td>
<td>Drosophila</td>
<td>4</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>Chick embryo cells</td>
<td>30</td>
</tr>
<tr>
<td>Pyrogens (e.g. LSD)</td>
<td>Rabbit</td>
<td>31</td>
</tr>
<tr>
<td>Recovery from anoxia</td>
<td>Drosophila</td>
<td>5</td>
</tr>
<tr>
<td>Stress</td>
<td>Rat</td>
<td>32</td>
</tr>
<tr>
<td>Sulfhydryl oxidants</td>
<td>Drosophila</td>
<td>21</td>
</tr>
<tr>
<td>Uncouplers of oxidative</td>
<td>Drosophila</td>
<td>4, 30</td>
</tr>
<tr>
<td>phosphorylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral infection</td>
<td>Adenovirus/HeLa cells</td>
<td>33</td>
</tr>
</tbody>
</table>

The processes of electron transport and oxidative phosphorylation would seem to be targets for many of the inducing agents listed in Table I. Indeed, early reports suggested that mitochondria were involved in the induction mechanism (34, 35). However, the precise role of mitochondrial functions in the heat shock response has yet to be determined.

Attempts have also been made to isolate pre-existing factors capable of inducing heat-shock genes. These include the mitochondrial factors that induce the heat-shock puffs in *D. hydii* (35) and a protease-sensitive, heat
labile factor that specifically activates Drosophila heat-shock genes in vitro (36, 37). These in vitro studies, where nuclei from normal cells are incubated with cytoplasmic extracts from heat-shocked cells, strongly support the possibility that protein factors may induce heat-shock genes (36-39). As to how these pre-existing factors are modified upon heat-shock remains to be determined.

The heat shock response is such a complex phenomenon that a simple one step induction mechanism has to be ruled out. A number of changes are observed soon after a heat-shock. One of these is the translocation of a major pre-existing cytoplasmic protein to the nucleus in heat-shocked Drosophila Kc cells (40). What role it may have, structurally or otherwise, is unknown. Three DNA binding proteins that are sequence specific for a 5'-noncoding region in the Drosophila hsp70 gene have been identified (41). However, since these proteins are present in both normal and heat-induced cells, their role in gene activation is not clear. Thus, although the presence of protein factors influencing the heat shock response are being identified, their mode of action or indeed how they were activated in the first place remains obscure.

Lee et al. (21) have recently suggested that the common factor among inducers of the heat shock response may be the development of an "oxidation stress" in cells. During the stress, adenylated nucleotides accumulate in the cell and may serve to trigger the heat shock response. This intriguing hypothesis could explain the widespread existence of hsps in both prokaryotic and eukaryotic cells, since the necessity for protection from the adverse physiological effects of excess intracellular oxygen presumably dates from early evolutionary times.
In addition to the pre-existing factors, there is evidence that the transcriptional and translational control of the heat-shock genes is autoregulatory, i.e. the concentration of functional hspS determines the activity of the heat-shock genes (42). The theory put forward is that in a normal cell a low amount of hspS is present, their function being to ensure that the heat-shock genes are not activated. However, if certain conditions inactivate the hspS or cause some cellular targets to increase the concentration and/or affinity of hsp binding sites, then there would be a rapid induction of the heat shock response. Presumably, as enough hspS are synthesized the activity of heat-shock genes would decrease. Other variables that affect the induction mechanism include the intensity, duration, and nature of the stress (42-44).

Finally, adding further complexity, pre-existing mRNAs are subject to translational control during induction of the heat shock response. Upon exposure to the stress, the pre-existing mRNAs are not degraded but maintained in an inactive state, enabling the heat-shock mRNAs to be rapidly translated (13, 45-47). Yeast cells are an exception in that they do not display translational control of pre-existing mRNAs and seem to degrade them (48). An interesting feature of the heat shock response in Xenopus oocytes is that the appearance of its hspS is due not to new transcriptional activity, but to the activation of translation of stored hsp mRNAs (47). Thus, it is evident that different cells have altered the heat shock response to suit their own needs.

1.4 Heat-Shock Proteins

The induction of protein synthesis by heat-shock has been primarily
studied in the fruit fly, *D. melanogaster*. However, this phenomenon has now been observed in a wide variety of organisms (see Table II).

**TABLE II. Occurrence of the Heat Shock Response**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference</th>
<th>Organism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoebae</td>
<td>49</td>
<td>Myogenic avian cells</td>
<td>56</td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>50</td>
<td>Salmon embryo cells</td>
<td>57</td>
</tr>
<tr>
<td>Chicken embryo</td>
<td>20, 52</td>
<td>Sea urchin embryos</td>
<td>58</td>
</tr>
<tr>
<td>fibroblasts</td>
<td></td>
<td>Slime mold</td>
<td>59</td>
</tr>
<tr>
<td>Chinese hamster</td>
<td>51</td>
<td>Soybean</td>
<td>60, 61</td>
</tr>
<tr>
<td>ovary cells</td>
<td></td>
<td>Tobacco tissue</td>
<td>61</td>
</tr>
<tr>
<td>Chironomus</td>
<td>53</td>
<td>culture cells</td>
<td></td>
</tr>
<tr>
<td>Drosophila</td>
<td>4</td>
<td>Tetrahymena</td>
<td>62</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>54</td>
<td><em>S. cerevisiae</em></td>
<td>63</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>55</td>
<td>Xenopus</td>
<td>47</td>
</tr>
<tr>
<td>Several mammalian</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell lines</td>
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</table>

The major hsps of most organisms fall into three classes: the small hsps (15 to 30 Kd), the hsp70-like (60 to 70 Kd), and the hsp83-like (80 to 90 Kd) polypeptides. These three classes of hsps are strongly conserved.

Antibodies against the hsp70 and hsp89 from chicken embryo fibroblasts cross-react with their counterparts from a wide variety of organisms (64, and E.A. Burgess, personal communication). Similarly, cross-hybridization of hsp70 genes from different organisms has been observed (50, 57, 65-68).

The small hsps from both *D. melanogaster* and *Caenorhabditis elegans* share extensive amino acid sequence homology with the mammalian α-crystallins (69, 70).

1.4.1 Heat-Shock Protein Variants

The number of hsps induced during stress has turned out to be greater than originally suggested. In *Drosophila*, when these proteins were
examined by high resolution 2-dimensional gel electrophoresis, the actual number of hsp variants turned out to be close to 50 or more (71). Similarly, more than 20 hsp variants have been observed in rainbow trout tissue culture cells (E. A. Burgess, personal communication). Most of these variants fall into one of the three classes mentioned above and may be a result of post-translational modification of an initial polypeptide. The existence of multigene families for particular hsp variants has been observed and thus a number of the protein variants could be products of the closely related genes. In either case, the large number of hsp variants shows the complexity of the heat shock response.

Wang et al. (72) have shown that hsp83 and hsp68 of arsenite treated chicken embryonic cells are methylated. They also suggest that this methylation of hsp variants is not due to an activation of pre-existing protein methylases. Another alteration of hsp variants is via phosphorylation (64, 73, 74).

1.4.2 Intracellular Localization of the hsp

In order to understand the functions of the hsp variants, their intracellular localization has been examined. Studies on this aspect have been done using a variety of techniques including sub-cellular fractionation by centrifugation (40, 75-77), microdissection of cells (53, 78), direct autoradiographic analysis (75, 78, 79), and immunofluorescence staining (80-82). In general, the small hsp variants are found in the nucleus, the hsp70-like proteins in both the cytoplasm and the nucleus, and the hsp83-like proteins exclusively in the cytoplasm. In Drosophila, the nuclear hsp variants are highly resistant to extraction with high salt concentrations and are associated with chromatin and nucleoli preparations (40, 75, 76). Upon return of the Drosophila cells to normal temperatures,
the nuclear hsp return to the cytoplasm (75). Although these early results suggested an association of the hsp with the chromatin, recent data imply a different nuclear role for some of these proteins. For instance, Sinibaldi and Morris (77) have reported that the binding of certain hsp appeared to be primarily to the nuclear scaffold rather than to the chromatin of heat-induced Drosophila Kc cells. In addition, immunofluorescent staining of chicken embryo fibroblasts with antibodies to their hsp suggests that chicken hsp89 is strictly cytoplasmic, whereas chicken hsp70 and hsp24 appear to be present throughout the cell in association with its cytoskeleton (80, 81). Indirect immunofluorescence techniques have shown the hsp100 of certain mammalian cells to be localized in the Golgi apparatus (82). The possible association of the heat shock response with electron transport was discussed earlier; results conflicting with this idea came from autoradiographic and electron microscopic analysis of hsp distribution in heat-induced Drosophila. Velazquez et al. (79) showed that insignificant amounts of the hsp are found in association with mitochondria.

1.4.3 Function of the Heat-Shock Proteins

Thus far, hsp characterization has not revealed the true nature of their roles; however, since these proteins occur in such diverse organisms, their roles are likely to be of a fundamental nature within cells. A general homeostatic function was postulated for the heat shock response fairly early in its study (83). In the past three to four years, studies on a variety of organisms have led to the conclusion that hsp confer thermotolerance upon cells (29, 84-88). Induction of hsp by means other than heat has also been shown to induce transient thermotolerance in
Chinese hamster fibroblasts (29). Additional support for the protective function of the hsps comes from studies on Dictyostelium, where a mutant strain unable to express hsps is also unable to induce thermotolerance (87). An interesting hypothesis was put forth by Minton (89), who suggested that hsps might contribute to enhanced thermotolerance in cells by non-specifically stabilizing stress-susceptible proteins.

Other functions for hsps include a regulatory role. In Drosophila, the absence of functional hsps (and particularly hsp70) prevents cells from attaining complete recovery after heat-shock (42).

1.5 Heat-Shock mRNAs

During the heat shock response, the dramatic alteration in the pattern of protein synthesis is accompanied by a parallel change in the distribution of ribosomes on polysomes (10). Pre-existing polysomes rapidly dissociate and the ribosomes then form new polysomes. Despite these changes, pre-existing mRNA is still found in the cytoplasm of heat-shocked cells and can be translated \textit{in vitro} (13, 45-47). Thus the selective translation of hsp mRNA in heat-shocked cells implies a specific recognition of hsp mRNA by the ribosomes.

Ballinger and Pardue (90) have shown pre-existing mRNAs are present in heat-shocked Drosophila cells, associated with ribosomes and RNP particles. They suggest that the translation of the 25°C mRNAs may be repressed by a selective inhibition of their elongation. The same conclusion was reached by Thomas and Matthews (91) from their studies on heat-shock mRNAs of HeLa cells. There is one reported structural difference between 25°C mRNA in control and induced Drosophila cells. The
25°C mRNAs in heat-shocked cells lack the ability to bind to oligo[dT]-cellulose, suggesting an absence of the normally present poly A+ tail (45). Unlike *Drosophila*, when yeast cells are induced by heat, the pre-existing mRNAs are not sequestered on polysomes but are allowed to degrade at their normal rates (48).

A characteristic of hsp mRNAs, at least in *Drosophila*, is the presence of an unusually long 5'-noncoding region. This region varies from 111 bp to 253 bp in length (92-94). The leader sequences of hsp70 and small hsp mRNAs from *Drosophila* have an unusually high (approximately 50%) adenosine content (92, 93); in contrast, the adenosine content of the 5'-noncoding region of hsp83 is much lower (94). The long leader sequences of hsp mRNAs may play a role in the selective translation of these messages.

Although the expression of all heat-shock genes appears regulated in a similar manner, there are exceptions. For instance, in *Drosophila*, the hsp83 polypeptide appears to be present in normally growing cells (43, 45, 71). In addition, the hsp83 gene is the only known *Drosophila* heat-shock gene to contain an intervening sequence (17). The only other known heat-shock gene to contain an intron is the hsp16 gene of *C. elegans* (R. Russnak, personal communication).

### 1.6 Organization of the Heat-Shock Genes

#### 1.6.1 The Heat-Shock Genes

Although the heat shock response has been studied in a number of organisms (see Table II), the organization of the hsp genes has been primarily studied in *Drosophila*. All the major heat-shock genes in *D. melanogaster* have been cloned, mapped, and sequenced (16-19, 69, 92-102).
These genes fall into three classes: the hsp83 gene which is present in a single copy at chromosomal locus 63BC (17), the hsp70 genes which are present in five copies, two at locus 87A and three at locus 87C (17, 98), and the small heat-shock genes coding for hsp22, 23, 26, 27, all present at locus 67B within an 11 Kb region (18, 19, 101). In addition, the hsp68 gene is present in a single copy at locus 95D (17). Each of the hsp70 genes is organized within a 2.5 Kb conserved element consisting of a 2.1 Kb mRNA coding region, and a 0.4 Kb 5' region which is not transcribed (96, 97, 99). The hsp70 genes at locus 87A are approximately 1.7 Kb apart and in opposite orientation (101, 103). Two of the hsp70 gene copies at 87C are in a tandem repeat separated from the third gene by about 40 Kb of DNA that contains the αβ-repetitive units (104). Due to the similarity of these five hsp70 genes in D. melanogaster, it has been suggested that the genes at 87A and 87C are not evolving independently and that gene conversion has occurred both within and between hsp70 loci (105).

As mentioned earlier, all four small hsp genes in D. melanogaster are present in single copies within an 11 Kb region at locus 67B. These four genes have partial homology among themselves (69) and may thus be a result of duplications of an ancestral gene.

Very little sequence information about the hsp genes from other organisms has been reported. The available information can be summarized briefly. The nucleotide sequence of an inducible hsp70 gene from yeast has been reported (65). It shows about 72% sequence homology to Drosophila hsp70 at the protein level. Very recently, Bardwell and Craig (68) reported the nucleotide sequence of the E. coli heat-inducible dnaK gene and showed it to be 48% identical at the polypeptide level to the hsp70 protein of Drosophila. The only other reported sequences for hsp genes is...
by Russnak et al. (70) who presented the cDNA sequences coding for hsp16 from *C. elegans*. Interestingly, the *C. elegans* hsp16 and the small hsp5 from *D. melanogaster* both share extensive amino acid sequence homology with the mammalian α-crystallins (68, 70). Study of the genomic organization of *C. elegans* DNA has revealed the presence of two (and possibly more) closely linked small heat-shock genes (R. Russnak, personal communication).

1.6.2 The Heat-Shock Cognates

Several reports have described the presence of heat-shock related genes that are expressed constitutively at some time during normal development. These genes are not necessarily induced by heat-shock. The developmentally regulated heat-shock genes will be described in a separate section.

Ingolia and Craig (68, 102) have reported a number of hsp70-like genes from *Drosophila* and *S. cerevisiae*. These cognate genes are not heat-inducible and are normally expressed during development. Unlike hsp70, some of these cognate genes have been shown to have large introns (102). The function of these cognate gene products remains to be determined.

1.6.3 Chromatin Structure of the Heat-Shock Genes

The changes in chromatin structure accompanying gene activation have been expressed in terms of sensitivity to certain nucleases (reviewed in 106). There are three broadly defined levels of nuclease sensitivity: low level sensitivity of bulk chromatin, moderate sensitivity of regions encoding gene sequences extending a few Kb in either direction, and finally
a hypersensitivity involving small domains usually in the 5' or 3' flanking regions of a transcriptionally active gene. A variety of nucleases have been used to measure the sensitivity of chromatin, the most utilized ones being deoxyribonuclease I (DNAase I) and micrococcal nuclease (MNase).

The chromatin structure of the major heat-shock genes of Drosophila has been analyzed in detail (summarized in 107). Using an indirect end-labelling technique, Wu (108) has demonstrated the presence of DNAase I hypersensitive sites 5' to the hsp70 and hsp83 genes in Drosophila embryos and tissue culture cells. These hypersensitive sites are present in both normal and heat-shocked cells; however, the DNAase I sensitivity of the whole gene is increased upon induction (198). Similar DNAase I hypersensitive sites have been demonstrated at or near the 5' end of each of the four small heat-shock genes in Drosophila (109). Upon heat induction, the heat-shock genes rapidly adopt an open chromatin structure and their sensitivity to nucleases is increased (110). The adoption of the open configuration may be auto-regulated by the presence or absence of functional hsps (42).

As far as nucleosome structure is concerned, Levinger and Varshavsky (111) have reported that the hsp70 genes of Drosophila contain heavily ubiquitininated nucleosomes. This is in contrast to the general population of nucleosomes. It is suggested that the nucleosomal proteins from the activated chromosomal region may be proteolytically removed and thereby cause the increased nuclease sensitivity of the DNA observed in actively transcribing genes (111).

Recently, Karpov et al. (112) have reported the selective removal of histones from the coding region of induced hsp70 genes in D. melanogaster. This, in addition to the lack of histones at the 5' hypersensitive sites,
may be the cause of the increased accessibility to nucleases of these regions.

Mace et al. (113) have demonstrated the presence of an S1 nuclease-sensitive structure associated with short direct repeats of DNA found in the 5' flanking regions of certain D. melanogaster heat-shock genes. Since this study was done on isolated plasmids containing the cloned genes, a precise role for these S1 nuclease-sensitive structures has not been determined.

1.7 Regulation of the Heat Shock Response

1.7.1 Transcriptional Control

In order to identify the various signals necessary for proper induction and transcription of Drosophila heat-shock genes, hsp70 sequences were introduced into a variety of heterologous systems. These included mouse cells (114), rat cells (115), monkey COS cells (116, 117), Xenopus oocytes (118-120), and yeast cells (121). The expression of these genes was heat-inducible indicating that the induction mechanisms and control signals for the heat shock response are conserved between Drosophila and the tested organism. In addition to the TATA box, an upstream promoter element was discovered. From deletion studies, a 70 bp stretch of sequence in the 5' flanking region of the hsp70 gene was determined to be necessary for heat-inducibility, the region spanning -47 to -66 being absolutely necessary (116, 117). Within this short stretch of DNA lies an imperfect inverted repeat (92). Pelham (117) searched for a similar upstream promoter in other Drosophila heat-shock genes and derived a consensus sequence for this element:
An imperfect inverted repeat is evident in the sequence and could play a role in protein recognition. Deletions in this region greatly decrease the transcription of the hsp70 gene during heat-shock (117). Pelham and Bienz (119) have constructed a synthetic heat-shock promoter element and used it to confer heat-inducibility on the herpes simplex virus thymidine kinase gene. Corces et al. (122) have constructed a fusion gene hybrid containing 1.3 Kb of the 5' sequence of the Drosophila hsp70 gene joined to the entire protein-coding region of a human growth hormone gene. This hybrid gene was found to be heat-inducible in mouse cells. The use of such heat-shock promoters in fusion genes may be particularly advantageous in the controlled expression of certain genes of medical or commercial interest.

Recently, the design and construction of an in-frame fusion between the S. cerevisiae hsp90 gene and the E. coli lacZ gene has been reported (123). When this fusion gene was introduced back into yeast on a multicopy plasmid vector, it still exhibited heat-inducibility. Moreover, the fused protein product had an active β-galactosidase activity. Similarly, a Drosophila hsp70 gene was fused to the E. coli β-galactosidase gene (124) and introduced back into the Drosophila germline by the P-element microinjection method of Rubin and Spradling (125). As expected, the β-galactosidase activity in the transformants is heat-inducible. These latter results should make it easier to investigate the transcriptional and translational regulation in the heat shock response. In addition, developmental and tissue specific expression of these genes can be more readily studied.

A system that seems to have a totally different transcriptional control mechanism for the heat shock response has been described for
Xenopus oocytes (47). In this system, heat-shock mRNA are present in normal cells but are stored in an inactive state by a unique control mechanism. Upon heat-shock, these mRNAs are rapidly activated and the heat shock response proceeds as usual. This form of control is presumably an adaptation necessitated by the very large size of the oocyte, and the resulting requirement for large amounts of hspS within a very short time period, which could not be supplied by transcription.

The mechanism of induction and the function of hspS can be elucidated more readily through genetic analysis. For this purpose, the heat shock response in *E. coli* will certainly attract more attention. As mentioned above, a response to heat has been observed in this microorganism (54). Further analysis has revealed the presence of a group of heat-inducible proteins which are all under the transcriptional control of a single gene called *hin* or *htpR* (126, 127). Mutations in this gene will prove invaluable in pin-pointing the mechanism of induction and the role of hspS. Four of the heat-inducible proteins from *E. coli* have been identified: a lysyl tRNA synthetase, and the *groEL*, *groES*, and *dnaK* gene products (127-129). The last three proteins are essential for growth of bacteriophage lambda and mutations in those genes render the bacteria temperature sensitive for growth at 43°C (130-133). Recently, Tilly et al. (134) showed that the *dnaK* protein may modulate the heat shock response of *E. coli*. They demonstrated that one of the roles for the *dnaK* protein was to shut off the response in *E. coli*. These latter results fit well with those of DiDomenico et al. (42) who suggested an autoregulatory role for the hsp70 of *Drosophila*. These results are even more striking in light of the report showing homology between the *dnaK* protein of *E. coli* and the *Drosophila* hsp70 (68).
1.7.2 Translational Control

One of the characteristics of the heat shock response is the rapid expression of the heat-shock mRNA. Upon heat-shock, pre-existing polysomes rapidly disaggregate and new polysomes are selectively formed on heat-shock mRNA (10). Thus the preferential translation of heat-shock mRNAs may explain the rapid appearance of hsp's in the stressed cell. The half-life of normal mRNA in yeast cells is much shorter than that in Drosophila and may explain the absence of translational control of pre-existing mRNAs in yeast (48).

In Drosophila, the return of heat-shocked cells to their normal temperature causes release of the translational control and pre-existing mRNAs are no longer selectively repressed (45). That these messages are not degraded was also demonstrated by in vitro translation of mRNA extracted from heat-shocked Drosophila cells (13, 45, 46). However, if lysates are prepared from heat-shocked Drosophila cells and used for the in vitro translation studies, heat-shock messages are preferentially translated (45, 46). In contrast, similar lysates from control cells will translate both normal and heat-shock mRNA (45, 46). The factors responsible for the preferential translation of heat-shock mRNA in heat-shock lysates have been localized to the ribosomal fraction (134). Addition of crude ribosomal fractions from control lysates to the heat-shock lysate releases the repression of normal mRNA translation (134). Glover (135) and Sanders et al. (136) have demonstrated that the heat-induced rapid dephosphorylation of an S6-like ribosomal protein in Drosophila closely parallels the heat-shock induced breakdown of polysomes, suggesting a possible relationship between the phosphorylation of this protein and translation. The response, however, seems to be heat-specific
since other chemical inducers do not cause the same dephosphorylation (187).

Lindquist (48) has shown that pre-existing mRNAs in *Drosophila* can be repeatedly sequestered and released through a number of heat-shock/recovery cycles; however, the synthesis of hsp5 gradually decreases in the presence of actinomycin D. Thus, although normal messages are protected during heat-shock, the reverse is not true, i.e. heat-shock mRNAs are degraded during recovery. It has also been demonstrated that the rates of initiation of mRNA translation are comparable for both normal and heat-shock mRNAs (48, 137). However, Ballinger and Pardue (90) have recently reported that the rates of both elongation and initiation of translation are reduced 15- to 30-fold on normal mRNAs compared to heat-shock mRNAs.

It should be noted that the regulation of transcription and translation during the heat shock response do not occur independently of each other but are in fact coordinated. It is also noteworthy that this stress response is not regulated in an all or none manner; the level of hsp induction is dependent on the severity of the stress. For instance, in *D. melanogaster*, the intensity of the response varies with the temperature of heat-shock and the time of exposure to the stress (42, 48). In addition, the rate of recovery depends on the strength of the initial stress (44). The heat-shock and recovery of *Drosophila* cells have been shown to be influenced by the concentration of functional hsp5s (especially hsp70); for instance, if the production of hsp5s is blocked, transcription of heat-shock mRNAs continues and their stability is increased (42). However, as soon as functional hsp5s are present, transcription of heat-shock mRNAs is reduced and their stability decreases (44).

From the above discussion it should be fairly apparent that the
regulation of the heat shock response is dependent on a variety of factors, including the intensity, duration, and nature of the stress, and the presence of functional hspS for autoregulation and recovery. In addition, pre-existing mRNAs are subject to translational control. It is also obvious that the rules governing this regulation are not rigid and that there are exceptions, e.g. storage of heat-shock mRNA during normal growth in *Xenopus* oocytes (47) and the absence of translational control on pre-existing mRNAs in heat-shocked yeast cells (48). Thus the regulation of the heat shock response is a complex process with manifestations at several stages of gene expression.

1.7.3 Developmental and Tissue Specific Expression of hspS

Until recently, it was thought that the heat shock response occurred in all tissues and at all stages of development. There is now evidence for developmental regulation of the hspS. For example, *Drosophila* preblastoderm embryos have a low inducibility of hspS (138, 139). Similarly, in developing sea urchins hps are only inducible in post-hatching stages (140). Recently, Zimmerman et al. (141) reported the accumulation of mRNAs for *Drosophila* hsps 83, 28, and 26 in adult ovaries. These messages were detected during normal development and were abundant in embryos until the blastoderm stage, suggesting the presence of differential control of heat-shock gene expression during development (141). Another such spontaneous expression of hsps is reported for mouse embryonal carcinoma cells, and for ectoderms from day 8 mouse embryos (142).

A link between hsps and viral transformation has also been found. For instance, mammalian hsp70 synthesis is induced by an early gene product of adenovirus (33) and by papovavirus infection (143). In addition the
chicken hsp89 has been shown to bind to tyrosyl protein kinases of avian sarcoma viruses (73, 74). The ecdysterone induced synthesis of the small hsp90 in Drosophila (28) emphasizes the differential control of the heat shock response and thus adds to the already complex nature of its regulation.

1.8 Heat-Shock Related Responses

1.8.1 Physiological Responses

Severe heat-shock of early Drosophila embryos results in developmental abnormalities called phenocopies. These are most likely caused by a disturbance in gene expression due to the heat-shock. Studies of this phenomenon can be used to identify critical steps in the regulation of morphogenesis. Attempts in this direction have been made by Mitchell and Petersen (144). A mild heat pretreatment of Drosophila larvae protects them from phenocopy induction (84). Other physiological responses to heat-shock include the adaptation to thermal stress which leads to a transient resistance to heat. This phenomenon has been discussed above.

1.8.2 Other Effects of Heat-Shock

A variety of changes caused by the heat-shock phenomenon have not yet been discussed. These include: protein modifications, metabolic perturbations, cell cycle synchronization, and disruption of cytoskeleton structure. One of the protein modifications already mentioned is the heat-induced dephosphorylation of an S6-like ribosomal protein in Drosophila (135, 136). This change has been correlated with the initial breakdown of pre-existing polysomes in heat-shocked cells. However, since
canavanine and sodium arsenite do not cause dephosphorylation of the S6-like protein (187), and yet produce the same translational regulation as heat, this protein modification may be an entirely separate response to heat. That protein phosphorylation may play a role in the regulation of gene expression is supported by the work of Caizergues-Ferrer et al. (145) who showed that ribosomal RNA synthesis was induced in Chinese hamster ovary cells recovering from heat-shock. The induction of RNA synthesis was correlated with the dephosphorylation of two nuclear proteins.

The effect of heat-shock on histones is quite specific. The rate of Drosophila H2B synthesis is increased while that of the other core histones is decreased (136, 145). In Tetrahymena heat-shock or deciliation induce the phosphorylation of histone H1 (147). The phosphorylation of H2A and H4 in heat-shocked Drosophila cells has also been reported (135). In addition, methylation of H2B and H3 is altered by heat-shock (148, 149), and extensive deacetylation of core histones is also observed (149). The latter change appears to be a consequence of the heat shock response rather than a part of the induction mechanism for hsp since hyperacetylation of the histones in trout cells does not prevent hsp synthesis (E.A. Burgess, personal communication). The variety and rapidity of heat-induced histone modifications suggests their possible involvement in regulation of the transcriptional response.

Under stressful situations, cells would be expected to economize and thus re-direct their metabolism towards energy conservation and/or production. In support of this expectation, Wilhelm et al. (150) have reported the accumulation of glycogen in heat-shocked Tetrahymena. In Drosophila and other mammalian cells, heat-shock blocks the assembly of heterogeneous nuclear RNA (hnRNA) into its normal nuclear ribonucleoprotein
(RNP) form (151). This block could be involved in the selective processing of heat-shock mRNAs.

Other effects of heat-shock include the inhibition of tubulin synthesis which may account for the induction of cell synchrony in Tetrahymena (152). In Drosophila and baby hamster kidney cells, the vimentin cytoskeleton disintegrates after heat-shock and aggregates at the nucleus (153).

1.9 The Present Investigation

At the time this project was initiated (late 1979), the heat shock response had been well characterized only in Drosophila (2). It had become apparent that this phenomenon in stressed cells was of great importance for their survival. Thus, its investigation in other systems would be invaluable in determining the induction mechanism of and functional role for the heat shock response. This would allow a comparison of the responses at both the transcriptional and translational levels in species from different phylogenetic backgrounds.

Therefore, an investigation of the heat-shock phenomenon in cultured cells of rainbow trout, Salmo gairdnerii, was undertaken. This cell line was used because of the ease with which it could be manipulated, especially for kinetic studies. Initially, the occurrence of the heat shock response in trout cells was characterized. The extent of its similarity to the Drosophila response was subsequently analyzed at several levels: hsp induction and turnover, hsp70 mRNA synthesis and degradation, translational control of pre-existing mRNAs, and hsp70 amino acid and nucleotide sequence homologies.
During the course of this study, the presence of a heat shock response in a wide range of organisms was becoming evident (1). The major theme emerging from these reports is the highly conserved nature of the heat shock response. In addition, the rapid kinetics of induction suggests that the chromatin of heat-shock genes is maintained in an "alert" state ready for immediate transcription.

The results presented in this thesis further emphasize the complex nature of heat shock response regulation, and support the conclusion that the hsp70 genes form a highly conservative gene family.
II. EXPERIMENTAL PROCEDURES

2.1 Cell Culture

2.1.1 Cell Line and Growth Conditions

The fibroblast-like line of RTG-2 cells was originally derived from mixed gonadal tissue of male and female rainbow trout, *S. gairdnerii* (154). The cells were grown in Eagle's minimum essential medium containing non-essential amino acids, Earle's basic salts, 100 U/mL of penicillin-streptomycin, and 10% fetal bovine serum (all from Gibco Ltd.). The cultures were maintained in disposable polystyrene tissue culture flasks of 25 cm$^2$ surface area, at a temperature of 22°C. For large-scale growth of cells, roller bottles with a surface area of 300 cm$^2$ were used.

2.1.2 Induction of Cells

Cells grown to near confluence were induced by two methods. In the first method, cells were subjected to higher temperatures either by partial immersion of the flask in a temperature-regulated water bath, or by placement in an air-incubator. Since the volume of medium in the flask was only 5 mL, temperature equilibration was rapid. In the second method, the cells were induced at 22°C by the addition of sodium arsenite to the medium. Cells from a single sub-culturing were used for each set of experiments. Details of the conditions of induction are described in the results section.
2.2 Protein Analysis

2.2.1 In vivo Labelling of Proteins

Following induction the cells were labelled for 1 hour with $^{[35}S]$methionine (1000 Ci/mmol, New England Nuclear) at 22°C. An average of 20-30 μCi/mL of $^{[35}S]$methionine was used per experiment. The label was added to 2 mL of medium lacking methionine (Selectamine kit from Gibco Ltd.) and this was substituted for the complete medium. Incorporation was terminated by removing the labelling medium and washing the cells with ice-cold saline-EDTA solution (137 mM NaCl, 0.5 mM EDTA, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 1.1 mM glucose). The cells were detached from the flask by a stream of saline-EDTA from a Pasteur pipet and pelleted by gentle centrifugation. The pellet was washed once in ice-cold isotonic saline (0.15 M NaCl). The cells were then suspended in Laemmli sample buffer (0.05 M Tris-HCl, pH 6.8, 1% SDS, 10 mM EDTA, 0.01% bromophenol blue, and 12% glycerol), and the proteins solubilized by boiling for 2 minutes.

2.2.2 Localization of Labelled Proteins

Following induction and labelling, cells were separated into the cytoplasmic and nuclear fractions. This was done essentially as described by Marushige and Bonner (155) with a few modifications. Cell pellets were suspended in 150 μL of TMKS buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 25 mM KCl, and 0.25 M sucrose) and 0.5% NP40. The suspension was freeze-thawed once on dry ice and homogenized in a glass-Teflon hand homogenizer. The sample was centrifuged at 3000 x g (10 minutes, 4°C) and the supernatant saved as the cytoplasmic fraction. The pellet was
rehomogenized in 500 μL of TMK buffer (same as TMKS without the sucrose) and 0.5% NP40. The sample was centrifuged as above and the nuclear pellet was washed in 500 μL of 10 mM Tris-HCl, pH 7.8, with mechanical agitation (Vortex mixer). The suspension was centrifuged at 12,000 x g (15 minutes, 4°C) to obtain a pellet of crude chromatin. Both the cytoplasmic and nuclear fractions were made 1X in Laemmli sample buffer.

2.2.3 SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

The cell extracts and fractionated proteins were analyzed on 10 or 12.5% polyacrylamide-SDS slab gels with a 4.5% stacking gel using the discontinuous buffer system of Laemmli (156). The gels contained an acrylamide:bisacrylamide ratio of 30:0.8 (w/w), in addition to 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.03% TEMED, 0.05% APS for the separating gel and 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.05% TEMED, 0.1% APS for the stacking gel. The gel electrophoresis buffer contained 25 mM Tris (pH about 8.3), 0.38 M glycine, and 0.1% SDS. Slab gels (0.08 x 7.5 x 10 cm) were run at 20 mA constant current for about 70 minutes and stained with 0.25% Coomassie blue in a methanol:glacial acetic acid:water (5:1:5 ratio, v/v) system. The microslab apparatus was as described by Matsudaira and Burgess (157). After destaining, the gels were dried and autoradiographed using Kodak X-Omat AR film for an average of 40 hours.

2.2.4 Densitometry Scanning of Autoradiographs

After autoradiography, the film was inserted into the holder of a Beckman DU-8 spectrophotometer and the profiles of [35S]methionine labelled proteins were determined by measuring the percentage transmittance of white light.
2.3 RNA Analysis

2.3.1 Isolation of Total RNA

RTG-2 fibroblasts, grown close to confluence, were induced with 50 μM sodium arsenite for 24 hours. The cells were subsequently harvested and the RNA was isolated essentially as described by Chirgwin et al. (158) with a few modifications. Briefly, the cell pellets were hand-homogenized on ice using a Gu*HCl buffer (6 M Gu*HCl, 20 mM sodium acetate, 0.1 M β-mercaptoethanol, pH 5.0). The homogenates were then carried through one cycle of freeze-thawing, followed by centrifugation at 12,000 x g (10 minutes, 4°C). To the supernatant was added 0.5 volume of 95% ethanol (-20°C). RNA was allowed to precipitate at -20°C for a few hours and subsequently pelleted by centrifugation at 12,000 x g (15 minutes, 0°C). The pellet was dissolved in 7.5 M Gu*HCl, 25 mM sodium citrate, 50 mM β-mercaptoethanol, pH 7.0 and the RNA was reprecipitated by the addition of 0.025 volume of 1 M acetic acid and 0.5 volume of 95% ethanol (-20°C). This cycle of reprecipitation was carried out two to three times. The final RNA pellet was washed once with 95% ethanol (-20°C), and then dried under a stream of nitrogen. The dried pellet was extracted three to four times with sterile water and the extracts were pooled. The RNA was precipitated once more with 0.1 volume of 2 M sodium acetate, pH 5.0, and 2 volumes of 95% ethanol (-20°C). The RNA was centrifuged at 12,000 x g (20 minutes, -10°C), dried under nitrogen and dissolved in sterile water at a final concentration of 2 mg/ml (assuming 20 A₂₆₀ units = 1 mg RNA).

Typical yields of total cellular RNA were about 1 mg per 5 roller bottles of cells. All glassware and solutions were treated with 0.1% diethylpyrocarbonate and baked or autoclaved, respectively, before use (a
standard procedure for all RNA analysis).

2.3.2 Purification of Polyadenylated RNA

Poly A⁺ RNA was separated from the total RNA by two passages through an oligo[dT]-cellulose column (Collaborative Research Inc.) using the procedure of Aviv and Leder (159). Total RNA was loaded onto the column in NETS buffer (0.3 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and 0.5% SDS) and the solution recirculated several times. The column was washed with more NETS buffer to elute off any unbound RNA. Poly A⁺ RNA was eluted off the column with a small volume of ETS buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and 0.05% SDS) or sterile water. The eluted poly A⁺ RNA was further purified by a second passage through the column. To the final eluant, containing the poly A⁺ RNA, was added 0.1 vol of 2 M NaAc, pH 5, and 2 vol of 95% ethanol (−20°C). The mixture was left for a few hours at −20°C and then centrifuged at 12,000 x g (30 minutes, −10°C). The pellet was washed once in 95% ethanol (−20°C), dried under nitrogen, and dissolved in sterile water.

The fate of the RNA through the purification procedure was monitored with an ISCO optical unit set at a wavelength of 260 nm. A typical purification profile is shown in Figure 1.

2.3.3 Sucrose Density Gradient Centrifugation of RNA

Sucrose gradients of 13 mL (15 to 35%, w/v) were made with the help of a Hoefer multiple sucrose gradient maker. The sucrose solutions contained 0.1 M NaCl, 1 mM EDTA, and 10 mM NaAc, pH 5. The RNA was heated to 80°C for 5 minutes and then chilled on ice. A total of 200 µg of RNA was loaded onto each gradient and centrifuged at 30,000 rpm (20 hours, 4°C) in
Figure 1. Purification of Poly A$^{+}$ on an Oligo[dT]-column. Poly A$^{+}$ RNA was separated from total RNA by affinity chromatography on oligo[dT]-cellulose. The arrows indicate various treatments of the column. The fate of the RNA was followed by absorption at 260 nm (ubf = unbound fraction, NETS = binding buffer, A$^{+}$ = purified poly A$^{+}$ RNA).

Note: one cycle through the column took one hour in a typical run.
a Beckman SW41 rotor. Fractions of 0.5 mL were collected by upward
displacement with 50% sucrose (w/v). RNA from each fraction was
precipitated with ethanol and dried in vacuo for 5 minutes. Individual
pellets were suspended in 8 µL of sterile water and stored at -20°C.

2.3.4 Cell-Free Protein Translation

RNA was translated in the rabbit reticulocyte system (NEN) as
described by Pelham and Jackson (160), with [35S]methionine as the
label. Polypeptides were fractionated and analyzed by SDS-polyacrylamide
gel electrophoresis and autoradiography.

2.3.5 Trout cDNA Libraries

The RTG-2 cDNA libraries were kindly supplied by Mr. Don Jones. Poly
A+ RNA from arsenite induced cells was used to synthesize complementary
DNA by one of two methods. The first method involved the use of
self-priming at the 3' ends of single-stranded cDNAs for second-strand
synthesis (161). These were cloned into the PstI site of plasmid pBR322
and yielded about 700 independent clones. The second method involved
addition of dC tails to the 3'-OH ends of single-stranded cDNAs and the use
of oligo-[dG] as primers for second-strand synthesis (162). These cDNAs
were also cloned into the PstI site of pBR322 and yielded about 2,000 and
5,000 independent clones in two separate attempts.

2.3.6 Screening of the cDNA Libraries

The cDNA libraries were screened either by the colony hybridization
method of Grunstein and Hogness (163) or by the high density plating method
of Hanahan and Meselson (164). In the former method, bacterial colonies
containing plasmids were individually transferred from a master plate to a NC filter (Schleicher and Schuell) and allowed to grow at 37°C on LB plates (1% bactotryptone, 0.5% yeast extract, 1% NaCl, and 1.4% agar) containing the antibiotics tetracycline (15 μg/mL) and streptomycin (25 μg/mL). When the colonies grew to a size of 1 mm, the filter was transferred to an LB plate containing 170 μg/mL of chloramphenicol, and the plasmids allowed to amplify overnight at 37°C.

In the second method, bacterial colonies from a glycerol stock of the cDNA library were plated at a density of about 5,000 colonies per plate onto a NC filter. These colonies were also grown under selection of tetracycline and streptomycin. The bacterial colonies were grown to a diameter of 0.1 mm and a replica made onto a second NC filter. Both the master and replica were returned to 37°C and the colonies grown to a diameter of 1 mm. At this stage the master plate was removed and stored at 4°C. The replica filter was transferred to a plate containing chloramphenicol and the plasmids amplified overnight.

Filters were then treated to obtain cell lysis. They were placed colony side up on a Whatman 3 MM filter saturated with 10% SDS (3 min). This was followed by successive washes in: (i) 0.5 M NaOH, 1.5 M NaCl, (ii) 1.5 M NaCl, 0.5 M Tris-HCl, pH 8, and (iii) finally in 2X SSPE (1X SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH 7.5, and 1 mM EDTA), all for 5 minutes. The filters were air dried and baked in vacuo at 80°C for 2 hours. After hybridization to a labelled DNA fragment, any positive signals were traced back to the master plate and appropriate colonies purified.
2.3.7 RNA Northern and Dot Blot Analysis

RNA was denatured in 1 M glyoxal, 10 mM sodium phosphate, pH 7 at 50°C for 1 hour according to the procedure of McMaster and Carmichael (165). The RNA samples were cooled to room temperature and 0.2 vol of a 5X loading buffer (1X loading buffer is 7% Ficoll, 10 mM sodium phosphate, pH 7, and 0.02% bromophenol blue) added. The denatured RNA was fractionated on a horizontal agarose gel. The gels were poured and run in 10 mM sodium phosphate, pH 7.0, buffer which was recirculated throughout the run. The glyoxalated RNA was transferred from the gel to a NC filter immediately after electrophoresis. The transfer was through 20X SSPE as described by Thomas (166). For the dot-blots, total RNA was placed in a small volume on a NC filter and allowed to dry. The filters were subsequently baked in vacuo at 80°C for 2 hours.

2.3.8 Cytoplasmic Quick Blots of RNA

Cytoplasmic extracts containing mRNA were blotted to nitrocellulose filters (Schleicher and Schuell, Inc.) as described by Bresser et al. (167), with a few modifications. Briefly, after appropriate treatments, cells from individual flasks were harvested as quickly as possible (to avoid inadvertant induction of hsp's) in 1.0 mL of ice-cold saline-EDTA solution. Cells were gently pelleted by a 5 minute centrifugation in a desk top centrifuge and resuspended in 0.5 mL of ice-cold saline-EDTA solution made 0.5% in SDS. Proteinase K (Boehringer Mannheim) was added to a final concentration of 0.2 mg/mL and the suspension was incubated at 37°C for 30 minutes. One-twentieth volumes of 10% Brij-35 and 10% sodium deoxycholate were added to the suspension, which was then incubated on ice for 5 minutes. To solubilize cellular contents, 0.81 volume of super-
saturated NaI (2.5 g NaI per mL of hot water), liquified at 75°C, was added to the suspension which was left at room temperature for 10 minutes. Dilutions were made into saturated NaI (0.81 volume of supersaturated NaI added to saline-EDTA solution). The solubilized extract and appropriate dilutions were passed at room temperature through a nitrocellulose filter with the aid of a Hybrid-Dot apparatus (Bethesda Research Lab, Inc.). Nitrocellulose filters were pretreated by soaking in water followed by a wash in 6X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). After filtration, nitrocellulose membranes were soaked successively in water, 70% ethanol, and finally acetic anhydride solution (100 mL of 0.1 M triethanolamine plus 0.25 mL of acetic anhydride prepared just prior to use), each for 10 minutes at room temperature. Filters were subsequently air dried and treated for hybridization. All solutions, except for ones containing NaI or proteins, were treated with 0.1% diethylpyrocarbonate and autoclaved before use.

2.4 DNA Analysis

2.4.1 Isolation of Plasmid DNA

Plasmid DNA was isolated by the method of Birnboim and Doly (168). For rapid small-scale isolation of plasmid, 1.5 mL of an overnight culture was transferred to an Eppendorf tube and the bacterial cells pelleted by centrifugation in a microfuge (Eppendorf). The cells were resuspended in 0.1 mL of lysis buffer I (50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA, and freshly added lysozyme at 4 mg/mL). The suspension was mixed for 30 seconds (Vortex) and left at room temperature. After 5 minutes, 0.2 mL of freshly made ice-cold lysis buffer II (0.2 N NaOH, 1% SDS) was added. To
the now viscous solution was added 0.15 mL of ice-cold 5 M potassium acetate, pH 4.8. The tube was inverted, mixed for 10 seconds (Vortex) and left on ice for 5 minutes. The bacterial cell debris and DNA were removed by a 5 minute centrifugation. The lysate, which contained the plasmid DNA, was subjected to extraction with 1 vol of phenol:CHCl₃ (1:1). The aqueous phase was treated with NaAc and ethanol to precipitate the plasmid DNA (room temperature, 2 min). The final pellet was dried and resuspended in 50 µL of sterile TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) plus 50 µg/mL of RNAase A (Sigma Ltd.).

For the large scale isolation of plasmid DNA, all the steps were scaled up. Usually, 0.5 L cultures of bacterial cells were used and processed to the lysate stage as above. Isopropanol (0.6 vol) was added to the lysate and the mixture left at room temperature for 15 minutes. The DNA was collected by centrifugation at 12,000 x g (20 minutes, 10°C) and the pellets washed once in 70% ethanol. The DNA was dried in vacuo and dissolved in 4.5 mL of TE buffer. One gram of solid cesium chloride was added per mL of solution. One mg of ethidium bromide was added and the mixture centrifuged at 12,000 x g (10 minutes, 10°C) to remove undissolved salt and RNA complexed to ethidium bromide. The cesium chloride solution of DNA was subjected to equilibrium density centrifugation at 50,000 rpm (20 hours, 15°C) in a Beckman VTi65 rotor. The DNA band from the gradient was collected and the ethidium bromide removed by several extractions with 1-butanol saturated with water. To prevent any cesium chloride from salting out, the aqueous phase was diluted 3-fold with water prior to addition of ethanol (6 vol). The DNA was pelleted by centrifugation at 12,000 x g (20 minutes, 0°C) and washed once in 70% ethanol. The final pellet was dried in vacuo and resuspended in 0.5 mL of sterile water.
2.4.2 Isolation of Genomic DNA

High molecular weight genomic DNA from trout testes was prepared by Dr. E.P.M. Candido following the method of Blin and Stafford (169). This involved grinding of the tissue in liquid nitrogen followed by extensive digestions with RNAse A and proteinase K. These digestions were interspersed with phenol:CHCl₃ (1:1) extractions and dialysis of the aqueous phase to remove all traces of phenol. High molecular weight DNA was wound out of the extract after addition of ethanol.

Genomic DNA samples from other sources were kindly provided by Elizabeth Burgess, Colin Hay, Jeff Leung, Ross MacGillivray and Roland Russnak.

2.4.3 Restriction Endonuclease Digestion of DNA

Digestion of DNA with restriction enzymes was carried out according to the directions of the supplier (BRL). Low (0 mM NaCl), medium (50 mM NaCl), or high (100 mM NaCl) salt buffers were used depending on the requirement of individual enzymes. Note that 20 mM KCl was substituted for NaCl when SmaI was used. For digestions of longer than 2 hours, 100 μg/mL BSA (ultrapure grade, BRL) was added. All digestions were at 37°C, except for TaqI (65°C), and the reactions terminated by incubation at 68°C for 5 minutes. The samples were cooled to room temperature and 0.2 vol of 20% Ficoll, 0.2% bromophenol blue, and 50 mM EDTA added prior to gel electrophoresis.

2.4.4 Agarose Gel Electrophoresis

Fractionation of DNA was by electrophoresis through horizontal agarose gels. Gels were poured and run in 1X TBE (89 mM Tris-borate, pH 8.3, and 2
mM EDTA) containing 0.1 mg/L of ethidium bromide. Gels were submerged during the electrophoresis (4 to 5 V/cm). The DNA bands were visualized under UV-light and photographs taken with a Polaroid camera.

2.4.5 DNA Southern Blot Analysis

DNA from agarose gels was transferred to NC filters following the principles described by Southern (170). The gel was pretreated for transfer as follows: two 15 minute washes in 1.5 M NaCl, 0.5 M NaOH followed by a short rinse in distilled water, then two 15 minute washes in 0.5 M Tris-HCl, pH 7.8, 1.5 M NaCl. The NC filter was pretreated by brief washes in distilled water and 20X SSPE. Transfer was overnight through 20X SSPE and the DNA immobilized by baking the filter in vacuo at 80°C for 2 hours.

2.5 Maxam and Gilbert DNA Sequencing

This method involved the base-specific modification and cleavage of end-labelled DNA (171, 172). The cleaved products were ordered according to size on an acrylamide gel and the sequence read from an autoradiogram.

2.5.1 End-Labelling of DNA Fragments

About 1 μg of purified DNA with unique 5' sticky ends was end-labelled with the appropriate [α-³²P]dNTP (40-50 μCi, 3,000 Ci/mmol, NEN) using the Klenow fragment of E. coli DNA polymerase I (Boehringer Mannheim). After 20 minutes at room temperature the reaction was terminated by addition of 0.2 vol of 20% Ficoll, 0.2% bromophenol blue, and 50 mM EDTA.
2.5.2 Preparative Acrylamide Gels

Single end-labelled fragments of DNA were purified on a 5 or 8% polyacrylamide (acrylamide:bisacrylamide ratio of 29:1) gel containing 1X TBE, 0.06% APS, and 0.1% TEMED. The DNA fragments of interest were identified by autoradiography, and electro-eluted from the gel into dialysis tubing. The final DNA pellet was Cerenkov counted (approximately 30% efficient) and suspended in sterile water at 1,000 - 2,000 Cerenkov cpm/μL.

2.5.3 Base-Specific Reactions on End-Labelled DNA

The protocol used for the base modification reactions on the end-labelled DNA is summarized in Table III. After the final lyophilization, the DNA was Cerenkov counted and taken up in 90% formamide, 25 mM EDTA, 0.02% xylene cyanol, and 0.02% bromophenol blue at a concentration of 2,000 Cerenkov cpm/μL (for the G and C base reactions) and 4,000 Cerenkov cpm/μL (for the G+A and T+C base reactions). The samples were heated at 95°C for 3 minutes and quick-chilled on ice prior to electrophoresis.

2.5.4 Sequencing Gels

The products of DNA sequencing reactions were analyzed on 6 or 8% polyacrylamide slab gels (0.035 x 15 x 35 cm). The gels contained an acrylamide:bisacrylamide ratio of 19:1 in addition to 8.3 M urea, 1X TBE, 0.06% APS, and 0.03% TEMED. Electrophoresis was in 1X TBE at a constant power of 30 W (approximately 1,600 V). The gels were dried onto Whatman filter paper and autoradiographed using Kodak X-Omat RP film.
TABLE III. DNA Base-Modification Reactions for M & G Sequencing

<table>
<thead>
<tr>
<th>Reaction Step</th>
<th>G</th>
<th>G + A</th>
<th>T + C</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>[{³²P}] DNA (µL)</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Carrier DNA (1mg/mL):</td>
<td>1 µL</td>
<td>1 µL</td>
<td>1 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>cacodylate buffer, 300 µL</td>
<td>dH₂O</td>
<td>dH₂O</td>
<td>5 NaCl</td>
<td></td>
</tr>
<tr>
<td>Mix &amp; Chill</td>
<td>DMS</td>
<td>formic acid</td>
<td>HZ</td>
<td>HZ</td>
</tr>
<tr>
<td>Add</td>
<td>2 µL</td>
<td>3 µL</td>
<td>30 µL</td>
<td>30 µL</td>
</tr>
<tr>
<td>Incubate</td>
<td>3', RT</td>
<td>10', 37°C</td>
<td>10', RT</td>
<td>15', RT</td>
</tr>
<tr>
<td>Add</td>
<td>G-stop</td>
<td>A-stop</td>
<td>Py-stop</td>
<td>Py-stop</td>
</tr>
<tr>
<td>50 µL</td>
<td>300 µL</td>
<td>300 µL</td>
<td>300 µL</td>
<td></td>
</tr>
<tr>
<td>+ 95% ethanol (-70°C)</td>
<td>1 mL</td>
<td>1 mL</td>
<td>1 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td>Store</td>
<td>-70°C, 15 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microfuge</td>
<td>5 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reprecipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash pellet &amp; dry in vacuo</td>
<td>2X in 70% ethanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resuspend in</td>
<td>100 µL 1.0 M piperidine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat to</td>
<td>90°C, 30 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cool on ice, add</td>
<td>100 µL dH₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyophilize</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Add</td>
<td>20 µL dH₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyophilize &amp; repeat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.6 M13-Dideoxy Sequencing of DNA

The second method of DNA base sequence determination utilized the 'dideoxy' chain termination procedure of Sanger (173, 174) as adapted to the M13 phage system by Messing et al. (175).

2.6.1 Cloning of DNA into M13 Phage

M13mp8 and M13mp9 RF DNA were kindly provided by Dr. E.P.M. Candido. About 100 ng of insert DNA, digested with restriction enzymes, was annealed and ligated to 50 ng of vector DNA (M13 RF) restricted at the appropriate site. The ligation reaction was at 15°C for 4 hours in 66 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM DTT, and 1 mM ATP, in addition to 1 Weiss unit of T4 DNA ligase (Boehringer Mannheim). The reaction was terminated by heating to 68°C for 10 minutes. The ligated DNA was transformed into JM101 or JM103 cells (strains obtained from Craig Newton). Essentially, host cells were grown in 20 mL YT medium (0.8% bactotryptone, 0.5% yeast extract, and 0.5% NaCl) to a density of 0.7 A₅₅₀ units and then chilled on ice for 30 minutes. The cells were pelleted by gentle centrifugation and resuspended in 10 mL of fresh ice-cold 50 mM CaCl₂. After storing on ice for 30 minutes the cells were pelleted (1,500 x g, 5 minutes, 4°C) and carefully resuspended in 2 mL of ice-cold 50 mM CaCl₂. Transformation reactions consisted of 0.3 mL of competent cells and 2 ng of ligated DNA; these were incubated on ice for 40 minutes. The mixtures were heat-shocked at 42°C for 2 minutes, and then left at room temperature for 5 minutes. Ten μL of 100 mM IPTG, 50 μL X-gal (2% in dimethylformamide), and 0.2 mL of a fresh culture of cells was added to the transforming mixture. The mixture was plated onto YT agar plates with 3 mL of soft agar. The plates were
incubated at 37°C and clear plaques identified as those containing recombinant phage.

2.6.2 Preparation of Single-Stranded Templates

Clear plaques (containing recombinant phage) were transferred with a sterile pasteur pipet, from a plate to a tube containing 2 mL YT medium and 20 μL of a fresh culture of JM101 or JM103 cells. The tube was incubated at 37°C with shaking for 6 to 7 hours. A 1.3 mL aliquot was poured into an Eppendorf tube and the cells pelleted (5 minutes in a microfuge). The supernatant was carefully transferred to a fresh tube containing 0.3 mL of 20% PEG, and 2.5 M NaCl. After mixing, the tube was left at room temperature for 15 minutes followed by a 5 minute centrifugation. The supernatant was aspirated off and the inside wall of the tube was wiped with tissue paper to ensure that all traces of PEG were removed. The phage pellet was dissolved in 0.2 mL of a low Tris buffer, LTB (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA), and then subjected to extractions with phenol, and phenol:CHCl₃ (1:1). The aqueous phase was transferred to a clean tube and the single-stranded DNA precipitated by the addition of 14 μL of 4 M NaAc, pH 5, and 0.5 mL of 95% ethanol (-20°C). The mixture was left at -70°C for 15 minutes and then centrifuged for 5 minutes. The barely detectable pellet was washed in 1 mL of 95% ethanol (-20°C), dried in vacuo for 5 minutes, and dissolved in 50 μL of LTB.

2.6.3 'Dideoxy' Chain Termination Reactions

Single-stranded template DNA (5 μL) from a recombinant M13 phage was mixed with 0.75 ng of M13-primer (17-mer, P-L Biochemicals) in 25 mM Tris-HCl, pH 7.5, 18 mM MgCl₂, and 150 mM NaCl in a total volume of 8 μL.
The mixture was transferred to a 50 µL glass capillary which was then sealed at both ends. The hybridization mix was placed at 68°C for 10 minutes and then gradually cooled to room temperature to allow proper annealing of primer and template DNA. After 15 minutes the capillary was broken open and the contents transferred to a tube containing 1 µL of 15 µM dATP and 1.5 µL of [α-32P]dATP (15 µCi of a 3,000 Ci/mmol stock, NEN). From the mixture, 2 µL aliquots were transferred to 'A' and 'T' tubes, while 2.5 µL aliquots were transferred 'G' and 'C' tubes. To these tubes were also added 1.5 µL of the appropriate dd/dNTP mix (see Table IV).

**TABLE IV. 'Dideoxy' Mix Composition***

<table>
<thead>
<tr>
<th></th>
<th>'G'</th>
<th>'A'</th>
<th>'T'</th>
<th>'C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddGTP</td>
<td>89</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ddATP</td>
<td>-</td>
<td>116</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ddTTP</td>
<td>-</td>
<td>-</td>
<td>547</td>
<td>-</td>
</tr>
<tr>
<td>ddCTP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>547</td>
</tr>
<tr>
<td>dGTP</td>
<td>7.9</td>
<td>111</td>
<td>158</td>
<td>158</td>
</tr>
<tr>
<td>dTTP</td>
<td>158</td>
<td>111</td>
<td>7.9</td>
<td>158</td>
</tr>
<tr>
<td>dCTP</td>
<td>158</td>
<td>111</td>
<td>158</td>
<td>10.5</td>
</tr>
</tbody>
</table>

* empirically derived by Dr. Joan McPherson

Reactions were started by addition of 0.2 units of the Klenow fragment of *E. coli* DNA polymerase I, and incubation at room temperature for 15 minutes. One µL of 0.5 mM dATP chase was added to each tube and incubation continued at room temperature for an additional 15 minutes. Reactions were terminated by the addition of 5 µL of 98% deionized...
formamide, 10 mM EDTA, 0.2% xylene cyanol, and 0.2% bromophenol blue. The samples were heated to 95°C for 3 minutes and quick-chilled on ice prior to electrophoresis. The sequencing gels were run as described under the methods to Maxam and Gilbert sequencing.

2.7 Some General Methods of DNA Analysis

2.7.1 Recovery of DNA from Agarose and Acrylamide Gels

DNA was recovered from gels by electro-elution into a dialysis tube. The DNA band of interest was sliced out of the gel and placed in a dialysis tube containing a small volume of 0.5X TBE. The tubing was clamped at both ends, taking care not to trap any air bubbles, and immersed in a shallow layer of 0.5X TBE in an electrophoresis tank. Elution of the DNA was generally achieved by 30-60 minutes at 100 V. The DNA was dislodged from the wall of the dialysis tube by reversing the current for 30 seconds, and the buffer in the bag recovered. The eluted DNA was either ethanol precipitated directly or purified further by ion-exchange chromatography.

2.7.2 Purification of DNA on Mini-Chromatography Columns

DNA eluted from agarose gels was further purified by chromatography either on DE-52 (Whatman Ltd.) or on RPC-5 analog (BRL) packed in 200 μL Eppendorf pipet tips. Both columns involved the initial loading of DNA in low salt (< 0.1 M NaCl) and eventual elution in high salt (> 0.5 M NaCl). The purified DNA was precipitated with ethanol and resuspended in sterile TE buffer.
2.7.3 Labelling DNA by Nick-Translation

High specific activity labelled DNA was obtained by nick-translating DNA according to the procedure of Rigby et al. (176). The 25 μL reaction mixture contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 100 μg/mL BSA, 0.2 mM CaCl₂, 10 μM each of dATP and dTTP, approximately 0.2 μg of purified DNA, and 15 μCi each of [α-³²P]dGTP and [α-³²P]dCTP (3,000 Ci/mmol, NEN). The reaction was started by the addition of 1 μL of DNAase I (diluted 1:40,000 from a 1 mg/mL stock) and 2 units of *E. coli* DNA polymerase I. The nick-translation was carried out at 14-15°C for 1 hour and terminated by the addition of 125 μL of stop buffer (50 mM EDTA, 100 μg/mL BSA). The labelled DNA was separated from the unincorporated dNTPs by centrifugation through a 1 mL column of Sephadex G-50. This spun-column was prepared by packing Sephadex G-50 beads into a disposable 1 mL syringe. In a standard nick-translation reaction, 150 μL of very high specific activity (approximately 10⁶ cpm/μg DNA) probe was recovered. An aliquot of the probe was denatured by heating to 100°C for 5 minutes and quick-chilling on ice, and used directly in hybridization mixes.

2.7.4 Hybridizations

All NC filters prepared for hybridizations were treated in essentially the same manner. Filters were heat-sealed in Seal-a-Meal bags (Sears Ltd.) to which were added prehybridization mix (5X SSPE, 50% deionized formamide, 5X Denhardt's reagent (177), 0.1% SDS, and 100 to 200 μg/mL of sheared, denatured calf thymus DNA) at 50 μL per cm² of filter. Prehybridization was at 42°C for 1 to 4 hours. Hybridizations were also carried out at 42°C in the mix described above except that 1X Denhardt's
reagent (0.02% each of PVP, BSA, and Ficoll), and a denatured $^{32}\text{P}$labelled DNA probe was used.

After hybridization (usually overnight), the filters were washed in two changes of 2X SSPE, 0.1% SDS followed by one change of 0.1X SSPE, 0.1% SDS all at room temperature. One final wash was carried out at 50°C in 0.1X SSPE, 0.1% SDS. The filters were fluorographed at -70°C using Kodak X-Omat AR film and a Dupont Cronex intensifying screen.

2.8 Construction and Screening of Trout Genomic Libraries

The variety of lambda vectors and bacterial hosts were kindly provided by the following: Terry Snutch (λCH4A, E. coli DP50$_{SF}$), Ross MacGillivray (E. coli Q358, E. coli Q359), and Michael Sung (λL47.1, E. coli K802).

2.8.1 Isolation of Bacteriophage Lambda DNA

Large-scale growth of phage lambda was based on the methods of Yamamoto et al. (178). An overnight culture of the appropriate bacterial host was infected with phage lambda at a multiplicity of infection of 0.1 (i.e. for a typical 0.5 L culture, 10$^{10}$ cells were used with 10$^9$ pfu). The phage was grown in 0.5 L of prewarmed (37°C) media in a 2 L flask. NZYDT (1% NZ-amine, 0.5% yeast extract, 0.5% NaCl, 0.2% CAA, 10 mM MgCl$_2$, 100 µg/L DAP, and 40 µg/mL thymidine, pH 7) was used for growing phage in the bacterial strain DP50$_{SF}$, whereas NZYC (1% NZ-amine, 0.1% yeast extract, 0.5% NaCl, 0.1% CAA, 10 mM MgCl$_2$, pH 7) was used if the host cells were from the strains K802, Q358, or Q359. The flasks were incubated at 37°C with aeration for 6 to 10 h until the bacterial cells lysed to
release the phage particles. Approximately 10 mL of CHCl₃ was added to the
flask and incubation continued for an additional 15 minutes to complete the
cell lysis. Bacterial debris was removed by centrifugation at 9000 x g (15
minutes, 4°C). Supernatants were carefully transferred to a clean flask
containing 29.2 g of NaCl (final concentration 1 M) and 50 g of PEG (final
concentration 10%). The contents were mixed and left overnight at 4°C.
The PEG precipitate was collected by centrifugation at 9,000 x g (20
minutes, 4°C) and the pellet resuspended in a total volume of 6 mL SM
buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 0.02%
gelatin). To the suspension was added 50 μL of 1 mg/mL DNAase I and 100
μL of 5 mg/mL RNAase A and the mixture incubated at 37°C for 30 minutes.
An equal volume of CHCl₃ was added to the phage suspension and the phases
separated by centrifugation at 1,500 x g (15 minutes, 4°C). The aqueous
layer was transferred to a fresh tube.

To purify the bacteriophage further, it was subjected to equilibrium
centrifugation in cesium chloride. For every 1 mL of phage, 0.75 mg of
cesium chloride was used. Centrifugation was at 45,000 rpm (15 hours, 4°C)
in a Beckman VTi65 rotor. The phage particles formed a tight bluish band
in the gradient and were collected in a fairly small volume (1 to 2 mL).
Extraction of the DNA from phage lambda was performed as follows: 0.1 vol
of 10X TE and 1 vol of deionized formamide was added to the cesium chloride
suspension of phage particles. The mixture was left at room temperature
for 1 to 2 hours and then 1 vol of distilled water and 6 vol of 95% ethanol
added. The DNA came out of solution within 5 minutes and was pelleted by a
30 second centrifugation. The DNA was rinsed in 70% ethanol, dried under
nitrogen, and resuspended in TE buffer. Finally, the DNA was subjected to
successive extractions twice with phenol:CHCl₃ (1:1), and twice with CHCl₃.
The DNA was reprecipitated with ethanol and taken up in TE buffer.

2.8.2 Preparation of Lambda 'Arms'

Lambda DNA was digested with the appropriate restriction endonuclease, e.g. EcoRI for CH4A (179, 180) and BamHI for L47.1 (181), to liberate the insert fragments. After extraction with phenol:CHCl₃ (1:1) and precipitation with ethanol, the DNA was dissolved in TE buffer supplemented with 10 mM MgCl₂ and incubated at 42°C for 1 hour to allow the cohesive ends of lambda to reanneal. This resulted in one large fragment (the right and left arms annealed together) and one or two much smaller insert fragments. The purification of lambda arms was done by one of two methods. In the case of CH4A, the annealed arms were separated from the insert fragments by fractionation on a 0.35% agarose gel. In the second method, L47.1 arms were purified by centrifugation through sucrose density gradients (182). Step gradients (40 mL) of 10, 20, 30, and 40% (w/v) sucrose in 1 M NaCl, 20 mM Tris-HCl, pH 7.7, and 5 mM EDTA were poured. Annealed L47.1 DNA (50 μg) was loaded onto each gradient and subjected to centrifugation at 26,000 rpm (23 hours, 15°C) in a Beckman SW27 rotor. Fractions (1 to 2 mL) were collected by upward displacement with 50% (w/v) sucrose and aliquots analyzed on a 0.5% agarose gel (Figure 2). Fractions containing the annealed arms were pooled, diluted with 3 vol of water and the DNA precipitated with ethanol. The dried DNA pellet was resuspended in TE buffer and stored at 4°C.

2.8.3 Preparation of 15-20 Kilobase Fragments of Trout DNA

For the CH4A library, trout testes DNA was digested with EcoRI at
Figure 2. Preparation of the 'arms' of phage lambda DNA by sucrose gradient centrifugation. λL47.1 DNA was digested with BamHI, annealed, and centrifuged through a 10-40% sucrose gradient. Fractions were collected by upward displacement with 50% sucrose and aliquoats were analyzed by electrophoresis through a 0.5% aragose gel. Size markers (M) are from a Hind III digest of wild type lambda DNA. The left arm (23.5 Kb, right arm (10.5 Kb), and insert ("stuffer") fragments (6.6 Kb) were identified. Fractions 22 to 29 containing the annealed arms were pooled.
varying enzyme:DNA ratios and for different durations by Dr. E.P.M. Candido. The DNA from various digests was pooled and fractionated on a 0.7% agarose gel. The 15-20 Kb region of the DNA on the gel was electro-eluted onto a Whatman filter paper backed dialysis membrane (183). The filter was washed in a small volume of TNE (10 mM Tris-HCl, pH 7.6, 10 mM NaCl, and 1 mM EDTA) plus 0.2% SDS. The fluid was removed to a fresh tube and the filter washed as before. The eluted DNA was precipitated with ethanol, dried under nitrogen, and resuspended in TE buffer.

Insert DNA for the L47.1 libraries was prepared by initially establishing conditions for partial digestion of trout DNA with \textit{MboI}. Digestion conditions ranged from 0.00038 to 0.025 units of \textit{MboI}/µg of trout DNA incubated for 15 or 60 minutes at 37°C. These digestions were analyzed on a 0.5% agarose gel. The optimum condition of digestion, to achieve maximum representation in the 15-20 Kb range, was determined to be 0.025 units \textit{MboI}/µg DNA incubated at 37°C for 60 minutes. For the large-scale preparation of partially digested trout DNA, half the optimum amount of enzyme was used. An equal aliquot of DNA was digested with the optimal amount of enzyme, but only for 15 minutes. The DNA from both digestions was pooled, extracted twice with phenol:CHCl₃ (1:1), and precipitated with ethanol. The dried pellet was resuspended in TE buffer. Prior to sucrose gradient centrifugation, the DNA was heated to 68°C for 10 minutes and cooled to room temperature. The centrifugation was as described for the preparation of vector DNA arms. Fractions from the gradients were analyzed on a 0.5% agarose gel (Figure 3). DNA in the 15-20 Kb size range was pooled (fractions 30-35), precipitated with ethanol, and resuspended in TE buffer.
Figure 3. Preparation of 15-20 Kb MboI fragments of trout DNA. Trout genomic DNA was partially digested with MboI and fractionated by centrifugation through a sucrose density gradient (10-40%). Fractions were collected by upward displacement with 50% sucrose and aliquots of selected fractions analyzed by electrophoresis through a 0.5% agarose gel. Fractions 30 to 35 were pooled and used subsequently to construct a library in λL47.1. Size markers (M) were from a HindIII digest of wild type lambda DNA. T = MboI partial digest of trout DNA prior to fractionation.
2.8.4 Preparation of *in vitro* Packaging Extracts

The procedure employs the lysogenic strains NS428 and NS433 (184) kindly provided by Terry Snutch. The two strains provide complementary extracts for efficient packaging of lambda DNA.

Colonies of NS428 and NS433 bacteria were grown on M9 (0.6% Na$_2$HPO$_4$, 0.3% KH$_2$PO$_4$, 0.5% NaCl, 0.1% NH$_4$Cl, 2 mM MgCl$_2$, 0.2% glucose, and 0.1 mM CaCl$_2$) plates at 30°C. Several colonies were checked for growth at 30°C and 42°C. Only colonies growing at 30°C, but not at 42°C, were used to innoculate 50 mL of M9 medium. NS428 and NS433 were grown in separate flasks at 32°C with shaking. The cultures were centrifuged at 1,500 x g (5 minutes, 4°C) and resuspended in 5 mL of M9 medium. Two 0.5 L cultures of NS433 and one of NS428 were innoculated to an initial $A_{600}$ of 0.05 and incubated at 32°C with aeration until an $A_{600}$ of 0.3 was reached. Bacteriophage growth was induced by transferring the flasks to a 45°C water bath for 20 minutes with frequent swirling, and then to a 37°C incubator with vigorous shaking for 2 hours. Contents from all 3 flasks were pooled and chilled in ice-water for 10 minutes. From this point everything was kept on ice to avoid inadvertant lysis of the cells. The cultures were subjected to centrifugation at 2,500 x g (10 minutes, 4°C) and the pellets resuspended in a total of 450 mL ice-cold M9 medium. The cells were recentrifuged as above and the supernatant drained completely. Six mL of CH buffer (40 mM Tris-HCl, pH 8, 10 mM spermidine-HCl, 10 mM putrescine-HCl, 0.1% $\beta$-mercaptoethanol, 7% DMSO, and 1.5 mM ATP) was stirred in with the pellets as quickly and gently as possible. Aliquots of 100 $\mu$L were transferred to Eppendorf tubes and these were immediately plunged in liquid nitrogen. The packaging extracts were stored frozen at -70°C. Packaging efficiencies varied from $10^7$ to $10^8$ pfu per $\mu$g of undigested lambda DNA.
2.8.5 Ligation and Packaging Reactions

For the ligation of lambda arms to insert DNA, a molar ratio of 2:1 (arms:insert) was used at a final DNA concentration of 0.2 μg/μL. Ligation was carried out at 12°-14°C for 12 to 16 hours in ligation buffer and 1 Weiss unit of T4 DNA ligase.

*In vitro* packaging of the ligated DNA was based on the method of Hohn and Murray (185). An aliquot of frozen packaging extract was thawed on ice for 3 minutes. Ligation mix (with about 2 μg total DNA), 1.5 μL of 0.1 M ATP, and 20 μL of CH buffer was added directly to the extract and mixed in with a heat blunted glass capillary. Packaging was carried out at 37°C for 1 hour. A second extract was thawed as above and 1 μL of 1 mg/mL DNAase I with 2.5 μL of 1 M MgCl₂ added to it. About 50 μL of this mix was added to the first packaging reaction and incubation continued at 37°C. After 30 minutes, 0.9 mL of SM buffer and 3 drops of CHCl₃ were added to the reaction mix. The debris was centrifuged out and the packaged phage was stored in a clean tube at 4°C.

2.8.6 Amplification of the Lambda Libraries

Both the CH4A and L47.1 libraries were amplified by plating out the packaging mixes on freshly poured plates. These were incubated at 37°C for 12 hours and then overlayed with 5 mL of cold SM buffer. Plates were left at 4°C for a few hours and the overlay collected. Chloroform was added and amplified libraries stored at 4°C in sealed tubes or flasks.

2.8.7 Screening of the Lambda Libraries

The phage lambda libraries were screened essentially as described by Benton and Davis (186). Phage were plated out at approximately 10-20,000
pfu per plate and grown at 37°C. A dry NC filter was placed neatly onto the surface of the top-agarose and orientation marks made. After 2 minutes the filter was peeled off and immersed DNA side up in 1.5 M NaCl, 0.5 M NaOH (3 min), then transferred to 1.5 M NaCL, 0.5 M Tris-HCl, pH 7.6 (3 min). The filter was subsequently rinsed twice in 2X SSPE and air dried prior to baking in vacuo at 80°C for 2 hours. After hybridization to a [³²P]labelled probe, any positives were traced back to the original plate and the plaque of interest was purified by a second and third screening.

2.8.8 Small-Scale Growth of Bacteriophage Lambda

DNA from phages identified as positives was prepared by growing a 20 mL culture in exactly the same manner as that described for the large-scale preparation. After lysis, the culture was transferred to a 30 mL Corex tube and centrifuged at 12,000 x g (10 minutes, 4°C) to remove the bacterial debris. To the supernatant was added 6 mL of 50% PEG and 3 mL of 5 M NaCl. The suspension was left at 4°C for several hours and then centrifuged 12,000 x g (10 minutes, 4°C). The pellet was resuspended in 0.3 mL of DNAase buffer (50 mM Hepes, pH 7.5, 5 mM MgCl₂, and 0.5 mM CaCl₂) and transferred to an Eppendorf tube. After addition of 5 µg DNAase I and 50 µg RNAase A, the mixture was incubated at 37°C for 30 minutes. The mixture was made 1X in SET (0.5% SDS, 10 mM Tris-HCl, pH 7.8, and 5 mM EDTA) and 100 µg of proteinase K added. The tube was incubated at 68°C for 30 minutes and the contents then extracted a number of times with phenol:CHCl₃ and finally with CHCl₃ alone. The phage DNA was ethanol precipitated and the dried pellet resuspended in 50 µL of TE buffer.
III. RESULTS

3.1 Characterization of the Trout Heat Shock Response at the Protein Level

3.1.1 The Heat Shock Proteins of Trout RTG-2 Cells

The effect of temperature elevation or sodium arsenite on trout fibroblasts is the production of a set of new polypeptides. These are referred to as the heat-shock polypeptides (hsp). The hsp of trout have been shown in the autoradiogram of Figure 4. These proteins were induced with sodium arsenite (panel A) or temperature elevation (panel B). Comparison with extracts of control cells (lane a or d) shows that at least six new proteins are synthesized. The molecular weights of the proteins are 87 K, 70 K, 62 K, 42 K, 32 K, and 30 K. The band at 62 K was sometimes resolved as a doublet (Figure 11). This band consistently appears after sodium arsenite induction but was not prominent if temperature elevation was used as the inducer (Figure 4B). The major hsp (70 K) was sometimes seen in control cells, but at a low level. This may be indicative of the condition of the cells at the time of harvesting in SDS sample buffer, i.e. even a slight degree of anoxia may be sufficient to cause induction of hsp70. A band at 100 Kd was sometimes observed by sodium arsenite induction and could also be a hsp. Densitometry scanning of the autoradiograms clearly showed the differences in protein synthesis patterns between control and induced cells (Figure 5).

The synthesis of normal cellular proteins could be reduced if the induction with arsenite was prolonged (Figure 4A, lane c); in this case almost all of the incorporation of label is into the hsp. It is interesting to note that hsp42 was no longer induced under this extended
Figure 4. The heat-shock proteins of trout RTG-2 cells. (A) Proteins inducible by Sodium Arsenite (SA): Lane (a) control, lane (b) 50 μM arsenite for 3 hours and recovery of 1 hour, lane (c) 50 μM arsenite for 24 hours. (B) Proteins inducible by temperature elevation (HS): lane (d) control, lane (e) 27°C for 5 hours and recovery at 22°C for 1 hour. All cells were labelled for 1 hour with [35S]methionine. Proteins were separated on a 10% SDS-polyacrylamide gel which was subsequently autoradiographed. The open triangle indicates another protein (100 Kd) that may also be inducible by arsenite.
Figure 5. Densitometry scans of autoradiographs from SDS-acrylamide gels. 

$[^{35}S]$methionine labelled proteins were fractionated on a 10% SDS-acrylamide gel and autoradiographed. The X-ray film was subsequently scanned in a Beckman DU-8 spectrophotometer. The scans from three separate lanes are shown: SA = 50 μM sodium arsenite exposure for 24 hours, HS = 28°C heat-shock for 1 hour, and control cells. Arrows indicate positions of hsp and actin (Ac) migration.
period of stress; hsp70 and hsp30 were the major species induced. The latter proteins were readily visible on gels with Coomassie blue staining when the cells were induced for 24 hours (Figure 6). The hsp32 is also visible under these conditions.

Samples of \textit{D. melanogaster} hsps (a gift of L. Moran) induced at 37°C were compared with the trout hsps induced with 50 μM sodium arsenite for 24 hours. The samples were fractionated on a 10% SDS-polyacrylamide gel and autoradiographed (Figure 7). The \textit{Drosophila} hsps are in lane a, while the trout hsps are in lane c. When samples from both were mixed and run together (lane b), it was seen that hsp70 from \textit{Drosophila} and trout had identical mobilities in this system. The major hsp from the nematode, \textit{C. elegans}, has also been found to co-migrate with the trout hsp70 (T. Snutch and R. Kothary, data not shown).

3.1.2 Temperature Profile of the Heat Shock Response

Our usual growth temperature for these cells was 22°C. Increasing the temperature by as little as 5°C resulted in the induction of hsps. The effect of a range of temperatures on hsp synthesis has been studied and the results shown in Figure 8. The cells were kept at the appropriate temperature for 1 hour, then returned to 22°C for another hour. This was followed by a 1 hour labelling period with $[^{35}\text{S}]$methionine. As shown in the autoradiogram (Figure 8), the response was present in all temperatures up to the lethal temperature of 34°C. Hsp70, hsp32, and hsp30 were present at all temperatures up to 34°C; hsp87 and hsp42 were present to 29°C. Heat shocks at higher temperatures, e.g. 37°C, will also elicit hsp synthesis if the duration of the induction period is short, e.g. less than 10 minutes (see Figure 8).
Figure 6. Detection of hsp70 with Coomassie blue staining. Cells were exposed to 50 μM sodium arsenite (SA) for 24 hours and the proteins from the cell extract were separated on a 10% SDS-acrylamide gel. These proteins were compared to those found in control cells (C).
Figure 7. Comparison of Trout and Drosophila hsp70s on an SDS-acrylamide gel. Lane (a) heat-shock induced (37°C) proteins of D. melanogaster (gift of Dr. L.A. Moran), lane (c) 24 hours 50 μM sodium arsenite induced proteins of trout, lane (b) a mixture of the trout and Drosophila hsp70s.
Figure 8. Temperature profile of the heat shock response. The cells were heat-shocked at the temperatures (°C) shown for 1 hour, then recovered at 22°C for 1 hour and finally labelled with \[^{35}\text{S}\text{]}\text{methionine for 1 hour at }22°C\). Note that the 37°C heat-shock was for 5 minutes only. The cell extract was fractionated on a 10% SDS-acrylamide gel and autoradiographed. The arrows indicated hsps 87, 70, 42, 32, and 30 from top to bottom respectively.
3.1.3 Effect of Duration of the Heat Shock

To examine the effect of prolonged temperature elevation, cells were incubated at 28°C for various lengths of time and then labelled with \(^{35}S\)methionine for 1 hour at 22°C. As the induction time at 28°C was increased from 1 hour to 16 hours, the level of incorporation into the hsps also increased (Figure 9). This was especially apparent for hsp70, hsp32, and hsp30. The incorporation rate at 16 hours of induction (Figure 9, lane f) was comparable to that at 5 hours (lane d) and 7.5 hours (lane e). These results suggest that the hsps are fully induced by 5 hours at 28°C.

3.1.4 Recovery from Heat Shock

To examine the reversibility of the heat shock response, cells heat shocked at 27°C for 1 hour were allowed to recover at 22°C for varying lengths of time and then labelled for 1 hour at 22°C with \(^{35}S\)methionine. The results are presented in Figure 10. Although the amounts of hsp87 and hsp30 were not altered significantly in the time span studied, hsp70 and hsp42 levels dropped after 3 hours at 22°C. This suggests either that the genes for hsp70 and hsp42 are more efficiently turned off in the absence of the inducing stimulus than are the hsp87 and hsp30 genes, or that the mRNAs for hsp87 and hsp30 are more stable.

3.1.5 Sodium Arsenite Concentration Study

As shown above (Figure 4A), sodium arsenite induced a spectrum of hsps in trout cells which were very similar to that induced by temperature elevation. The only significant difference was the extra band at 62K daltons which was induced by sodium arsenite but not by temperature shift. A study was performed to determine the optimum arsenite concentration
Figure 9. Time study of heat-shock. Cells were heat-shocked at 28°C for various lengths of time and then labelled at 22°C for 1 hour with [\textsuperscript{35}S]methionine. These proteins were separated on a 10% SDS-acrylamide gel and autoradiographed. Lanes are: (a) control, (b) 1 hour heat-shock, (c) 3 hours heat-shock, (d) 5 hours heat-shock, (e) 7.5 hours heat-shock, (f) 16 hours heat-shock. The triangles indicate the hsp 87, 70, 42, 32 and 30 from top to bottom respectively.
Figure 10. Recovery from heat-shock. Cells were heat-shocked at 27°C for 1 hour and allowed to recover at 22°C for different lengths of time. They were then labelled with [35S]methionine for 1 hour at 22°C. The cell extracts were run on a 12.5% SDS-acrylamide gel and autoradiographed. The recovery times were as follows: lanes (a) 0 hours, (b) 1.5 hours, (c) 3 hours, (d) 4.5 hours, and lane (e) control cells.
for hsp induction. The range of final concentrations used was from 1 to 600 μM (Figure 11, lanes b-l). The cells were induced for 3 hours with the appropriate amount of sodium arsenite. This was followed by incubation in fresh medium for 2 hours, after which they were labelled for an hour with [35S]methionine. The induction, recovery, and labelling were all done at 22°C. As shown in the autoradiogram of Figure 11, different hspS varied in their inducibility as a function of the arsenite concentration. Hsp70 and hsp87 induced very readily with an optimum at about 50 μM (lane i). The doublet at 62K daltons was also very readily induced but decreased in amount after 50 μM. Hsp42 was induced equally well at all the concentrations up to 300 μM (lane k). Hsp32 increased from 5 to 50 μM arsenite and then disappeared again. The most dramatic effect was on hsp30. Its level was specifically enhanced at arsenite concentrations of 15 to 100 μM (lanes g-j), with a peak of 50 μM. Since all the hspS were inducible at an arsenite concentration of 50 μM, this level was used in subsequent experiments.

3.1.6 Recovery from Sodium Arsenite Induction

Since the effect of sodium arsenite was similar to that of temperature elevation in the induction of the hspS, the similarity of the reversal of the induction was examined. As shown above (Figure 10), the synthesis of hsp70 and hsp42 returned to normal control levels following recovery from temperature shock. To study recovery from arsenite induction, trout cells were initially induced for 3 hours with 50 μM sodium arsenite and then allowed to recover in fresh medium for different lengths of time. This was followed by a 1 hour period of labelling with [35S]methionine. All the hspS were still present 2 hours after the arsenite had been removed (Figure
Figure 11. Hsp synthesis at varying sodium arsenite concentrations. Cells were induced with various concentrations of arsenite for 3 hours and then left to recover in fresh medium for 2 hours. Labelling was for 1 hour with $[^{35}\text{S}]$methionine. Cell extracts were separated on a 10% SDS-acrylamide gel and autoradiographed. Concentrations (μM) of arsenite used were as follows: (a) 0, (b) 1, (c) 2.5, (d) 5, (e) 7.5, (f) 10, (g) 15, (h) 25, (i) 50, (j) 100, (k) 300, and (l) 600.
12, lane c). This suggests that the translatable heat shock mRNA level was at a maximum at this time. Again the 100 Kd protein was present with the others and the 62Kd band was now clearly resolved as a doublet. As with recovery from temperature shock, hsp42 was the first to disappear; here its synthesis was terminated between 2 and 6 hours of recovery. Production of hsp70 and the doublet of hsp62 was halted before 10 hours of recovery. Hsp87 synthesis was terminated after about 15 hours of recovery. Although the level of hsp30 dropped after 10 hours of recovery, it was still detectable even after 28 hours of recovery. Thus, the majority of the heat shock mRNAs are either not present or present but not translated after 6 to 10 hours from the end of induction. As in the case of heat shock, the different recovery times for hsps following arsenite induction may be attributed to differences in the rates of repression of the hsp genes, to variations in the hsp mRNA stabilities, or both.

3.1.7 Localization of the hsps

RTG-2 cells were induced at 28°C for 1 hour, recovered at 22°C for 2 hours, then labelled with \[^{35}\text{S}]\)methionine at 22°C for 1 hour. The label was chased for 0 or 40 minutes in fresh medium and the cells harvested as usual. For the control situation, uninduced cells were labelled with \[^{35}\text{S}]\)methionine at 22°C for 1 hour and harvested without any pulse-chase. The cells were separated into the cytoplasmic and nuclear fractions and subsequently analyzed on a 10% SDS-polyacrylamide gel. The autoradiogram is shown in Figure 13. The presence of both hsp70 and hsp30 was easily detected in the nuclear fraction of the induced cells (Figure 13, lanes e, f). However, substantial amounts of hsp70 and hsp30 were still present in the cytoplasmic fraction. An interesting trend was the relative
Figure 12. Recovery from sodium arsenite induction. Cells were induced with 50 μM arsenite for 3 hours and then left to recover in fresh medium for different lengths of time. Following the recovery period, cells were labelled with \[^{35}\text{S}]\text{methionine}\) for 1 hour. Cell extracts were separated on a 10% SDS-acrylamide gel and autoradiographed. The recovery periods were: (b) 0 hours, (c) 2 hours, (d) 6 hours, (e) 10 hours, (f) 15 hours, (g) 20 hours, (h) 24 hours, (i) 28 hours, (j) 32 hours; lane (a) is the control. The solid triangles designate the hsp 87, 70, 62, 42, 32, and 30 from top to bottom respectively. The open triangle indicates the 100 Kd protein.
Figure 13. Subcellular localization of the hsp s. Following induction and labelling, cells were separated into the cytoplasmic and nuclear fractions as described in the Experimental Procedures. Conditions used were as follows: 28°C heat-shock for 1 hour followed by a 2 hours recovery period at 22°C. Labelling with $[^3S]$methionine was at 22°C for 1 hour. Cytoplasmic and nuclear fractions were subjected to SDS-acrylamide gel electrophoresis and autoradiography. Lanes: (a) control cytoplasm, (b) control nuclei, (c) heat-shocked cytoplasm, 0 minute chase, (d) heat-shocked cytoplasm, 40 minute chase, (e) heat-shocked nuclear fraction, 0 minute chase, (f) heat-shocked nuclear fraction, 40 minute chase.
increase in hsp70 and hsp30 in the nuclear fraction when a 40 minute pulse-chase was done. Hsp87 was found exclusively in the cytoplasmic fraction. Though definite conclusions about the localization of the trout hsp87 cannot be made, the results suggest an increase in levels of hsp70 and hsp30 in nuclei with time. The large amount of hsp70 and hsp30 associated with the cytoplasmic fraction may be reflecting the condition of the cell, i.e. in the recovering cell hsp87 may not be required in the nuclei, and may have already relocated in the cytoplasm by the time the labelling was completed at 22°C.

3.2 Translational Regulation of the Heat Shock Response

3.2.1 In vitro translation of mRNA

Total RNA isolated from control cells or from induced cells (50 µM sodium arsenite for 24 hours) was translated using a reticulocyte cell free protein synthesis system. The translated products were fractionated on a 10% SDS-polyacrylamide gel. From the results (Figure 14), it was clear that the induced mRNA contained the messages for the hsp87, hsp70, hsp62, hsp32, and hsp30, whereas the control mRNA did not. This indicated that the induction of the hsps was controlled at the transcriptional rather than the translational level. Comparison of the in vivo labelled proteins (after 24 hr, 50 µM sodium arsenite induction) in Figure 4A (lane c) with the in vitro labelled proteins (Figure 14, lane c) showed that the normal cellular proteins, which were not synthesized in vivo during hsp induction, are synthesized in vitro. Thus, although normal mRNAs are present in the induced cells, their translation is inhibited.
Figure 14. *In vitro* translation products of trout mRNA. RNA was translated in the rabbit reticulocyte system, with $[^{35}\text{S}]$methionine as the label. Lane (a) water control, (b) control mRNA, (c) induced mRNA (24 hours, 50 μM arsenite).

Note: Translation products were fractionated on a 10% SDS-acrylamide gel and autoradiographed.
3.2.2 Sucrose Gradient Fractionation of RNA

Total RNA from induced trout cells (24 hours, 50 μM sodium arsenite) was fractionated according to size on a 10-40% (w/v) sucrose gradient. The resulting $A_{260}$ profile of the gradient is shown in Figure 15. RNA from selected fractions were translated in vitro and the products analyzed on a 12.5% SDS-polyacrylamide gel. The autoradiogram is shown in Figure 15. The majority of the RNA was collected from fractions 5 to 12. Separation of the small hsp mRNA from the hsp70 mRNA was evident and this could be a useful step in the purification of heat-shock messages. If need be, certain enriched fractions could be used as RNA probes against a total cDNA library to narrow down the number of clones to be screened. However, since a heterologous probe for hsp70 was obtained, the RNA enrichment approach was not pursued.

3.3 Isolation of the Trout Hsp70 cDNA Clones

3.3.1 Screening of the Trout cDNA Library

A trout cDNA library, made by the methods of Wickens et al. (161) was screened for the presence of hsp70 sequences. From a total of about 700 independent clones, two trout hsp70 cDNA sequences, pTHS70.7 and pTHS70.14, were isolated. The identification of these two clones was based on their homology to a Drosophila hsp70 gene from the clone 132E3 (provided by Dr. L.A. Moran, ref 96).

3.3.2 Preliminary Examination of pTHS70.7 and pTHS70.14

Plasmid DNA for the two trout hsp70 clones was isolated and subjected to Southern blot analysis after digestion with several different
Figure 15. Sucrose gradient fractionation of RNA. Total RNA from induced RTG-2 cells (50 μM sodium arsenite, 24 h) was fractionated on a 15-35% sucrose gradient. Fractions (0.5 mL) were collected by upward displacment with 50% sucrose. RNA in individual fractions was precipitated with ethanol and aliquots translated in vitro. The translation products were separated on a 10% SDS-acrylamide gel and autoradiographed. C = water control, T = total induced RNA.
restriction enzymes. Hybridization was to a \(^{32}P\)labelled 1.0 Kb PstI fragment of the \textit{Drosophila} hsp70 gene. The results are shown in Figure 16 and homology between the \textit{Drosophila} gene and both pTHS70.7 (lanes a - d) and pTHS70.14 (lanes e - h) is evident. DNA from pBR322 was used as the control and very little hybridization to the \textit{Drosophila} gene was observed (lanes i - k). Upon further examination of pTHS70.7, it was discovered that the right half of the cDNA was not homologous to the \textit{Drosophila} hsp70 gene, and did not code for an inducible mRNA in trout cells. This anomaly was analyzed separately and the results presented in the appendix.

3.4 Further Analysis of THS70.7 and THS70.14

3.4.1 Nucleotide Sequences for THS70.7 and THS70.14

THS70.7 and THS70.14 were further analyzed by nucleotide sequencing. Partial restriction maps and the sequencing strategy are presented in Figure 17. The two cDNAs proved to be incomplete copies of hsp70 messages, and were from different regions, with an overlap of about 250 nucleotides. The primary nucleotide sequences of THS70.7 and THS70.14 are shown in Figure 18. The GC content in the coding regions of both THS70.7 (57.7\%) and THS70.14 (50.3\%) is relatively high. The overall GC content of the rainbow trout genome has been reported to be 43\% (188). Both cDNAs have one long open reading frame (Figure 18). The THS70.7 sequence contains information for a 278 amino acid long region of hsp70. This corresponds to amino acids 128 to 406 of the \textit{Drosophila} hsp70 (92). A seryl residue at position 213 of the \textit{Drosophila} hsp70 is deleted from THS70.7. A similar deletion has been reported in yeast hsp70 (65). The THS70.14 sequence contains information for the first 213 amino acids of hsp70, assuming
Figure 16. Southern blot analysis of Trout hsp70 cDNA clones. DNA from pTHS70.7, pTHS70.14, and pBR322 was isolated and digested with several different restriction enzymes. The digestion products were fractionated on a 1.0% agarose gel and DNA transferred to a NC paper. The blot was probed with a $^{32}$P-labelled fragment from the *Drosophila* hsp70 gene (a 1.0 Kb PstI fragment from 132E3, ref. 96). Digests were as follows: (a) and (e) PstI; (b), (f), and (j) EcoRI/PstI; (c), (g), and (k) BamHI/PstI; (d) and (h) SalI; (i) EcoRI. Note that pTHS70.14 DNA from this preparation was resistant to digestion by restriction enzymes, however hybridization to the hsp70 gene was not inhibited. Size markers are from a HindIII digest of phage lambda DNA.
Figure 17. Partial restriction map and strategy used to determine the nucleotide sequences of THS70.7 (A) and THS70.14 (B) cDNAs. Arrows represent the direction of sequencing from Klenow-labelled fragments, using either the chemical cleavage method (squares) or the dideoxy termination method (circles). The lengths of the arrows represent the actual number of nucleotides sequenced from each site. The areas used as hybridization probes have been indicated by a dashed line, above the maps. The boxed regions represent the cDNA sequences whereas the thin lines represent pBR322 sequences. The hatched area within the boxes indicates the region of overlap between the two cDNAs. This overlap region has a 73.3% homology at the level of nucleotide sequence. The restriction sites are: A, AvaII; B, BamHI; Bg, BglI; D, DdeI; H, HaeIII; P, PstI; S, SauIIIA; T, TaqI.
Figure 18. Nucleotide sequences for THS70.7 (A) and THS70.14 (B) cDNAs with their predicted amino acid sequences. The overlap region between the two cDNAs has been indicated by a line above the respective amino acid sequences. The single letter amino acid code is A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, Lysine; L, Leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
that it starts at the first methionine. The predicted amino terminal sequence for THS70.14 differed from that of the *Drosophila* gene, in that it contained an extra three amino acids.

3.4.2 Comparison of Hsp70 from Trout, *Drosophila*, and Yeast

The predicted amino acid sequence of a complete *Drosophila* hsp70 gene and of an inducible yeast hsp70 gene (YG100) have been published (92, 65). Figure 19 compares a section of these sequences to that of THS70.7. Amino acids that differ have been indicated. The highly conserved nature of the three hsp70 sequences is evident. Many of the differences in the three hsp70 sequences occur in the same positions. Conservative amino acid changes account for approximately 55% of the differences between the trout and *Drosophila* sequences, and 48% of those between trout and yeast.

The extent of homology between THS70.7, THS70.14, *Drosophila* hsp70 (92) and YG100 (65) is summarized in Figure 20. In all cases, the percentage homology was calculated for the total sequence information available for the overlap regions. As expected, the degree of sequence divergence at the nucleotide level is greater than that at the amino acid level. Interestingly enough, the nucleotide sequences of THS70.7 and THS70.14 are only 73.3% homologous in their overlap region, yet their homology at the amino acid level is quite high (88%). Thus even within trout, codon preference varies between the two genes in the regions analyzed. Compared with the *Drosophila* hsp70 gene, YG100 has less homology to both THS70.7 and THS70.14 at the nucleotide level. However, when the amino acid sequences are compared, YG100 and the *Drosophila* hsp70 show similar degrees of homology to the trout hsp70 (see also Figure 19).
Figure 19. Comparison of the predicted amino acid sequences of THS70.7, a 
Drosophila hsp70 gene (taken from Ingolia et al., ref. 92), and 
yeast hsp70 gene YG100 (taken from Ingolia et al., ref. 65). 
Regions compared are amino acids number 128 to 406, based on the 
Drosophila hsp70 numbering. Mismatches in the sequences have 
been boxed in. The single dashes represent deleted amino acids 
relative to the compared sequence. The solid line in the yeast 
sequence represents information not yet available.
Figure 20. A matrix summarizing the nucleotide homology (A) and the amino acid homology (B) between THS70.7, THS70.14, *Drosophila* hsp70, and YG100. The homologies are given as percentages from the total available sequence information in the overlap regions.
3.5 Synthesis and Turnover of Trout Hsp70 mRNA

3.5.1 RNA Northern and Dot Blot Analysis

To ensure that pTHS70.7 and pTHS70.14 were coding for induced hsp70 species, RNA from control and sodium arsenite treated RTG-2 cells was subjected to Northern and RNA dot blot analysis (Figure 21). The induction of hsp70 mRNA (approximate size 2.2 Kb) in the arsenite-induced cells is evident. Control cells contained very little (if any) message for hsp70. A heterologous probe from *Drosophila* (Figure 21C) resulted in a much weaker hybridization compared to that of THS70.7 (Figure 21A) and THS70.14 (Figure 21B). This is more obvious when the two sets of RNA dot blots are compared. Here, equal amounts of control and induced RNA were blotted on both filters. The THS70.7 probe gave a much stronger hybridization signal, even at shorter exposure times. Multiple bands in the induced mRNA were revealed by hybridization to labelled THS70.7 (Figure 21A). This indicates the existence of either an hsp70 multigene family in trout or of splicing intermediates from a single transcript. As will be seen from evidence presented below, the former possibility is more likely.

3.5.2 Induction of Hsp70 mRNA

The major inducible heat shock protein in trout RTG-2 cells is a 70,000 dalton species. The kinetics of induction of this hsp have been shown to depend on the intensity of stress. Here, a study was undertaken to determine whether similar kinetics are observed at the level of transcription. The cytoplasmic quick-blots were hybridized to a 0.7 Kb PstI fragment from a trout hsp70 cDNA, THS70.7. This fragment codes for amino acids 128 to 348 of hsp70, based on the reported *Drosophila* hsp70
Figure 21. Northern blot analysis of trout RTG-2 RNA from control (c) and sodium arsenite induced (i) cells. Either 2 μg (A and B) or 0.2 μg (C) of poly A\(^+\) RNA was glyoxalated, separated by electrophoresis on a 1.4% (A and B) or 1.2% (C) agarose gel, and transferred to nitrocellulose filters. The insets to A and C are dot blots of total RNA, 5 μg on each spot. Hybridization was to \(^{32}\)P-labelled THS70.7 (A), THS70.14 (B), or a 1.0 Kb PstI fragment from a Drosophila hsp70 gene (C) at 42°C in 50% formamide. The size was estimated by comparison to glyoxalated HindIII digested DNA of phage lambda. Note the differences in exposure times required for the different hybridization probes: (A) and (B), 1 hour, (C) 7 days. The exposure times for the dot-bLOTS have been indicated.
sequence (92).

RTG-2 cells, grown at 22°C, are induced to synthesize hsp70 at 27°-28°C. When these cells were placed at 28°C, the level of hsp70 mRNA increased dramatically for the first 2 hours (Figure 22). After 2 hours, a gradual decrease in hsp70 mRNA levels was seen. This suggests that either the cells begin dying off under these conditions, or that some autoregulation of mRNA synthesis occurs. Since there is no decrease in protein synthesis in these cells after 16 hours at 28°C (see Figure 9), the latter explanation is more likely. When 50 μM sodium arsenite was used as the inducer, no decrease in hsp70 mRNA at long exposure times was observed (Figure 23); the rate of increase in hsp70 mRNA levels was similar to that seen with temperature induction. After 24 hours of exposure to arsenite, the level of hsp70 mRNA in the cells was maintained, and even rose slightly. This agrees with our previous observations on rates of hsp synthesis, i.e. maximal rates of hsp synthesis occur following long exposures (e.g. 24 hours) to 50 μM sodium arsenite, where translation of pre-existing mRNAs is also maximally inhibited.

The induction of hsp70 by sodium arsenite is rapid, occurring within minutes. The level of hsp70 expression was followed immunologically using antibody to chicken hsp70. This antibody cross-reacts strongly with the trout hsp70 (E.A. Burgess, personal communication). Trout hsp70 was detectable at low levels in uninduced cells by this technique. This may be due to the presence of hsp70-like genes in trout that are expressed constitutively at a low level. Increased levels of hsp70 were detected as early as 5 minutes after sodium arsenite induction with the largest accumulation occurring after 1 hour of induction.

As mentioned above, the intensity of the response to heat-shock
Figure 22. Induction of hsp70 mRNA by heat-shock. RTG-2 cells were treated with a 28°C heat-shock for various time periods. No recovery time was allowed. Cells were harvested and cytoplasmic quick-blots of RNA were performed as described under Experimental Procedures. The quick-blots were hybridized to a $^{32}$P-labelled trout hsp70 cDNA, washed, and subsequently fluorographed. The inset shows the hybridization signals obtained at the various time points. To quantify the signals, spots on the nitrocellulose filter corresponding to bound RNA were cut out and the amount of radioactivity hybridized to each was determined. The same procedure was used to obtain the results in Figures 23-26. HS = heat-shock.
Figure 23. Induction of hsp70 mRNA with sodium arsenite. RTG-2 cells were exposed to 50 μM sodium arsenite for various times. No recovery was allowed. The cells were processed in the same manner as described in the legend to Figure 22. SA = sodium arsenite.
reflects the level of stress applied. For instance, when trout cells were 
induced by increasing concentrations of sodium arsenite, the levels of 
hsp70 mRNA rose until a lethal exposure limit was reached (Figure 24). The 
decrease in hsp70 mRNA, after a peak at 50 μM sodium arsenite, 
corresponds to the general decrease in protein synthesis at higher arsenite 
concentrations.

3.5.3 Repression of Hsp70 mRNA Synthesis

After an initial stress, cells were allowed to recover under normal 
growth conditions and the level of hsp70 mRNA was examined. Recovery from 
a 1 hour heat shock (28°C) was very rapid, with hsp70 mRNA reaching control 
levels by 3 to 4 hours (Figure 25). The efficient repression of hsp70 mRNA 
synthesis and its rapid degradation during recovery from heat shock 
indicate that both transcription and stability of these messages are 
subject to precise control. This is especially striking since after 1 hour 
at 28°C the cells were still actively synthesizing hsp70 mRNA (see Figure 
22).

The recovery of RTG-2 cells from a 2 hour, 50 μM sodium arsenite 
exposure (Figure 26) was slightly different than the recovery from heat 
shock. The level of hsp70 mRNA continued to rise even after the arsenite 
had been removed. This may be explained by the persistence of 
intracellular arsenite during this period, or it may reflect a slower 
inactivation of the hsp70 gene. In either case, the decrease in hsp70 mRNA 
levels following recovery from arsenite treatment is apparent, although not 
as rapid as in the case of recovery from heat shock.
Figure 24. Induction of hsp70 mRNA under different sodium arsenite concentrations. RTG-2 cells were exposed to different concentrations of arsenite for 3 hours followed by 2 hours of recovery. The cells were processed in the same manner as described in the legend to Figure 22. SA = sodium arsenite.
Figure 25. Hsp70 mRNA levels during recovery from heat-shock. RTG-2 cells were heat-shocked at 28°C for 1 hour and then allowed to recover at 22°C for different lengths of time. The cells were processed in the same manner as described in the legend to Figure 22. C = control cells, no heat-shock.
Figure 26. Hsp70 mRNA levels during recovery from sodium arsenite shock.
RTG-2 cells were treated with 50 μM arsenite for 2 hours and then allowed to recover in fresh medium for different lengths of time. The cells were processed in the same manner as described in the legend to Figure 22. C = control cells, no arsenite shock.
3.6 DNA Southern Blot Analysis

3.6.1 Detection of Multiple Hsp70 Genes in the Trout Genome

Genomic DNA was isolated from trout testis and subjected to analysis by the Southern blot technique. Several restriction digests were performed and the DNA was fractionated on agarose gels. After transfer of the DNA to nitrocellulose filters, hybridizations were carried out under stringent conditions to hsp70 probes. The results in Figure 27A were obtained using a nick-translated THS70.7 fragment which spans amino acids 128 to 348 of the hsp70, and contains a PstI site at the 3' end. Strong hybridization to two bands was detected when PstI was used to cleave the genomic DNA (Figure 27, lane 1), suggesting the presence of at least two hsp70 genes. Hybridization to BamHI cleaved DNA (Figure 27, lane 2) revealed a larger fragment of approximately 8 Kb. The existence of two hsp70 genes on this BamHI fragment is possible, since the digestion of trout DNA with both BamHI and PstI resulted in the disappearance of the large BamHI fragment and the appearance of the two smaller PstI fragments (Figure 27, lane 6). However, the existence of two different BamHI fragments, each containing an hsp70 gene, is also a possibility. Hybridization to EcoRI cleaved trout DNA (Figure 27, lane 3) revealed a number of bands of different intensities, possibly due to incomplete digestion of the DNA. Genomic trout DNA was also subjected to double digests and hybridized to the THS70.7 fragment (Figure 27, lanes 4-6) or to the 1.0 Kb PstI fragment from a Drosophila hsp70 gene (Figure 27, lanes 7 and 8) spanning amino acids 1 to 312 in addition to some 5' non-coding sequence. Both probes produced similar results but THS70.7 resulted in much better signals, as expected. The detection of two hsp70 sequences in the trout genome does not rule out
Figure 27. Southern blot analysis of genomic DNA isolated from trout testis. Approximately 6 µg of the genomic DNA was digested with PstI (lane 1), BamHI (lane 2), EcoRI (lane 3), PstI and EcoRI (lanes 4 and 7), BamHI and EcoRI (lanes 5 and 8), or PstI and BamHI (lane 6), and separated by electrophoresis on a 1.2% (A) or 0.9% (B) agarose gel. The DNA was then transferred to nitrocellulose filters. Hybridization was to $^{32}$P-labelled THS70.7 (A), or a 1.0 Kb PstI fragment from a Drosophila hsp70 gene (B), at 42°C in 50% formamide. The size markers are from a HindIII digest of phage lambda DNA.
the presence of additional hsp70 genes. Sequence divergence, and the incomplete nature of the cDNA probes used, might allow other hsp70 sequences in the genome to go undetected. It is worth noting, however, that washing filters at lower stringency yielded similar results (Figure 28).

3.6.2 Identification of Hsp70-like Sequences in other Genomes

To investigate interspecies hsp70 sequence homology, genomic DNA from a variety of sources (trout testis, RTG-2 cells, HeLa cells, C. elegans, bovine liver and D. melanogaster) was cleaved with PstI and subjected to Southern blot analysis. Hybridization was to either THS70.14 (Figure 29, lane 1) or to the THS70.7 fragment described above (Figure 29, lanes 2-7). It should be noted that the filters containing trout DNA were exposed to film for a much shorter time than the filters containing the other DNAs. The THS70.14 fragment (spanning amino acids 1 to 210 and some 5' non-coding region) hybridized to the same two PstI fragments in trout DNA as those detected by the THS70.7 probe. In addition, two larger PstI fragments were evident, revealing hsp70 sequences that were not detected by the THS70.7 probe. Cross-hybridization of the trout hsp70 sequences with sequences in the genomes of other organisms was also evident (Figure 29, lanes 4-7). Multiple bands are evident in HeLa cell DNA, bovine liver DNA, and D. melanogaster DNA. Hybridization to the C. elegans DNA was very weak and may be due to the relatively high AT content (64%) of its genome (189).
Figure 28. Effect of washing stringency on signal detection from genomic Southern blots. Approximately 6 μg of trout DNA was digested with either PstI (P) or BamHI (B). The cleaved DNA was fractionated by electrophoresis through a 1.0% agarose gel, and transferred to NC paper. Hybridization was to a $^{32}$P-labelled 0.7 Kb PstI fragment from pTHS70.7 (see Figure 17). The washing conditions were as follows: (1) 2XSSPE/0.1% SDS, twice for 15 minute at room temperature; (2) same as in (1) plus a 15 minute room temperature wash in 0.1XSSPE/0.1% SDS; (3) same as in (2) plus a 15 minute 50°C wash in 0.1XSSPE/0.1% SDS.
Figure 29. Southern blot analysis of PstI digested genomic DNA from various sources: 6 μg trout testis DNA (lanes 1 and 2), 4 μg RTG-2 DNA (lane 3), 6 μg HeLa cell DNA (Lane 4), 3 μg C. elegans DNA (lane 5), 8 μg bovine DNA (lane 6), and 3 μg D. melanogaster DNA (lane 7). The DNA was cleaved, fractionated by electrophoresis through a 1.0% agarose gel, and transferred to nitrocellulose filters. Hybridization was to [\(^{32}\)P]labelled THS70.14 (lane 1) or THS70.7 (lanes 2 to 7), at 42°C in 50% formamide. The hsp70 related sequences have been indicated by triangles next to appropriate bands. Note that the filters containing trout DNA were exposed for 2 days to X-ray film, compared with 8 days for the other filters. Size markers were from a HindIII digest of phage lambda DNA.

Note: lane 3 was from a different electrophoresis run.
3.7 Trout Genomic DNA Libraries

3.7.1 Screening of the CH4A Lambda Library

To ensure 99% probability that the entire trout genome (approximately $2.8 \times 10^9$ bp) is represented, one would need to package and obtain approximately $10^6$ pfu in vitro. The trout CH4A library contained only $0.25 \times 10^6$ individual clones; despite this, a screen was carried out. Approximately $0.5 \times 10^6$ pfu from the amplified library were grown at a density of 20,000 pfu/plate. These were subsequently probed with the Drosophila hsp70 gene. Four clones that hybridized to the probe were purified. DNA from three of these ($\lambda$2b, $\lambda$C2, and $\lambda$C3) were isolated, digested with EcoRI, and subjected to Southern blot analysis (Figure 30). All three clones contained inserts with EcoRI restriction sites; with $\lambda$2b and $\lambda$C2 having a common 4.6 Kb EcoRI fragment that hybridized to the Drosophila hsp70 gene, and $\lambda$C3 containing a 5.5 Kb fragment that also hybridized to the hsp70 gene (Figure 30).

Although the initial results looked promising, further analysis of these clones showed no hybridization to trout mRNA from control or induced cells. An interesting point was the similarity of the 4.6 Kb EcoRI fragments from $\lambda$2b and $\lambda$C2. Partial nucleotide sequencing of these fragments showed them to be identical although no similarity to the Drosophila hsp70 sequence was evident (data not shown).

A second attempt at screening the CH4A library, using THS70.7 as the probe, resulted in no positive hybridizations.

3.7.2 Screening of the L47.1 Lambda Libraries

The two L47.1 libraries contained $0.5 \times 10^6$ pfu and $3 \times 10^6$ pfu,
Figure 30. Southern blot analysis of trout genomic clones. DNA from clones λ2b, λC2, and λC3 was digested with EcoRI, fractionated on a 0.7% agarose gel and Southern blotted to NC paper. The DNA was hybridized to a \[^{32}P\]labelled 2.0 Kb SalI fragment from a Drosophila hsp70 gene (clone 56H8, ref. 96). Hybridizing bands have been indicated and size assigned by comparison to fragments from a HindIII digest of phage lambda DNA.
respectively. However, the amplified versions of these libraries may not have been entirely representative since the plaque size obtained from growth on Q359 was very small (note that Q359, a P2 lysogen, was used since recombinants in λL47.1 are rendered Spi⁻ and thus may be selected by their ability to grow in P2 lysogens of E. coli). Prior to screening of the libraries, the 15-20 Kb fractionated DNA of the MboI partial digests was restricted with BamHI and subjected to Southern blot analysis. Hsp70-like sequences were detected in both the total MboI partials and the 15-20 Kb MboI partials (Figure 31). However, after two separate attempts at screening the L47.1 libraries, no positives were detected.
Figure 31. Southern blot analysis of MboI partial fragments from trout genomic DNA. Both 15-20 Kb fractionated MboI partials (A) and total MboI partials (B) of trout DNA were digested with BamHI and fractionated on a 1.0% agarose gel. After transfer to NC paper, the DNA was probed with a $^{32}$P-labelled 0.7 Kb PstI fragment from pTHS70.7 for hsp70-like sequences. Size markers are from a HindIII digest of phage lambda DNA.
IV. DISCUSSION

The results presented in this thesis, on the heat shock response in trout cells, provide a base for the study of this phenomenon in a vertebrate system. The various experiments have helped to further emphasize the complex nature of the regulation of the heat shock response with controls at several stages of gene expression. In addition, the comparison of hsp70 from trout to those of other organisms supports the conclusion that hsp70 genes form a highly conservative gene family.

The relevance of the data presented is discussed in terms of the importance of the response to the organism. The maintenance of the heat shock response in both vertebrates and invertebrates suggests a fundamental role for it. In addition, the conserved nature of this phenomenon implies a similar function in all organisms. Thus the heat shock response of trout has been discussed with emphasis on the above mentioned aspects.

4.1 The Heat Shock Response in Trout Cells

The response of cultured cells of rainbow trout, *S. gairdnerii*, to either temperature elevation or incubation in the presence of sodium arsenite is very similar to that of *Drosophila* (2). A novel set of polypeptides (the hsp70) is rapidly induced and depending on the severity of the stress applied, normal protein synthesis can also be decreased. For instance, exposing cells to 50 μM sodium arsenite for 24 hours resulted in a dramatic reduction of normal protein synthesis with only the hsp70 being produced (Figure 4). It has become fairly obvious that the heat-shock response is a reaction to stress in general and not temperature
perturbation alone. In support of this, the response of trout cells to heat or arsenite was observed to be similar in most respects. Some differences were observed however: some hsps were induced by arsenite but not heat, repression of normal protein synthesis varied between the two inducing agents, induction and/or stability of hsp70 mRNA differed during extended exposures to arsenite or heat, and degradation and synthesis of hsp70 mRNA also differed upon return of cells to normal conditions. These differences will be discussed separately.

Molecular weights of the hsps were assigned by comparison to standard proteins. They were determined to be 87 Kd, 70 Kd, 62 Kd (arsenite induced only), 42 Kd, 32 Kd, and 30 Kd. An additional protein of 100 Kd (arsenite induced only) may also be considered an hsp. As with most organisms, the trout hsps fall into three classes: hsp83-like, hsp70-like, and small hsp-like polypeptides.

Optimum conditions for the induction of trout hsps were determined to be 27°C to 29°C (compared with the normal growth temperature of 22°C) for heat-shock or 15 to 100 uM for sodium arsenite exposure. It should be noted that there was no trigger point for the induction of the heat shock response; in fact the rate of hsp synthesis was proportional to the severity of stress. Consider sodium arsenite: as its concentration was increased the levels of hsps also increased. This pattern continued until a lethal concentration was reached, after which the cells began to die off. A similar reaction was observed with temperature shocks. Thus the cell must be able to monitor the amount of discomfort and respond correspondingly. The response of trout cells to sodium arsenite was very similar to that found in chick embryo fibroblasts (52), i.e. different hsps become induced at different arsenite concentrations. For instance, hsp70
was readily induced at all concentrations between 1 and 300 μM whereas hsp30 could only be induced between 15 and 50 μM to any extent. Thus, although the heat-shock genes are coordinately regulated, they seem to be differentially expressed.

One of the features of the heat shock response is its rapid induction. The entire cell protein synthesis apparatus seems to be geared towards an increased production of hsps during a stress response. This shift is so efficient that several hsps become major constituents of the cell after a few hours of induction. In trout, hsp70 and hsp30 were readily visible on Coomassie blue stained SDS-polyacrylamide gels of proteins from cells induced for extended periods. Thus, the presence of hsps in such large numbers implies an important function for them, otherwise one would not expect the cell to expend unnecessary energy in times of stress. As mentioned, hsp70 is one of the two major hsps of trout. This is also the case for most organisms studied to date (1). Since the heat shock response is observed in all organisms and hsp70 is the major hsp of these organisms, logic dictates that some conservation of this protein would be maintained through the different species. Several experiments were done to compare the trout hsp70 with that of Drosophila to investigate the above expectation. One of these involved the mobility of trout and Drosophila hsps on an SDS-polyacrylamide gel (Figure 7). The only hsp to have identical mobility in the two systems was the hsp70. Other similarities were determined at the structural level and will be discussed later.

One approach that can help to determine the function of hsps is to localize these proteins to certain parts of the cell. In this thesis however, the localization of trout hsps was not extensively investigated.
The limited results obtained from pulse-chase experiments seemed to suggest that both hsp70 and hsp30 were translocated to the nucleus. However, the bulk of the labelled hsps was localized in the cytoplasmic fraction. The ambiguous results may have been due to the conditions of the experiment. Although the induction of the trout cells was at an elevated temperature, the pulse and chase portions were done at normal growth conditions after a recovery period of 2 hours. Velazquez and Lindquist (190) have recently reported the stress dependent translocation of hsp70 in *Drosophila* cells. Their use of indirect immunofluorescence with monoclonal antibodies to hsp70 has helped to localize the protein predominantly in the nuclei of heat-shocked cells. During recovery, the hsp70 was seen to migrate back into the cytoplasm. Thus in the case of the pulse-chase experiment described in this thesis (Figure 13), the results may actually be reflecting the situation in a recovering cell. Further experiments would need to be done to confirm the above suspicion.

4.2 Regulation of the Heat Shock Response

The complex nature of the regulation of the heat shock response has become apparent in the last few years. Several levels of control seem to be coordinated in some manner to produce a rapid response to certain stressful agents. In some organisms, one type of control occurs at the level of normal protein synthesis. It has been known for some time that normal cellular mRNAs are not degraded during heat-shock, but rather are selectively repressed (13, 45-47). Upon return of the cells to normal conditions, the pre-existing mRNAs are released from this inhibition. Yeast cells do not exhibit the same control on pre-existing mRNAs but
rather allow them to be degraded at the normal rate (48). In vitro translation of trout mRNA show that normal cellular mRNAs are present but not translated during induction of the heat-shock genes. Thus it would be reasonable to assume that translational control occurs in trout as it does in most other organisms. The purpose for this translational control may be to allow selective translation of heat-shock mRNAs and thus rapidly and efficiently express the hsp. This suggestion was supported by evidence showing the rapid induction of hsp in trout cells. For instance, the two major hsp of trout (hsp70 and hsp30) were readily detected in cells induced for short periods at 37°C. The detection was based on the incorporation of \[^{35}\text{S}\]methionine into the proteins. Since the labelling was carried out at 22°C for 1 hour, one cannot rule out the possibility that the entire hour was necessary for hsp synthesis. However, the rapid appearance of trout hsp70 following induction has also been monitored by immunological methods (E.A Burgess, personal communication), and an increase in hsp70 was detected as early as 5 minutes after the addition of arsenite to the cells.

The expression of heat-shock genes is also regulated at the level of transcription. As with most organisms studied, the induction of hsp in trout cells is dependent on new transcription. This was determined by analyzing transcripts from both normal and induced cells. The levels of hsp70 mRNA in sodium arsenite induced RTG-2 cells were much greater than in control cells (Figure 21). The multiple bands for hsp70 mRNA observed on Northern blots most probably represent different species of hsp70 message in trout. To further study transcriptional control in trout, the kinetics of induction and recovery of cells from heat-shock or sodium arsenite exposure were examined by measuring hsp70 mRNA levels. There are at least
two and probably more hsp70-like genes in trout and since only one of the trout hsp70 cDNAs (THS70.7) was used in these studies, definite conclusions can only be made concerning the induction and recovery of one hsp70 mRNA species. However, since the levels of total hsp70 polypeptides seem to parallel the mRNA levels quite closely, the conclusions can probably be generalized to include all inducible hsp70 mRNAs in these cells.

Induction of trout hsp70 was rapid (of the order of a few minutes) when cells were subjected to either heat or sodium arsenite stress. This parallels the findings in Drosophila, where complete hsp70 mRNAs are detected 4 minutes after temperature elevation (43). As mentioned earlier, during sodium arsenite induction of trout cells, immunological detection of hsp70 within 5 minutes after start of the induction is possible (E.A. Burgess, personal communication). This is in contrast to results obtained by hsp70 cDNA hybridization to mRNA from these cells which do not show significant levels of hsp70 mRNA until 15 minutes after start of induction. The disparity between the appearance of the protein and the presence of the transcript may reflect the sensitivity of the two techniques. Since one technique relies on the detection of the protein whereas the other on detection of a transcript, results from both cannot be quantitatively compared. More than one gene may code for the trout hsp70 and transcripts from other genes may not hybridize to the hsp70 cDNA clone under conditions of high stringency. It should be noted that trout hsp70 was detected immunologically with antibody to chicken hsp70. The cross-reaction of the hsp70s from these two species further emphasizes the highly conserved nature of this protein. The rapid rate of induction suggests that the heat-shock genes, although inactive in uninduced cells, are maintained in an "alert" state ready for immediate transcription. One
major difference was observed between the induction pattern obtained by heat-shock versus sodium arsenite. Prolonged exposure to arsenite resulted in continued synthesis and/or maintenance of trout hsp70 mRNA levels (Figure 23). In contrast, continuous heat-shock at 28°C for longer than 2 hours caused a decrease in hsp70 mRNA levels (Figure 22). Since protein synthesis was still occurring in cells heat-shocked for up to 16 hours (Figure 9), cell death is not a likely cause of the latter behaviour. This leaves the possibility of autoregulation and may also reflect a type of cellular adaptation to thermal shock. In addition to transcriptional control differences, hsp70 mRNA stability may be affected differently by heat-shock and arsenite exposure.

The level of heat-shock mRNA induction seems to reflect the intensity of the inducing stimulus. The levels of hsp70 mRNA varied with the concentration of sodium arsenite used (Figure 24); hsp70 mRNA levels rose until a concentration of approximately 50 µM arsenite was reached, after which the cells began to die. This behaviour was also reflected at the level of hsp synthesis (Figure 11). This relationship between the intensity of the stress and the magnitude of the response has also been reported in Drosophila (42, 43).

Finally, the control of the recovery from heat-shock was monitored in trout cells. As was the case with induction, the repression of the hsp70 mRNA synthesis was very rapid, especially during recovery from temperature stress (Figure 25). Recovery from sodium arsenite differed in that hsp70 mRNA continued to be synthesized for approximately 30 minutes after removal of the inducer (Figure 26). After the initial lag period, hsp70 mRNA levels decreased, but more slowly than during recovery from temperature stress. The short lag may be due to the persistence of intracellular
arsenite or may reflect a separate mechanism for repressing hsp70 mRNA synthesis. Rapid recovery of cells from stress situations has also been observed in Drosophila (42, 44) and the actual kinetics of recovery varies with the severity of the initial heat-shock (44). An interesting feature of this complex pattern of recovery is the requirement for functional hsps. In Drosophila, absence of functional hsps prevents the cells from attaining complete recovery after a heat-shock (42).

The regulation of the heat shock response is dependent on a variety of factors, including: the intensity, duration and nature of the stress, and the presence of functional hsps for autoregulation and recovery. In addition, pre-existing mRNAs are subject to translational control (13, 45-47). It is evident that regulation of the heat shock response is a complex process with manifestations at several stages of gene expression (Figure 32).

4.3 The Conserved Nature of the Heat Shock Response

The presence of the heat shock response has been observed in a wide range of organisms, spanning all three primary kingdoms. For this phenomenon to be maintained through evolution, it must have been under considerable selective pressures. An interesting suggestion made by Lee et al. (21) was that the development of an "oxidation stress" in cells may be a common factor among inducers of the heat shock response. Since protection from excess intracellular oxygen would have been a necessity early in the evolutionary time scale, one would have expected the cell to have developed a stress response. It may have followed that this response was maintained and modified to include other stress situations.
Figure 32. Model for the regulation of the Heat Shock Response. The diagram summarizes some of the steps involved in the regulation of the heat shock response. Briefly, the inducer (1) disrupts pre-existing polysomes and sequesters the mRNA. These are kept under translational control and are only expressed when the cell is returned to normal conditions. The inducer could act directly on the heat-shock genes but more likely through an intermediate (2). The identification of this secondary messenger has yet to be determined. Rapid transcription of the heat-shock messages occurs (3) followed by processing and transport to the cytoplasm (4). Polysomes form on these newly made messages (5) and selective translation (6) results in the synthesis of hsp5 (hatched boxes). These proteins could in turn affect the regulation of the response by controlling the rate of recovery of the cell (7), by binding to the cytoskeletal structure (8), by inactivating the secondary messenger (9), and by binding to heat-shock DNA (10) or RNA (11). C = cytoplasm, N = nucleus.
Although the precise role for this phenomenon has yet to be determined, it is becoming evident that it has an overall protective function. This is supported by studies showing that a mild heat-shock preceding a normally lethal heat-shock confers thermotolerance on cells (29, 85, 86). Such thermotolerance can be conferred upon cells by other inducers of the response, such as sodium arsenite (29). The migration of the major Drosophila hsp to the nucleus during heat-shock, and its association with decondensed chromatin would also imply a protective function (190). Since functional properties of hsps have been suggested to be similar in all organisms, it should follow that structural properties of hsps also be conserved. One approach in this respect has been to compare the major hsp (hsp70) of all organisms. Methods such as proteolytic cleavage, immunological cross-reactivity, or cross-hybridization at the nucleic acid level have been used. However, the best method of comparison is by determining the sequence of these proteins. Thus far, the only reported sequence information for a eukaryotic hsp70 is for Drosophila (92) and yeast (65). They show 72% homology at the nucleotide level. Recently, the sequence for the E. coli dnaK gene was reported (68). It shows 57% identity at the nucleotide level to the Drosophila hsp70 gene. Such conservation is striking and a similar homology with trout hsp70 would enhance these findings and help to narrow down conserved domains which may reveal its functional properties.

Two hsp70 cDNAs, THS70.7 and THS70.14 from rainbow trout, S. gairdnerii have been identified and analyzed in this thesis. The predicted amino acid sequences from these cDNAs are very similar to those reported for the hsp70 genes of D. melanogaster (92) and S. cerevisiae (65). The presence of a multigene family of hsp70 sequences in the trout genome has
been inferred from the hybridization of genomic DNA blots to the hsp70 cDNAs. The existence of multiple hsp70 genes in trout is also supported by the presence of multiple spots on two dimensional polyacrylamide gels of protein samples from in vitro translated, hybrid-selected mRNA for trout hsp70 (E.A. Burgess, personal communication).

The nucleotide sequences of the two cDNAs were determined and compared to hsp70 sequences from Drosophila and yeast (Figure 20). The extent of homology is striking, especially at the amino acid sequence level. The amino acid sequences coded for in the trout cDNAs are approximately 79% homologous with both Drosophila and yeast hsp70. When compared to the E. coli dnaK protein (68), the THS70.7 shows 55% identity. For organisms representing phyla that diverged early in the evolutionary time scale, this degree of sequence conservatism is remarkable. Hsp70-like proteins have been observed in many other organisms, and a high degree of sequence conservatism can now be predicted on the basis of the following observations: the similarities of the induction process in most organisms studied (1), the cross-reaction of a chicken hsp70 antibody with similar proteins in widely divergent species (64), the cross-hybridization of hsp70 genes from one organism to the genomic DNA and RNA of other organisms (50, 57, 65-67). The conservatism of the heat shock response is not limited to the hsp70-like proteins; similarities among different hsp83-like proteins (64) and among the small hsp8 (69, 70) have been reported. The results presented here support the conclusion that the hsp70 genes form a highly conservative gene family.
4.4 Genomic Organization of Hsp70 Genes

The organization of hsp70 genes has been most extensively studied in *Drosophila* (2). These genes are present in multiple copies in the *Drosophila* genome and are found clustered within a few kilobases. Similarly, an hsp70 multigene family has been identified in yeast (65). The occurrence of hsp70 multiple gene copies in the trout genome has been supported by experiments presented in this thesis. In addition, the analysis of genomic DNA from different organisms has revealed the presence of multiple bands on Southern blots probed with hsp70 sequences. For instance, the presence of at least two and possibly more hsp70-like genes in trout was observed. Why does the cell need extra copies of the hsp70 gene? This question has not really been answered, however, several suggestions have been made. The presence of multiple copies of the hsp70 gene may help produce the rapid response to stress i.e. a lot more transcripts can be made in a short time. Another possibility is that the different hsp70 genes code for slightly different hsp70s having different targets in the cell.

Although the construction of lambda libraries containing trout genomic DNA was partially successful, screening of these libraries ended in failure to isolate hsp70 genes. For a genome the size of trout (3 x 10^9 bp), one would need approximately 10^6 clones with inserts of 15 to 20 Kb in length to represent the entire genome. We obtained more than 3 x 10^6 clones from two attempts at constructing a trout genomic library in λ47.1 phage, yet no positives for hsp70 sequences were identified. Since the 15-20 Kb *MboI* partials used to construct the libraries contained the hsp70 sequences (Figure 31), the only explanation for the lack of any positives was the
possibility of under-representing the genome after the library amplification stage. In the case of the CH4A library, incomplete representation of the genome may have been due to the low number (2.5 x 10^2) of individual clones obtained. Although no hsp70 genes were isolated from this library, several clones were found to contain inserts that showed some hybridization to the *Drosophila* hsp70 gene even under stringent conditions (Figure 30). In addition, two of these clones have been shown to contain identical sequences based on restriction mapping, Southern blot analysis (Herb Chang, personal communication) and partial sequence analysis. However, hybridization of these sequences to trout RNA on Northern blots have failed to reveal any bands. Thus, the possibility exists that these sequences represent pseudogenes or cognate genes for hsp70 in trout. The presence of some repetitive sequence in the clones causing hybridization to the *Drosophila* hsp70 gene cannot be ruled out either.

4.5 Conclusions and Future Prospects

The results presented in this thesis have shown the heat shock response of trout to be very similar to other organisms. Different aspects of this response were analyzed and are summarized in Table V.
TABLE V. Summary of the Heat Shock Response in Trout Cells

<table>
<thead>
<tr>
<th>Heat-inducible hsps</th>
<th>87, 70, 42, 32, and 30 (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium arsenite inducible hsps</td>
<td>100, 87, 70, 62, 42, 32, and 30 (Kd)</td>
</tr>
<tr>
<td>Translational control on pre-existing mRNAs</td>
<td>Yes</td>
</tr>
<tr>
<td>Transcriptional control</td>
<td>Yes</td>
</tr>
<tr>
<td>Response reversible</td>
<td>Yes</td>
</tr>
<tr>
<td>hsp70 sequence conservation</td>
<td>Yes</td>
</tr>
<tr>
<td>hsp70 multigene family</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The characterization of the heat shock response in trout should provide a suitable base from which to investigate other aspects of this phenomenon. Of immediate concern however, would be to isolate genomic copies of the trout hsp70 sequence. This would facilitate, among other things, the analysis of control regions flanking the gene. Expression in heterologous systems would allow for the study of hsp70 regulation under controlled conditions. How well the expression is regulated would allow one to better understand the conserved nature of the response. Similarly, one can study the structure of actively transcribing hsp70 chromatin since fairly rigid control can be maintained by introducing or removing the inducing agent. Beside the use of the heat shock response as an ideal model system for the study of gene regulation, other aspects of the phenomenon can also be investigated. For instance, the linking of heat-inducible promotors onto genes coding for medically or commercially
important proteins would enable one to control the expression of these
genes and thus make these proteins more accessible.

Another area of the heat shock response that seems to be gaining more
attention is hsp function. Although we can be fairly sure that hsps serve
a protective function, the exact manner in which they accomplish it has yet
to be determined. Why does the cell need different types of hsps and hsp
variants? Thus analysis of the structure of other trout hsps and in
particular hsp30 may prove invaluable in answering some of the questions
raised above. It is my feeling however, that since the heat shock response
is so complex, answering one question will probably raise ten others.
V. BIBLIOGRAPHY


VI. APPENDIX - Is T31 an IS-Element?

As mentioned earlier, when the insert to pTHS70.7 was sequenced and analyzed, an unusual feature became evident. The total insert of about 2.2 Kb length consisted of two separate and unrelated sequences. One half of the insert (850 bp) had partial sequence information for a trout hsp70. The hsp70 cDNA came to an abrupt halt and the rest of the insert (1370 bp) was totally unrelated to it. The hsp70 coding region was further analyzed and discussed earlier in this thesis.

Due to the unusual nature of the insert in pTHS70.7, two other trout cDNA libraries were screened for the presence of other such "fusion" cDNAs. The probe used for this purpose consisted of 170 bp of hsp70 sequence and 690 bp of the unassigned sequence from pTHS70.7. The two new cDNA libraries were made, from mRNA of arsenite-induced RTG-2 cells, using the double tailing method.

A high density screen of these libraries revealed the presence of a number of clones homologous to the probe. All of these clones were analyzed by restriction mapping which proved them to be identical to the unassigned sequence from pTHS70.7. One of the new clones, pT31, was studied further by nucleotide sequencing. The partial restriction maps and sequencing strategies for the right half of THS70.7 and T31 are shown in Figure 33. The similarity of the two clones was further enhanced when their nucleotide sequences were compared. There was base for base sequence identity between T31 and the right half of THS70.7, except that the ends of these sequences varied in length by a few bases. For this reason, only the total base sequence for T31 is presented here (Figure 34). In addition, the 5' and 3' ends of the two sequences are compared in Figure 35. An interesting
Figure 33. Partial restriction map and strategy used to determine the nucleotide sequences of THS70.7 and T31 cDNAs. Arrows represent the direction of sequencing from Klenow-labelled fragments, using either the chemical cleavage method (squares) or the dideoxy termination method (circles). The lengths of the arrows represent the actual number of nucleotides sequenced from each site. The boxed regions represent the cDNA sequences whereas the thin lines represent pBR322 DNA. The hatched area represents part of the hsp70 coding region from THS70.7. The restriction sites are: A, AvaII; B, BamHI; P, PstI; S, SauIII; Sm, SmaI; T, TaqI.
Figure 34. Nucleotide sequence for T31 with its predicted amino acid sequence. The single letter amino acid code has been given in the legend to Figure 18.
Figure 35. The inverted repeat of T31. The length of the perfect repeat is at least 25-31 bp. The unusual number of GC pairs at both the 5' and 3' ends may explain how this element was readily cloned into the PstI site of pBR322.
finding was the indentification of a perfect inverted repeat at the ends of the T31-like sequences (Figure 35). One of the characteristics of mobile elements is the occurrence of an inverted repeat at the ends of a region of DNA. This may explain how the T31-like sequence inserted itself within the hsp70 sequence of THS70.7. However, since no duplication is observed at the site of integration, the latter explanation may not be valid.

Although no promoter like sequences were identified in the T31 element, one long open reading frame was present (Figure 34). This 375 amino acid long region contained both an ATG start site and a TAA stop codon. No polyadenylation signal (AAUAAA) could be identified on the 3' side of the stop codon. The hypothetical protein sequence for T31 was sent to the National Biomedical Research Foundation at the Georgetown University Medical Center in Washington, D.C. A search was conducted in their protein sequence database for homology to the T31 hypothetical protein. Although no extensive homologies were found in the 2538 sequences searched, the best homology was to the IS4 hypothetical protein I from E. coli. The IS-like qualities (see Table VI) of T31 would suggest that the limited homology to the E. coli IS4 was not a coincidence.

<table>
<thead>
<tr>
<th></th>
<th>T31</th>
<th>E. coli IS4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>1347 bp</td>
<td>1428 bp</td>
</tr>
<tr>
<td>Major ORF</td>
<td>365 aa</td>
<td>442 aa</td>
</tr>
<tr>
<td>Inverted Repeat</td>
<td>31 bp</td>
<td>18 bp</td>
</tr>
</tbody>
</table>

T31 was subjected to further analysis by using it as a probe against trout DNA and RNA (results not shown). Hybridization was not detected in
either case. The most likely explanation for these negative results is that the T31-element did not originate in the trout genome, but from the chromosome of the host strain (E. coli RR1) during the transformation procedure. To further investigate this phenomenon, E. coli B genomic DNA (Sigma Ltd.) was digested with several different restriction enzymes and Southern blot analysis carried out. When probed with the T31 sequence, several distinct bands were revealed indicating the presence of T31-like elements at several locations in the E. coli genome (Figure 36). In conclusion, the T31 sequence has been tentatively identified as a prokaryotic IS-element. These results should also serve to warn other researchers about one of the several artefacts that can be encountered during cloning procedures.

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Figure 36. Southern blot analysis of *E. coli* genomic DNA. DNA from *E. coli* (lanes 1-5, 8, and 9) and pT31 (lanes 6 and 7) was digested with a variety of restriction enzymes, fractionated on a 0.7% agarose gel, and transferred to NC paper. The probe used was $^{32}$P-labelled PstI fragments from T31. The digests in (A) were performed such that fragments from within T31 were liberated. In contrast, the digests in (B) were chosen to liberate fragments containing whole copies of T31. The restriction enzymes (with expected internal fragment size) are as follows: (1) Hinfl, 283 bp; (2) PstI/RsaI, 316 bp; (3) PstI/BamHI, 483 bp; (4) DdeI, 599 bp; (5) BamHI/RsaI, 800 bp. Lanes (6) BamHI/RsaI and (7) PstI were control digests of pT31. Lanes (8) HindIII and (9) PvuI indicate that T31 is present in 3 copies in the *E. coli* genome. Size markers are from a HindIII digest of phage lambda DNA. (Southern blot courtesy of D. Jones).