A MOLECULAR AND GENETIC ANALYSIS OF THE HEAT SHOCK RESPONSE OF

CAENORHABDITIS ELEGANS

by

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Molecular and Genetic Analysis of the Heat Shock Response of

Caenorhabditis Elegans.

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ABSTRACT

My objectives were to study the heat shock response in
Caenorhabditis elegans and to characterize the structure and
expression of its 70 kilodalton heat shock protein (hsp70)
genes.

Exposure of C. elegans to temperatures above 28°C results
in the preferential synthesis of nine polypeptides with
molecular weights between 16 and 81 kilodaltons. The synthesis
in vivo of most other polypeptides is reduced. This reduction
appears to be regulated at the level of translation. Extensive
similarities between the heat shock response of C. elegans and
the heat shock response of Drosophila are shown.

In order to study the C. elegans genes coding for hsp70,
sequences homologous to the Drosophila hsp70 gene were isolated
from a C. elegans Charon 4 genomic library. These sequences,
which constitute a small multigene family, include three
prominent single copy sequences: the class A, B and C hsp70
genes. Of these three, only the class A hsp70 gene shows heat
shock stimulated transcription. Transcription of the class C
hsp70 gene appears to be developmentally regulated. Transcripts
of the class B hsp70 gene have not been detected.

In order to place the cloned genes on the C. elegans
genetic map, the regions flanking the class A, B and C hsp70
genes were examined for sequence variation between the Bristol
and Bergerac strains of C. elegans. No sequence variation was
detected in 40 kilobases of DNA flanking the class B and C hsp70

genes. In contrast, analysis of DNA flanking the heat shock inducible class A gene shows an unusually high amount of sequence variation (7.5%) between the two strains. These variations are not the result of large DNA rearrangements but are probably the result of numerous point mutations. An hypothesis is proposed, correlating these variations with the special status of the class A hsp70 gene and its surrounding chromatin in the germline of C. elegans.

The precise mapping of the class A gene shows that it lies to the far right end of chromosome IV, approximately 0.1 map units to the left of dpy-4.

DEDICATION

To Mops and Pops

ACKNOWLEDGEMENTS

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A. General Introduction

The development of most metazoans proceeds from a fertilized egg through to the adult stage via a complex set of biochemical and morphological changes. The elucidation of how these alterations occur and what factors regulate the synchronous pattern of events that each developing embryo follows will provide insight into the molecular and cellular foundations of the developmental process.

The initial pursual of these goals was through a detailed genetic and biochemical analysis of prokaryotes. These studies showed that the expression of genes could be regulated at a variety of points along the pathway defined by the central dogma (DNA - RNA - Protein). The expression of prokaryotic genes can be regulated at the level of initiation of transcription, as for the bacteriophage lambda cro and cI genes (Johnson et al, 1981; Ptashne et al, 1980) and the E. coli lactose operon (Miller and Rezinkoff, 1980). Alternatively, gene expression can be controlled at the level of transcription termination as in the lambda N gene product (Hershey, 1971). Other, elegant examples of how prokaryotes synchronize genome expression to their growth requirements include, the attenuation system (Oxender et al, 1980) of the tryptophan operon and the feedback regulation of ribosomal protein synthesis (Nomura et al, 1980).

Many of the regulatory mechanisms described for prokaryotic gene expression rely on inherent features, such as operons and

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coupled transcription - translation, which probably do not occur in eukaryotes. Among other things, mechanisms describing the regulation of eukaryotic gene expression must take into account the packaging of DNA into nucleosomes and looped domains, the existence of various types of repetitive DNA elements and the feedback which must occur between transcription in the nucleus and translation and cellular metabolism in the cytoplasm. It was therefore necessary that model eukaryotic systems be developed and studied.

The puffing patterns of polytene chromosomes of _Diptera_ indicated that different genes were being turned on and off during development (Beerman, 1956). Subsequent analysis of _Drosophila_ and other eukaryotes has shown that differential gene expression is probably the primary control point for the regulation of the developmental process. Transcription of genes occurs in both temporal and spatial specific fashions. An elegant example of this is the vertebrate globin gene family, in which specific members of the alpha and beta globin gene families are coordinately expressed in different tissues at different times during development (reviewed by Proudfoot et al, 1980). The elucidation of the molecular mechanisms responsible for tissue specific and temporal expression of genes will undoubtedly be answered through a molecular genetical approach. This relatively new approach combines classic genetic analysis and rapid DNA sequencing (Sanger et al, 1977; Maxam and Gilbert, 1977) and molecular cloning techniques (Murray and Murray, 1974;

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Blattner et al, 1977; Maniatis et al, 1978).

Initial results indicate that eukaryotic gene expression may be regulated at a large number of control points (reviewed by Brown, 1981; Darnell, 1982). Unlike prokaryotes, the transcription of eukaryotic genes is under the primary direction of three distinct DNA dependent RNA polymerases. RNA polymerase I (pol I) transcribes rRNAs, pol II the mRNAs and pol III the 5S rRNA, pre-tRNAs, small viral RNAs and some other cellular RNAs. While little is known about the promoters on which pol I acts, a significant amount is known about the promoters with which pol II and pol III interact. The dissection of eukaryotic promoters has been made possible through the in vitro mutagenesis of cloned genes and subsequent assay for their correct transcription either in vivo or in vitro. Pol III acts upon intragenic promoters and requires the presence of positive transcription factors which are specific for the various pol III class genes (Sakonju et al, 1980; Bogenhagen et al, 1980; Engelke et al, 1980; Hofstetter et al, 1981; Galli et al, 1981; Honda and Roeder, 1980). Pol II promoters on the other hand, are located 5' to the transcription initiation point. McKnight and Kingsbury (1982) have identified three distinct promoter regions within 105 base pairs 5' of the herpes simplex thymidine kinase gene which affect the accuracy and efficiency of transcription.

Analysis of the primary structure of eukaryotic structural genes and their corresponding mRNA transcripts has shown a number of unique features. Many genes contain noncoding

intervening sequences dispersed between protein coding regions.
These introns are cut out of the primary mRNA transcript and the
protein coding regions are rejoined prior to translation
(reviewed by Chambon, 1981). In the adenovirus, some primary
transcripts contain multiple splice sites, allowing different
proteins to be synthesized, depending upon the specific introns
removed (Ziff, 1980). As transcription is occurring, a
methylated cap is enzymatically added to the 5' end of the mRNA.
After transcription, 100 to 200 residues of adenylic acid are
enzymatically added to the 3' end of most mRNAs. A conserved
sequence in the 3' noncoding region has been suggested as a
poly(A)+ addition signal (Proudfoot and Brownlee, 1976).

Several features of eukaryotic genomes indicate that
certain elements are capable of movement from one genomic
location to another. The generation of immunoglobulins involves
the somatic rearrangement of various heavy and light chain
segments (Lui et al, 1980; Davis et al, 1980; Tucker et al,
1980). Other eukaryotic elements, transposons, exhibit some
structural and functional similarities to bacterial
translocatable elements (Kleckner, 1977; Calos and MIller,
1980). These elements occur in a variety of eukaryotes
(McClintock, 1956; Cameron et al, 1979; Finnegan et al, 1978;
Emmons et al, 1983), and in Drosophila account for a large
proportion of the middle repetitive DNA. While these results
indicate that many potential control points exist for the
regulation of eukaryotic gene expression, the primary site of

control is at the level of transcription (Derman et al, 1981).

A model system which provides a unique opportunity to study gene regulation, structure and function is the 'heat shock' response. In addition to the puffing patterns noted during development, Ritossa (1962, 1964) demonstrated that a unique set of puffs could be induced in Drosophila melanogaster by subjecting larvae to elevated temperatures. Spradling et al (1977) extensively studied the RNA species induced by heat shock and found that six of the species are detected only in cells subjected to heat shock and hybridize in situ to the heat induced puffs. Further there are changes in the pattern of protein synthesis which parallel the changes in puffing patterns, resulting in the synthesis of seven to nine polypeptides (Tissieres et al, 1974). Since the initial studies in Drosophila, the 'heat shock' response has been shown to be inducible by a large variety of chemical and physical insults (reviewed by Ashburner and Bonner, 1979). In general, a 'heat shock' response is characterized by the enhanced synthesis of a small set of polypeptides and an accompanying decrease in synthesis of most polypeptides normal to development. A similar response to elevated temperatures has been reported in a wide range of organisms, including vertebrates (Kelly and Schlesinger, 1978), yeast (Miller et al, 1979), Dictyostelium (Loomis and Wheeler, 1980), E. coli (Yamamoni et al, 1978) and plants (Barnett et al, 1980).

The large amount of current interest in the heat shock response stems from the variety of molecular and cellular problems which may be addressed through dissection of this response. The main advantage of this system for molecular analysis is that a specific group of genes can be rapidly induced with little time, effort or expense on the part of the investigator. In addition, the induction is readily reversible, allowing for the analysis of many aspects of transient gene expression. Questions which need to be addressed include; What DNA and protein signals regulate the coordinate induction of the heat shock genes? How are the heat shock genes of organisms other than _Drosophila_ organized in their genomes? What is the function of the various heat shock peptides? What is the physiological relationship between the large number of agents which can induce a 'heat shock' response? What aspects of heat shock response are involved in the generation of phenocopy mutants? What is involved in the selective translation of heat shock mRNAs over nonheat shock mRNAs?

These and other aspects of the heat shock response are currently being addressed, mainly in _Drosophila_ _melanogaster_ (see Schlesinger _et_ _al_, 1982). The ubiquitous nature of the heat shock response suggests a need to characterize it in several species. Such a comparison would provide valuable insight into the conservation and identification of the regulatory elements involved in the expression of these coordinately expressed genes.

Caenorhabditis elegans is an excellent model organism for
the biochemical and genetical analysis of eukaryotic gene
regulation (Brenner, 1974; Riddle, 1980). This free living
nematode possesses many favorable characteristics, including; a
3.5 day generation time in which about 300 progeny are produced
from a single adult; the ability to maintain stocks indefinitely
in liquid nitrogen; the smallest genome size of any known
animal; transparency, allowing the detailed analysis of its
anatomy; and most importantly, it's ammenable to genetic
analysis.

The fact that C. elegans is a self-fertilizing
hermaphrodite allows the easy maintenance of homozygous stocks.
Genetic manipulations are possible however since hermaphrodites
will also reproduce by mating with males, which arise
spontaneously by nondisjunction of the X chromosome (Brenner,
1974; Hodgkin and Brenner, 1974). The adult hermaphrodite
possesses five pairs of autosomes and two sex chromosomes (XX)
while the male has five pairs of autosomes and a single X
chromosome. Both the embryonic and post-embryonic cell lineages
have been completely determined for C. elegans (Sulston and
Horovitz, 1977; Sulston et al, 1983). Adult worms have slightly
less than 1000 somatic cells and about 2500 gonadal nuclei
(Sulston and Horovitz, 1977). While its anatomy is relatively
simple, C. elegans contains a variety of differentiated tissue
types, including, hypodermis, nerve, gonad, muscle and gut.

The use of C. elegans for genetic studies is well documented (Brenner, 1974; Riddle, 1978; Herman et al, 1976, Rosenbluth and Baillie, 1981). The most detailed of these studies have concentrated on specific genes which affect muscle structure and function (Epstein et al, 1974; MacLeod et al, 1977; Rose and Baillie, 1980; Moerman and Baillie, 1979, Moerman et al, 1982; Waterston et al, 1982; Rogalski et al, 1982; Rogalski, 1984).

C. elegans is also well suited for molecular analysis. Its small genome size (8×10^7 bp) and high proportion of single copy sequence (83%) simplify the construction and screening of genomic DNA libraries (Sulston and Brenner, 1974). An elegant example of the potential of C. elegans for molecular genetic dissection is the unc-54 locus which encodes the gene for the major body wall myosin heavy chain (MacLeod et al, 1977). In this case, the properties of an internal unc-54 deletion were used to isolate the clones containing the unc-54 gene (MacLeod et al, 1981). Subsequent sequence analysis of unc-54 wild type and mutant alleles has allowed the alignment of the genetic and DNA sequence maps (Karns et al, 1983).

The initial objective of this thesis was to demonstrate the feasibility of using C. elegans as a model organism in which to study the heat shock response. At the time this project was initiated, the biochemical and physiological effects of a transient high temperature shock had not been reported in the phylum Nematoda. A second objective, relying on the results of

the first, was to clone, characterize and genetically map members of the C. elegans hsp70 gene family. It was thought that a comparison of the hsp70 genes and their flanking sequences between Drosophila and C. elegans might identify conserved sequences which are involved in the coordinate expression of the hsp genes.

The specific objectives were to: 1) determine the conditions under which C. elegans synthesized a set of hsps and then to analyse these polypeptides both in vivo and in vitro 2) determine the homology of the Drosophila hsp70 gene to C. elegans genomic DNA sequences using both genomic DNA blots and RNA blots 3) construct a C. elegans genomic DNA library and screen for the hsp70 gene(s) utilizing the Drosophila probe 4) characterize the isolated genes by restriction mapping and genomic DNA hybridizations 5) analyse the expression of the genes by Northern blots and the isolation of cDNAs 6) search for restriction fragment length differences in regions flanking the genes and then use them to map the cloned genes to their chromosomal locations.

The thesis follows this general outline, designating individual chapters for the in vivo heat shock response of C. elegans, the isolation and characterization of hsp70 genes, and the molecular genetic mapping of one of the cloned genes. In addition, a fourth chapter deals with an unusually high amount of strain specific sequence divergence flanking one of the cloned genes.

B. Chapter One

The Heat Shock Response of C. elegans

I. Introduction

All organisms tested respond to a sudden elevation of temperature by inhibiting the synthesis of proteins and RNA normal to development while simultaneously synthesizing a small group of proteins, called heat shock proteins (hsps; reviewed by Ashburner and Bonner, 1979; Schlesinger et al, 1982). In addition to heat shock, a large number of chemical and physical insults are known to induce a 'heat shock' response. These include, uncouplers of oxidative phosphorylation, inhibitors of electron transport, amino acid analogues, heavy metals, trauma, virus infection and anoxia (Ashburner and Bonner, 1979; Tanguay, 1983). The diverse nature of these inducers makes it difficult to determine a common pathway of induction. In addition, not all agents induce the hsps in all organisms.

In _Drosophila_, repression of the synthesis of proteins normal to development occurs very rapidly; prior to the detection of hsp synthesis (Velazquez et al, 1980). This repression requires neither new protein synthesis nor RNA synthesis, indicating that the factors regulating this aspect of the heat shock response preexist in the cell. A clue to the induction of the heat shock response may be found in the analysis of the cytoskeleton. The vertebrate intermediate filament protein, vimentin, is related to a 46 kd _Drosophila_ protein. In _Drosophila_, disruption of oxidative phosphorylation or heat shock causes the collapse of these filaments and results

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in the rapid migration of the 46 kd protein to the nucleus (Tanguay and Vincent, 1982; Biessmann et al, 1982). Translocation of the protein is accompanied by its phosphorylation. Biessmann et al (1982) have proposed that if the mRNAs normal to development are translated on polysomes linked to these filaments, then any disruption of the filament structure could account for the disintegration of pre-existing polysomes observed upon heat shock (McKenzie et al, 1975).

Two groups of researchers believe they have found a common pathway of activation for all heat shock response inducing agents. Wilhelm et al (1982) suggest that all inducers can act as protein synthesis inhibitors by blocking the function or synthesis of aminoacyl-tRNAs. In this scenario, heat shock acts by reducing the synthesis of GTP, which is normally required for the ribosome initiation complex; aminoacyl-tRNA, elongation factor I and GTP. They suggest, but have no evidence for, the presence of an unusual nucleotide which would act as a messenger between protein synthesis and transcription.

Independently, B. Ames and coworkers, working with Salmonella typhimurium, have found that heat shock induces the rapid synthesis of a family of adenylated nucleotides (ApppppA, ApppGpp, ApppppG, ApppG and ApppA; Lee et al, 1983b). High levels of these nucleotides accumulate within five minutes of heat shock and do not require the synthesis of the hsps. This fact has lead to the suggestion that they are the primary inducers of hsp synthesis. Their previous results (Lee et al, 1983a) showed

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that these nucleotides are synthesized in response to oxidative stress, which, they suggest, is the mechanism of action of all heat shock response inducers. In agreement with Wilhelm's group, they argue that the primary sensors of stress are either tRNAs or aminoacyl-tRNA synthetases. Significantly, AppppA is also found in a variety of eukaryotic cells (reviewed by Lee et al, 1983b).

In an effort to determine the cellular functions of the various hsps, attempts have been made to study their intracellular locations. A general conclusion from these studies is that the hsps are not equally distributed throughout the cell, but fall roughly into three classes. In Drosophila (Arrigo et al, 1980; Velazquez et al, 1980; Tanguay and Vincent, 1982), Chironmus (Vincent and Tanguay, 1979), Tetrahymena (Guttman et al, 1980; Loomis and Wheeler, 1982) and chicken (Schlesinger et al, 1982), hsp70 is found both in the nucleus and cytoplasm. In Drosophila, monoclonal antibodies have been used to demonstrate that newly synthesized hsp70 is rapidly transported to the nucleus during heat shock (Velaquez and Lindquist, 1984). Upon return to normal temperatures, hsp70 gradually leaves the nucleus, reappearing in the cytoplasm. However, not all the hsp70 is transported to the nucleus during heat shock. Some portion remains in the cytoplasm, apparently associated with the cytoskeleton (Schlesinger et al, 1982).

A second class of hsp is found almost entirely in the nucleus. These include, the Drosophila hsps 22, 23, 25, 28 and

34 (Arrigo et al, 1980; Tanguay and Vincent, 1982), the

Chironomus hsp34 (Vincent and Tanguay, 1979), the Tetrahymena

hsp29 (Guttman et al, 1980) and the Dictyostelium hsp26 to hsp32

class (Loomis and Wheeler, 1982). In addition, the mammalian

hsp100 is normally associated with the Golgi apparatus and upon

heat shock migrates to the nucleus (Lin et al, 1982). A

correlation of hsp function with nuclear location is difficult

as several reports are conflicting. For example, the Drosophila

low molecular weight hsps have been reported in one case to be

associated with chromatin (Arrigo et al, 1980) and in another

case to be part of the nuclear matrix (Levenger and Varshavsky,

1981).

The third class of hsp is found almost entirely within the

cytoplasm. These include the Drosophila hsp83 (Arrigo et al,

1980; Tanguay and Vincent, 1982) and Dictyostelium hsp82 (Loomis

and Wheeler, 1982).

The Drosophila heat shock response involves the rapid

induction of hsp synthesis and a concomitant decrease in both

the transcription and translation of proteins normal to

development. The regulation of these events appears to involve

an intricate pathway of transcriptional and translational

control mechanisms. While the induction of the various hsps

initially appeared to be coordinate, it is now known that each

hsp has distinct induction characteristics (Lindquist, 1980).

For example, while hsp83 synthesis is at its maximum at 33°C,

the synthesis of hsp70 and hsp26 are maximum at 35 and 37°C,

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respectively. In addition, the rate of hsp synthesis depends upon the severity of the heat·shock; at high shock temperatures all hsps show similar kinetics but at intermediate temperatures, individual hsps are synthesized at different rates (Lindquist, 1980).

When cells are returned to lower temperatures a gradual decrease in hsp synthesis and a return to normal protein synthesis occur. Control of these events appears to be by hsp self-regulation at both the transcriptional and translational levels (DiDomenico et al, 1982). For example, upon return to 25°C after a heat shock in the presence of inhibitors of the synthesis of functional hsps, hsp synthesis continues and the normal pattern of protein synthesis remains obstructed. In agreement with these results, as functional hsps accumulate to an appropriate level (in regards to the severity of the stress), further hsp mRNA transcription is inhibited and their cytoplasmic mRNAs are degraded (DiDomenico et al, 1982). The apparent involvement of the hsps in their own regulation and the fact that some hsps are capable of binding to nucleic acids leads to the possibility that some of the hsps can interact directly with their own genes and mRNAs (Valequez and Lindquist, 1984).

Translational control of the mRNAs normal to development occurs in response to heat shock. Preexisting polysomes are broken down and a new set of polysomes appear (McKenzie et al, 1975). The mRNAs normal to development remain present in the

cytoplasm of heat shocked cells but are not translated (Storti
et al, 1980; Scott and Pardue, 1981; Kruger and Benecke, 1981).
Specifically, the pre-existing mRNAs are found associated with
an apparently normal number of ribosomes (Ballinger and Pardue,
1983). While the specific mechanism of the selective translation
of the hsp polysomes over the non-hsp polysomes is not known, it
appears to act at the level of polypeptide elongation (Ballinger
and Pardue, 1983; Thomas and Mathews, 1982). A clue however, may
be in the specific and rapid dephosphorylation of the
cytoplasmic ribosomal protein, S6, that occurs upon heat shock
(Glover, 1982). Phosphorylated S6 may be required for normal
25°C mRNA translation but not for hsp mRNA translation. The
question remains however, of how the hsp mRNA and non-hsp mRNAs
are distinguished by the ribosomes.

The selective translation of hsp mRNA does not appear to be
universal. For example, in Xenopus and chicken fibroblasts the
mRNAs normal to development compete with the newly synthesized
hsp mRNA for ribosomes (Bienz, 1982; Schlesinger et al, 1982).
In yeast, the synthesis of hsps is accompanied by the
degradation of pre-existing mRNAs (Lindquist, 1981). Finally,
upon heat shock, Xenopus oocytes appear to utilize two distinct
translational control mechanisms. One mechanism is similar to
that found in Drosophila; pre-existing mRNAs are translationally
sequestered during heat shock. The second mechanism is unique to
the synthesis of hsp70. Xenopus oocytes contain massive amounts
of stored hsp70 mRNA which is unmasked and translated in

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response to heat shock (Bienz and Gurdon, 1982).

While the biological significance of the hsps is unknown, their ubiquity and complex regulation implies that they confer a distinct selective advantage. The first step in analysing this interesting phenomenon in C. elegans was to demonstrate their existence and then to compare it to the well characterized response of Drosophila.

II. Materials and Methods

Culturing of Nematodes

Caenorhabditis elegans var. Bristol, strain N2 was
originally obtained from S. Brenner (MRC, Cambridge, England).
Other strains were either constructed at SFU or obtained from
the Caenorhabditis Genetics Centre at the University of
Missouri. Nematodes were maintained on NGM plates supplemented
with E. coli strain OP50 as a food source (Brenner, 1974). For
large scale DNA and RNA preparations, worms were cultured on
high peptone plates seeded with a lawn of E. coli strain B as
described by Rose et al (1982). C. elegans var. Bergerac and
hybrid strains containing Bergerac chromosomes were maintained
at 18°C.

RNA Isolation

Worms were collected off plates with 0.04 M NaCl and
pelleted lightly. The pellet was washed twice with 0.04 M NaCl.
Ten volumes of cold 6 M guanidine hydrochloride, 0.2 M sodium
acetate, 0.1 M B-mercaptoethanol (pH 5.0) was added and the
suspension was immediately passed twice through a French Press
at 12,000 psi (pounds per square inch; 1 psi = 6.894757 kPa)
onto ice. After spinning out the worm carcasses, half a volume
of 95% ethanol was added and the RNA was precipitated overnight

at -20°C. Nematode RNA was subsequently purified as described by Chirgwin et al (1979). The final RNA pellet was washed twice with cold 95% ethanol, dried under air and dissolved at a concentration of between 1 and 2 mg/ml in sterile water.

Poly(A)+ heat shock and control RNA was isolated from total RNA by passage through oligo(dT) essentially as described by Lee et al (1978). The yield of poly(A)+ RNA was about 5% after one passage through the oligo(dT) column. After a second passage, the yields varied between 0.4% and 1.2% of the total RNA applied.

Cell Free Protein Synthesis

Total RNA (2 ug) was translated in a cell free rabbit reticulocyte lysate (Pelham and Jackson, 1976) as described by the supplier (New England Nuclear). Translation was monitored by the incorporation of [^{35}S]methionine into TCA precipitable material. The reaction was terminated by the addition of RNase followed by incubation at room temperature for 10 minutes. Translation samples were mixed 1:1 with loading buffer and approximately equal numbers of TCA precipitable counts were electrophoresed on 10 or 12.5% SDS-polyacrylamide gels as described by Laemmli (1970).

Heat Shock

For RNA preparations, worms were collected from plates in 0.04 M NaCl and immersed in water baths at the desired temperatures. After 15 minutes the worms were spotted onto prewarmed NGM plates and transferred to an incubator for various times. RNA was purified as described in RNA isolation.

Initially, for the detection of in vivo heat shock polypeptide synthesis, worms were collected and spotted onto plates containing ^{35}S-labelled E. coli and allowed to feed at 20°C for 45 minutes. At time zero, they were collected, immersed into a water bath for 15 minutes and then spotted onto prewarmed plates containing labelled E. coli for the duration of the heat shock. Following heat shock, the worms were spotted onto nonradioactive E. coli at 20°C for 30 minutes. Finally, worms were washed off, pelleted and boiled in 50 ul of Laemmli loading buffer. Approximately equal numbers of TCA precipitable counts of material was separated on 10 or 12.5% acrylamide slab gels as described by Laemmli (1970). After electrophoresis, gels were stained in 0.25% Coomassie blue, destained, dried and autoradiographed. After these initial experiments indicated under conditions which an in vivo heat shock response could be detected, the procedure was modified somewhat. Worms were collected, washed twice with 0.04 M NaCl and spotted onto NGM plates with no food source for 30 minutes. At time zero the worms were collected and immersed into water baths of 20°C for control or 35°C for heat shock for 10 minutes. The worms were

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then spotted onto NGM plates with labelled E. coli and transferred to incubators at either 20 or 35°C for 2 hours. After this time the worms were collected and spotted onto NGM plates with nonradioactive E. coli and allowed to recover at 20°C for 90 minutes. The in vivo labelled peptides were then analysed as described above.

Radioactive Labelling of E. coli

Radioactive E. coli were prepared by growing strain B in 100 mls of low sulphate medium supplemented with 10 mCi of [^{35}S]sulphate (25 Ci/mg) as described by Bretscher and Smith (1972) except that the medium was also supplemented with 1 mM CaCl2, 20 ug/ml thymidine and 4 mg of each amino acid except cysteine and methionine. Incorporations ranged from 10 to 60%.

Isolation of Synchronous Worms

Synchronous populations of worms were obtained by growing larvae from isolated eggs. Eggs were isolated by dissolving gravid adults in 2% sodium hypochlorite, 0.05 M NaOH for 15 minutes at room temperature. Eggs were pelleted, washed twice with 0.04 M NaCl and spotted onto NGM plates. The larval stages were estimated by measurement of heat killed larva with an ocular micrometer at 50x magnification. Labelled eggs were isolated by dissolving gravid adults, which had grown from the L3 stage on labelled E. coli, in 2% sodium hypochlorite, 0.05 M NaOH as described by Emmons et al (1979).

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C. elegans dauer larvae were isolated by washing worms from NGM plates on which the food supply had been exhausted for at least 5 days. The worm pellet was dissolved for 20 minutes in 1% SDS. Only dauer larvae survive this treatment. The pellet was washed twice with 0.04 M NaCl and the purified dauer larvae were spotted onto NGM plates without food. Control and heat shock RNA was isolated as described for growing worms.

III. Results

Heat Shock Peptides of C. elegans

Short exposure of C. elegans to temperatures 10 to 15°C
above normal 20°C culture conditions caused dramatic changes in
the pattern of peptide synthesis. Figure 1.1 illustrates the
results of the in vivo peptide synthesis of worms cultured at
various temperatures. There were several striking aspects of the
temperature response. First, as compared with control worms,
29°C resulted in the appearance of a 70 kdalton hsp (hsp70)
which was slightly visible at 20°C and which appeared to
increase in relative abundance as the temperature was increased
from 29°C to 35°C. Also noted at 29°C was the appearance of two
smaller peptides of 16 and 18 kdaltons, which sometimes appear
in controls and were enhanced by heat shock. Second, as the
culture temperature was increased from 29 to 35°C, different
peptide expression patterns developed. Peptides of 81, 41, 38,
36, 29 and 19 kdaltons appeared. Three of these peptides (36, 29
and 19) did not appear in control worms while the remainder (81,
41 and 38) were visible prior to heat shock and appeared to be
enhanced with increasing temperature. Finally, the synthesis of
many peptides normal to development decreased with elevated
temperatures. The decrease in non-hsp synthesis was not due to
protein catabolism, as worms which were prelabelled at 20°C and

Figure 1.1 SDS gel electrophoresis of polypeptides labelled in vivo in worms exposed to various temperatures. A population of worms mixed with respect to age was allowed to feed on ^{35}S-labelled E. coli at 20°C for 45 minutes. At time 0 the worms were suspended in 0.04 M NaCl at the indicated temperature for 15 minutes, then spotted onto prewarmed plates and transferred to an incubator at that temperature for 4 hours. After heat shock the worms were allowed to feed on non-radioactive E. coli for 30 minutes, boiled, then loaded onto a 12.5% SDS-acrylamide slab gel, electrophoresed and autoradiographed. Lane 1 shows control worms at 20°C, while Lanes 2-5 show worms incubated at 29, 31, 33, and 35°C respectively.

Lane labels (top): 1 2 3 4 5

Molecular weight markers (right):
- ◄ 81 000
- ◄ 70 000
- ◄ 41 000
- ◄ 38 000
- ◄ 29 000
- ◄ 19 000
- ◄ 18 000
- ◄ 16 000

24b
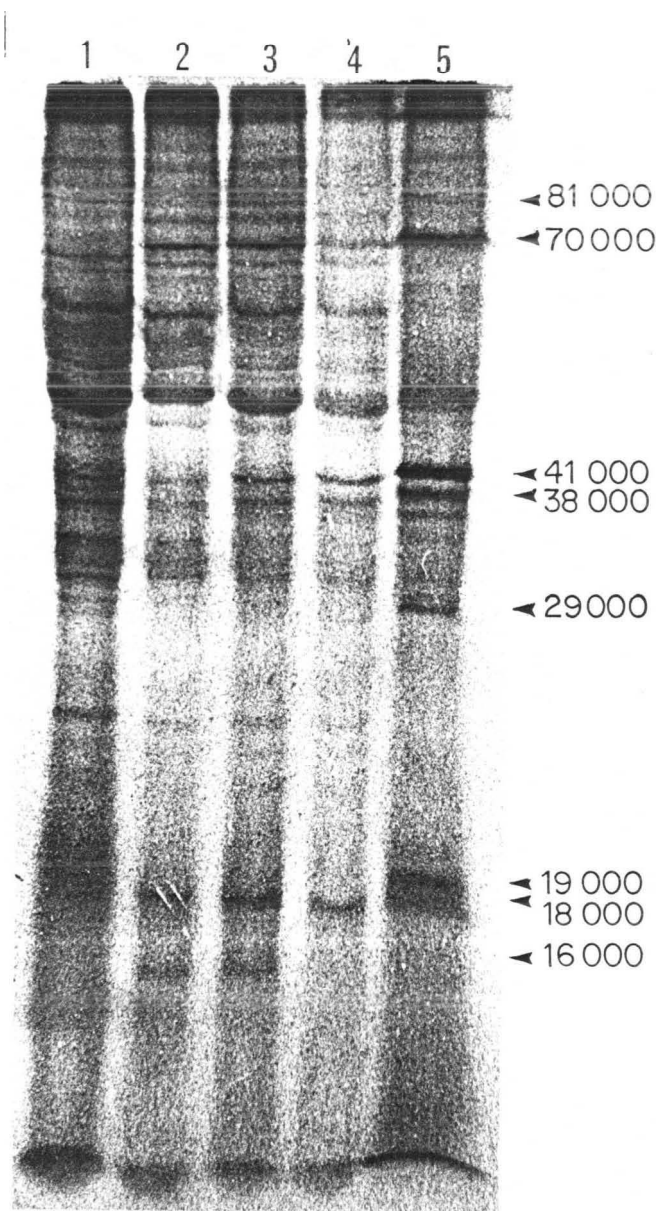
then shifted to 35°C on unlabelled E. coli showed no differences from the control in vivo peptide pattern. Also, supporting this argument is the fact that in vitro translation of heat shock RNA produces the same set of hsps (Figure 1.4).

Specifically, the nematode heat shock response varies, depending upon the severity of the heat shock, but always includes the induction of hsp70. The hsp70 is the major in vivo labelled peptide at 35°C. In fact, staining of the in vivo gels with Coomassie blue showed that hsp70 becomes readily visible after a 2 hour heat shock at 35°C.

Survival and Behaviour of C. elegans at Elevated Temperatures

The conditions under which heat shock was carried out for further experiments were achieved by culturing C. elegans at a variety of temperatures. Ten to 20 adult hermaphrodites were placed on each of ten NGM plates and their condition was monitored both immediately following heat shock and 18 and 48 hours after return to 20°C. At temperatures of 28°C and higher the worms stopped growing and laying eggs. The time required to kill 50% of the worms on a plate at 35, 42 and 45°C was 5.5, 1.2 and 0.4 hours, respectively. Even though half of the worms survive these conditions, it was determined that they were not suitable for in vivo labelling experiments since many of the survivors were severely uncoordinated, sterile and did not actively feed. The heat shock conditions which allowed approximately 100% survival of the worms throughout the heat

shock and for 18 hours post heat shock at 20°C, while showing an intense in vivo heat shock response was between 2 and 4 hours at 35°C.

Elevation of the culture temperature to 35°C produced several behavioural modifications. For approximately the first 2 hours at 35°C the worms behaved normally, actively feeding and moving about the plate. Within 3 to 4 hours they were observed to become quite sluggish, pharyngeal pumping decreased and adults stopped laying eggs. As the incubation period was increased past 4 to 5 hours the worms completely stopped feeding and lay motionless on the plate.

Return of the worms to 20°C initiated the slow recovery of normal behavioural patterns. Within 1 to 3 hours the worms had started to feed and slowly move about the plate. After about 6 hours at 20°C most worms were feeding normally but adults had not yet resumed egg laying. Within 12 hour to 18 hours the worms were fully recovered and L1's were hatched from newly layed eggs. The developmental progress of these worms was about 12 hours behind control worms and fewer progeny were produced from the heat shocked adults.

Heat Shock During Development

The life cycle of C. elegans involves development from the egg, through four larval stages (L1-L4) to the adult, over a 3.5 day period at 20°C. To test whether the heat shock response was unique to any particular developmental stage, synchronous

populations of worms were pulse labelled with ³⁵S labelled E. coli at each stage. The developmental stages were estimated by measuring the length of heat killed worms. Figure 1.2 shows that at 33°C hsp induction is not dependent upon a given developmental stage; that is they exhibited no stage specific banding pattern differences.

Comparison With the Drosophila Heat Shock Response

The well documented heat shock response of Drosophila indicates that the changes in gene expression following heat shock provide a good model system for studying the regulation of eukaryotic gene expression (Ashburner and Bonner, 1979). Figure 1.3 compares the heat shock response of C. elegans to that of Drosophila melanogaster. The heat shock responses of the two organisms show a number of similarities. Both Drosophila and C. elegans produce a unique set of polypeptides, which accounts for the majority of protein synthesis during heat shock. Similarities also exist between the electrophoretic mobilities of the induced hsps of Drosophila and C. elegans. In both cases, a 70 kdalton hsp accounts for the majority of the heat shock polypeptide synthesis. Both also show a larger molecular weight hsp (81 kd in C. elegans, 83 kd in Drosophila) and a set of smaller hsps. While the heat shocked Drosophila essentially synthesize only hsps, the nematode has a slightly higher background of other proteins. This is probably the result of the prelabelling with radioactively labelled E. coli in the case of

Figure 1.2 Heat shock induction at each developmental stage.
Synchronous populations of worms were grown from eggs isolated
by dissolving gravid adults in 2% sodium hypochlorite, 0.05 M
sodium hydroxide for 10 minutes, then filtering through a 50 um
Nitex filter. Heat shock was at 33°C as described in Fig. 1
legend. Labelled eggs were obtained by growing worms from the L3
stage to adult on ^{35}S-labelled $\underline{E.}$ $\underline{coli}$ and isolating the eggs as
described. Lanes 1 and 8 show control worms, while Lanes 2
through 7 are adult, L4, L3, L2, L1 and egg stages,
respectively.

1　2　3　4　5　6　7　8

←70 000

←18 000

←16 000
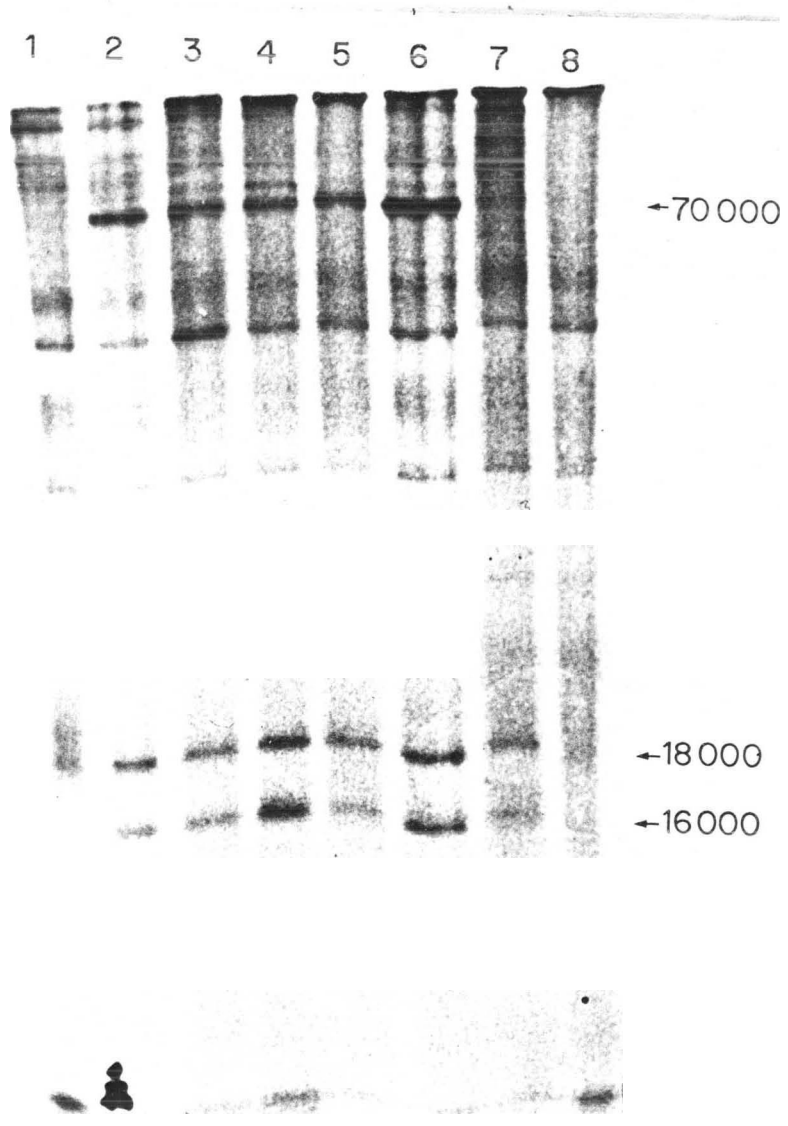
Figure 1.3 Comparison of C. elegans and Drosophila melanogaster heat shock patterns. Lane 1 shows worm control peptide pattern (20°C) while Lane 4 shows Drosophila control (25°). Lanes 2 and 3 are C. elegans and Drosophila heat shock respectively. Worms were heat shocked at 35°C while the Drosophila shock was at 37°C. Drosophila samples were obtained from L. Moran.

81 000 →

70 000 →

← 84 000

← 70 000
← 68 000

41 000 →
38 000 →

29 000 →

← 26 000

← 23 000

← 22 000

19 000 →
18 000 →
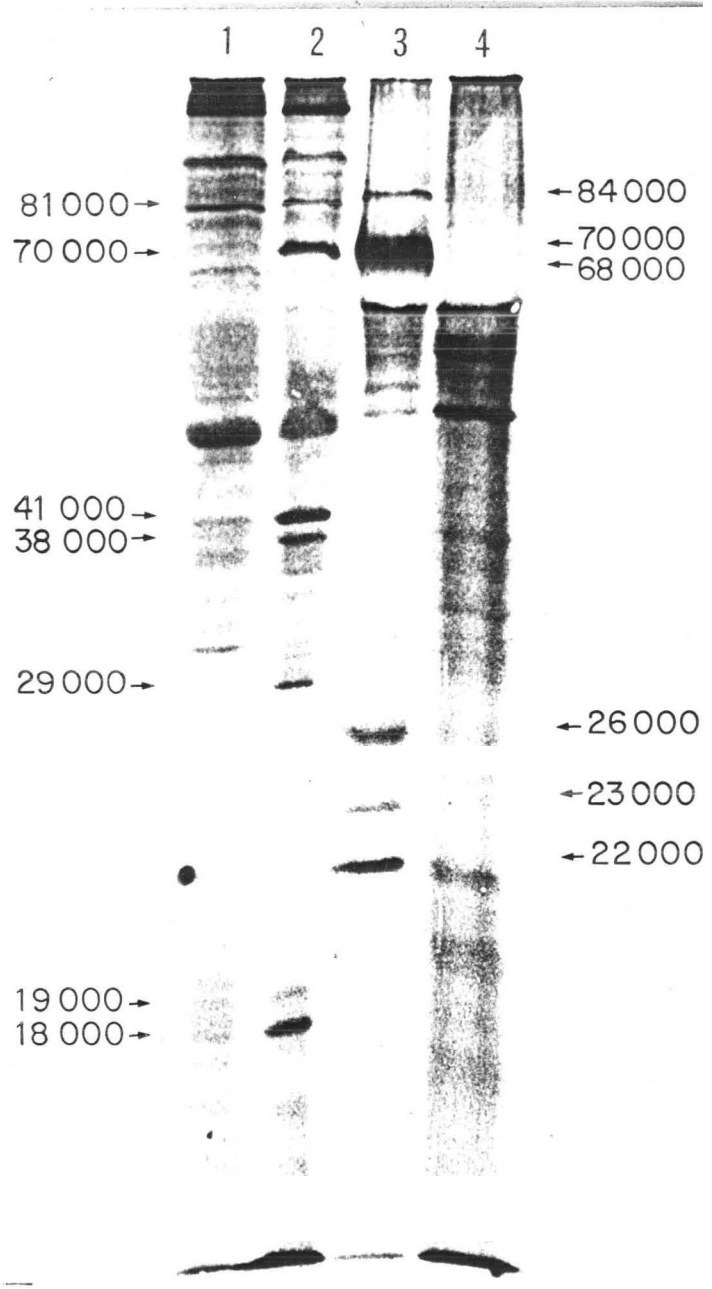
the C. elegans heat shock sample.

Translation of Heat Shock and Control Messages In Vitro

To study the mechanisms which control the reduction of
non-heat shock polypeptide synthesis and the induction of hsp
synthesis during heat shock, translatable mRNAs extracted from
control and heat shocked worms were analysed. Figure 1.4 shows
the products of mRNA translated in a rabbit reticulocyte lysate.
Lane 1 shows a single band around 55 kdaltons when no message
was added and corresponds to the reticulocyte endogenous protein
synthesis. Lanes 2 and 7 show the in vitro translation products
of total RNA isolated from worms grown at 20°C, while lanes 3-6
show the products of total RNA isolated from worms heat shocked
at 35°C. There was no detectable difference in the non-heat
shock mRNA products between heat shocked and control worms.
Thus, the in vivo reduction of non-heat shock polypeptide
synthesis during heat shock is not the result of degradation of
the non-heat shock mRNA, but probably due to a suppression of
its translation.

The presence of the non-heat shock mRNA makes the analysis
of mRNA coding for the low molecular weight hsps difficult.
Figure 1.4 shows, however, that there was little translatable
mRNA coding for hsp81 and hsp70 at 20°C, but that they appeared
at 35°C. In this experiment, translatable amounts of these mRNAs
appeared within 60 minutes of heat shock. While hsp81 was barely
detectable in in vivo labelled heat shocked worms, it was

Figure 1.4 SDS gel electrophoresis of _in vitro_ translation products of total RNA from heat shocked and control worms. Heat shock and RNA purification were as described in Materials and Methods. Purified RNA (2 micrograms) was translated in a cell free rabbit reticulocyte lysate for 60 minutes. Translated samples were mixed 1:1 with loading buffer and approximately equal numbers of TCA-precipitable counts were electrophoresed. Lane 1 shows the endogenous RNA synthesis in the reticulocyte system, Lanes 2 and 7 show control RNA at 20°C, while Lanes 3-6 show heat shock at 35°C for 3, 2, 1, and 0.5 hours respectively.

←81000
←70000

31b
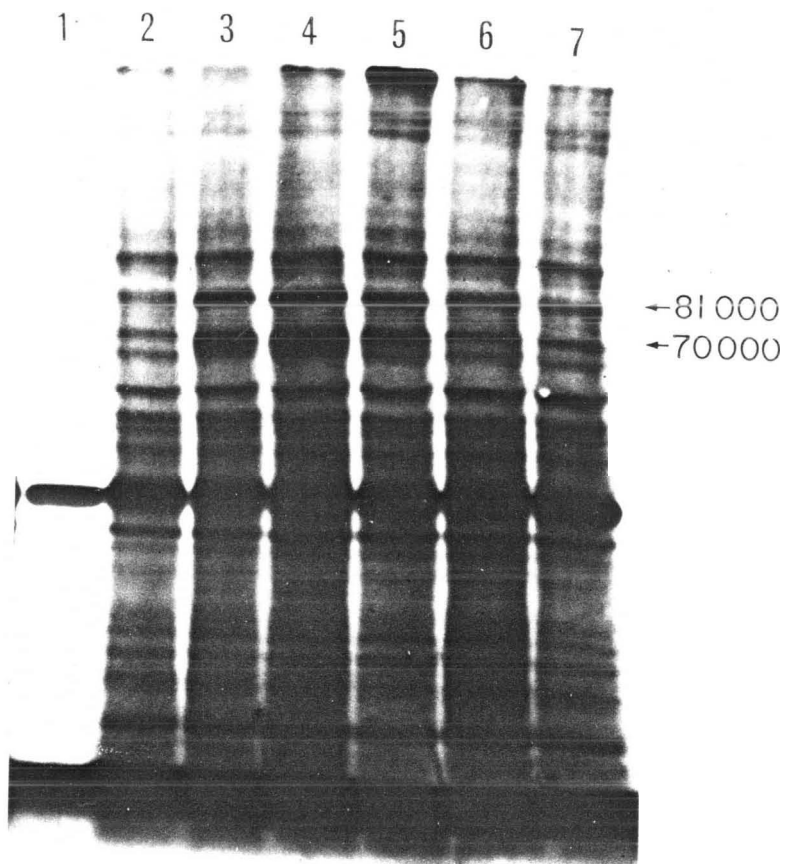
readily detected in vitro.

An analysis of RNA translated in vitro from worms returned to 20°C after a 4 hour heat shock at 35°C showed that the hsp70 mRNA was present in slightly higher amounts after one hour of recovery than immediately after a 4 hour heat shock. Detectable amounts of translatable hsp70 mRNA were still present up to 8 hours after return to 20°C. However, its' relative abundance decreased with recovery time. Between 8 and 14 hours after return to 20°C translatable hsp70 mRNA completely disappeared from message extracts.

Heat Shock of C. elegans Dauer Larva

The C. elegans dauer larva is a facultative larval stage which is formed when environmental conditions are unfavorable. Since the dauer larvae cannot feed, they probably have a limited energy supply and might not respond to heat shock as would growing worms. To test this, 7 to 10 day dauer larvae were subject to a 4 hour heat shock and total RNA was isolated and translated in vitro.

Figure 1.5 shows the results of the in vitro translation of control and heat shocked dauer larvae RNA. Several aspects of the results were interesting. First, compared with control dauer larvae it was apparent that a new set of mRNAs appeared in the heat shocked worms. The fact that worms in this stage will spend some of their limited energy on the production of the heat shock mRNAs possibly indicates the importance of the heat shock

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Figure 1.5 SDS gel electrophoresis of in vitro translation products of control and heat shocked dauer larvae RNA. Heat shock and RNA purification were as described in Materials and Methods. Purified RNA (2 micrograms) was translated in a cell free rabbit reticulocyte lysate for 60 minutes. Translated samples were mixed 1:1 with loading buffer and approximately equal numbers of TCA-precipitable counts were electrophoresed. Lane 1, control dauer larvae; Lane 2, heat shocked dauer larvae; Lane 3, no RNA.
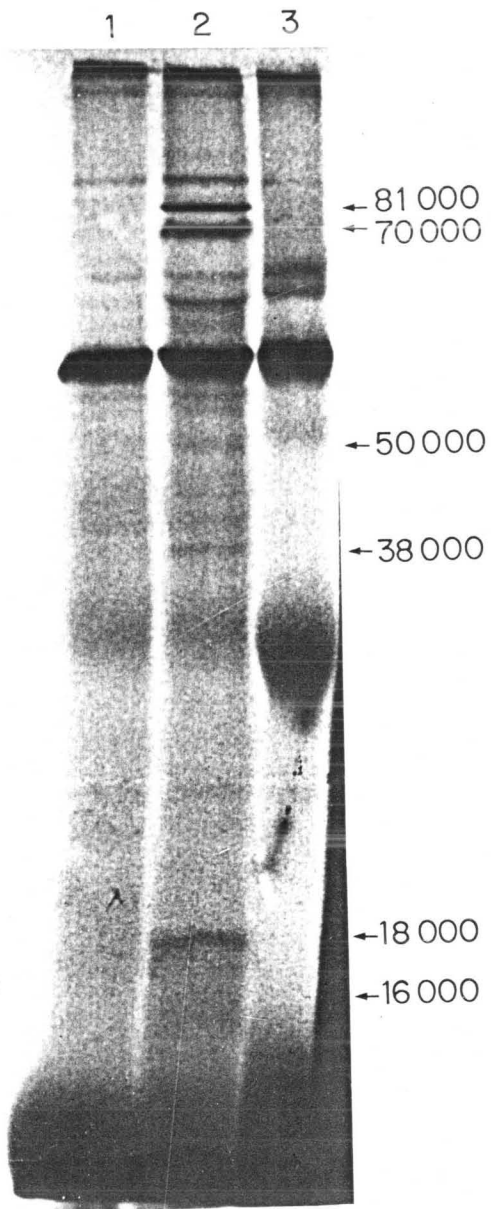
response to the survival of the organism. Second, relative to growing worms (Figure 1.4), the control dauer larvae did not appear to be actively synthesizing or accumulating translatable amounts of mRNA. Third, the low content of non-heat shock mRNA in this system allowed detection of heat shock mRNA coding for the low molecular weight hsps. Finally, the heat shocked dauers showed at least one inducible polypeptide (hsp50) which had not been previously detected in growing worms.

In Vivo Heat Shock Without Prelabelling

Several in vivo heat shock experiments have been completed using an E. coli food source labelled to specific activity about 10 fold higher than the initial heat shock experiments. This eliminated the need for the prelabelling period prior to heat shock. Figure 1.6 shows the in vivo response of Bristol worms labelled only throughout a 2 hour heat shock at 35°C. Compared to Figure 1.1, in which prelabelled worms were heat shocked, Figure 1.6 shows a much more dramatic decrease in non-heat shock peptide synthesis. It was also possible to detect hsp50, which was previously only detected in the dauer larva RNA preparations.

As will be demonstrated in Chapter III, a high level of nucleotide sequence variation exists between the Bristol and Bergerac strains of C. elegans in regions flanking one member of the hsp70 gene family. Analysis of the heat shock response of the Bergerac strain showed an identical hsp induction pattern to

Figure 1.6 SDS gel electrophoresis of polypeptides labelled in vivo without prelabelling. A population of worms mixed with respect to age was starved for 30 minutes and then spotted onto NGM plates with ^{35}S-labelled E. coli and maintained at either 35°C (lane 1) or 20°C (lane 2) for two hours. After this time the worms were spotted onto NGM plates with nonradioactive E. coli for 90 minutes and the proteins were analysed on a 12.5% SDS-acrylamide slab gel.
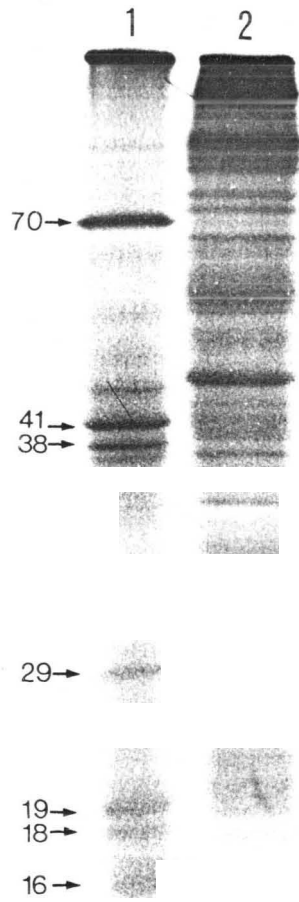
that of the Bristol strain.

IV. Discussion

Heat shock of C. elegans induced the preferential synthesis of at least nine polypeptides of 81, 70, 50, 41, 38, 29, 19, 18 and 16 kdaltons. Induction of the heat shock response was observed under which conditions as mild as 29°C for two hours and for times as short as 20 minutes at 35°C. The worms did not grow when shifted from 2C to 35°C but died slowly, with a half-life of about 5.5 hours. At temperatures above 35°C heat shock severely impaired the worm's ability to feed, making the analysis of in vivo synthesized polypeptides difficult. The optimum conditions for inducing the heat shock response were found to be between two and four hours at 35°C followed by a 30 to 60 minute recovery at 20°C.

The synthesis of the various hsps varied with the severity of the heat shock. For example, the synthesis of hsp70, hsp18 and hsp16 were detected after incubation at 29°C, while hsp19 and hsp29 did not appear until the culture temperature reached 35°C. These results are similar to those of Drosophila (Spradling et al, 1977; Lindquist, 1980) and Dictyostelium (Loomis and Wheeler, 1980). Thus, it may be a general feature of the heat shock response that the synthesis of individual hsps is correlated to the severity of the heat shock.

Hsp synthesis was inducible at all four larval stages and in adult worms. The results also showed that the hsps were inducible in egg preparations. However, since in this instance

the term 'egg' refers to everything from early embryos to L1
larvae just prior to hatching, I cannot rule out the possibility
that early cleavage embryos of some early embryonic stages may
not respond to heat shock. A precedent for this scenario exists
in Drosophila, where the hsps are inducible at all stages of
development except in the zero to three hour preblastoderm
embryonic stage (Graziosi et al, 1980; Dura, 1981).

A translational control of the heat shock response, similar
to that described in Drosophila (Storti et al, 1980; Scott and
Pardue, 1981; Kruger and Benecke, 1981), may also exist in C.
elegans. While non-heat shock polypeptide synthesis was reduced
in vivo during heat shock, analysis of in vitro translation
products showed that the RNA coding for these polypeptides was
still present and functional. In this respect, the C. elegans
heat shock response more closely resembles the heat shock
response of Drosophila than of yeast, where the non-heat shock
mRNAs are degraded upon heat shock (Lindquist, 1981). While
hsp70 was the major in vivo labelled hsp, the in vitro analysis
here showed that both hsp70 and hsp81 were translated in almost
equal amounts. This suggests that translational control of hsp81
synthesis occurs in vivo. Similarly, in Drosophila, the
synthesis of hsp83 appears to be under translational control in
vivo (Buzin and Petersen, 1982).

One-dimensional gel electrophoresis shows that most
organisms respond to heat shock by synthesizing a set of between
five and ten hsps. Thus, the heat shock induction response of C.

elegans corresponds well with other organisms. However, the
actual number of C. elegans hsps will await analysis of the in
vivo heat shock response by two-dimensional gel electrophoresis.
Buzin and Petersen (1982) have shown that most of the Drosophila
hsps are each represented by between three and nine isoelectric
point variants. In Drosophila, since most hsps are encoded by
single genes, the variants are probably due to
post-translational modifications.

Dauer larvae have been shown to be resistant to a variety
of chemical insults, osmotic shock, anoxia and thermal stress
(Cassada and Russell, 1975; Anderson, 1978). The possibility
that at least part of this resistance is related to hsp
synthesis has not been addressed. It is however, very
interesting that the normally quiescent dauer larvae will react
to heat shock by expending energy to actively synthesize the hsp
mRNAs. In addition to emphasizing the widespread nature of the
heat shock response, these results open up the possibility of
studying several aspects of gene regulation using the dauer
larvae system. The elucidation of the molecular mechanisms
involved in maintaining very low levels of non-hsp mRNA while
retaining the ability to specifically synthesize the hsp mRNAs
upon heat shock should be of general interest. Compared to
growing worms, the dauer larvae are greatly enriched for hsp
messages. Thus, for the purpose of obtaining molecular probes
for the C. elegans heat shock genes, the enriched dauer larvae
preparation should allow direct construction of a cDNA library

enriched for the hsp gene transcripts.

Table 1.1 is a list of heat shock elicited polypeptides from a variety of organisms. While any individual species induces a set of hsps which is different in molecular weight from that of any other species, a general consistency is evident. In fact, the major hsps of most organisms can be assembled into three classes distinguishable by their relative molecular weights. Several lines of evidence suggest that grouping the hsps into these three classes actually reflects their biological significance. Firstly, immunological data shows that antibodies raised against chicken hsp70 crossreacts with a similar molecular weight polypeptide in all eukaryotes (Kelly and Schlesinger, 1982). Secondly, sequencing of hsp70 genes from yeast and Drosophila and the heat shock inducible E. coli dnaK gene shows a high degree of sequence conservation between these distantly related organisms (Craig et al, 1982). Thirdly, in almost all organisms, hsp70 is the first hsp to be detected upon heat shock and accounts for the majority of hsp synthesis. Finally, in those instances where the intracellular location of the hsps has been studied, a strong correlation exists between the molecular weight and location. The low molecular weight hsps are concentrated in the nucleus (Arrigo et al, 1980; Tanguay and Vincent, 1982; Guttman et al, 1980; Vincent and Tanguay, 1979), the hsp80 class in the cytoplasm (Arrigo et al, 1980; Loomis and Wheeler, 1982) and the hsp70 class are found both in the nucleus and cytoplasm (Vincent and Tanguay, 1979; Arrigo et al, 1980;

Guttman et al, 1980; Tanguay and Vincent, 1982; Schlesinger et al 1982; Valazquez et al, 1980).

It would appear, therefore, that specific hsps have been conserved throughout evolution and that the members within each class probably perform similar heat shock related functions.

TABLE 1.1

MAJOR CLASSES OF HEAT SHOCK PROTEINS *

Species	Low Molecular Weight Class	Hsp70 Class	Hsp80 to Hsp90 Class
Trout	30, 32	62, 70	87
Chicken	24, 35	70	81
Dictyostelium	26 to 32	70	82
Polysphondylium	33	74	87, 105
Tetrahymena	23 to 30	73, 75	91
C. elegans	16,18,19,29	70	81
Soybean	16,19,21	72, 75	80, 94
E. coli	16, 34	64,73,76	81, 87
Mammals	37	72, 73	80,90,100,110

* sizes are in kilodaltons

C. Chapter II

Cloning the Hsp70 Related Genes of C. elegans

I. Introduction

An important question which needs to be addressed in regards to the basis of eukaryotic development is how different genes which are not closely linked in the genome are coordinately regulated. The molecular study of the heat shock response provides an excellent opportunity to analyse many aspects of eukaryotic genes structure, organization and regulation (see Ashburner and Bonner, 1979 for review). In Drosophila, genes encoding the major hsps have been cloned and sequenced (Holmgren et al, 1979; Moran et al, 1979; Ingolia et al, 1980; Ingolia and Craig, 1982; Karch et al, 1981). The hsp genes can be grouped into three classes; the four low molecular weight hsp genes, the hsp68 and hsp70 genes and the hsp83 gene. The three classes of genes are separated from each other in the Drosophila genome but are transcribed in response to heat shock and other stresses. In addition to their heat shock activity, several hsp genes are also expressed at low levels during development (Ireland and Berger, 1982; Sirotkin and Davidson, 1980; Cheney and Shegan, 1983). The elucidation of the regulatory mechanisms responsible for the developmental and coordinate induction of hsp genes will advance the understanding of the developmental process.

In addition to transcriptional control, the heat shock response has been shown to involve several types of translational control. While the hsps are induced at high

temperatures, mRNAs normal to development are translationally sequestered until there is a return to normal temperatures (Storti et al, 1980; Kruger and Benecke, 1981; Lindquist, 1981). The hsps appear to be involved in their own transcriptional and translational regulation as well as that of the previously existing mRNAs (Lindquist et al, 1982). In *Xenopus* oocytes, hsp70 mRNA is stored in a translationally repressed state until induction by heat shock (Bienz and Gurdon, 1982).

The spectrum of hsps induced varies from organism to organism but there appears to be conservation of the major hsp70 throughout evolution (Kelly and Schlesinger, 1982; Bardwell and Craig, 1984). Isolation of the genes coding for this hsp from *D. melanogaster* and *S. cerevesiae* has demonstrated that in both species they exist as part of a small multigene family (Craig et al, 1982). It was therefore interesting to clone and analyse the structure and expression of hsp70 related genes in *C. elegans*.

II. Materials and Methods

Construction of Nematode Genomic DNA Libraries Into Lambda Charon 4

a) Genomic DNA Partial Digestions. Four nematode genomic DNA libraries were constructed into the lambda vector, Charon 4 (Williams and Blattner, 1979). These included two of C. elegans Bristol (strain N2), and one each of C. elegans Bergerac (strain BO) and C. briggsae (strain G16). For each library, in an 80 ul reaction mix each of the following was added to three separate 1.5 ml Eppendorf tubes; 25 ug genomic DNA, 8 ul of 10 x EcoRI buffer, 8 ul of 1 mg/ml BSA and water up to 80 ul. At time zero 2.5 Units of EcoRI was added to tube A (final = 0.1 Units/ug DNA), 12.5 Units of EcoRI to tube B (0.5 Units/ug DNA) and 25 Units of EcoRI to tube C (1.0 Units/ug DNA). At times of 5, 15, 30 and 60 minutes, 20 ul was withdrawn from each of the tubes A, B and C. Each aliquot was pooled and the solution was made 20 mM for EDTA and heated to 65°C for 10 minutes. In total, 12 different EcoRI digestions were pooled to construct each library. The pooled DNA was heated to 65°C for 5 minutes just prior to separation on a 0.5% agarose gel run at 15 Volts for two days. Lambda DNA digested with BamHI giving a 17 kb marker and with EcoRI giving a 21 kb marker provided suitable size markers. After two days the material between approximately 15

and 23 kb was cut out of the gel and electroeluted in dialysis membrane at 40 Volts overnight. Contaminating agarose was removed by centrifugation for 5 minutes in an Eppendorf microfuge. The supernatant was collected and 0.1 volumes of 3 M sodium acetate and 1 volume of isopropanol was added and the DNA precipitated overnight at -20°C. The DNA was then pelleted for 10 minutes in an Eppendorf microfuge, washed twice with 70% ethanol and air dried briefly. The DNA pellet was dissolved in 40 ul of TE buffer and precipitated as described above. The final DNA pellet was then dissolved in 5 ul of TE buffer. The concentration of DNA was estimated using DNA standards applied to an agarose plate containing 0.5 ug/ml of ethidium bromide.

b) Ligation of genomic DNA to Charon 4 arms. Purified Charon 4 arms DNA was generously supplied by I. Kovesdi. The optimal conditions for the formation of concatamers of annealed lambda arms and insert DNA is a 2:1 molar ratio of arms: insert DNA (Dugaiczyk et al, 1975). Assuming an average insert size of 19 kb and knowing the lambda arms are 31 kb, the ratio of arms to insert required can be calculated. The ligation reaction of one of the C. elegans (Bristol) libraries constructed in this study contained 1.5 ug of Charon 4 arms and 0.5 ug of insert DNA. The other three libraries were constructed by ligating 0.85 ug of Charon 4 arms to 0.25 ug of insert DNA. The ligations were in 66 mM Tris pH 7.5, 5 mM MgCl2, 5 mM DTT, 1 mM ATP and 1 Unit of T4 ligase in a 10 ul reaction mix at 14°C overnight. To test the completeness of ligation, 1 ul of the ligation material was

run on a 0.4% mini-agarose gel. The ligated material runs at least the size of intact lambda DNA.

c) In vitro packaging of the ligated DNA. In vitro packaging for each library was essentially as described by Maniatis et al. (1982). One 50 ul packaging extract was thawed on ice about 3 minutes. Immediately, 1.5 ul of 100 mM ATP, 20 ul of CH buffer and the ligated DNA sample was added and the reactants mixed very well. This was then incubated at 37°C for 60 minutes. After this time a second extract was thawed and 10 ul of 0.1 mg/ml DNase and 2.5 ul of 1 M MgCl2 was added to the preparation. Twenty-five ul of this mixture was added to the first extract, mixed and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 900 ul of lambda diluent and 4 drops of chloroform. The solution was spun in a microfuge and the supernatant collected and titred. The titres of the four libraries ranged from 1.2 to 3.4 x 10^6 pfu/ml. Assuming the average insert size is 19 kb and knowing the genome size of C. elegans, then the packaged DNA represents between 285 and 807 genome equivalents. The background of intact Charon 4 and those hybrid phage containing internal lambda 7.0 and 8.0 kb fragments was estimated by plating 17600 pfu on LB plates containing 40 ug/ml of X-gal. The background of phage containing a functional B-galactosidase gene was 0.57%. Considering that the internal 8.0 kb fragment of Charon 4 is not detected in this assay, this figure was doubled to give a maximum background of 1.04%.

Preparation of In Vitro Packaging Extracts

The Charon 4 - C. elegans hybrid molecules prepared in this study were introduced into E. coli using the in vitro packaging system (Becker and Gold, 1975). In vitro packaging extracts were prepared from the E. coli lambda lysogens NS428 (N205:lambda Aam11 b2 red3 cIts857 Sam7) and NS 433 (N205;lambda Eam4 b2 red3 cIts857 Sam7) (Sternberg et al. 1977). The two lysogens provide complementing functions required for the packaging of lambda DNA into viable phage particles. These hybrid phage can then be used to infect E. coli and establish clone banks.

Prior to preparation of the packaging extracts the NS428 and NS433 strains were streaked out in duplicate on M-9 agar plates. The presence of the cIts857 mutation was tested by incubating one set of plates at 32°C and one set at 42°C overnight. As expected, neither strain grew at 42°C. Single colonies were picked from the plates grown at 32°C and used to innoculate 30 ml of M-9 buffer and grown overnight at 32°C. The bacteria were then collected by centrifugation and resuspended in 3 mls of M-9 buffer. The OD600 was measured and for the NS433 strain, 25 OD units of cells was added to each of two 500 ml cultures of M-9 buffer prewarmed to 32°C. To a third 500 ml flask of M-9 buffer, 25 OD units of NS428 was added and all three cultures were grown at 32°C until mid-log phase (OD600 = 0.3 to 0.4). The cultures were heat shocked for 15 minutes at 45°C with occasional swirling and then grown at 37°C for 2.5 hours with vigorous aeration. After this period, the cultures

were pooled and allowed to stand on ice for ten minutes and then the bacteria were pelleted, washed in cold M-9 minus the Casamino acids and repelleted. The bacterial pellet was drained of liquid and resuspended in six mls of CH buffer (CH buffer is 10 mM putrescine, 10 mM spermidine hydrochloride, 0.1% B-mercaptoethanol, 7% DMSO, 40 mM Tris pH 7.5, 1.5 mM ATP). Quickly, the suspension was aliquoted into Eppendorf tubes (50 ul/tube) and dropped into liquid nitrogen. For long term storage the packaging extracts were stored at -80°C.

Subcloning

DNA fragments were subcloned from lambda clones in order to obtain smaller more manageable fragments for restriction mapping and to obtain specific regions to use as hybridization probes. Portions of the 6.6 kb class A EcoRI fragment subcloned into pUR2 were further subcloned into pUC12 and pUC13.

All lambda class A, B and C clone EcoRI fragments were subcloned into the E. coli plasmid vector, pUR2 (Ruther, 1980). This vector simplifies subcloning by allowing the detection of recombinant plasmids without the need for replica plating. Insertion of foreign DNA into the EcoRI site within plasmid DNA coding for the B-galactosidase alpha-peptide prevents complementation with a Z gene deletion peptide synthesized by the host E. coli strain. Since the pUR2 plasmid also contains the gene for ampicillin resistance, insertion of foreign DNA is detected by growth of white colonies on

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5-bromo-4-chloro-indolyl-B-D-galactoside (X-gal),

isopropyl-thiogalactoside (IPTG), ampicillin (Amp) plates over a

background of blue colonies which represent plasmids either not

containing an insert or which contain an insert that does not

interrupt the alpha-peptide reading frame and introduces no

harmful sequences (Ruther, 1980).

Cloning from lambda phage into pUR2 was as follows. In a

1.5 ml Eppendorf tube one ug of phage DNA was mixed with 0.2 ug

of pUR2 DNA, one ul of 10 x EcoRI buffer, 5 Units of EcoRI and

water to a final volume of 10 ul and the mixture was incubated

at 37°C for 2 hours. The reaction was stopped by heating to 65°C

for 10 minutes. To this 6 ul of 2 x Ligation Mix and 1 Unit of

T4 ligase was added and the mixture incubated at 15°C for two

hours (2 x Ligation Mix is: 2.5 mM ATP, 250 ug/ml BSA, 25 mM

DTT, 25 mM MgCl2, 125 mM Tris pH 7.4). After 2 hours 104 ul of 1

x Ligation Mix and 0.5 Units of T4 ligase was added and the

reaction was left overnight at 15°C.

Transformation of the ligated material and preparation of

fresh competent cells was carried out using the procedure of

Lederberg and Cohen (1974) except that the cells were pelleted

in 10 mM NaCl and incubated in 30 mM CaCl2. Competent cells were

routinely frozen at -80°C to be used at a later date (Morrison,

1979).

In this study, plasmid pUR2 was transformed into E. coli

strain 7902 while the pUC plasmids were transformed into E. coli

strain JM83 (ara, lac-, pro, strA, thi, 080d lacZ M15).

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Positive transformants were selected on L broth plates containing 50 ug/ml Amp, 40 ug/ml X-gal and 160 ug/ml IPTG. Clones were restreaked twice on Amp plates and maintained at 4°C on Amp plates (50 ug/ml).

Isolation of Phage DNA

a) Liquid lysates. Approximately 1 x 10^{10} bacteria (fresh overnight of C600) were incubated with 1 x 10^8 phage (moi=0.01) for 15 minutes at room temperature. The mixture was used to inoculate a 2 litre flask containing 500 mls of NZY broth supplemented with 10 mM NaCl and grown with heavy aeration until the bacteria lysed (usually 4 to 5 hours). The lambda phage were purified from the lysate according to Yamamoto et al (1970) except that the DNase and RNase steps were omitted. Phage were further purified by a single equilibrium centrifugation in 0.75 g/ml cesium chloride at 45 k rpm for 16 to 18 hours (Maniatis et al, 1982).

b) Plate lysates. Approximately 1 x 10^5 to 2 x 10^5 phage were incubated for 15 minutes in 60 ul of a C600 overnight culture that had been resuspended in 0.5 volumes of lambda diluent. The solution was mixed with 2.5 mls of NZY top agar, poured onto fresh NZY agar plates and incubated in closed plastic containers lined with wet paper towels. The plates were incubated until confluent lysis occurred (usually 8 to 10 hours) and placed in the cold with 5 mls of lambda diluent overnight (Davis et al, 1980). The supernatant was collected and phage

isolated by precipitation in 1 M NaCl, 10% poly ethylene glycol followed by CsCl ultracentrifugation as described above.

Phage DNA was isolated directly from CsCl using the formamide method of Davis et al (1980b) except that an additional wash with 70% ethanol was performed to ensure no formamide remained in the DNA pellet. It was found that yields from the liquid lysates varied extensively, sometimes yielding between 100 and 400 ug DNA/litre culture while at other times no DNA at all. In contrast, plate lysates consistently yielded between 100 and 250 ug DNA from 10 plates.

Restriction Enzyme Digestions

Genomic and plasmid DNA samples were digested with a large variety of restriction enzymes obtained from either Bethesda Research Laboratories or New England Nuclear. Single enzyme digests were performed in either high, medium or low salt buffers as described by Davis et al (1980b). Generally, twice the recommended amount of enzyme was added and the reaction was allowed to proceed for twice the recommended time. These conditions usually assured complete digestion of the DNA. DNA samples which were digested with two or three restriction enzymes were carried out in BRL 'core' buffer and all enzymes were added simultaneously. These reactions were allowed to proceed for three times the recommended time with twice the recommended amount of each enzyme. Digests were stopped by addition of 0.1 volumes of loading buffer (loading buffer is 20%

glycerol 0.05% bromophenol blue, 178 mM Tris, 178 mM borate and
5 mM EDTA).

Agarose Gel Electrophoresis

Neutral agarose gels were prepared in 1 times TBE and
electrophoresed in this same buffer at 28 Volts for 12 to 15
hours (TBE is 89 mM Tris, 89 mM borate, 2.5 mM EDTA). After
boiling of the agarose and prior to pouring the gel, ethidium
bromide was added to a final concentration of 1.0 ug/ml. The
concentration of agarose gels varied from 0.5 to 1.5%, depending
upon the fragment lengths under study. Marker DNA was lambda DNA
(strain cI857 Sam7) digested with restriction enzymes which give
known fragment lengths and plasmid pBR322 DNA digested with
HpaII for small fragment length markers. The separated DNA
fragments were visualized and photographs were taken under a 360
nm wavelength transilluminator.

Labelling of DNA Probes

DNA probes were nick-translated to a specific activity of
approximately 2×10^7 to 2×10^8 cpm/ug using [alpha-32P]dATP or
[alpha-32P]dCTP singly or both simultaneously, essentially as
described by Rigby et al (1977). The reaction was allowed to
proceed for 90 to 120 minutes at 12°C and then terminated by
addition of one volume of TE saturated phenol:Sevag (1:1).
Unincorporated nucleotides were removed by passage of the
aqueous supernatant through a 1.5 ml Sephadex G25 column. Prior

to hybridization the probe was denatured by immersion in a
boiling water bath for ten minutes and then rapidly cooled in
ice-water.

Transfer of DNA to Nitrocellulose

DNA samples were completely digested with restriction
enzymes and 4 ug per lane was loaded onto 0.5 to 1.5% agarose
gels. Following electrophoresis the gels were soaked in 0.25 M
HCl for 30 minutes and then DNA was transfer from the gel to two
pieces of nitrocellulose (Schleicher and Schuell, No. BA85, 0.45
um pore diameter) by the bidirectional transfer method of Smith
and Summers (1980).

Transfer of RNA to Nitrocellulose

Total RNA (17 ug) or poly(A)+ RNA (10 ug) was denatured in
deionized 1 M glyoxal, 50% dimethyl sulphoxide, 10 mM sodium
phosphate (pH 7.0) at 50°C for 60 minutes and then
electrophoresed through a 1.1% agarose gel and transferred to
nitrocellulose using the method of Thomas (1980). Alternatively,
total RNA was denatured in 2.2 M formaldehyde, 50% formamide, 1
mM EDTA, 5 mM sodium acetate, 20 mM morpholinopropane sulphonic
acid (pH 7.0) for 15 minutes at 55°C and electrophoresed through
a 1.1% agarose gel containing 2.2 M formaldehyde, 1 mM EDTA, 5
mM sodium acetate, 20 mM morpholinopropane sulphonic acid (pH
7.0). Electrophoresis was at 70 Volts for 5 hours. Marker lanes
were cut off and stained in 0.5 ug/ml ethidium bromide in 0.1 M

ammonium acetate, 0.1 M E-mercaptoethanol as described by
Maniatis et al. (1982). The rest of the gel was soaked in water
for 30 minutes and transferred to nitrocellulose as described by
Thomas (1980). The transfer of RNA from formaldehyde gels was
about twice as efficient as from glyoxal gels.

Hybridization of DNA Probes to RNA and DNA

DNA and RNA filters were prehybridized at the hybridization
temperature in 5 x SSPE, 0.02% SDS, and 2.5 x Denhardts (1 x
SSPE is 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4;
1 x Denhardts is 0.02% BSA, 0.02% Ficoll and 0.02% polyvinyl
pyrrolidone). Hybridization was carried out in a fresh aliquot
of the above solution and included the denatured,
nick-translated probe. The temperature of hybridization and
subsequent washing of the filters varied according to the
stringency desired for each experiment. Hybridizations were
usually for 18 to 36 hours, except where noted.

Amplification of Genomic Libraries

The titre of the lambda libraries was increased about 1000
fold by amplification on LB plates. Approximately 5000 phage per
plate were plated on E. coli strain C600 (F-, thi-1, thr-1,
leuB6, lacY1, tonA21, supE44) and grown at 37°C until plaques
were almost confluent. The plates were then placed in the cold
and overlayed with 5 mls of lambda diluent and left overnight.
The supernatant was collected, bacterial debris removed by

centrifugation and the supernatnat stored over chloroform at 4°C. In the case of one of the Bristol libraries, 15 genome equivalents (about 75,000 pfu) was amplified. For the other three libraires, 20 genome equivalents (about 100,000 pfu) was amplified.

Chromosomal Walking In The Class A Region

In order to determine the nature of the genome flanking the class A gene an attempt was made to 'walk' in both the 5' and 3' directions. To walk in 5' direction from the class A gene the 2.0 kb EcoRI fragment sutcloned into pUR2 (subclone HSL-135-26) was used to screen the Charon 4 Bristol library. From six genome equivalents, 21 positives were selected and rescreened, resulting in the final purification of 11 positives (about 2 per genome equivalent). DNA was prepared from four of these. To walk in the 3' direction the 4.8 kb fragment (subclone HSL-151-18) was nick translated and used to screen six genome equivalents. Thirteen positives were selected and rescreened, resulting in the final purification of 5 positives. DNA was purified from 3 of these. All of the EcoRI fragments comprising both the 5' and 3' extended phage were subcloned into pUR2. The fragment furthest from the class A gene in each case has subsequently been used to walk further (K. Beckenbach, unpublished results).

Isolation of DNA Fragments From Agarose Gels

Initially, DNA fragments were separated on Low Melting
Point Agarose (BRL) and the desired fragments were cut out, the
agarose melted at 65°C and the DNA purified using the
cetyltriammonium bromide method of Langridge et al (1980). This
procedure was found to be very inefficient for the purification
of large DNA fragments and to be very time consuming. Subsequent
DNA fragment purifications were performed by slicing the desired
fragment from a regular agarose gel, placing the gel slice into
dialysis tubing, adding approximately 1.0 mls of TBE and
electroeluting the DNA at 50 to 60 Volts for 3 hours. After this
time, the current was reversed for two minutes, the supernatant
collected and the DNA precipitated in 0.1 volumes of 3.0 M NaCl
and 2.0 volumes of 95% ethanol. Precipitations were routinely
for 20 minutes on dry ice followed by centrifugation in a
microfuge for five minutes.

Screening of Lambda Libraries

Lambda libraries were screened according to the procedure
of Benton and Davis (1977). The number of clones required to
screen with a 99% probability of obtaining a single copy
fragment was calculated from the relationship derived by Clarke
and Carbon (1977):

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where, N = number of clones needed to screen with a probability,
P = 0.99 and where, f = fraction of genome which average phage

insert occupies. In the case of C. elegans,

$$f = \frac{x}{8 \times 10^7}$$

and x = average Charon 4 insert size.

If the average Charon 4 insert size is assumed to be 17,000 bp, N equals about 22,000 plaques.

Positives from the first screen were picked with the small end of a sterile pasteur pipette and resuspended in 0.5 mls of lambda diluent. The phage were plated at 50 to 100 pfu per plate and rescreened with the labelled probe. The positive furthest from other plaques was picked and subsequently used as a stock for further manipulations.

Hybridization Criteria

The following three sets of hybridization and washing conditions were employed throughout this thesis:

a) low stringency - hybridization : 57 to 60°C

   - washing : 47°C, 2 x SSPE

b) moderate stringency - hybridization : 62 to 68°C

   - washing : 62 to 68°C, 2 x SSPE

c) high stringency - hybridization : 68°C

   - washing : 68 to 75°C, 0.2 x SSPE

III. Results

Homology Between C. elegans and D. melanogaster Hsp Genes

The similarities in types of proteins and the regulatory
mechanisms involved in the C. elegans heat shock response to
those described for D. melanogaster suggested that there may
also be conservation at the DNA level. To test this,
nick-translated DNA probes for each of the D. melanogaster hsps
were hybridized under low stringency conditions to C. elegans
genomic DNA restricted with either EcoRI or HindIII (Figure
2.1). The results are of interest in several respects. Firstly,
all of the D. melanogaster genes showed homology to sequences in
the C. elegans genomic blots. Apparently, some segment of each
gene has been conserved between the two species. In support of
this finding, two C. elegans cDNAs coding for hsp16 have been
sequenced and show strong homology with the four low molecular
weight D. melanogaster hsp genes (Russnak et al, 1983).
Secondly, hybridization cf the genes encoding the four low
molecular weight hsps of D. melanogaster showed that three of
them (hsp22, hsp23 and hsp26) hybridized to the same set of
restriction fragments on the C. elegans genomic blots. The
pattern for the fourth gene, that coding for hsp28, was quite
different in that it has a large number of apparently homologous
sequences, most of which did not overlap with the set of bands

Fig. 2.1. Hybridization cf ^{32}P-labelled <u>Drosophila</u> hsp genes to
HindIII (lane 1) and EcoRI (lane 2) digested <u>C. elegans</u> DNA.
Hybridization stringency was low. Markers are HindIII digested
lambda DNA. The filter was exposed at -80°C for 2 days to Kodak
Blue Brand film with an intensifying screen.
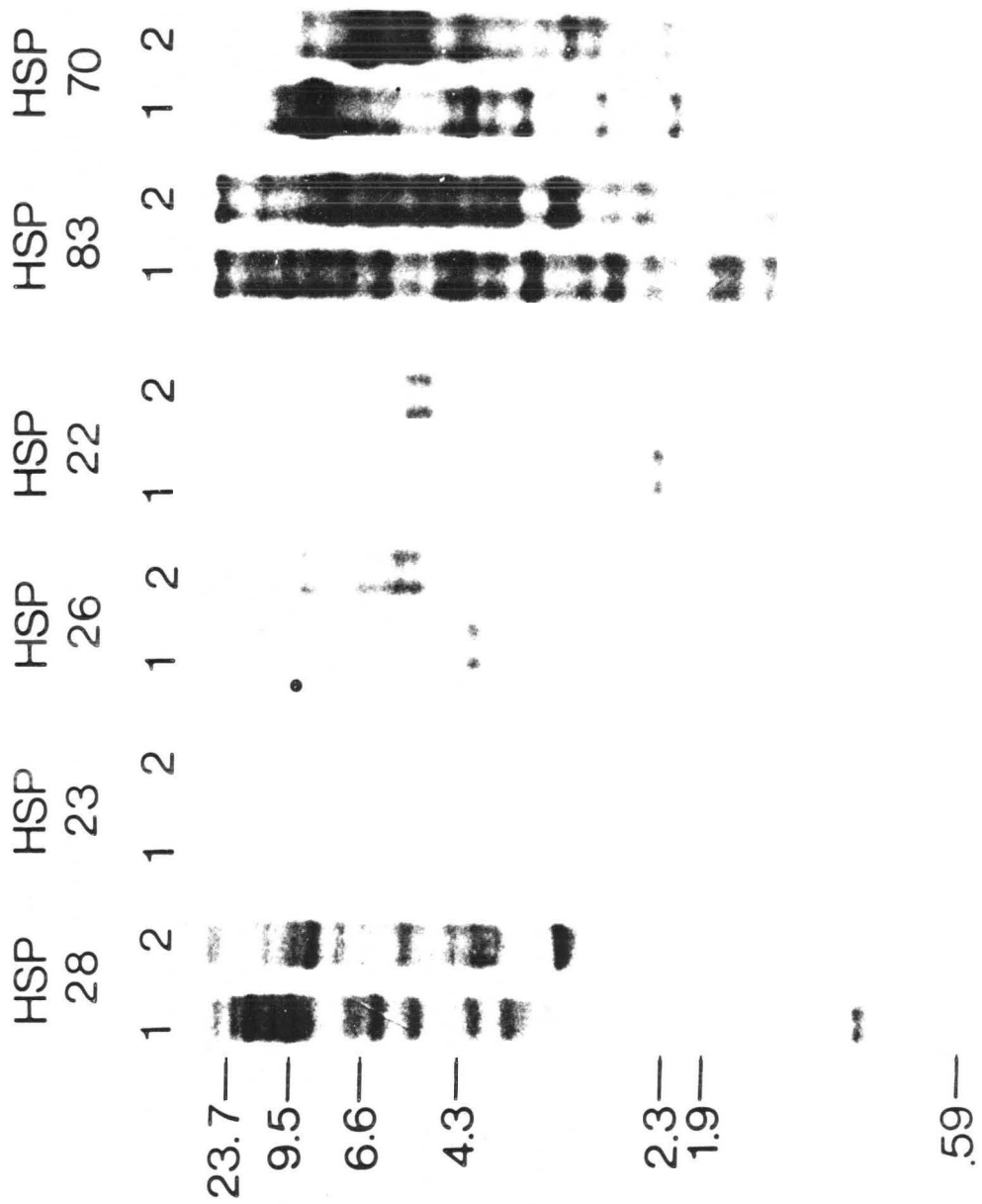
seen for the other three hsps. Thirdly, the hsp28, hsp83 and hsp70 genes showed a great number of homologous sequences in C. elegans. Under higher stringency conditions each gene hybridized strongly to only a small number (three to five) of restriction fragments.

Further evidence that the hsp70 gene is conserved between D. melanogaster and C. elegans is shown in Figure 2.2. Control and heat shock poly(A)+ RNAs were hybridized to the Drosophila hsp70 probe using a Northern blot. The result indicated that the probe was specific for a heat inducible poly(A)+ mRNA of approximately 2400 nuclectides. Also shown is the hybridization to total RNA from heat shocked dauer larvae. The increase in hsp70 homologous mRNA in the heat shocked RNAs suggests that expression of this gene is under transcriptional control.

Cloning of the C. elegans hsp70 Related Sequences

In order to study the structure, expression and evolution of hsp70 genes and their related sequences, two different C. elegans (strain Bristol) genomic libraries were screened with the D. melanogaster hsp70 gene probe. An initial screening of an EcoRI total digest library constructed in lambda 607 (a gift of S. Bektesh) resulted in the final purification of seven positives. Phage DNAs were isolated, digested with EcoRI and the clones analysed by visualization in agarose gels and by hybridization to the Drosophila probe. Table 2.1 summarizes these results. While five of the seven phage clones contained

Fig. 2.2. Hybridization of ^{32}P-labelled Drosophila hsp70 gene to heat shock and control RNA. Lane 1, total RNA (40 micrograms) from heat shocked dauer larvae; lane 2, poly A+ RNA (10 micrograms) from control growing worms; lane 3, poly A+ RNA (10 micrograms) from heat shocked growing worms. Sizes are in nucleotides. Hybridization stringency was low. The filter was exposed at -80°C for 5 days to Kodak Blue Brand film and an intensifying screen.

1　2　3

←2.4

.63b
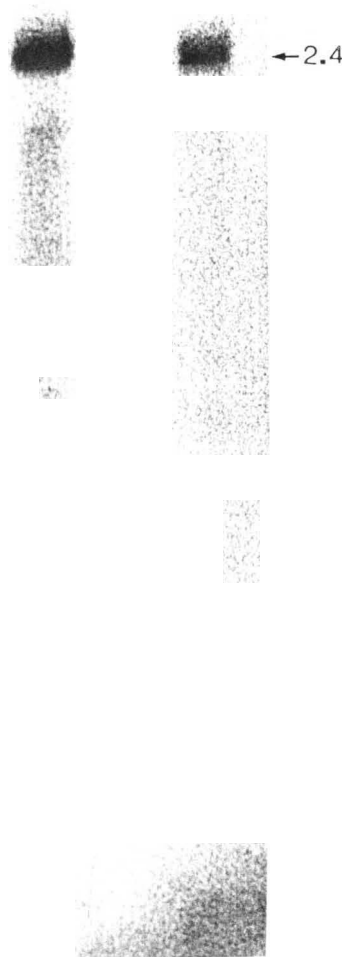
TABLE 2.1

<u>HSP70 POSITIVES FROM LAMBDA 607 LIBRARY</u>

<u>Clone</u>	<u>Insert Size (kb)</u>	<u>Homology to hsp70 (+/-)</u>
B1	a) 2.9	+
	b) 1.6	−
B3	a) 1.7	+
B4	a) 2.9	+
	b) 1.6	−
	c) 1.3	−
B5	a) 2.6	+
	b) 0.4	−
B6	a) 2.6	+
B8	a) 2.9	+
	b) 1.6	−
	c) 1.3	−
B9	a) 2.5	−
	b) 1.7	+
	c) 0.5	−

more than one insert, a single EcoRI fragment was homologous to the _Drosophila_ probe in each case. Three different size EcoRI fragments related to the _Drosophila_ probe were found (1.7, 2.6 and 2.9 kb). All three represent minor homologies when compared to a genomic DNA blot with the same probe.

Hybridization of the phage inserted EcoRI fragments not homologous to the _Drosophila_ probe to genomic DNA blots showed that they were not adjacent to the hsp70 related fragments in the _C. elegans_ genome. The B4 1.3 kb EcoRI fragment contained a repeated element present between 20 to 30 times in the _C. elegans_ genome. The B4 1.6 kb EcoRI fragment contained a repeated element present between 50 to 100 times in the _C. elegans_ genome. These elements have not been studied further.

In order to isolate the _C. elegans_ genomic regions which showed the most homology to the _Drosophila_ probe, an EcoRI partial digest library was constructed into Charon 4 (see Materials and Methods). Screening of five genome equivalents resulted in the final purification of nine recombinant phage. Figure 2.3a shows the ethidium bromide stained pattern of these phage after restriction with EcoRI and electrophoresis. Hybridization of blots of the gels with the _Drosophila_ hsp70 probe showed that all nine phage fell into three distinct classes of hsp70 related sequences (Figure 2.3b). These were designated as class A, B and C clones. The class A clones consisted of four phage containing a common 6.6 kb EcoRI fragment with homology to the _Drosophila_ hsp70 probe. Two of the

Figure 2.3. Isolation of hsp70 related clones from a C. elegans genomic library. A) Ethidium bromide stained pattern of the nine isolated phage digested with EcoRI B) Hybridization of ^{32}P-labelled Drosophila hsp70 gene to a blot of the gel shown in A. Markers are HindIII/EcoRI digested lambda DNA. The filter was exposed to Kodak Blue Brand film for 6 hours.
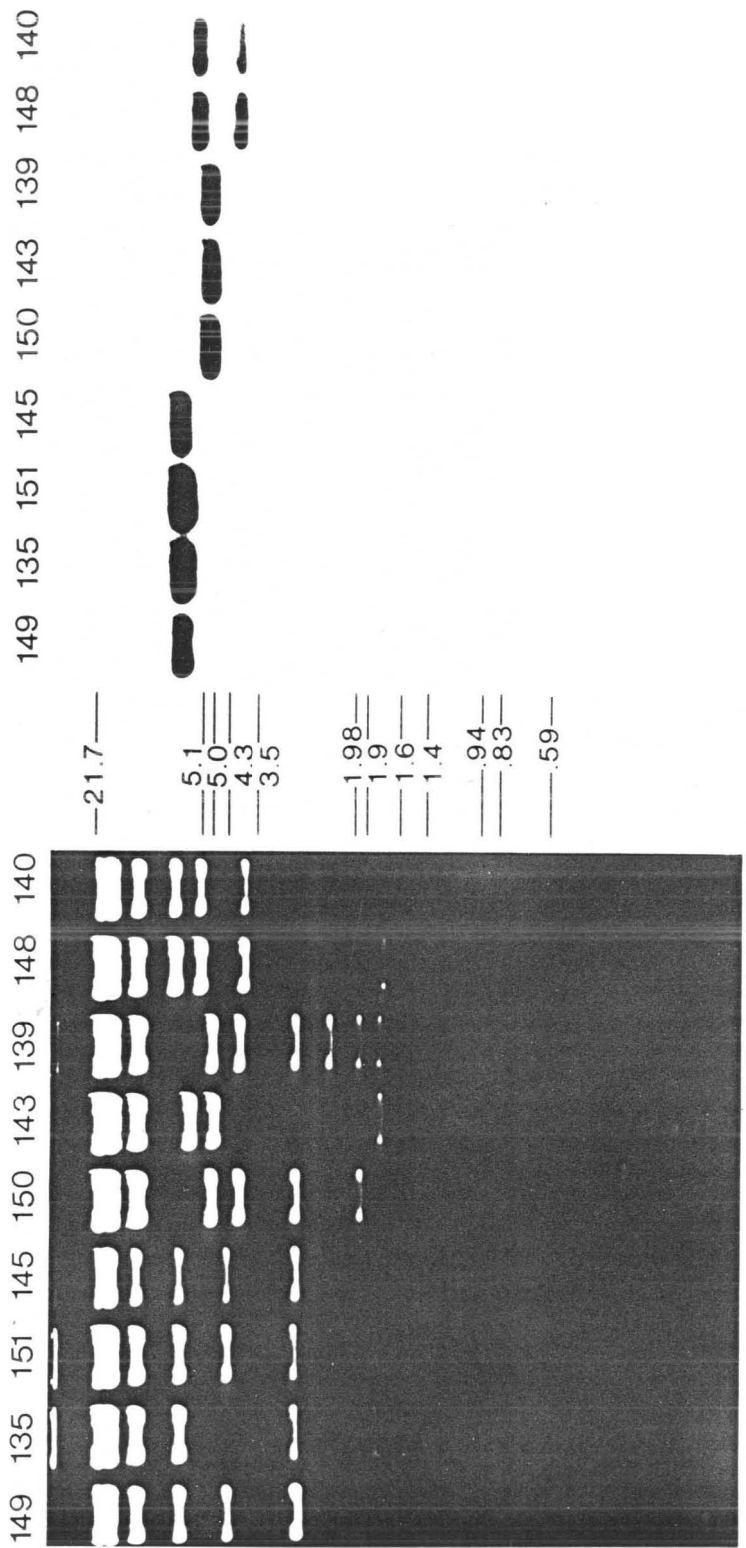
phage were identical (149 and 145). The class B clones consisted of three overlapping phage with a 5.2 kb EcoRI fragment in common and homologous to the _Drosophila_ probe. The remaining two, the class C, had 6.2 and 4.3 kb fragments which showed homology to the probe. Comparison of the hybridization patterns of the _D. melanogaster_ hsp70 gene to _C. elegans_ genomic DNA and to the fragment sizes of the class A, B and C homologous bands revealed that these four genomic EcoRI fragments accounted for the majority of the _C. elegans_ genomic sequences homologous to the _Drosophila_ hsp70 probe.

Genomic Organization of the hsp70 Related Sequences

Phage from each class of Charon 4 hsp70 clone were digested with EcoRI and subcloned into the plasmid vector, pUR2. The individual subclones provided more manageable size fragments to carry out subsequent restriction mapping and provided a more convenient source of DNA for preparation of labelled probes. From the class A lambda clones, eight subclones representing 23 kb of DNA were derived. Similarly, seven subclones from the class B and four from the class C, representing 24 and 20 kb, respectively, were obtained. In total, 67 kb of DNA representing about 0.1% of the _C. elegans_ genome were subcloned.

Restriction mapping and subsequent blot hybridizations showed that all three classes contained sufficient regions homologous to the _Drosophila_ hsp70 probe to encode a 70 kilodalton peptide (Figure 2.4). Based on their respective

Figure 2.4. Restriction maps of the class A, B and C hsp70 related clones. The top line for each class is the restriction map representing the entire cloned region. Individual clones are shown below. R(Right) and L(Left) indicate the orientation of the insert within the Charon 4 vector. The heavy line represents homology to the _Drosophila_ hsp70 gene. H, HindIII; E, EcoRI; X, XbaI; O, XhoI; S, SalI; K, KpnI; B, BamHI.

CLASS A

CLASS B

CLASS C

5'

3'

1kb

HSL 151
HSL 135
HSL 149, HSL 145

HSL 143
HSL 150
HSL 139

HSL 148
HSL 140

68b
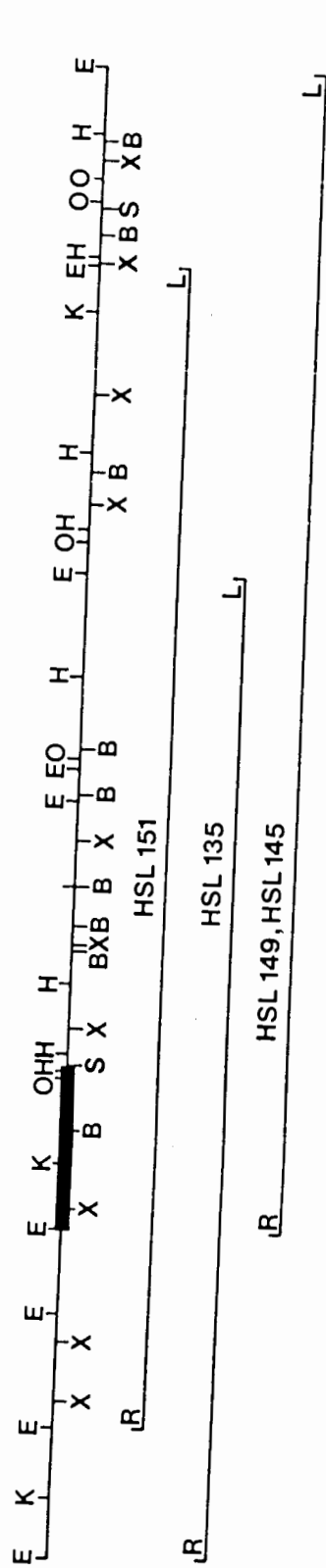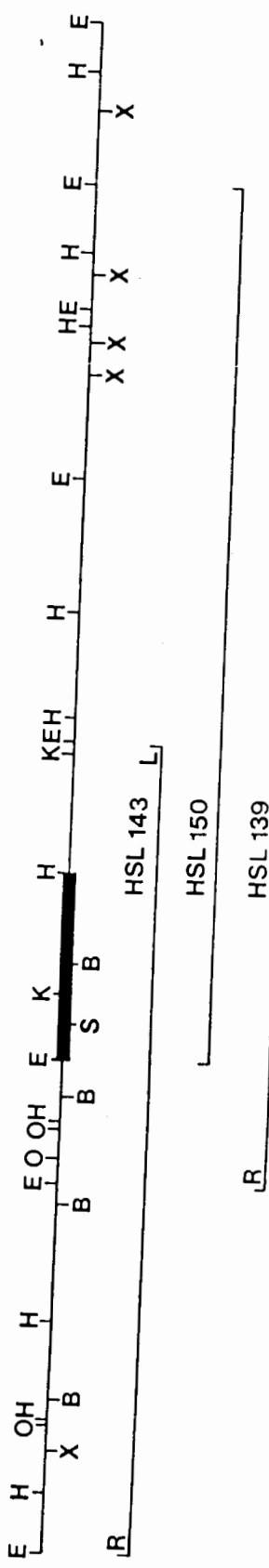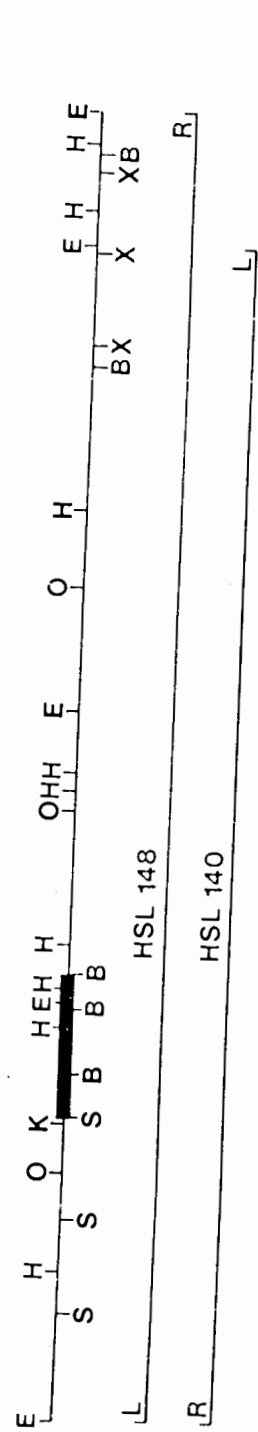
restriction maps, the three genes show distinctive differences and thus are unique. At this level of detection, similarities can be found between the class A and B genes. Both contain an internal 500 bp KpnI - BamHI fragment. Also, the 5' EcoRI - KpnI distance is similar between the two except that the class B distance is about 100 bp larger.

Figure 2.5 shows the results of hybridizing each of the class A subclones to blots of genomic DNA digested with several restriction enzymes (the 3.0 kb fragment 3' to the 4.8 kb fragment is not shown). In addition to confirming the restriction mapping, these hybridizations demonstrated that, except for the 6.6 kb and 4.8 kb fragments, the individual subclones did not contain any major repetitive elements. On a longer exposure of the autoradiograph, the 4.8 kb fragment was found to contain an element that was repeated 10 to 12 times in the C. elegans genome (see Figure 3.3). The 6.6 kb fragment and the Drosophila hsp70 gene both hybridize to the same set of genomic EcoRI and HindIII fragments, suggesting that, other than hsp70 related sequences, the 6.6 kb fragment contains no other repeated elements. A similar analysis of the class B and C gene flanking regions demonstrated that these regions are also essentially single copy DNA.

From the restriction maps and hybridization of individual subclones from any one class to all the other classes, it is apparent that none of the three classes are closely linked with each other in the C. elegans genome.

Figure 2.5. Hybridization of subcloned EcoRI fragments derived from class A clones to C. elegans genomic DNA. C. elegans DNA was digested with either BamHI (B), HindIII (H) or EcoRI (E). Hybridization was low stringency. Markers are HindIII digested lamoda DNA.
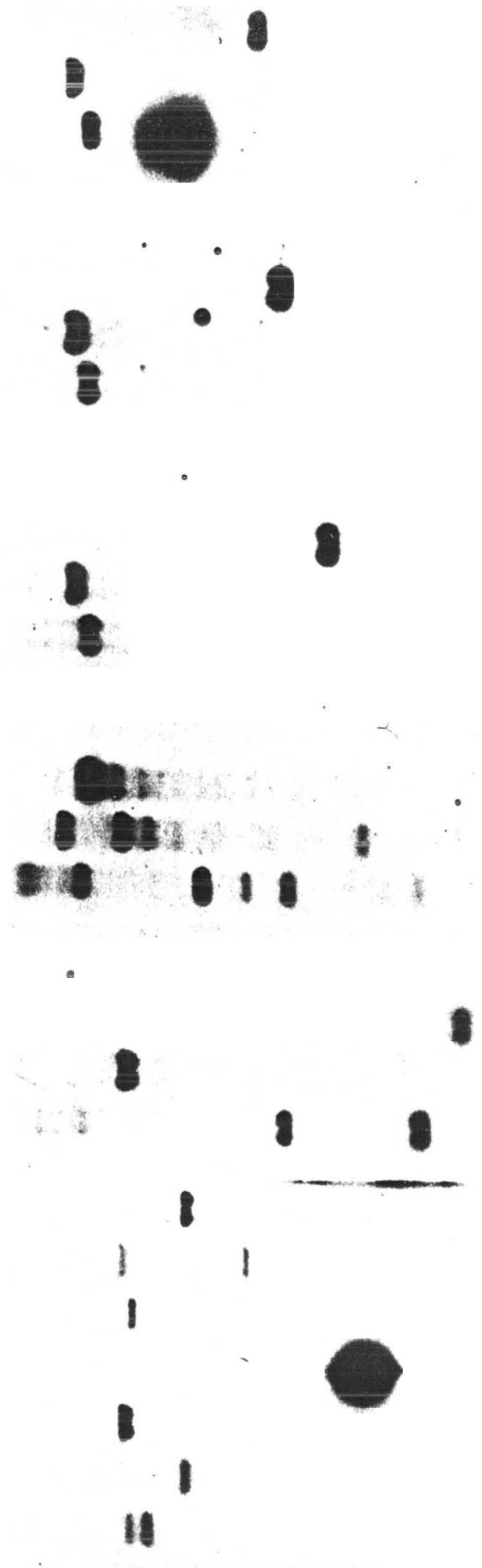
Each hsp70 Related Gene is Present Once Per Haploid Genome

From the genomic blot hybridization it was apparent that the different EcoRI fragments hybridized to different extents to the _Drosophila_ hsp70 probe. Stronger hybridization signals may represent: (1) the presence of more than one copy of the homologous element (2) more than one copy of the fragment itself (3) a higher degree of homology to the probe. Weaker hybridization signals may represent: (1) the presence of restriction sites within the homologous element (2) the presence of introns or insertions within the homologous element (3) a lesser degree of homology to the probe. To distinguish between these, the physical structures of the three classes of clones was determined.

The restriction map analysis showed that each of the class A, B and C coding regions was present only once in each cloned region. In order to detect whether these elements are found at other locations in the _C. elegans_ genome, each of the EcoRI subclones containing the hsp70 homologous region was hybridized to blots of genomic DNA digested with various restriction enzymes. The results indicated that, in each case, all homologous fragments in the genome can be accounted for by examination of the restriction maps of the three genes. In addition, comparison of the hybridization intensities of coding and flanking sequences indicated that there was no difference in intensity. Thus, each of the class A, B and C hsp70 related

genes is a single copy element.

An additional experiment confirmed that the class A gene is single copy. A second laboratory strain of C. elegans, Bergerac, is known to differ from the wild type Bristol strain by about 1% in nucleotide sequence (Emmons et al, 1979; Rose et al, 1982). When cloned DNA fragments from one strain are hybridized to genomic DNA blots of the other, the 1% sequence difference appears as restriction fragment length differences (RFLDs). Using the individual subclones from the class A phage the Bergerac strain was searched for RFLDs. Hybridization of the 6.6 kb subclone (containing the class A coding region) to Bristol and Bergerac genomic DNA showed that a new EcoRI site appeared in the Bergerac strain, creating fragments of 3.7 and 2.9 kb. No fragment of 6.6 kb was seen in the Bergerac strain. If there were more than one copy of the 6.6 kb fragment in the C. elegans genome, it would be highly unlikely that point mutations would be introduced in all copies at identical positions. I interpret this to mean that C. elegans contains only one copy of the class A hsp70 gene.

From the above results it is apparent that the differences in hybridization intensity of the Drosophila hsp70 probe to the various C. elegans EcoRI fragments are probably due to homology differences (as compared to copy number). The 6.6 kb fragment shows the greatest degree of homology under the conditions employed.

Hybridization of C. elegans Clones to the Drosophila hsp70 Gene

To determine the homology of the various cloned fragments with the Drosophila hsp70 gene, the 3.0 kb SalI fragment of the Drosophila plasmid 132E3 (Moran et al, 1979) was gel purified and digested with XhoI and BamHI. Each of the seven cloned C. elegans EcoRI fragments exhibiting hsp70 homology were nick-translated and hybridized to blots of the Drosophila fragments. Figure 2.6 represents the extent to which the EcoRI fragments hybridized to various Drosophila hsp70 genomic regions. Each C. elegans fragment showed a unique relationship to the Drosophila hsp70 gene. The 6.6 kb class A fragment hybridized strongly to both the 5' and 3' hsp70 coding regions and less strongly to the 5' noncoding region. In contrast, the class B 5.2 kb fragment was related only to the 5' and 3' coding regions. No reaction was detected to the 5' noncoding region. The 6.2 kb class C fragment hybridized to the Drosophila 5' coding region, while the adjacent 4.3 kb fragment was related to the 3' coding region. The B9 1.7 kb fragment and the B5 2.6 kb fragment EcoRI fragments both showed homology only to the 3' coding region. The B4 2.9 showed strong homology to the 5' coding regions and weak homology to both the 3' coding and 5' noncoding Drosophila hsp70 gene. The intensity of hybridization of the 6.6 kb class A and HSL B4 2.9 fragments to the Drosophila 5' noncoding region was very weak and may in fact be the result of non-specific hybridization.

Figure 2.6. Homology of the C. elegans clones to the Drosophila
hsp70 gene. Each of the indicated C. elegans cloned fragments
was hybridized to the Drosophila hsp70 gene which had been
separated into 5' non-transcribed, 5' noncoding + 5' coding and
3' coding portions. The dashed line represents minor homology.

5′		3′	
0	0	B	S

DROSOPHILA HSP70

6.6 kb class A

5.2 kb class B

6.2 kb class C

4.3 kb class C

HSL B9 1.7

HSL B2 2.9

HSL B5 2.6
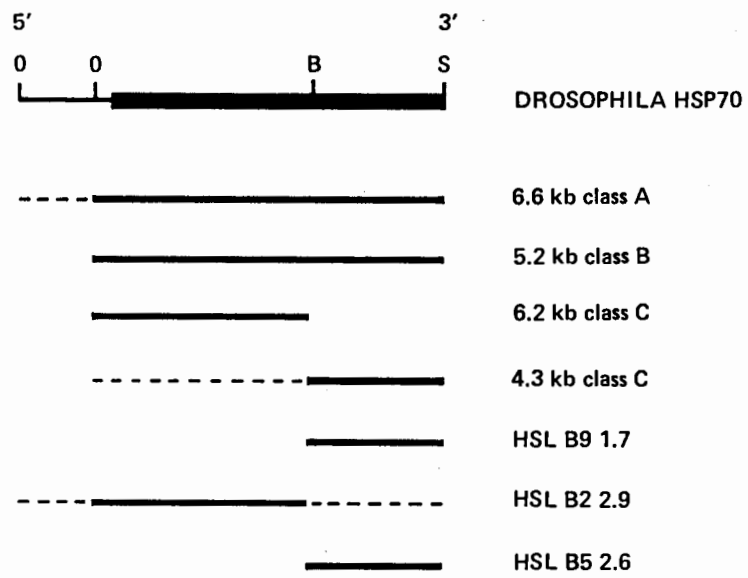
Crossreaction of the Class A, B and C Hsp70 Related Clones

To determine the relationships between the class A, B and C clones, they were nick-translated and hybridized to C. elegans genomic DNA digested with either EcoRI or HindIII. One half of the double blot was washed under low stringency conditions and the other half under high stringency conditions. Figure 2.7 shows that there is a great difference between the hybridization patterns between high and low stringency washes. For example, the class A 6.6 kb fragment hybridizes to about 15 different EcoRI bands at low stringency, but only to itself and the 5.2 kb class B fragment at high stringency. The amount of crossreaction was determined by cutting the appropriate band out of the nitrocellulose filter and counting in scintillation fluid. Under high stringency conditions the class A and B clones crossreacted at about 15% the level self-hybridization. The class A and C clones and the class B and C clones did not crossreact at all under high stringency conditions. These results suggested that for the purpose of Northern blots, it should be possible to distinguish between classes A and C and class B and C mRNAs, while classes A and B mRNAs will crossreact. In order to distinguish between the latter two it would probably be necessary to use a 5' or 3' nontranslated spacer as a probe.

Figure 2.7. Hybridization of ^{32}P-labelled class A, B and C probes to Bristol DNA. <u>C. elegans</u> genomic DNA was digested with either HindIII (lane 1) or EcoRI (lane 2). A) Hybridization and washing under low stringency conditions. a) 6.2 class C subclone b) 5.2 class B subclone c) 4.3 class C subclone d) 6.6 class A subclone. B) Hybridization and washing under high stringency conditions. a) 1.8 kb cDNA b) 2.3 kb cDNA c) 6.2 class C subclone d) 5.2 class B subclone e) 4.3 class C subclone f) 6.6 class A subclone. Sizes are in kilobasepairs.

High stringency hybridization conditions (described in Materials and Methods) were defined as those hybridization and washing conditions whereby the class A and C and the class B and C hsp70 homologous regions did not detectably crossreact. For these determinations, each probe was labelled to a specific activity of between 1 - 2 x 10^8 cpm/ug and hybridized to genomic DNA blots at the described conditions for 18 to 24 hours. The filters were exposed to Kodak Blue Brand film at -80°C for 48 hours with an intensifying screen.

76b

B

a   b   c   d   e   f

76c
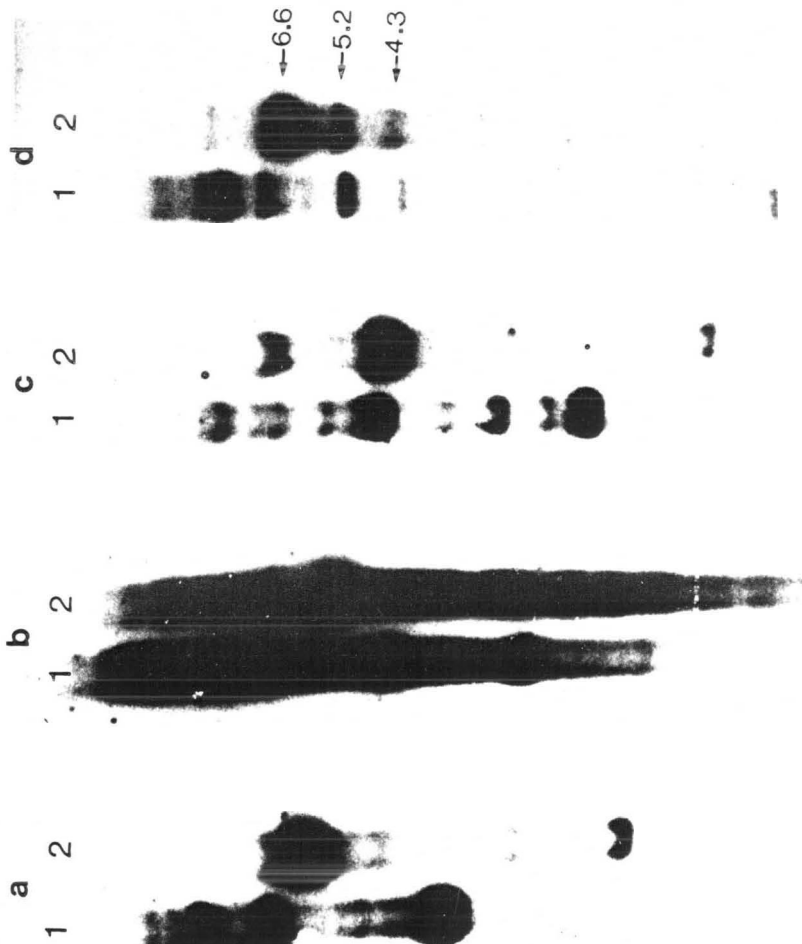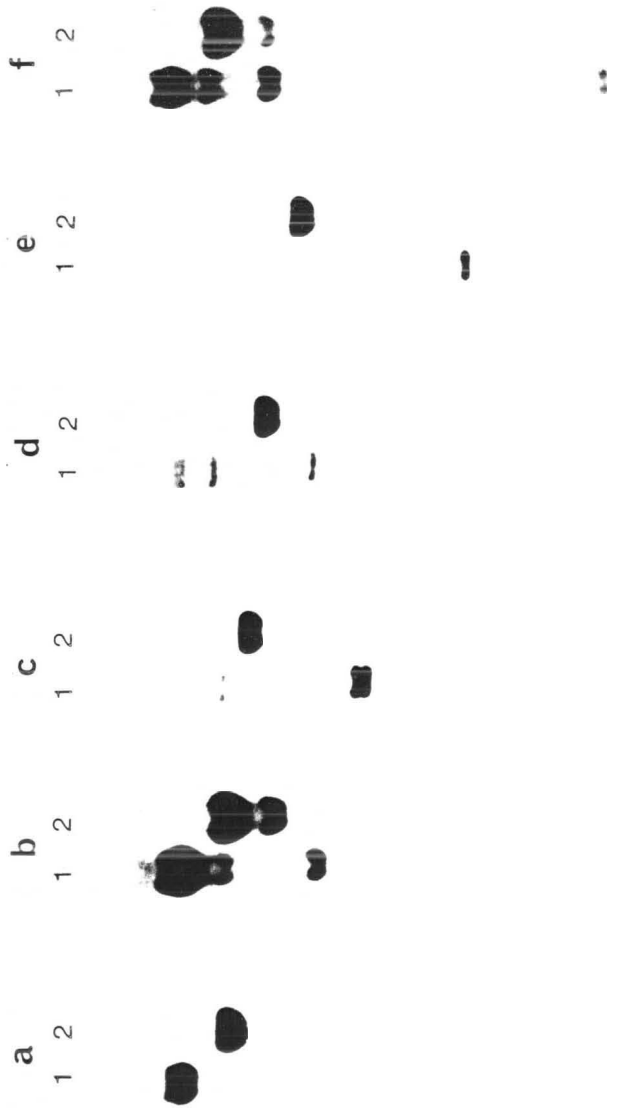
Screening of a C. elegans lambda gt10 cDNA Library

The expression of the class A, B and C genes was analysed by screening a cDNA library constructed in lambda gt10 (a gift from B. Meyers). About 32,000 phage were screened with each of the class A, B and C probes, resulting in the final purification of four positives. Two of the phage contained a cDNA insert of 2.3 kb in size, while the remaining two had 1.8 kb inserts. Hybridization of these cDNAs to EcoRI and HindIII genomic DNA digests revealed that all four were homologous to the class A hsp70 gene (Figure 2.7). The two classes of cDNA were subcloned into the plasmid pUC13. Restriction mapping of the subclones confirmed that they were related to the class A hsp70 gene (Figure 2.8). The 1.8 kb cDNA lacks the internal KpnI site and sequences 5' to this site. The larger 2.3 kb cDNA contains the KpnI site and about 500 bp 5' to this site. From the restriction map data, the 1.8 and 2.3 kb cDNAs have similar 3' termini, suggesting that transcription of the class A gene terminates downstream of the SalI site and prior to the HindIII site of the genomic clone. If this is the case, and the 2.3 kb cDNA is about 100 bp shorter than the intact mRNA, then transcription of the class A gene should initiate about 100 bp downsream from the XbaI site. This scenario depends upon there being no introns in the 5' region of the class A gene.

Figure 2.8. Comparison of the class A hsp70 genomic region and the 1.8 (pCe 5.2) and 2.3 (pCe 6.2) kb cDNAs. The region between the arrows of the class A genomic clone represents the region homologous to the _Drosophila_ hsp70 gene. Restriction enzymes; X, XbaI; B, BamHI; H, HindIII; S, SalI; E, EcoRI; K, KpnI; O, XhoI.

class A genomic region

p Ce 6.2   cDNA

polylinker

p Ce 5.2   cDNA

polylinker

1 kb
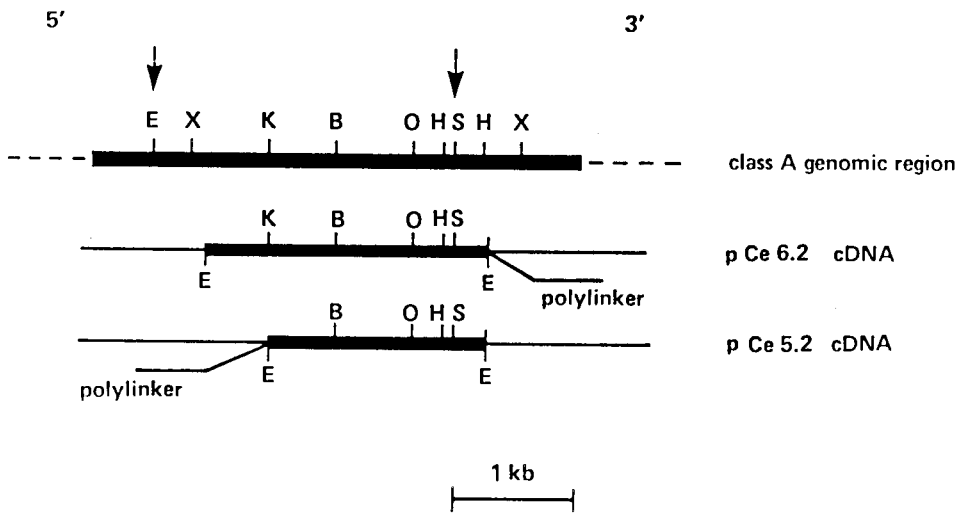
The class A cDNAs are represented at a frequency of about 1/8000 phage in this library, which was constructed from mRNA isolated from worms grown at 20°C in liquid (B. Meyers, pers. comm.). Recent evidence suggests that the frequency of cDNA inserts in this library is about 10% (D. Holm, unpublished results). Thus, the class A hsp70 cDNAs are probably present at a frequency of about 1/800 cDNAs. To date I have not detected cDNAs of the class B and C hsp70 related genes.

Northern Blot Analysis

The expression of the three hsp70 class genes and of the three EcoRI hsp70 homologous fragments were analysed by hybridization to total RNA from heat shocked and control worms (Figures 2.9 and 2.10). The class A gene was expressed in control worms and was increased in response to heat shock. In both heat shocked and control RNA the message was 2.4 kb in size. The pattern of hybridization was identical to that seen for hybridization of the Drosophila hsp70 gene to C. elegans RNA (Figure 2.2; Snutch and Baillie, 1983). Class C gene transcripts were found in control and heat shock RNA samples, but no heat inducible increase was observed. The major message class was 2.6 kb in size. A minor band, possibly a primary transcript, was also seen at 2.8 kb. Transcripts of the class B gene have not been detected. The B9 1.7 and B4 2.9 kb EcoRI fragment also appeared to be heat shock inducible, synthesizing messages of 2.4 and 2.7 kb, respectively. Transcripts of the B5

Figure 2.9. Hybridization of class A, B and C subclones to
control (lane 1) and heat shock total RNA (lane 2). Total RNA
(17 ug) was denatured and electrophoresed through a 1.1% agarose
gel containing formaldehyde as described in Materials and
Methods. The RNA was transferred to nitrocellulose as described
by Thomas (1980) and hybridized to the indicated hsp70 probe
labelled to a specific activity between 1-2 x 10^8 cpm/ug.
Hybridization was at 68°C for 36 hours. The filters were washed
under high stringency criteria. Quantification of the
hybridizations was achieved by cutting the bands out of the
nitrocellulose filter and counting in scintillation fluid. A)
the class A 6.6 kb subclone B) the class B 5.2 kb subclone C)
the class C 4.3 and 6.2 kb subclones. Sizes are in kilobases.
Hybridization was high stringency. The filter was exposed to
Kodak blue brand film at -80°C for 3 days with an intensifying
screen.

Figure 2.10. Hybridization of the β9 1.7, B5 2.6 and B4 2.9 EcoRI fragments to heat shock (lane 1) and control (lane 2) total RNA. Total RNA (17 ug) was denatured and electrophoresed through a 1.1% agarose gel containing formaldehyde as described in Materials and Methods. The RNA was transferred to nitrocellulose as described by Thomas (1980) and hybridized to the indicated hsp70 probe labelled to a specific activity between 1-2 x 10^8 cpm/ug. Hybridization was at 68°C for 36 hours. The filters were washed under high stringency criteria. Quantification of the hybridizations was achieved by cutting the bands out of the nitrocellulose filter and counting in scintillation fluid. A) the β9 1.7 kb EcoRI fragment B) the B4 2.9 kb EcoRI fragment C) the B5 2.6 kb EcoRI fragment. Sizes are in kilobases. The filter was exposed to Kodak blue brand film at -80°C for 3 days with an intensifying screen.
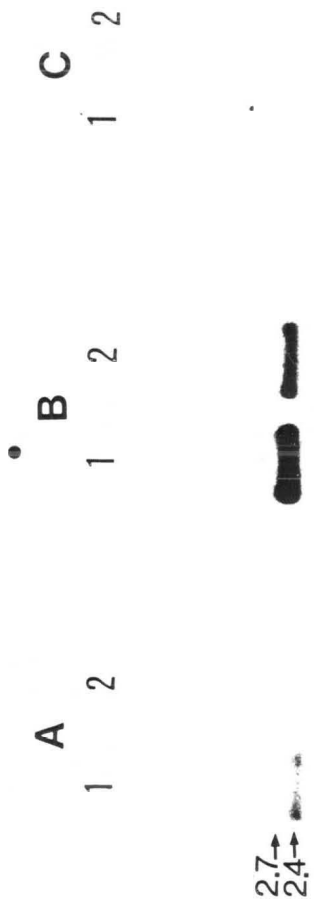
2.6 kb EcoRI fragment have not been detected.

The relative expression of the class C gene was analysed at various stages of development in worms grown at 20°C. Figures 2.11 and 2.12 show the relative abundance of class C gene transcripts compared to tropomyosin and actin transcripts. The relative level of expression of each gene was determined by cutting the bands out of the nitrocellulose filter and counting in scintillation fluid (Table 2.2). The three genes each showed a distinct developmental expression pattern. Transcipts of the tropomyosin gene decreased about ten fold between the L1 and L2 stages and remained constant at this level throughout the rest of development. The class C gene transcripts decreased about two fold from L1 to L2, increased at mid-larval stages and then dropped in the adult stage. Actin gene expression increased throughout larval development and then decreased dramatically in the adult stages.

Genome Walking in the Class A Region

In the initial screen of the Charon 4 library, three different phage representing 24 kb of DNA were isolated in the class A region. In order to extend the molecular analysis of this region, the end fragments of these clones were used to rescreen the Charon 4 library. Several new phage were isolated, extending the region 13 kb in the 5' direction and 3 kb in the 3' direction. The end fragments of these new phage were subcloned and used to walk further. To date, about 60 kb of DNA

Table 2.2

Relative abundance of class C hsp70, actin and tropomyosin transcripts in total RNA

Gene	Stage	Counts per minute Background	Above Background	Percentage of L_1 RNA
Class C hsp70		60.0		
	L_1		201.5	100.0
	L_2		114.3	56.7
	$L_{3,4}$		139.4	69.2
	Adult		33.9	16.8
Actin		61.5		
	L_1		104.8	100.0
	L_2		120.6	115.0
	$L_{3,4}$		158.1	150.8
	Adult		36.2	34.5
Tropomyosin		55.9		
	L_1		110.4	100.0
	L_2		13.0	11.8
	$L_{3,4}$		11.0	10.0
	Adult		8.3	7.6

Note: Bands from Figure 2.11 were cut from the nitrocellulose
filters and counts per minute measured by scintillation
counting.

Figure 2.11. Developmental expression of the class C hsp70 gene. Hybridization of the class C hsp70 gene (A), a putative C. elegans tropomyosin gene (B) and a Drosophila actin gene (C) to Northern blots of 17 ug of total RNA isolated from the larval stages L1 (1), L2 (2), L3+L4 (3) and adult worms. Sizes are in kilobases. Hybridization was high stringency. The filter was exposed to Kodak XAR-5 film for 24 hours at -80°C with an intensifying screen.

Figure 2.12. Relative developmental stage specific expression of the class C hsp70 gene, the tropomyosin gene and the actin genes of C. elegans. The bands shown in Figure 2.11 were cut out of the nitrocellulose filters and counted in scintillation fluid. Each gene type is plotted relative to it's mRNA concentration in L1 larvae. The level of expression between the three classes of genes cannot be directly compared in this experiment. The bars indicate the 95% confidence limits of cpm.

DEVELOPMENTAL EXPRESSION OF CLASS C HSP70, ACTIN AND TROPOMYOSIN GENES
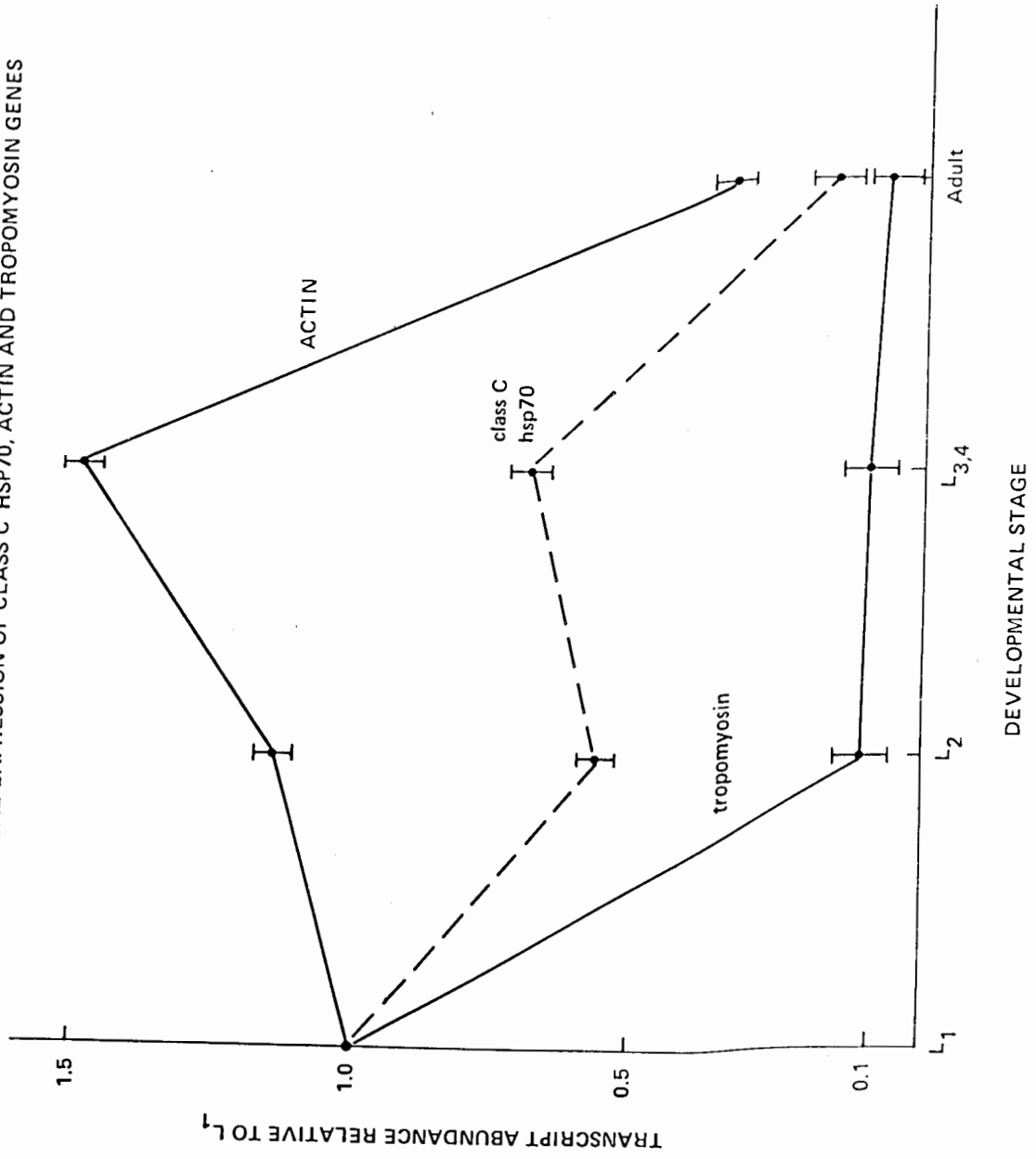
has been isolated in this region (T. Snutch and K. Beckenbach, unpublished results).

The molecular structure of the region has been analyzed in several ways. Individual phage were nick-translated and hybridized to genomic DNA blots. The results showed that the region consists almost entirely of single copy sequences. Probing of this region with the six Drosophila heat shock genes showed that only the hsp70 class A gene is represented in the 60 kb. To determine whether any other transcribed genes are present in the region, phage representing the 60 kb were nick-translated and hybridized to Northern blots of heat shock and control RNA. Figure 2.13 shows that only the lambda phage containing the class A hsp70 gene reacts with the RNA.

Conservation of the hsp70 Gene Family Between Nematode Species

A second species of Caenorhabditis, C. briggsae, is morphologically identical to C. elegans. It was therefore interesting to ask how the hsp70 gene family was conserved between the two species. Figure 2.14 shows the results of hybridization of the six different hsp70 family members to C. briggsae DNA. The results are quite interesting. It is apparent that C. briggsae, like C. elegans, has a family of hsp70 related sequences. Surprising, however, is the fact that not all C. elegans hsp70 genes have a corresponding sequence in the C. briggsae genome.

Figure 2.13. Northern blots with lambda phage representing 60 kb of cloned DNA in the class A hsp70 region. Individual lambda clones were nick translated and hybridized to control (lane 1) or heat shock (lane 2) total RNA (17 ug). Sizes are in kilobases. The filter was exposed to Kodak XAR-5 film at -80°C for 24 hours with an intensifying screen. Lambda 151 contains the class A heat shock gene.

λ_{40} λ_{37} λ_{151} λ_{33} λ_{30}

←2.4
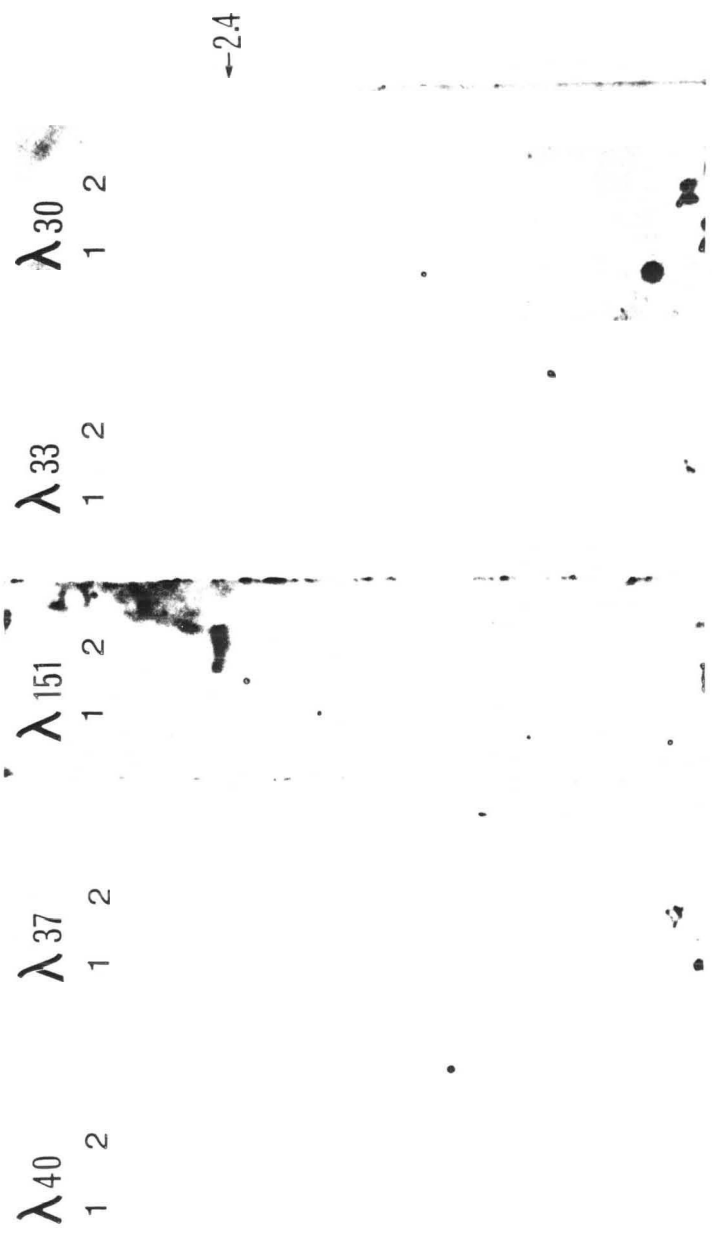
Figure 2.14. Conservation of the hsp70 gene family between nematode species. Each class of hsp70 related fragment was nick translated and hybridized to C. elegans Bristol (lane 1), Bergerac (lane 2) and C. briggsae (lane 3) genomic DNA which had been digested with EcoRI. A) class A 6.6 kb fragment B) class B 5.2 kb fragment C) class C 6.2 + 4.3 kb fragments D) B4 2.9 kb EcoRI fragment E) B9 1.7 kb EcoRI fragment F) B5 2.6 kb EcoRI fragment G) C. elegans putative tropomyosin gene.

The order of the lanes is reversed for some probes because the gel was a bidirectional transfer. The filter was exposed to Kodak blue brand film at -80°C for 3 days with an intensifying screen.

88b
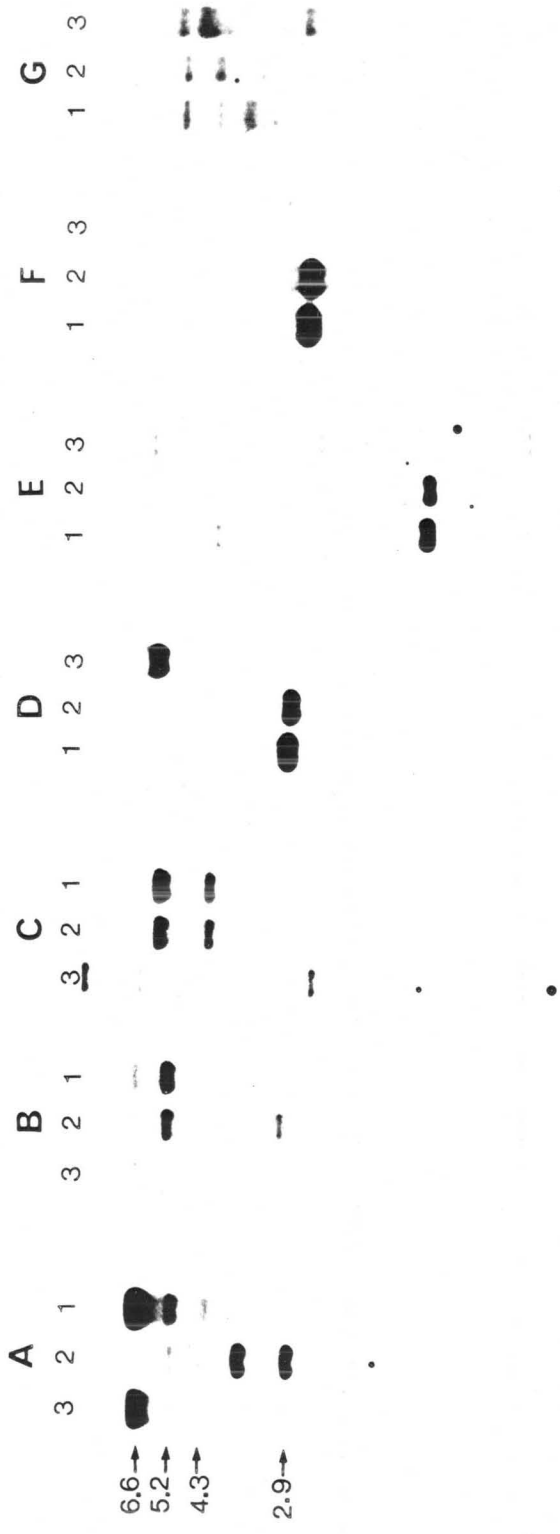
The C. elegans class A gene hybridized strongly to a 6.5 kb EcoRI fragment in C. briggsae. This gene is expressed in C. briggsae as a heat shock inducible mRNA 2400 nucleotides in size (see Figure 3.9). Similarly, the C. elegans class C gene and the B9 1.7 and B4 2.9 kb EcoRI fragments showed homology to sequences in the C. briggsae genome. In contrast, the C. elegans class B gene only showed cross-hybridization to the 6.5 kb class A fragment in C. briggsae. No other homology was detected in the C. briggsae genome. Similarly, the B5 2.6 kb EcoRI fragment showed no corresponding sequence in C. briggsae. Construction of a C. briggsae genomic library and screening for the class A, B and C homologous sequences resulted in the purification of clones representing only the class A and C genes. This supports the evidence that no class B gene is present in C. briggsae.

Probing of the C. briggsae genome with subclones representing the flanking regions of the class A, B and C genes showed that most of the probes did not hybridize to C. briggsae DNA under moderate stringency conditions. The exceptions were the 3' flanking 3.0 and 4.8 kb class A EcoRI fragments, which showed partial homology to the C. briggsae genome.

IV. Discussion

Using antibodies against chicken hsp70 and hsp89, Kelley and Schlesinger, (1982) showed the conservation of these proteins across a wide variety of organisms. I have extended this analysis by the hybridization of clones representing six different D. melanogaster heat shock genes to C. elegans genomic DNA. The results indicate that the observed similarities between the in vivo heat shock responses of the two organisms can be extended to include conservation at the DNA level. All Drosophila heat shock genes tested showed hybridization to sequences in the C. elegans genome. That the homologous C. elegans sequences are heat shock genes is supported by several lines of evidence. Firstly, the Drosophila hsp70 gene also hybridized to a C. elegans heat shock inducible poly(A)+ mRNA of 2.4 kb. The size of the hsp70 mRNA is conserved between D. melanogaster, C. elegans and a second species of nematode, C. briggsae. Secondly, Russnak et al (1983) have shown that two different cDNAs encoding the C. elegans hsp16 share considerable sequence homology to the four Drosophila low molecular weight hsps. Finally, under conditions of slightly higher stringency, the Drosophila hsp83 gene hybridizes strongly to a single 3.0 kb EcoRI fragment in C. elegans. Charon 4 phage containing this fragment (potentially the C. elegans hsp81 gene) have recently been isolated. Thus, screening the genomes of other organisms with cloned heat shock genes may demonstrate a more general

conservation of individual hsps which was not evident by protein analysis.

The fact that the Drosophila hsp22, 23 and 26 genes showed a different hybridization pattern to C. elegans DNA than did the hsp28 gene was of some interest. These four genes are clustered in a 12 kb region in Drosophila (Craig and McCarthy, 1980; Corces et al, 1980). A more extensive analysis of this region has identified three additional genes which appear to be developmentally regulated (Sirotkin and Davidson, 1982). It may be that the hsp28 subclone also contains portions of another, previously unidentified gene that is also conserved between these species. Alternatively, the hsp28 subclone may contain an element that is repeated in the C. elegans genome.

In order to further study the hsp70 gene family I have cloned C. elegans sequences related to the Drosophila hsp70 gene. Under low stringency hybridization conditions the Drosophila probe hybridizes to about 15 C. elegans EcoRI fragments. Under moderate stringency the Drosophila probe is related to only four C. elegans EcoRI fragments. Screening of two C. elegans genomic DNA libraries resulted in the purification of seven of the homologous EcoRI fragments, including the four most closely related to the Drosophila probe.

Hybridization of each cloned fragment to the Drosophila hsp70 gene separated into 5' nontranscribed, 5' coding and 3' coding regions showed that each C. elegans fragment is uniquely related to the Drosophila gene. The class A, B and C clones and

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the B4 2.9 kb EcoRI fragment show crossreaction to both the hsp70 coding regions. In addition, the class A clone reacts positively with 5' nontranscribed region. The B9 1.7 and B5 2.6 kb EcoRI fragments showed homology only to the 3' coding region. Assuming that these two cases represent genes with an internal EcoRI site, then C. elegans possesses at least six and probably not more than nine hsp70 related genes.

Molecular analysis of the class A, B and C hsp70 clones showed that each is present once per haploid genome. The degree to which each of these genes is related to the Drosophila heat shock inducible hsp70 gene can be summarized as A > B > C. The hsp70 gene most closely related to the Drosophila gene (the class A) is heat shock inducible as judged by Northern Blots. In Drosophila and yeast, members of the hsp70 gene family which are not transcriptionally activated by heat shock are termed heat shock cognates (hsc; Ingolia and Craig, 1982). The Drosophila hsc genes are also structurally different from hsp70 genes in that two of the three cognates contain intervening sequences. The C. elegans class C hsp70 gene appears to be a hsc gene; transcription is not increased in response to heat shock; it is developmentally regulated; and it may possess an intron of about 200 bp. In many respects the results reported here for C. elegans are similar to those found in Drosophila and yeast. All three species possess a family of hsp70 related genes in which transcription of some members is inducible by heat shock, while others are trancribed at normal temperatures and are

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developmentally regulated (Ingolia and Craig, 1982; Ingolia et
al, 1982; Craig et al, 1983).

The available evidence suggests that the class B hsp70 gene
is neither heat shock inducible nor is it required at any stage
during development. Class B transcripts are not detected even
under conditions (ie. heat shock) whereby nonheat shock gene
transcripts such as those of the class C and tropomyosin genes
are readily detected. Also, arguing against any developmental
role for the class B gene product, is the fact that it is not
found in the genome of the closely related species, C. briggsae.
The class B hsp70 gene may be a recent addition to the C.
elegans genome. This could be the result of a duplication event
involving all or part of the class A gene, or by a gene
conversion event between the class A gene and another locus.
Alternatively, this gene may have been present in the ancestor
of C. elegans and C. briggsae and has been recently lost from
the C. briggsae genome. In any of these scenarios, the class B
gene may be a pseudogene.

Transcription studies on cloned Drosophila hsp70 genes have
identified two upstream promoter regions. One of these, the TATA
box, is found about 30 bp 5' to the initiation of transcription
of all pol II genes (Benoist et al, 1980). The second promoter
element is centred about 20 bp upstream of the TATA box and
appears to be required for the positive control of hsp70
transcription. Pelham (1982) has derived a consensus 14 bp
sequence which is found upstream of the transcription initiation

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point of most _Drosophila_ heat shock genes. The promoter element consists of a small conserved inverted repeat which is in turn centred in a larger, more variable inverted repeat. A positive transcription factor which binds to the promoter and is required for heat shock inducible transcription of the hsp70 gene has been identified (Parker and Topol, 1984). The fact that transcription of the _Drosophila_ hsp70 gene can be accurately controlled in a variety of heterologous systems suggests that the regulatory mechanisms governing the heat shock response have been conserved throughout evolution (Corces _et al_, 1981; Burke and Ish. Horowicz, 1982; Pelham, 1982). The _Drosophila_ heat shock promoter consensus sequence contains the recognition sequence for the restriction enzyme XbaI (TCTAGA). Examination of the _C. elegans_ class A, B and C potential coding regions showed that the class A gene has an XbaI site about 700 bp 5' to the internal KpnI site. This site is about 100 bp upstream from the start of transcription estimated by the analysis of the class A cDNAs and mRNA transcripts. It is possible that this XbaI site marks the class A hsp70 promoter region. This region is now being sequenced by the Maxam and Gilbert (1977) method. The class B and C genes, which show no evidence of heat shock inducibility, do not possess an XbaI site in their 5' flanking regions.

The expression of the hsp70 gene family has been analysed by Northern blots and screening of a cDNA library. Transcripts of the class A gene were detected in the cDNA library at a

frequency of 1/800 cDNAs. If the cDNA library accurately
reflects the population of mRNAs in vivo, then the class A hsp70
transcripts account for about 0.13% of the poly(A)+ transcripts.
Under high stringency hybridization conditions I have been able
to distinguish between class A, B and C transcripts. Under these
conditions the class A and B genes crossreacted to about 15 % of
the level of self hybridization. However, in heat shock RNA
class B transcripts are not found at more than the level that
can be accounted for by crosshybridization to with the class A
transcripts. It is therefore likely that this gene is not heat
shock inducible. The class C gene does not detectably
cross-hybridize with the class A or B genes at high stringency
and synthesizes a mRNA that is larger than the class A gene
transcript. It is transcribed at all stages of development and
shows a different developmental expression pattern than either
of actin or tropomyosin genes. It will be interesting to
determine whether tissue specific expression of this gene
occurs.

The class A and B4 2.9 and B9 1.7 kb EcoRI clones all show
an increase in transcription in response to heat shock.
Irrespective of the RNA preparation the expression of the B4 2.9
kb fragment does not increase more than 2 to 3 fold in response
to heat shock. A similar type of hsp70 gene, which is 94%
homologous to the major heat shock inducible hsp70 gene and
whose transcription is increased about two to three fold in
response to heat shock has been reported in yeast (B. Craig,

pers. comm.). In contrast to B4 2.9, detection of the class A and B9 1.7 transcripts in heat shock and control RNA depends significantly upon the RNA preparation being probed. This appears to be due to at least two factors. First, when probing RNA isolated from a mixed population of worms, I find that age of the majority of worms is significant. Transcripts of both the class A and B9 1.7 clones were detected in higher relative amounts (2 to 4 fold) in heat shocked larvae than in heat shocked adults. Second, how the worms were handled during RNA isolation greatly affects the amount of hsp70 RNA in control worms. I believe that the processes of collecting worms off NGM plates and concentrating them by light centrifugation is stressful enough to initiate the 'heat shock' response. Unfortunately, alternatives such as growing the worms in liquid medium or freezing them rapidly are not viable solutions to this problem.

In summary, while each hsp70 related gene showed a unique expression pattern as judged by Northern blots, it is possible that some cross-hybridization does occur between the various clones. Quantitative assessment of the expression of these genes will await further experiments using either kinetic analysis, cDNA primer extension or Northern blots with gene specific probes.

The genomic organization of the C. elegans hsp70 gene family is quite different from that of D. melanogaster. C. elegans has a single class A gene within a 60 kb region, and

including the class B and C genes, none of these genes are linked within 100 kb. In contrast, most Drosophila strains contain five nearly identical copies of the hsp70 gene in addition to several divergent hsc genes. At the 87A locus two hsp70 genes are divergently arranged between a spacer region of about 1.5 kb. At the 87C locus three hsp70 genes are found within a 55 kb distance. Two genes exist as tandem repeats and the third in the opposite orientation about 38 kb away (reviewed by Ish-Horowicz and Pinchin, 1980). The three Drosophila hsc genes are dispersed at three separate cytological locations, including one located about 8 kb from the xanthine dehydrogenase gene (Ingolia and Craig, 1982; Craig et al, 1983). The C. elegans class A gene is flanked essentially by single copy DNA and in which no other heat shock inducible genes are found. In D. melanogaster, the spacer region at 87C is occupied by a number of tandem copies of middle repetitive elements which are transcribed but not translated in response to heat shock (Lis et al, 1978). The genomic organization of the yeast hsp70 family has not been reported.

A number of other genes have been cloned from C. elegans. Most represent multigene families in which members are both clustered and dispersed throughout the genome. These include the yolk protein (Blumenthal et al, 1984), the actin (Files et al, 1983), the major sperm protein (Burke and Ward, 1983) and the collagen (Kramer et al, 1982) gene families. In each of these cases, the isolation of the gene family was made possible by the

availability of either a cloned gene from another species or a cDNA which could be constructed from a developmental stage enriched for the gene product. For the majority of genes regulating the development of C. elegans, cloned probes will not be available. The cloning and molecular analysis of these interesting genes will require unconventional methods. Rose et al (1982) have demonstrated the feasibility of using strain DNA polymorphisms to clone regions of the C. elegans genome for which gene specific probes do not exist. However, the identification of genes in a cloned region is not always easy. Northern blots are not sensitive enough to detect low abundance transcripts and the screening of cDNA libraries is both tedious and depends upon the gene in question being represented in the library.

I believe that this problem can be addressed by utilizing the hybridization of cloned C. elegans probes to genomic blots of C. briggsae DNA. The evidence presented here indicates that some portions of the C. elegans genome have diverged significantly from C. briggsae. It is reasonable to assume that the divergent regions include mostly noncoding DNA sequences while the conserved regions code for the proteins necessary for nematode growth and reproduction. For example, hybridization of those hsp70 clones which appear to be transcribed (class A, class C, B4 2.9 and B9 1.7) to C. briggsae DNA shows homologous sequences are present in the C. briggsae genome. In contrast, neither those hsp70 clones for which transcriptional evidence is

lacking nor the class A and C flanking regions appear to be represented in the C. briggsae genome. A convenient two step procedure for identifying unknown coding regions would be to first hybridize the C. elegans probes to C. briggsae genomic DNA under moderate stringency conditions. If the fragment proved to be conserved between the two species, it could subsequently be used to probe Northern blots and screen cDNA libraries. This would significantly simplify the process of searching for genes on large regions of cloned DNA. This will be especially valuable in C. elegans, where the small genome size and relative lack of repetitive DNA make genome walking feasible.

D. Chapter III

A Region of High Sequence Divergence Flanking the Class A Hsp70

Gene

I. Introduction

Genetic variation between populations has traditionally been studied by analysis of protein structural variation. The evolutionary importance of maintaining large amounts of genetic polymorphism in populations has been widely discussed (for a general review see Nei and Koehn, 1983). The advent of recombinant DNA analysis has significantly increased both the number of genetic markers available and the precision with which genetic variation can be determined. In particular, analysis of DNA polymorphisms of mitochondrial genomes has provided a useful model system for studying both the evolution of eukaryotic DNA and the biology of mitochondria (Brown, 1983; Avise and Lansman, 1983).

In the nuclear genome, DNA polymorphisms have been used to characterize regions flanking known cloned genes and for randomly cloned regions. In humans, the prenatal diagnosis and evolution of B-thalassemias has been facilitated through the analysis of polymorphic restriction endonuclease sites (Kan and Dozy, 1978; Little et al, 1980; Kazazian et al, 1983). The linkage of restriction site polymorphisms with loci known to be associated with disease is a convenient method for both identifying carriers of the disease and for the molecular cloning of the locus (for example, the Huntington's disease gene, Gusella et al, 1983). Restriction fragment length polymorphisms have also been used to increase the number of

available genetic markers and to establish genetic linkage maps (Bostein et al, 1980; Naylor et al, 1984; Solomon and Bodmer, 1979). Rose et al (1982) have detailed the genetic and evolutionary problems which may be addressed through the study of genetic variation in nematode strains.

Analysis of a cloned gene is greatly facilitated if the genomic location of the cloned gene and its neighbours are known. In order to map the cloned hsp70 genes genetically, a search for restriction fragment length differences (RFLDs) was initiated between two closely related strains of C. elegans. Previous studies into the sequence variation between the Bristol and Bergerac strains of C. elegans have shown the presence of a number of RFLDs (Emmons et al, 1979; Rose et al, 1982). For randomly cloned DNA fragments a divergence of about 1% in nucleotide sequence must be assumed in order to explain the observed frequency of RFLDs. The absence of detectable protein polymorphism between the Bristol and Bergerac strains (Butler et al, 1981) suggests that the 1% sequence difference is distributed mainly in noncoding DNA regions. Using Bristol and Bergerac interstrain crosses, the RFLDs can be mapped to chromosomal locations. This method involves a combination of standard genetic manipulations and hybridization of cloned probes to genomic DNA blots (Emmons et al, 1979; Rose et al, 1982).

In an attempt to identify RFLDs in regions flanking the cloned hsp70 genes I have found a region of the C. elegans

genome in which RFLDs are highly clustered. The results suggest that some regions of the genome accumulate mutations at a rate 10 to 20 fold higher than other regions.

II. Materials and Methods

Screen of Bergerac Strain for RFLDs

Four ug per lane of Bristol or Bergerac genomic DNA were digested with an excess of EcoRI, separated on 0.6% agarose gels and transferred to nitrocellulose using the method of Smith and Summers (1980). Filters were hybridized under moderate stringency conditions to each of the class A, B and C EcoRI subclones. Subsequent probing for RFLDs employed Bristol and Bergerac genomic DNA completely digested with SalI, XhoI, BamHI, PstI, SmaI, XbaI or KpnI and hybridized against various class A, B and C probes.

Northerns

C. elegans, strain Bergerac, and C. briggsae, strain G16 worms were heat shocked at 35°C for four hours then allowed to recover at 20°C for 90 minutes prior to isolation of total RNA. Control worms were maintained at 20°C. Formaldehyde RNA gels containing 17 ug of total RNA per lane were run as described in Chapter II.

Screening of Bergerac Library for Class A Charon 4 Clones

The construction of a Bergerac strain library into Charon 4 was described in Chapter II. Five genome equivalents were screened with the Bristol class A 6.6 kb EcoRI fragment. Thirty-one positives were isolated on the first screen and 29 of these retested positive on the second screen. In hopes of analysing only the class A clones, seven of the positives with strongest hybridization signal were grown up and phage DNA was isolated.

Estimation of Sequence Divergence

For each strain, the number of restriction site differences was divided by the total number of hexanucleotide restriction sites multiplied by six. This value was calculated for each strain and the average was taken (see Bender et al, 1983a).

III. Results

Figures 3.1, 3.2 and 3.3 show the results of hybridization of the class A, B and C region subclones to EcoRI digests of Bristol and Bergerac strain DNA. For the class B and C genes, comparison of the Bristol and Bergerac genomes for RFLDs showed that the flanking regions were identical for EcoRI sites. In contrast, comparison of the class A clones showed a surprising result: every EcoRI subclone showed a RFLD between the two strains. The nature of the RFLD varied from fragment to fragment. For example, some Bristol strain fragments shifted to larger fragments (the 2.0, 1.7, 1.3 and 0.5 kb), while others were split into two or more fragments in the Bergerac strain (the 6.6, 3.0 and 4.8 kb). A summary of the various RFLDs is shown in Table 3.1.

Several aspects of the class A blot are worth noting. First, the 4.8 kb Bristol fragment contained a repeated element. If it is assumed that each EcoRI fragment contains one copy of the element then there are about 15 copies in the C. elegans genome. The element does not appear to be highly mobile. Other than the 4.8 kb shifted to 3.7 and 4.0 kb fragments, only one other EcoRI fragment differs between the two strains. Second, none of the minor bands to which the 6.6 kb fragment hybridizes

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Figure 3.1. Hybridization of subcloned EcoRI fragments derived from regions flanking the class B hsp70 gene to Bristol (N2) or Bergerac (BO) DNA digested with EcoRI. The 4.8 kb fragment contains the hsp70 homologous region. Markers are HindIII digested lambda DNA. Hybridization was carried out under low stringency conditions.

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Figure 3.2. Hybridization of subcloned EcoRI fragments derived from regions flanking the class C hsp70 gene to Bristol (N2) or Bergerac (BO) DNA digested with EcoRI. The 6.2 and 4.3 kb fragments contain the coding element. Markers are HindIII digested lambda DNA. Hybridization was carried out under low stringency conditions.
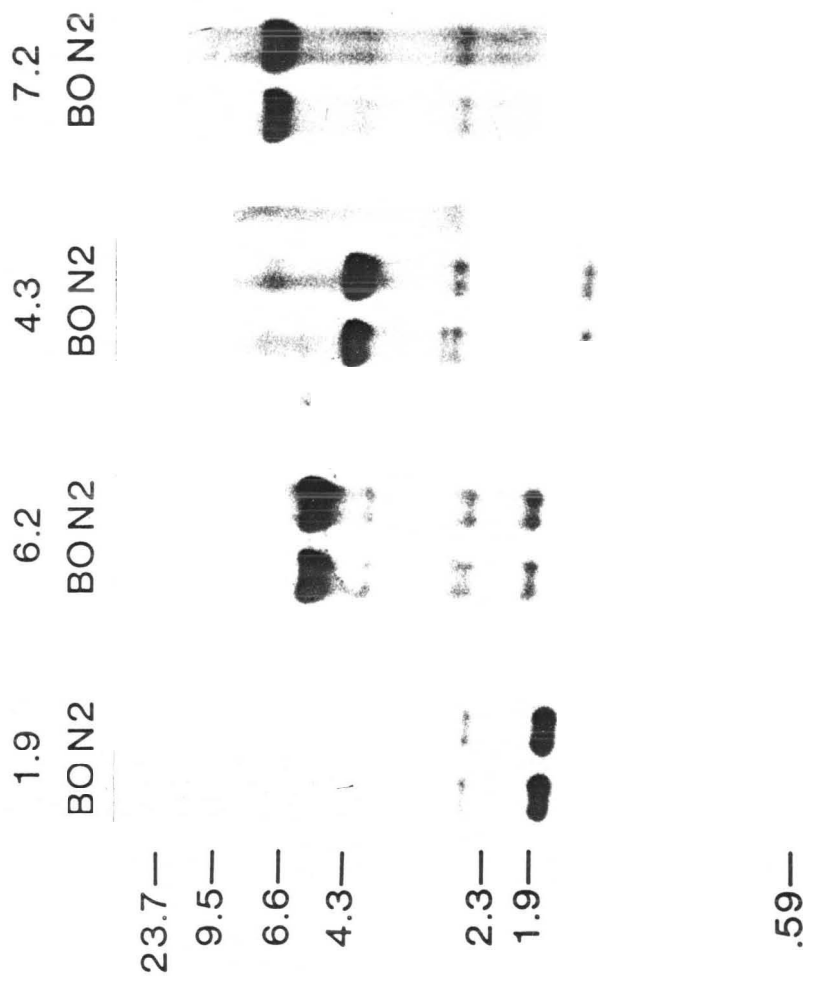
Figure 3.3. Hybridization of subcloned EcoRI fragments derived from regions flanking the class A hsp70 gene to Bristol (N2) or Bergerac (BO) DNA digested with EcoRI. The 6.6 kb fragment contains the coding element. Markers are HindIII digested lambda DNA. Hybridization was carried out under low stringency conditions.

2.0 BON2 1.7 BON2 BON2 1.3 BON2 BON2 6.6 BON2 0.5 BON2 BON2 3.0 BON2 BON2 4.8 BON2 BON2

23.7—
9.5—
6.6—
4.3—
2.3—
1.9—

0.59—

109b

TABLE 3.1

ECORI RFLDS IN CLASS A HSP70 REGION

Bristol Fragment (kb)	Bergerac Fragment (kb)
2.0	2.5
1.7	3.2
1.3	3.2
6.6	2.9
	3.7
0.5	2.3
3.0	2.3
	0.5
	3.7
4.8	3.7
	4.0
3.0	4.0

were shifted between the Bristol and Bergerac genomes. Third, when the Bristol fragments were aligned 5' to 3', the Bergerac RFLDs appear to show a linear relationship. For example, both the 0.5 and 3.0 kb fragments which are adjacent in the Bristol genome, hybridized to a 2.3 kb Bergerac fragment. Similarly, both 1.3 and 1.7 kb Bristol fragments hybridized to a 3.0 kb fragment in Bergerac. This suggested that despite the unusually high number of RFLDs, the genomic organization in the class A region is similar in the two strains.

Extent of RFLDs in the Class A, B and C Regions

Bristol and Bergerac genomic DNA was digested with a variety of restriction enzymes and the class A, B and C regions were probed with the EcoRI subclones. The class B and C flanking regions did not show any RFLDs with any of the enzymes or probes tested. From the number of nucleotides screened for RFLDs (174 bp in the class C region and 192 bp in the class B region) it can be estimated that the class B and C regions are less than 1% divergent between the Bristol and Bergerac strains.

Figure 3.4 shows the hybridization of the 3.0 kb class A probe to Bristol and Bergerac DNA digested with various enzymes. Several RFLDs are obvious. For example, the BamHI 4.6 kb Bristol fragment shifted to a 4.3 kb fragment in Bergerac. The Bristol PstI is shifted from about 19 to 22 kb. The Bristol XhoI 4.0 kb is shifted to about 10 kb in Bergerac and the 5.5 kb Bristol XbaI fragment is shifted to about 12 kb in Bergerac. Similar

Figure 3.4. Hybridization of the 3.0 kb class A subclone to Bristol (N2) and Bergerac (BO) DNA digested with various restriction enzymes. N2 and BO DNA was cut with either SalI, XbaI, SmaI, XhoI, PstI, or BamHI. Four of the six digests show clear RFLDs. Markers are HindIII digested lambda DNA. Hybridization was carried out under low stringency conditions.

Sal   Xba   Sma   Xho   Pst   Bam
BO N2   BO N2   BO N2   BO N2   BO N2   BO N2

23.7 —
9.5 —
6.6 —
4.3 —
2.3 —
1.9 —
.59 —
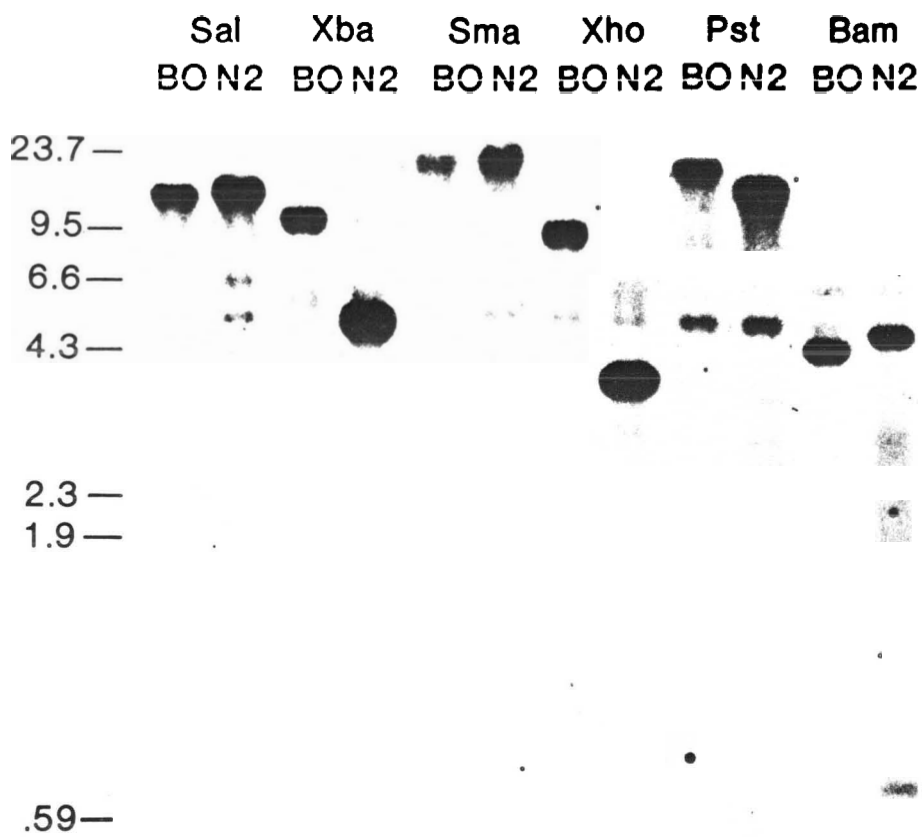
hybridizations with other class A probes showed that about 50%
of the hexanucleotide restriction sites were altered in the
class A region of the Bristol and Bergerac strains. Overall, 15
RFLDs were detected in the 192 bp screened. This can be equated
to a minimum of an 8% nucleotide difference in the flanking
regions (assuming that the RFLDs are due to point mutations.

Analysis of Charon 4 Clones of the Bergerac Class A HSP70 Region

The large number of RFLDs in the class A hsp70 region may
be due to a number of genome alterations, including; point
mutations, deletions, inversions and insertion of mobile genetic
elements. The possibility that DNA rearrangements have occurred
has been tested by examination of cloned DNA from the Bergerac
strain. A Bergerac library was constructed into Charon 4 and
seven class A hsp70 clones were isolated by homology to the
Bristol 6.6 kb EcoRI fragment. Digestion of the seven phage with
EcoRI indicated that all seven represented a single overlapping
region. Two of the seven were represented twice, resulting in
five distinct but overlapping clones. Figure 3.5 shows the
ethidium bromide stained pattern of the Bristol and Bergerac
class A phage. It is evident that none of the EcoRI fragments is
the same size between the two strains. All five Bergerac phage
had 3.7 and 2.9 kb fragments in common. Significantly, the sum
of these fragments equal the 6.6 kb Bristol fragment.

Figure 3.6 shows the hybridization of several Bristol
subclones to blots of the gel shown in figure 3.5. The 3.0 kb

Figure 3.5 Ethidium bromide stained gel of two Bristol strain class A phage (lanes 1 and 2) and five Bergerac strain class A phage (lanes 3 to 7). Markers are HindIII/ EcoRI digested lambda DNA.

Figure 3.6 Autoradiogram of blots of the gel shown in Figure 3.5 after hybridization to the 3.0, 0.5, 6.6 and 1.3 kb class A subclones. Due to the overlapping nature of the lambda phage, only the 6.6 kb probe hybridizes to all seven clones. Markers are EcoRI/HindIII digested lambda DNA.

3.0   0.5   6.6   1.3

1 2 3 4 5 6 7   1 2 3 4 5 6 7   1 2 3 4 5 6 7   1 2 3 4 5 6 7

21.7—

5.1—
5.0—
4.3—
3.5—

1.98—
1.9—
1.6—
1.4—

.94—
.83—

.59—

115b

Bristol fragment hybridized to 3.7, 2.3 and 0.5 Bergerac

fragments. The 0.5 kb Bristol·subclone hybridized to a 2.3 kb

fragment and the 6.6 kb subclone containing the class A coding

element hybridized to the 3.7 and 2.9 kb fragments. The 3.7 kb

fragment that the 6.6 kb probe hybridized to is different from

the 3.7 kb fragment to which the 3.0 Bristol probe hybridized.

The hybridizations indicate that while a large number of

RFLDs have occurred between the two strains, the majority of the

sequences remains the same. The RFLDs present in the lambda

phage were identical to those which occurred in the genome.

Furthermore, the linear arrangement of EcoRI sites in the

Bergerac and Bristol phage showed the class A hsp70 regions of

these two strains to be colinear.

Restriction Mapping of the Bergerac Class A hsp70 Region

The 22 kb of Bergerac class A DNA was restriction mapped

with six hexanucleotide recognition endonucleases and compared

to the Bristol class A region (Figure 3.7). The results showed

that there is an overall sequence divergence of about 6% over

the 19 kb compared. However, the sequence divergence was not

equally distributed throughout the region. Figure 3.7b shows

that 5 kb of DNA flanking the gene 5' and 12 flanking 3' have

diverged by 8 and 7%, respectively, between the two strains. In

contrast, no sequence difference was detected within the hsp70

coding element. The nearest detected sequence alteration was the

introduction of an EcoRI site about 150 bp 3' to the coding

Figure 3.7. Restriction map of the N2 and BO class A heat shock genes. A, Linear arrangement of the overlapping BO phage. B, Comparison of the restriction maps of the N2 and BO class A gene region. Asterisks mark sites of an RFLD caused by either generation or loss of a restriction site. Inverted triangle 5' to the coding element represents site of a 200 bp insertion. The thick line represents the hsp70 coding region. H, HindIII; E, EcoRI; X, XbaI; O, XhoI; S, SalI; k, kpnI.
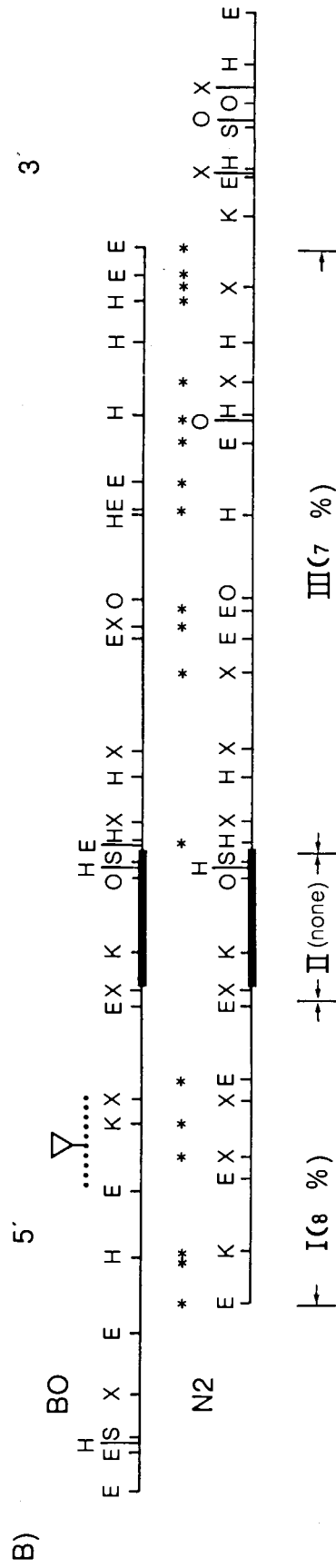
element. The nearest detected alteration in the 5' flanking
region is the loss of an EcoRI site about 1.5 kb from the coding
element. Comparison of the flanking regions shows that about 53%
of the hexanucleotide restriction sites are identical between
the two strains. The colinearity of the overall region indicates
that most of the restriction site changes are not the result of
large DNA rearrangements.

Search for a Specific Transposable Element in the Bergerac Class
A hsp70 Region

C. elegans, like many organisms, contains mobile genetic
elements, thus, it possible that the RFLDs result from
transposon insertion or deletion events. The Bristol and
Bergerac strains contain approximately 25 and 250 copies,
respectively, of the transposon, Tc1. Hybridization of a Tc1
element (Tc1 Hin variant a gift of A. Rose) showed that neither
strain carried this element in the class A hsp70 region (Figure
3.8).

The Bergerac Class A Gene is Transcribed in Response to Heat
Shock

At elevated temperatures the Bristol and Bergerac strains
synthesize similar amounts of hsp70 in vivo, indicating that the
Bergerac strain has an active, inducible, hsp70 gene
(unpublished results). Figure 3.9 shows the results of
hybridization of the class A gene to heat shock and control RNA
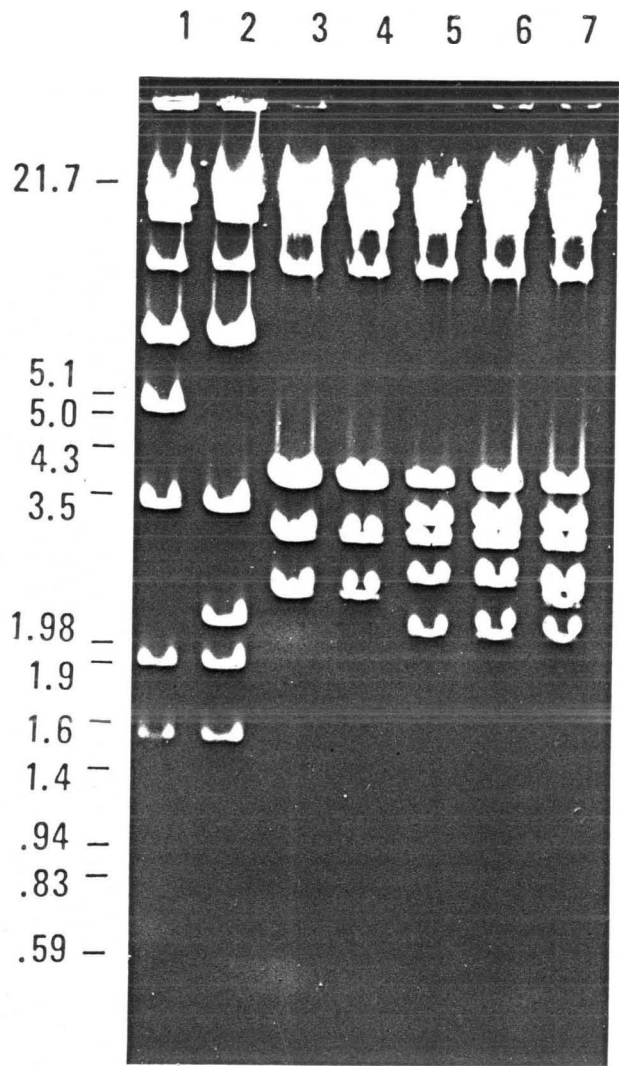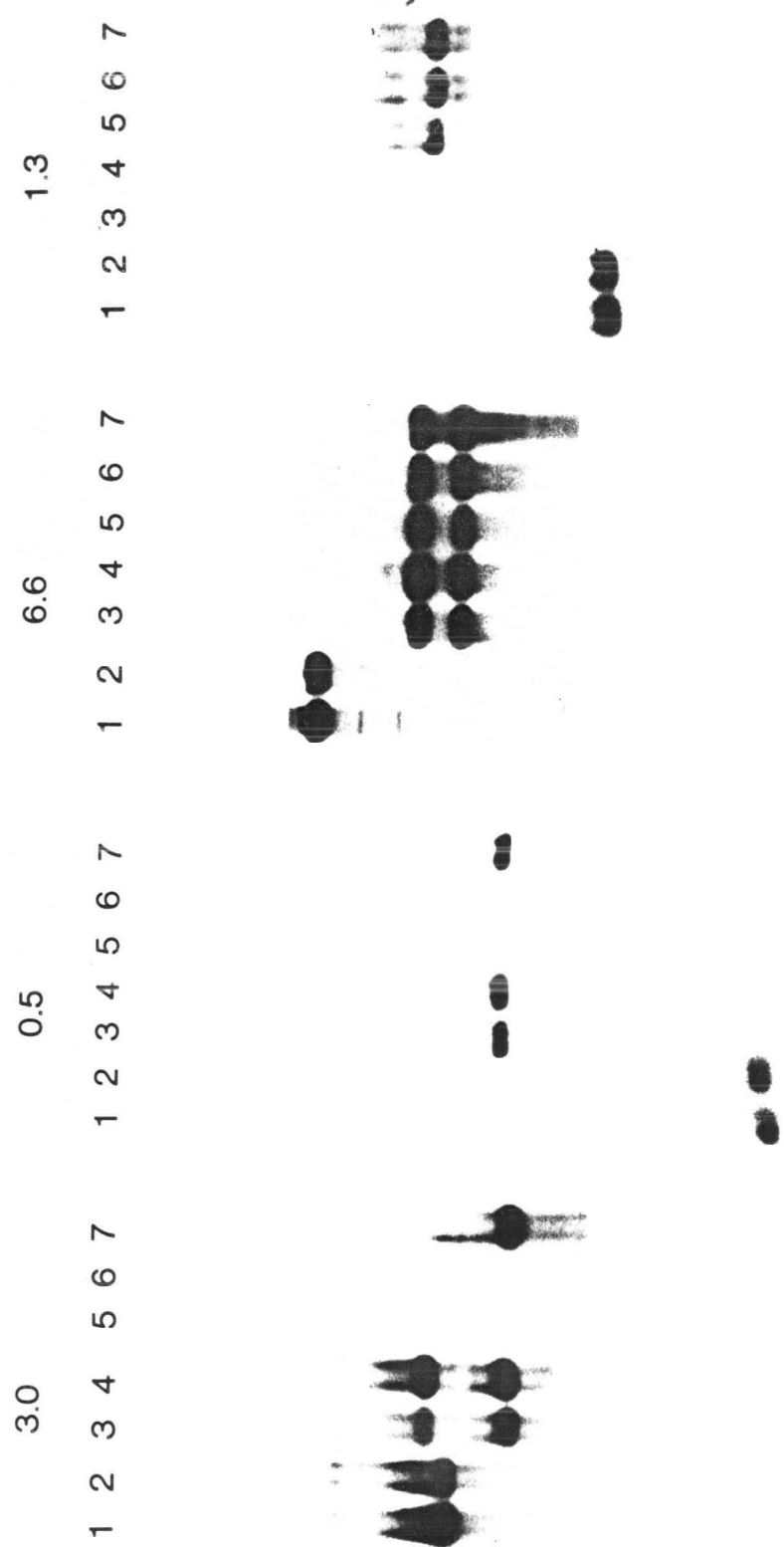
Figure 3.8. Autoradiogram of blots of the gel shown in Figure 3.5 after hybridization to the 4.8, 1.7 and 2.0 kb class A subclones and to the C. elegans transposon, Tc1. Markers are HindIII/ EcoRI digested lambda DNA.

TC1

4.8   1.7   2.0

1 2 3 4 5 6 7   1 2 3 4 5 6 7   1 2 3 4 5 6 7   1 2 3 4 5 6 7

21.7 —

5.1 —
5.0 —
4.3 —
3.5 —

1.98 —
1.9 —
1.6 —
1.4 —

.94 —
.83 —

.59 —

1196
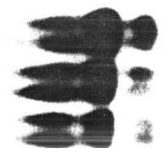
Figure 3.9. Hybridization of the Bristol class A hsp70 gene to C. elegans Bristol (A) and Bergerac (B) and C. briggsae (C) heat shocked (lane 1) and control (lane 2) RNA. Heat shock was carried out at 35°C for 2.5 hours and followed by recovery at 20°C for 60 minutes. Sizes are in kilobases. The filter was exposed to Kodak blue brand film at -80°C for 2 days with an intensifying screen.
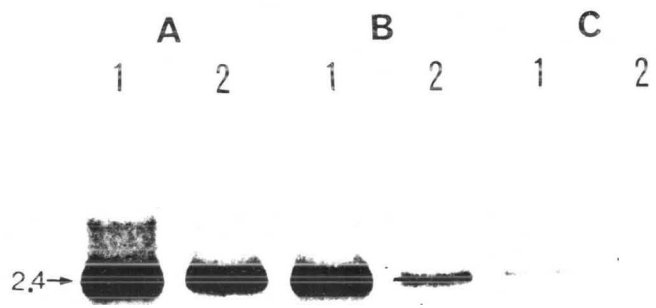
from Bristol, Bergerac and C. briggsae worms. Both C. elegans strains and the closely related C. briggsae synthesize a heat shock inducible message 2400 nucleotides in size. I conclude that the Bergerac class A gene is actively transcribed in response to heat shock.

IV. Discussion

The sequence variation between two strains of C. elegans
has been compared for three cloned regions of the genome.
Unexpectedly, the DNA flanking the class A hsp70 gene showed a
sequence divergence of about 7.5%. Comparable sequence
differences have been described for organisms which are
separated for millions of years (for example, cow and sheep,
11.2%, Kohne et al, 1972; mouse and rat, 9%, McCarthy and
Farquhar, 1972).

In the D. melanogaster Oregon R and Canton S strains a
nucleotide difference of about 0.4% exists (Bender et al,
1983a). Two previous studies have used randomly cloned DNA
fragments to analyse the sequence divergence between the Bristol
and Bergerac strains of C. elegans (Emmons et al, 1979; Rose et
al, 1982). These studies concluded that a nucleotide difference
of about 1% exists. Emmons et al (1979) have previously noted
that this difference is rather high for two closely related
strains but that most of the 1% nucleotide divergence can be
accounted for by the high number of Tc1 insertions in the
Bergerac strain. The results reported here suggest that the
reported 1% sequence difference between the Bristol and Bergerac
strains of C. elegans vary by as much as ten to twenty fold in
some regions of the genome. For example, in the class B and C
hsp70 regions of the genome no detectable divergence occurred.
Also, other researchers have not detected any RFLDs in large

regions of the C. elegans genome flanking three cloned myosin genes (G. Benian and R. Waterston, pers. comm.). In contrast, the class A hsp70 gene flanking regions showed a high degree of sequence divergence. In contrast to the two previous studies of sequence divergence between the Bristol and Bergerac strains, none of the RFLDs detected in the class A hsp70 region was the result of Tc1 insertions.

RFLDs may be generated by any of several mechanisms (Kazazian et al, 1983). Point mutations may alter the recognition sequence of restriction enzymes, causing either the loss or generation of a restriction site. In this case, the RFLD appears as a change from a single restriction fragment to two smaller fragments whose total length is equal to the larger. A clear example of where this has occurred in the class A hsp70 region was the 6.6 kb Bristol EcoRI fragment shifted to 2.9 and 3.7 kb fragments in Bergerac. Many other examples of point mutations seemed to have occurred in this region; however the high number of RFLDs makes their identification more difficult. For example, the loss of the EcoRI site defining the 3' end of the 0.5 kb Bristol fragment would normally have resulted in the adjacent 3.0 kb fragment becoming 3.5 kb in Bergerac. In fact, two additional EcoRI fragments were generated 0.5 kb apart and in addition, the distal 3.0 kb EcoRI site was lost in the corresponding Bergerac fragment. This generated three new fragments of 2.3, 0.5 and 3.7 kb in the Bergerac strain. The fact that other restriction enzyme sites were conserved in this

region argues that the RFLDs observed are due to an unusually high number of point mutations.

RFLDs may also be generated by insertion or deletion events. In these instances, RFLDs appear as the generation of a new band either larger or smaller than the original fragment. A possible instance of where this type of event occurred in the class A hsp70 region was 5' to the hsp70 coding element. The loss of the EcoRI site between the Bristol 1.7 and 1.3 kb fragments would be expected to create a 3.0 kb fragment in Bergerac. In fact, a 3.2 kb fragment was observed, suggesting the possibility of a 200 bp insertion. Leigh Brown (1983) has shown that a high frequency of insertion/deletion events has occurred 5' to the coding elements at the 87A heat shock locus in natural populations of D. melanogaster. One class of insertion elements consists of 100 to 200 bp fragments. The second class is comprised of known transposable elements.

RFLDs may also be generated by the unequal crossover between tandomly repeated elements (Spritz, 1981) or by gene conversion events (Slighton et al, 1980). The detection of these types of RFLDs in the class A hsp70 region would require extensive DNA sequence analysis. In summary, the majority of RFLDs in this region appear to be the result of many point mutations with the possibility that one 200 bp insertion event has occurred 5' to the coding region.

Results markedly similar to those reported here have recently been shown to exist at the maize alcohol

dehydrogenase-1 (Adh-1) locus (Johns et al, 1983). Comparison of
20 kb of DNA flanking seven Adh-1 alleles showed that any two
alleles varied by between 5 and 12% in nucleotide sequence. As
shown here for the class A hsp70 region, no sequence difference
was detected in the Adh-1 coding region. The authors suggest
four hypotheses to explain the high level of sequence divergence
in the Adh-1 flanking regions. I will discuss these
possibilities with regards to the C. elegans class A hsp70
region.

Firstly, the authors suggest that it is possible that the
Adh-1 gene is located in a region of mobile DNA elements. In
yeast, it has been shown that transposable element mobilization
is associated with DNA rearrangements such as deletions,
translocations, duplications and inversions (Chaleft and Fink,
1980; Roeder and Fink, 1980). Since no copies of the transposon,
Tc1 (thought to be the only transposable element in C. elegans;
Emmons et al, 1983) exist in the C. elegans class a hsp70
region, the mobilization of transposable elements does not
explain the high sequence difference. In addition, the only
repetitive element in this region, that found in the Bristol 4.8
kb EcoRI fragment, does not appear to be highly mobile.

Secondly, Johns et al (1983) suggest that perhaps they have
inadvertently selected for the high sequence divergence by
selecting for study, seven Adh-1 alleles which exhibit unique
molecular and biochemical properties. In the case of the C.
elegans class A hsp70 gene, the Bristol and Bergerac strains

were not selected for study because of any particular phenotypic differences. They are the two common laboratory strains of C. elegans.

The third and fourth explanations suggested by Johns et al (1983) are that the domestication of maize involved major genomic rearrangements of noncoding DNA or that the high level of sequence divergence reflects a large amount of variation in the progenitors of maize. In both of these cases it would be expected that many other regions of the genome would equally show a high amount of sequence divergence. In the case of C. elegans, this is clearly not the case. No RFLDs are detected between the Bristol and Bergerac class B and C hsp70 regions, nor in a large number of other cloned regions (A. Rose, K. Beckenbach, D. Baillie, pers. comm.).

I have attempted to rule out several other mechanisms for the occurrence of this high sequence divergence. Isolation of the corresponding class A hsp70 gene from a Bergerac genomic library and hybridization of Bristol DNA flanking probes showed that the hsp70 coding element was surrounded by essentially the same DNA in both strains. This indicated that no localized DNA rearrangements have occurred. In addition, I have mapped the class A hsp70 gene to chromosome IV in both strains, indicating that no major DNA rearrangments have occurred (see Chapter IV).

Two recent studies on D. melanogaster have analysed the alcohol dehydrogenase (Kreitman 1983) and the 87A heat shock loci (Leigh Brown, 1983) for sequence variation in a large

number of individual chromosomes. Both have concluded that different regions of the genome may be subject to different mutation rates or selective constraints. We propose that the increased frequency of RFLDs around the C. elegans class A hsp70 gene is a reflection of its unique chromatin domain in gonadal tissue (Snutch and Baillie, 1984).

It is generally thought that eukaryotic DNA exists as a 200 to 300 A° chromatin fibre which is folded into looped domains (for review see Chambon, 1978). Evidence suggests that each looped domain, containing between 45 and 90 kb of DNA (Benyajati and Worcel, 1976; Pinon and Salts, 1977), may correspond to individual transcription units (Korgee, 1977; Lamb and Daneholt, 1979). It has also been shown by electron microscopic and biochemical analysis, that genes which are active or potentially active have altered properties suggesting that these chromatin domains exist in a decondensed, extended form (Weintraub and Groudine, 1976; Garel and Axel, 1976; Howard et al, 1981; Andersson et al, 1980; Foe, 1978). Transcription of the hsp70 gene has been shown to be heat shock inducible in a large number of species (see Chapter I). It is found in all adult tissues tested, including significantly, the gonad (Lewis et al, 1975; Zimmerman et al, 1983; Currie and White, 1983). It would therefore be expected that this particular domain is in an active or potentially active configuration at all times. We propose that maintenance of the active configuration or actual transcriptional activity in the germline tissue will be

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conducive to increased mutational events which can be passed onto the next generation.

If this proposal is correct, we would predict that the increased accumulation of mutational events would cease abruptly at the boundaries of the hsp70 domain. Screening for RFLDs in the region obtained by walking 5' and 3' from the original class A cloned region (Chapter II) showed that, as predicted, the occurrence of RFLDs falls off dramatically in the limits of this region (K. Beckenbach, unpublished results). The region of high sequence divergence is about 30 kb in length, with the class A hsp70 coding region located at about the midpoint of this distance.

If this proposal is correct, we would also expect that other wild type strains of C. elegans would also show a high amount of sequence divergence in their class A flanking regions. A limited comparison of the sequence divergence between Bristol, Bergerac and seven wild type strains showed that the Bristol strain and the wild type strains possessed identical restriction patterns for the class A hsp70 flanking regions. While this analysis was limited, it appears that the sequence divergence in the class A region is not as great in other strains as it is in Bergerac.

It may be, therefore, that the Bergerac genome possesses some unique features which have allowed the RFLDs to accumulate in this region or that the proposed theory (Snutch and Baillie, 1984) is correct and reflects some unusual and severe stress

that has occurred on the Bergerac strain. It will be interesting
to know whether other germline expressed genes show a similar
phenomenon.

E. Chapter IV Molecular Mapping of the Class A HSP70 Gene

I. Introduction

The relative simplicity of molecular cloning techniques has produced a wealth of information concerning the structure, expression and evolution of a large number of eukaryotic genes. Ideally, the dissection of eukaryotic genes is through a molecular genetic approach whereby mutations in a gene are correlated to the corresponding DNA sequences. In many cases, however, the physical analysis of cloned genes has proceeded independently of standard genetic analysis.

The main obstacle to this combined approach in organisms amendable to both molecular and genetic analysis is in correlating cloned genes to genetic loci and vice versa. In Drosophila, this obstacle has been overcome by the in situ hybridization of labelled nucleic acids to polytene chromosomes (Gall and Pardue, 1969). Elegant examples of the potential of a molecular genetic approach include the bithorax complex (Bender et al, 1983a) the xanthine dehydrogenase gene (Bender et al, 1983b; A Chovnick pers. comm.) and the dopa decarboxylase gene (Gilbert et al, 1984). Once the chromosomal location and orientation of a cloned probe is known it is possible to 'walk' into neighbouring regions, extending the molecular genetic analysis (Bender et al, 1983a,b).

In other genetically well defined organisms such as S. cerevisiae and C. elegans, in situ hybridization to chromosomes is impractical. In these instances, linked restriction fragment

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length differences have been used to correlate cloned genes to genetic markers (Petes and Bostein, 1977; Olson et al, 1979; Rose et al, 1982; Files et al, 1983).

In C. elegans, the assignment of cloned probes to specific chromosomal locations requires two factors. First, a cloned fragment of DNA which exhibits a RFLD between the Bristol and Bergerac strains. Second, it requires the construction of hybrid strains which are homozygous for either Bristol or Bergerac at a known loci while remaining randomly heterozygous at all other regions of the genome.

In this study, four separate experiments were performed in an attempt to localize the class A hsp70 gene on the C. elegans genetic map. This work was carried out for several reasons: (1) to determine whether the class A hsp70 gene is related to any previously defined gene; (2) to mark its chromosomal position in order that flanking genes could isolated and characterized; (3) to provide an initiation point for walking into genetically well defined regions; (4) to mark its chromosomal position in order that point mutations of the hsp70 class A gene be isolated and characterized.

II. Materials and Methods

Initially, in order to place the class A RFLDs to a
specific chromosome, the 3.0 kb class A probe was blotted to DNA
representative of individual Bristol chromosomes. These specific
chromosomal DNAs were constructed by a number of coworkers for
use in a previous study (Rose et al, 1982). Essentially, each
chromosome specific strain was constructed by crossing Bristol
males heterozygous for a recessive visible mutation to wild type
Bergerac hermaphrodites. The progeny from this cross were
selfcrossed and F2 progeny showing the visible mutation were
selected. These worms were homozygous for the Bristol chromosome
in the region of the visible mutation and may be homozygous or
heterozygous Bristol and Bergerac for all other regions of the
genome. To ensure the other regions of the genome were randomly
represented, about 200 individuals were grown up and they were
pooled prior to isolation of DNA (Rose et al, 1982). When
hybridized to EcoRI digested DNA of individual chromosomal
preparations the 3.0 kb probe showed both the Bristol and
Bergerac patterns in equal amounts for all chromosomes tested
except for chromosome IV. This indicated linkage of this cloned
region to chromosome IV.

In order to more precisely map the class A gene on
chromosome IV, three factor crosses were carried out between the
Bristol strain unc-22 unc-31 and the Bergerac strain, between
the Bristol strain unc-31 dpy-4 and the Bergerac strain and

between the Bristol strain unc-26 dpy-4 and the Bergerac strain.

Figure 4.1 outlines the general procedure followed in this type
of analysis. For example, in the case of the unc-26 BO(+)/
unc-26 BO(+) recombinants, each recombinant contains Bergerac
strain chromosome IV DNA to the right of the crossover point and
Bristol strain chromosome IV DNA to the left. If the RFLD is
located between the two markers, then some recombinants will
show the Bristol pattern while others will show the Bergerac
pattern. The relative frequency of each RFLD type reflects the
map distance between the markers and the RFLD. If all
recombinants show one or the other pattern, then the RFLD is
located either very close to the markers on the inside or beyond
them at an undetermined distance.

To position the cloned fragment relative to unc-22 (the
marker used to construct the chromosome IV specific DNA) the 3.0
kb probe was blotted to DNA from recombinants in the unc-22 to
unc-31 region. The construction of these strains has been
described (Rogalski, 1984). Briefly, from a cross between
unc-22(s7) unc-31(e169)/ + + Bristol (N2) males and Bergerac
(BO) hermaphrodites, F1 unc-22 unc-31/ BO(+) BO(+) individuals
were selected. These were allowed to self and BO(+) unc-31/
unc-22 unc-31 recombinants were selected using nicotine. From
these, six individual BO(+) unc-31/ BO(+) unc-31 recombinants
were selected and DNA was isolated from the recombinants which
were pooled just prior to DNA preparation. The 3.0 kb probe was
hybridized to this DNA, positioning it relative to unc-22 and

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Figure 4.1. The genetic protocol for constructing 3-factor
crosses. The example shown is for the unc-26 to dpy-4 region on
chromosome IV. See Materials and Methods for details. The DNA
from individual unc-26's and dpy-4's is digested with EcoRI and
separated by agarose gel electrophoresis. The DNA is transferred
to nitrocellulose and probed for the restriction fragment length
difference (RFLD). If the RFLD is between the two visible
markers, then some recombinants will show the Bergerac pattern
and others will show the Bristol pattern.

BRISTOL   BERGERAC

unc-26   dpy-4   BO (+)   BO (+)

P_0 ────────────────── ♂   ～～～～～～～～～～ ♀
       ──────────────────   ～～～～～～～～～～

         +   +   BO (+)   BO (+)

                              ↓

                    unc-26   dpy-4
F_1 ──────────────────   ♂ ⟲
                    ～～～～～～～～～～

                    BO (+)   BO (+)

                              ↓

F_2 Pick Unc-26 and Dpy-4 recombinants

       unc-26   BO (+)   BO (+)   dpy-4
      ─────～～～～～─────   and   ～～～～～─────────
      ──────────────────   ──────────────────
       unc-26   dpy-4   unc-26   dpy-4

       For each recombinant: progeny test five F_2's and select a single F_3 homozygote

                              ↓

         unc-26   BO (+)   BO (+)   dpy-4
        ───────～～～～～   ～～～～～─────────
F_3 ───────～～～～～   ～～～～～─────────
         unc-26   BO (+)   BO (+)   dpy-4

              ↓   ↓

         prepare DNA   prepare DNA
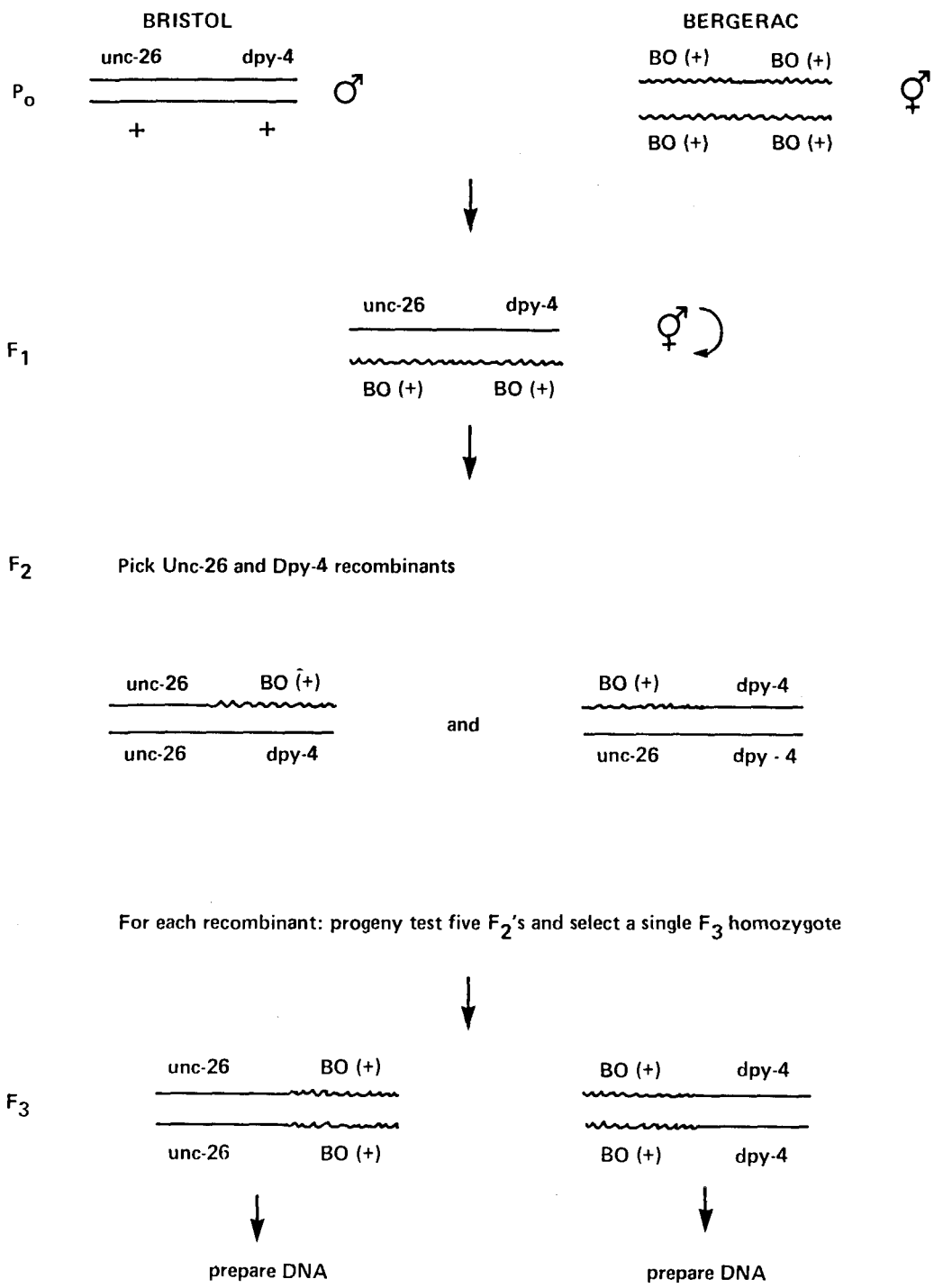
unc-31.

To position the class A 70 kd hsp gene region relative to
unc-31 and dpy4, 21 recombinant strains were constructed as
follows. Bergerac hermaphrodites were crossed to unc-31(e169)
dpy-4 (e1166)/ + + N2 males and virgin F1 hermaphrodites were
picked and selfcrossed. Those plates which showed no Dpy Unc's
in the F2 were discarded. Those which segregated Dpy Unc's were
screened and unc-31 BO(+)/ unc-31 dpy-4 recombinants were
selected. These were selfcrossed and six F3 worms having the
unc-31 phenotype were set up individually from each recombinant
(potential genotypes were unc-31 BO(+)/ unc-31 dpy-4 or
homozygous unc-31 BO(+)/ unc-31 BO(+)). Each plate was then
screened for Dpy Unc's and if present the plate was discarded.
One plate from each set of six which showed only unc-31 BO(+)/
unc-31 BO(+) was used as a stock for DNA preparations. Twenty
one individual recombinants were isolated. For each, 13 high
peptone plates were set up with individual worms and cultured at
18°C until most of the food was exhausted. DNA was prepared from
six of the plates while the remaining seven plates were pooled
together and the DNA isolated through CsCl. The class A 3.0 kb
probe was hybridized to DNA from ten individual recombinants and
to the pooled sample of all 21 recombinants.

To position the class A region relative to unc-26 and
dpy-4, sixty recombinant strains were constructed as follows.
Bergerac hermaphrodites were crossed to unc-26(e205)
dpy-4(e1166)/ + + N2 males and virgin hermaphrodites were picked

and selfcrossed. Plates which did not show any Dpy Unc's were
discarded. Those containing Dpy Unc's were screened and both
unc-26 BO(+)/ unc-26 dpy-4 and BO(+) dpy-4/ unc-26 dpy-4
recombinants were selected. These were selfcrossed and five
phenotypically Unc or Dpy were set up from each Unc or Dpy
recombinant, respectively. Any plate which segregated Dpy Unc's
were discarded. One plate from each set which segregated only
unc-26 BO(+)/ unc-26 BO(+) or BO(+) dpy-4/ BO(+) dpy-4 was
selected and one worm picked and used to establish a stock.
Thirty unc-26 BO(+)/ unc-26 BO(+) and thirty BO(+) dpy-4/ BO(+)
dpy-4 recombinants were isolated. For each, ten high peptone
plates were set up with individual worms and cultured at 18oC
until the food supply was almost exhausted. DNA preparations
were carried out on 54 of these recombinants. The DNA was
digested with EcoRI and probed with the class A 3.0 kb probe.
DNA samples were separated through 0.8% agarose gels and
transferred to two pieces of nitrocellulose using the
bidirectional transfer method (Smith and Summers, 1980). The
filters were probed with the 3.0 kb class A fragment that was
nick-translated to a specific activity of between 1 - 2 x 10^8
cpm/ug.

III. Results

Linkage of the Class A HSP70 Gene to Chromosome IV

Of the many RFLDs in the class A region, the 3.0 kb EcoRI

fragment was chosen for use as a probe. This fragment represents

single copy DNA (unlike the 6.6 kb or 4.8 kb fragments) and

shifts to three bands in Bergerac, two of which are smaller than

the 3.0 and therefore making an easy distinction between

complete and partial genomic digests. Figure 4.2 shows the

results of hybridization of the 3.0 kb probe to DNA homozygous

for individual Bristol chromosomes. The pattern showed both the

Bristol and Bergerac patterns in about equal amounts for

chromosomes I, III, V and the reciprocal translocation eT1. The

chromosome IV lane however showed that the Bristol pattern was

present in a far greater intensity than the Bergerac pattern.

Position of the Class A Gene Relative to unc-22 and unc-31

The hybridization pattern of the 3.0 kb class A probe to

the chromosome IV DNA suggested that this probe was not closely

linked to unc-22. The position of the class A region probe was

next positioned relative unc-22 and unc-31 on the right arm of

chromosome IV. From a cross between Bergerac hermaphrodites and

unc-22 unc-31/ ± ± N2 males, T. Rogalski isolated six

recombinant worms from the F3 generation which had the genotype

Figure 4.2 Hybridization of ^{32}P-labelled Bristol 3.0 kb class A probe to DNA homozygous for individual Bristol chromosomes. I, chromosome I; III, chromosome III; IV, chromosome IV; V, chromosome V; eT1, translocation between chromosome V and chromosome III (Rosenbluth and Baillie, 1981); unc 31, pooled DNA from six BO(+) unc-31/ BO(+) unc-31 recombinants. N2, Bristol; BO, Bergerac. Hybridization was moderate stringency. The filter was exposed at -80°C for 2 days to Kodak Blue Brand film and an intensifying screen.
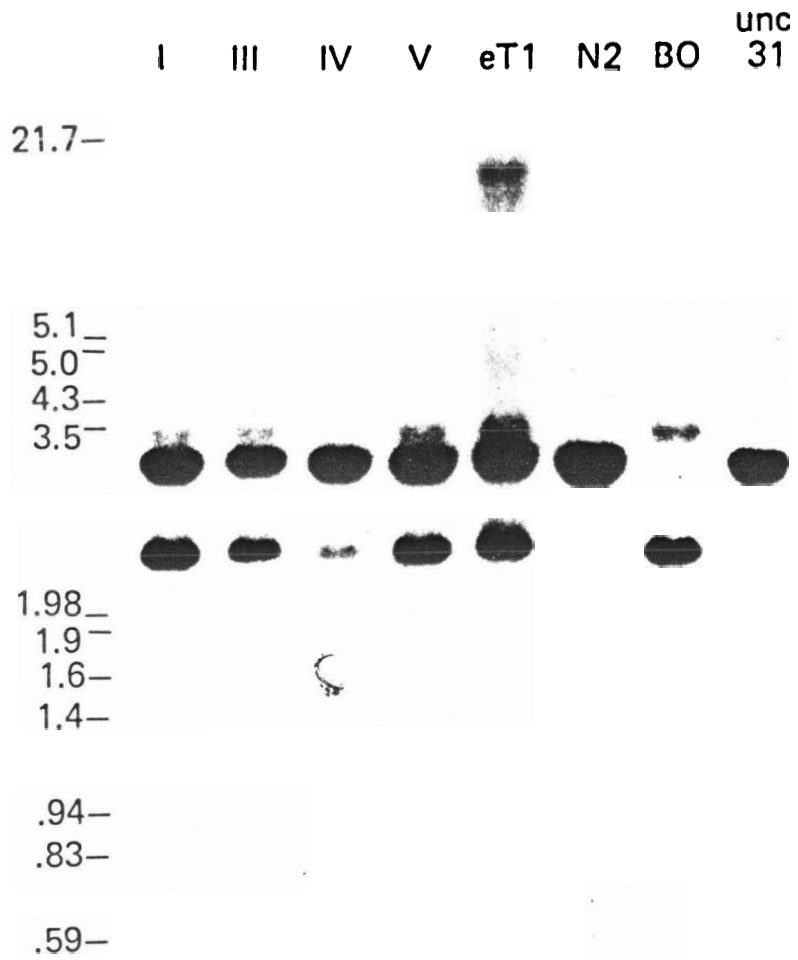
BO(+) unc-31/ BO(+) unc-31. Each of these strains contains
Bergerac chromosome IV to the left of the crossover point and
Bristol chromosome IV to the right. The worms should be random
Bergerac and Bristol for all other chromosomes. Each recombinant
was grown on plates separately and the worms pooled just prior
to the isolation of DNA. Figure 4.2 shows the results of
hybridization of the 3.0 kb probe to the pooled DNA (labelled
unc-31). Only the Bristol pattern occurred. This indicated that
the class A region was located either very close to the left of
unc-31 (less than 1/6 of the unc-22 to unc-31 distance) or to
the right.

Position of the Class A Gene Relative to unc-31 and dpy-4

The class A gene was further positioned on chromosome IV by
probing recombinants in the region unc-31 to dpy-4. Twenty one
recombinants with the genotype unc-31 BO(+)/ unc-31 BO(+) were
isolated, grown up on plates and DNA was prepared from 12
individual recombinants and a pooled sample from all 21
recombinants. Figure 4.3 shows the hybridization pattern of the
3.0 kb probe to eight individual recombinants and the pooled DNA
sample. All eight individual recombinants showed the Bergerac
pattern. In a separate experiment the DNA from 2 other
recombinants was probed and also showed the Bergerac pattern.
Hybridization to the pooled DNA sample from all 21 recombinants
showed mostly the Bergerac pattern, but a distinct 3.0 kb
fragment representing the Bristol pattern also occurred.

Figure 4.3. Hybridization of the Bristol class A 3.0 kb probe to unc-31 Bo(+)/unc-31 Bo(+) recombinants. Bristol, (N2); Bergerac, (Bo); pooled DNA from 21 recombinants (lane 1); individual recombinants, lanes 2-9. Note the small amount of Bristol pattern in the pooled sample. Hybridization stringency was moderate. The filter was exposed at -80°C for 5 days to Kodak Blue Brand film and an intensifying screen. Sizes are in kilobasepairs.
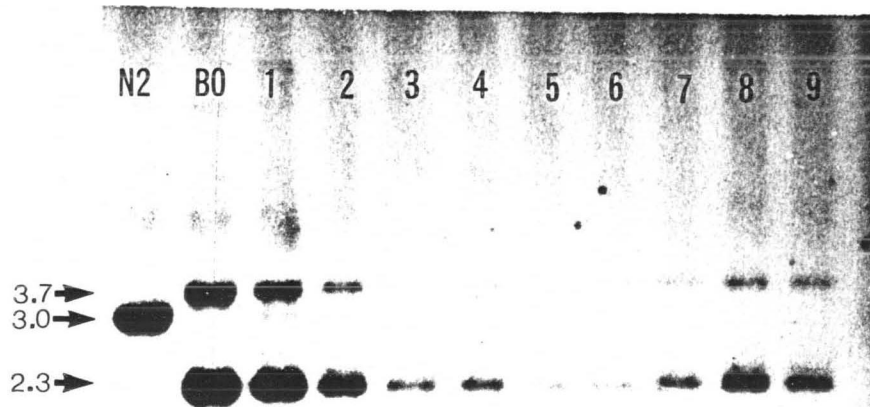
N2 BO 1 2 3 4 5 6 7 8 9

3.7→
3.0→
2.3→

141b

These results suggested that the class A gene region was far to the right of unc-31 and to the left of dpy-4 (see Discussion).

Position of the Class A Gene Relative to unc-26 and dpy-4

In order to map the class A gene to an accurate position near dpy-4, a large recombination experiment was set up. In the previous two mapping experiments only one set of possible recombinants was isolated. In this analysis both unc and dpy recombinants were selected. Thirty BO(+) dpy-4/ BO(+) dpy-4 and 24 unc-26 BO(+)/ unc-26 BO(+) recombinants were isolated and DNA was prepared from 10 plates of each. Figure 4.4 shows the results of hybridization of the 3.0 kb probe to 12 unc-26's and 12 dpy-4's. Similar probing of all 54 recombinants showed the following results. First, all 30 dpy-4's showed Bristol pattern. Second, 2 of 24 unc-26's showed the Bristol pattern, the other 22 having the Bergerac pattern. These results suggested that the class A gene was 1/27 of the unc-26 to dpy-4 map distance, nearest to dpy-4.

Figure 4.4. Hybridization of 3.0 Bristol class A probe to unc-26 Bo(+)/unc-26 Bo(+) and Bc(+) dpy-4/ Bo(+) dpy-4 recombinants. A) Twelve individual dpy-4 recombinants (lanes 1-12) All show the Bristol (N2) pattern. B) Twelve individual unc-26 recombinants (lanes 1-12) Two of twelve show the Bristol pattern. Hybridization was at high stringency. The filters were exposed at -80°C for 2 days to Kodak XAR-5 brand film and an intensifying screen. Sizes are in kilobasepairs.
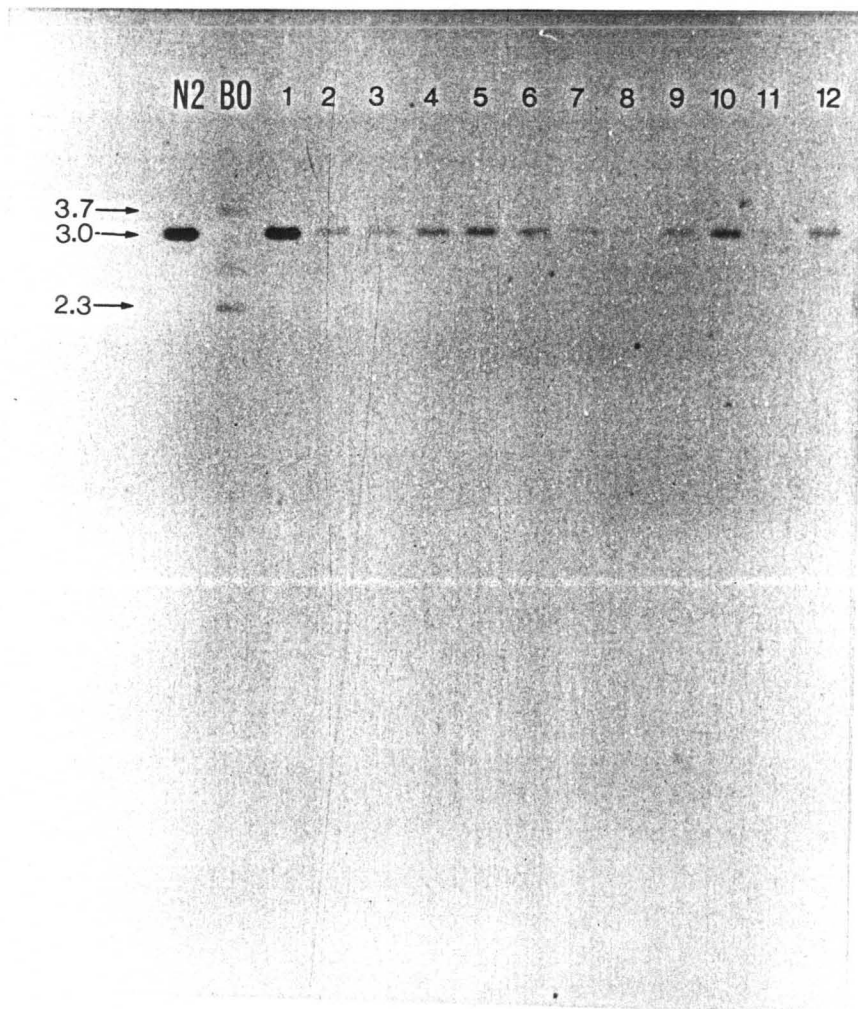
A

B

143c
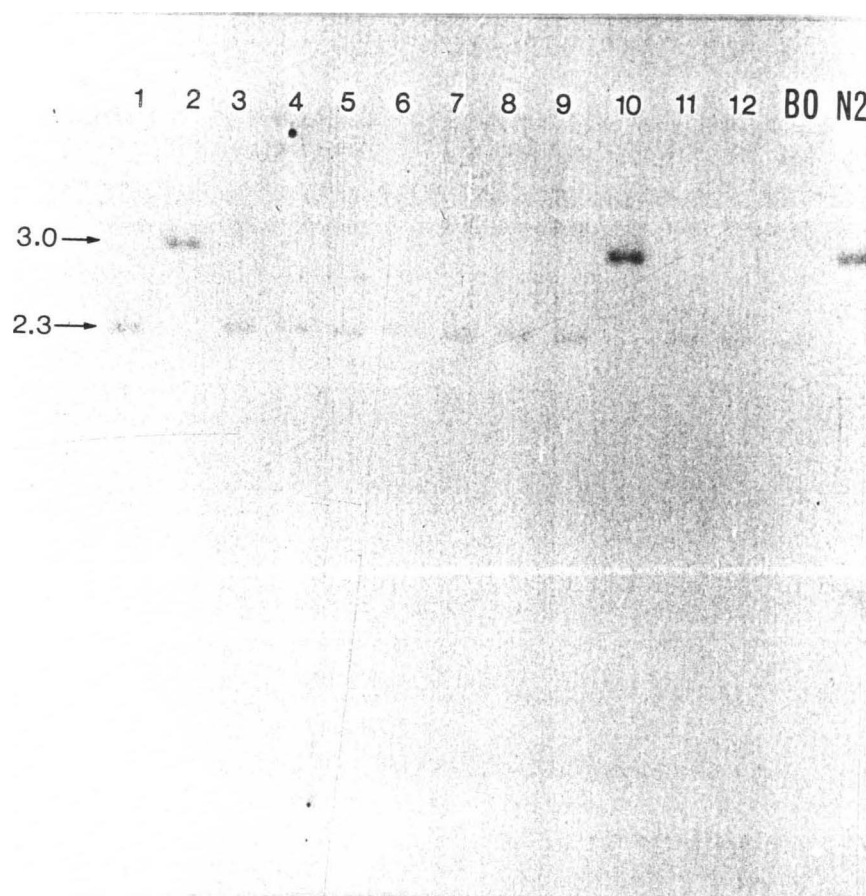
IV. Discussion

The results clearly show linkage of the class A hsp70 gene to chromosome IV. All of the chromosomes tested except for chromosome IV showed both the Bristol and Bergerac patterns (the X was not tested), indicating random assortment. The small amount of Bergerac fragment present in the Bristol chromosome IV DNA preparation suggested that the gene was not closely linked to the genetic marker used for the selection of chromosome IV DNA (unc-22). Using a similar approach, no recombination away from the marker was seen for the actin gene cluster which maps about two map units from dpy-11 (Files et al, 1983).

To test whether the class A gene was to the right or left of unc-22, a three factor cross between Bristol unc-22 unc-31 and the Bergerac strain was carried out. In this case since all six BO(±) unc-31/ BO(±) unc-31 recombinants showed the Bristol pattern, the 3.0 kb probe is either within approximately 0.18 map units (1/6 of the unc-22 to unc-31 map distance) to the left of unc-31 or to the right of it.

The 3.0 kb probe was further positioned relative unc-31 and dpy-4. The DNA from ten individual unc-31 BO(+)/ unc-31 BO(+) recombinants and a pooled DNA sample from 21 recombinants was probed with the class A 3.0 kb probe. All ten individuals showed the Bergerac pattern while the pooled DNA showed mostly the Bergerac along with a small amount of the Bristol pattern. This indicated that at least one and probably not more than two of

the unc-31 recombinants which were not analysed individually had the Bristol pattern. Two conclusions can be made from these data: (1) that the class A gene is between unc-31 and dpy-4 (2) the gene is within about 2/21 of the unc-31 dpy-4 distance, closest to dpy-4 (about 0.4 map units to the left of dpy-4).

In the final mapping experiment between unc-26 and dpy-4, both the unc-26 BO(+)/ unc-26 BO(+) and BO(+) dpy-4/ BO(+) dpy-4 recombinants were isolated and analysed. The results showed that while all 30 dpy-4 recombinants had the Bristol pattern, two of 24 of the unc-26 recombinants had the Bristol pattern. From these results, it is apparent that the class A gene maps quite close to dpy-4. The distance of the class A gene from dpy-4 can be estimated to be about 0.1 map units (95% confidence limits; 0.01 to 0.4). Knowing that the C. elegans genetic map consists of about 300 map units and that the haploid genome size is 8 x 10^7 b.p., the number of base pairs per map unit can be estimated to be about 267,000. This would place the class A hsp70 gene about 27,000 base pairs to the left of dpy-4. If this is correct, then we may have already cloned the dpy-4 locus in the 60 kb of isolated DNA in this region. Northern blot analysis did not detect any transcripts other than those of the class A gene in this region, however, low abundance transcript would not have been detected in this analysis.

It should be noted that there is an alternate explanation for the finding that two of the 24 unc-26 recombinants possess the Bristol pattern. It is possible that the class A hsp70 gene

is to the far right of dpy-4 and a second recombination event produced the two Bristol patterns. For such an event to have occurred with a frequency of 2 in 24, the class A gene would be approximately 29 map units to the right of dpy-4 (95% confidence limits; 4.0 to 92.0). This is unlikely since dpy-4 is the most distal marker found on chromosome IV. At most the chromosome probably extends only two to three map units past dpy-4.

The use of RFLDs in mapping cloned genes has become very valuable in correlating genetic and molecular data in C. elegans. In addition to the previously mentioned actin genes, the 5S rDNA cluster has been mapped on chromosome V near unc-42 (D. Nelson, pers. comm.) and two yolk protein genes, YP3 and YP4, have been localized to the X chromosome (T. Blumenthal, pers. comm.). In addition, many copies of the transposon Tc1 have been mapped to various chromosomal positions (A. Rose; K. Beckenbach; D. Moerman, pers. comm.).

Knowing the chromosomal location of the class A hsp70 gene greatly facilitates its potential for genetic analysis. The region near dpy-4 can be screened for lethal, visible and conditional mutations in an attempt to select mutants in the hsp70 gene. If successful, this type of analysis would help identify both the biochemical and developmental functions of the class A gene product.

Figure 4.5. The region around unc-22 IV in C. elegans. dpy-4 in the most distal marker on the right chromosome IV. The relative positions of nonessential genes and several deficiencies and duplications is shown. The position of the class A hsp70 gene (with 95% confidence limits) is shown.

1 map unit

unc-43   unc-22   let-68   unc-31   unc-30   unc-26   lev-1   dpy-4

class A
hsp 70

sDf2

sDf21

sDf22

sDf24

sDf23

sDp7

sDp6

147b
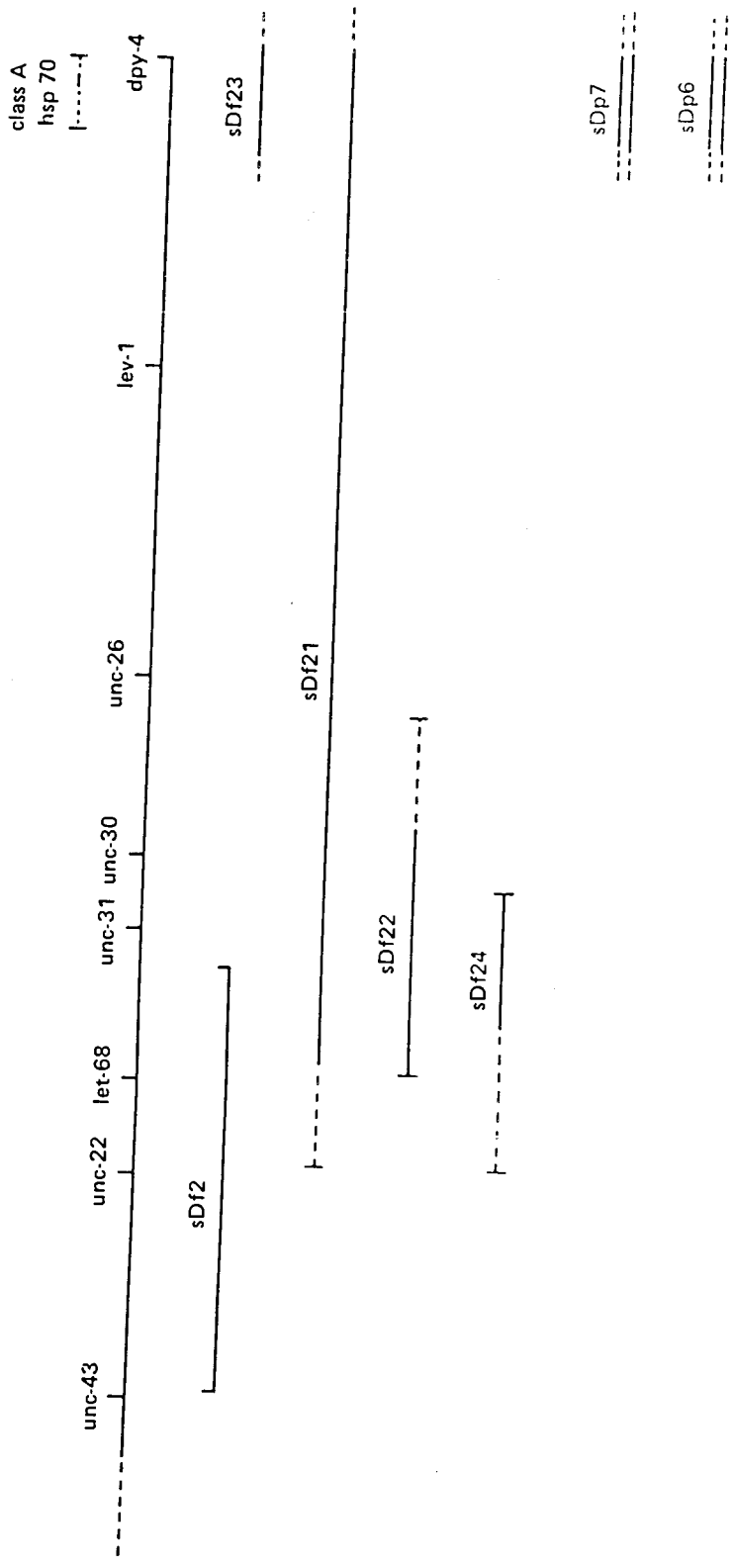
Genes coding for seven of the _Drosophila melanogaster_ hsps have been mapped cytologically by hybridization of cloned probes to polytene chromosomes (Spradling _et al_, 1977; Henikoff and Meselson, 1977; Artavanis-Tsakonas _et al_, 1979; Holmgren _et al_, 1979; Corces _et al_, 1980; Craig and McCarthy, 1980). Attempts to isolate mutations in the _Drosophila_ hsp genes have not succeeded. Gausz _et al_ (1981) could not detect visible, lethal or temperature sensitive mutations in the hsp70 gene. Similarly, using three different mutagens, Mohler and Pardue (1984) could not detect any visible or lethal mutations affecting the 93D heat shock locus. Several explanations for the failure to detect mutations at these loci include: (1) that the hsp genes are nonessential (2) that other loci can compensate for the loss of the hsp gene product (3) the screening experiments were not carried out under conditions in which the gene products are required.

The conservation of a 93D-like locus in distantly related _Drosophila_ species and the conservation of hsp70 from prokaryotes to higher eukaryotes argues against a nonessential role. The explanation for these results may involve a combination of the last two explanations. It is possible that mutations in the hsp70 gene can be compensated for by other loci. For example, by the increased synthesis of the hsp68 gene product or perhaps by a slight increase in either the synthesis or stability of the hsp70 cognate gene products. Alternatively, Loomis and Wheeler (1982) have isolated several _Dictyostelium_

mutants which have lost the ability to show a thermotolerance effect. These mutants do not synthesize the lower molecular weight hsps. It seems, therefore, that under the right selection conditions, mutations which affect hsps synthesis can be isolated.

The region on chromosome IV defined by the deficiency sDf2 is the most extensive genetically characterized region in C. elegans (Moerman, 1980; Moerman and Baillie, 1979; Rogalski, 1984; Rogalski et al, 1982). The 1.5 map unit region contains an estimated 34 genes, including the muscle gene, unc-22. About 70 alleles and several internal deletions of unc-22 have been isolated, making this gene an excellent candidate for molecular genetic dissection (Rogalski, 1984). While it is possible to walk the estimated 1.5 x 10⁶ bp from the cloned heat shock region to unc-22 by picking up a series of overlapping clones, a quicker method is to 'jump' across large distances by the use of deficiences or inversions (Bender et al, 1983a,b). Along this line, a number of deletions in the unc-31 to dpy-4 region have been isolated and coworkers are continuing to isolate more (Figure 4.4; G. Wild, D. Baillie, L. Donati pers. comm.). Once the right hand break point of the deletion is found, a total digest EcoRI library can be constructed into an insertion vector such as lambda Gt10. This library would be screened for the recombinant fragment containing the deletion break point and the walk could be continued into the unc-22 region. The orientation of the walk could be monitored by blotting cloned fragments

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against any of the deficiency DNAs whose break points flank unc-22.

Another benefit of determining the location of the class A hsp70 gene is that it provides a chromosomally positioned cloned marker. J. Sulston and coworkers at Cambridge are attempting to physically map the entire C. elegans genome using randomly isolated cosmids. In order to correlate the vast amount of restriction map data to the genetic map, specifically mapped cloned markers are required. Towards this end, the class A hsp70 gene will provide a definitive marker on the right arm of chromosome IV.

F. Summary and Conclusions

The major aim of this thesis was to demonstrate the possibility of utilizing C. elegans as a model system for the study of the heat shock response in a higher eukaryote. My results indicate that C. elegans is a suitable organism in which to analyse many aspects of the heat shock response, including, hsp gene organization, regulation and evolution.

Culturing of C. elegans of temperatures above 29°C resulted in the increased synthesis of at least nine polypeptides. The major inducible hsp has an apparent molecular weight of 70 kilodaltons. A similar hsp is found in all organisms, including prokaryotes. The conservation of hsp70 throughout evolution suggests that the stress related function of this polypeptide is fundamental to the structure and/or regulation of all cells.

The isolation of sequences homologous to the Drosophila hsp70 gene from a C. elegans genomic library showed that C. elegans possesses a small multigene family of hsp70 related sequences. The Northern blot analysis of hsp70 gene expression conducted in this thesis work taken together with the study of hsp16 gene expression carried out by Russnak et al (1983), suggests that the heat shock response is regulated, at least in part, at the level of transcription. Transcripts of the heat shock inducible class A, B4 2.9 and B9 1.7 genes were also detected in worms cultured at 20°C. It is possible that this result is due to crossreaction of these probes with heat shock

cognate transcripts or that these transcripts are synthesized as a result of the stresses involved in collecting worms off agar plates. It is also possible that these gene products are required during nematode development or that the transcripts are not translated at control temperatures. An exact quantitative and qualitative determination of the extent to which each of these genes is transcribed under control and heat shock conditions will require methods whereby no crossreaction between family members can occur (for example, cDNA primer extension). In addition, further analysis of hsp induction could be carried out utilizing inhibitors of RNA and protein synthesis (for example, Lindquist, 1980).

At least one family member, the class C gene, is not transcribed in response to heat shock and appears to be developmentally regulated. Taken together with the finding that some hsp70 family members of yeast and Drosophila are also expressed during development (Ingolia et al, 1982; Ingolia and Craig, 1982; Craig et al, 1983), these results emphasize the biological importance of maintaining distinct types of hsp70 genes.

The finding that the class B (and possibly the B5 2.6 EcoRI fragment) hsp70 gene is not conserved between C. elegans and C. briggsae is very interesting. There are several possibilities for this finding, including that the class B gene is a pseudogene. The class B gene may be a recent additon to the C. elegans genome through a gene conversion or duplication event.

Alternatively, this gene may be quite ancient and has recently been lost from the C. briggsae genome. A distinction between these possibilities will await the nucleotide sequencing of this gene.

The fact that most C. elegans sequences do not hybridize to the C. briggsae genome under moderate stringency conditions is also interesting. While these two species have been referred to as twin species (Dougherty, 1953), little is known about their true evolutionary separation. It is possible that either these two species diverged tens of millions of years ago or that the rate of nucleotide substitution is higher in nematodes than in other organisms (Emmons et al, 1979).

The discovery of the high level of nucleotide sequence difference between the Bristol and Bergerac strains of C. elegans in regions flanking the class A gene is interesting. The actual extent of this divergence should be determined by DNA sequencing. The theory proposed for this unusual occurrence (Snutch and Baillie, 1984) should be tested by determining whether the class A gene is indeed inducible in germline tissue and by comparing the sequence variation between the Bristol and Bergerac genomes for other heat shock genes.

Several regions of the C. elegans genome have been extensively characterized genetically (Rose, 1979; Moerman, 1979; Rogalski, 1984). The determination of the location of the class A hsp70 gene on chromosome IV near dpy-4, should permit the genetic analysis of this region.

I. Proposals for Further Research

The experiments carried out in this thesis provide the
foundation for futher molecular genetic analysis of the C.
elegans heat shock response. The following experiments are
suggested as a means of furthering this analysis.

1. Sequence the 5' noncoding regions of the class A, B and
C genes, searching for sequences which resemble the Drosophila
consensus heat shock promoter.

2. Sequence the entire coding regions of the class A, B and
C clones in order to determine both their relationships with
each other and with the yeast and Drosophila hsp70 gene
families.

3. Determine the transcription initiation points of the
transcribed hsp70 genes. This could be accomplished by using the
techniques of S-1 mapping or cDNA primer extension.

4. Determine the individual expression patterns of the
hsp70 related genes under both heat shock and control
conditions. This could be accomplished using gene specific
probes for Northern blots or by cDNA primer extension.

5. Isolate overlapping clones in the class B and C regions
and 'walk' in both directions. As the walk progresses, screen
the Bergerac strain for RFLDs and then use them to place these

genes on the C. elegans genetic map.

6. Continue walking in the 5' and 3' directions in the class A region. Search for other genes using the technique of screening the C. briggsae genome for homologous regions and then using these fragments as probes for Northern blots and for screening cDNA libraries.

7. Screen the Charon 4 library for lambda clones representing the B9 1.7, B5 2.6 and B4 2.9 kb EcoRI fragments and any remaining members of the C. elegans hsp70 gene family. Characterize the purified genes as for the class A, B and C clones.

8. Use deficiencies in the dpy-4 region as a means of cloning the genetically well defined unc-22 region.

9. Isolate lethal, sterile and conditional mutations flanking dpy-4 as a means of identifying other genes in this region. Correlate the genetic map with the cloned DNA restriction map.

10. Sequence the class A regions where the strain specific high level of sequence divergence drops off significantly.

11. Determine the tissue and developmental expression of patterns of all hsp70 family members.

12. Analyse the chromatin structure of the hsp70 gene family using DNAse-1.

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