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ANOXIA INDUCES CHANGES IN TRANSLATABLE mRNA POPULATIONS IN
Trachemys scripta elegans:
A POSSIBLE ADAPTIVE STRATEGY FOR ANOXIA TOLERANCE

by

DONNA NABEHA DOUGLAS

B.Sc. (Hons.) Carleton University, 1991

*A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfillment of the
requirements for the degree of
Masters of Science
in the department of
Chemistry*

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ANOXIA INDUCES CHANGES IN TRANSLATABLE mRNA POPULATIONS IN
Trachemys scripta elegans:
A POSSIBLE ADAPTIVE STRATEGY FOR ANOXIA TOLERANCE

Submitted by Donna N. Douglas (B.Sc.)

in partial fulfillment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chair, Department of Chemistry

ABSTRACT

A crucial first step in examining the anoxia-induced changes in mRNA in tissues from *Trachemys scripta elegans* is the extraction of total RNA. This study assesses the efficacy and reliability of a method of total RNA isolation compared to the guanidine HCl/CsCl ultracentrifugation of Chirgwin *et al.* (1979). The method described by this thesis isolates pure undegraded RNA of comparable yield and purity to that isolated by the guanidine HCl/CsCl ultracentrifugation procedure, only in considerably less time and without the use of an ultracentrifuge. This method was used to isolate total RNA from various tissues from 16 h anoxic turtles and their control tissue counterparts. Polyadenylated RNA was prepared from the total RNA using affinity chromatography on oligo(dT) cellulose. These RNAs were then translated *in vitro* in a wheat germ lysate system and the synthesized labeled polypeptides were separated by SDS-PAGE. The transition into anoxia induced both quantitative and qualitative changes in the *in vitro* translatable mRNA populations. An approximate 27-kDa primary translation product was observed in liver and heart; a novel 20-kDa and higher levels than control of an 80-kDa translation product was observed in liver; a novel 38-kDa transcript was observed in red muscle; and a novel 33-kDa transcript was observed in kidney. The results presented here indicate that anoxic exposure in *T. s. elegans* triggers a rapid cellular response by modifying translatable mRNA populations, and this response may be key adaptations that support natural anoxia tolerance in this species.

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INTRODUCTION

Metabolic Depression: A Survival Strategy

Metabolic depression is commonly seen in nature as a characteristic feature marking the dormant life stages of many organisms (e.g., eggs, cysts, seeds, and spores) (Hochachka and Guppy 1987; Busa and Nuccitelli, 1984). This capacity is also used facultatively by many species in order to survive periods of environmental stress. Many species must enter into a hypometabolic, or even an ametabolic state in order to escape the biological hazards associated with suboptimal physiological conditions such as low oxygen tension, low temperature and low water availability (Storey and Storey, 1990; Hochachka and Guppy, 1987). As such, metabolic rate depression is an essential mechanism in facultative anaerobiosis, anhydrobiosis, hibernation, estivation, torpor, and diapause.

By lowering metabolic rate an organism can maximize the efficient use of fuels and energy reserves when confronted with an environmental stress. For example, hibernating ground squirrels utilize only 12 % of the energy that they would normally expend to remain euthermic throughout the winter season (Wang, 1978). Respiratory rate in diapausing insects is one-quarter to one-third that of nondiapausing individuals at the same life stage, while a 20-fold metabolic rate reduction has been measured during winter dormancy of adult insects (Wigglesworth, 1972). In response to dehydration, *Artemia* cysts reduce metabolic rate to the point where no metabolic activity is detectable below a water content of 0.1 g/g cysts (Clegg, 1981).

Mechanisms of Metabolic Rate Depression

There are many triggers that can stimulate escape to a hypometabolic state. However, the consequences for cellular metabolism are quite similar and are characterized by reductions in various physical, physiological and nonessential cellular functions (e.g. feeding, movement, heart beat, breathing, or ventilation rate, anabolism and growth) and the re-establishment of essential metabolic functions into a new balance. The regulatory controls used for metabolic depression must meet certain criteria by being: (1) rapidly initiated to minimize metabolic damage; (2) easily reversed; (3) applicable to many cellular processes; (4) readily coordinated to maintain the balance between essential processes, and (5) responsive to external stimuli.

The inability of nontolerant species to survive when extreme physiological stress is imposed on them reinforces the necessity for such coordinated regulation (Siesjo, 1981; Hansen, 1985; Hochachka, 1986). Thus when mammalian organs are exposed to hypothermia or ischaemia, the balance between ATP-producing, ATP-utilizing, and passive processes in the cell is disrupted. The most detrimental consequence of the inability to maintain or re-establish this balance appears to be the disruption of ion homeostasis; membrane depolarization is caused by an imbalance between energy-driven ion pumps and ion movement through ion-specific channels. When imbalances in Na^+ and K^+ influx-efflux lead to a critical level of membrane depolarization, voltage-dependent Ca^{2+} channels are activated resulting in an uncontrolled influx of Ca^{2+} into the cell and a variety of Ca^{2+} -dependent disruptions ensue. In mammalian brain, irreversible damage occurs after only a few minutes of ischaemia.

Metabolic Depression and Anoxia Tolerance

The well-developed anoxia tolerance of many species is based upon metabolic rate depression in the anoxic state. For instance, freshwater turtles have a remarkable ability to withstand prolonged periods of anoxia while diving or hibernating underwater. They spend most of their time under water and, except for periods of basking, only occasionally come to the surface to breathe (Belkin, 1964). Species of the genera *Trachemys* and *Chrysemys* can survive submergence for up to 2 weeks at elevated temperatures and as long as 3 months at 3°C (Robin *et al.*, 1964; Ultsch and Jackson, 1982). These species have a minor capacity to extract dissolved oxygen from water, but their primary survival strategy involves well-developed mechanisms for enduring hypoxia and anoxia. For example, adjustments in the cardiovascular system are made (Steggerda and Essex, 1957; Belkin, 1964; Millen *et al.*, 1964; White and Ross, 1966) which preferentially route blood to the brain, heart, and sites of aquatic oxygen uptake via extrapulmonary routes (Root, 1949; Dunson, 1960; Girgis, 1961; Belkin, 1968; Gatten, 1980). An apparently more important factor in the survival of turtles encountering a limited oxygen supply is due to extremely well developed metabolic arrest mechanisms (Belkin, 1986; Brooks and Storey, 1989; Ultsch, 1985; Ultsch and Jackson, 1982). Diving turtles combat low oxygen availability by lowering metabolic rate to only 15 % of the standard metabolic rate in air (Jackson, 1968). Once body O₂ supplies are depleted, turtles exhibit a remarkable ability to survive for many additional hours utilizing anaerobic glycolysis to generate ATP (Belkin, 1963; Robin *et al.*, 1964; Jackson, 1968; Cark and Miller, 1973). The main metabolic endproduct of this anaerobic metabolism in turtles is lactate, although some succinate, alanine, and pyruvate also accumulate (Arillo and DeGiuli, 1970; Penney, 1974; Hochachka and Storey, 1975; Hochachka *et al.*, 1975). When a turtle is submerged,

there is an initial respiratory acidosis (CO₂ buildup) that develops as remaining oxygen is used up and this is combined, as anoxia is prolonged, with a metabolic acidosis due to the accumulation of lactate. Blood lactic acid concentrations in turtles typically reach 20 to 50 mM in experimental dives of long duration (Robin *et al.*, 1964; Jackson and Silverblatt, 1974; Penney, 1974), and the combined effects of respiratory and metabolic acidosis may result in a decrease in blood pH values as low to as 6.7. The ability of turtles encountering a limited oxygen supply to utilize anaerobic metabolism to produce energy is the result of several factors including: 1) large glycogen stores (Dau *et al.*, 1967; Beall and Privitera, 1973); 2) special properties of brain, heart and muscle enzymes involved in the generation of ATP via anaerobic pathways (Miller and Hale, 1968; Altman and Robin, 1969; Simon and Robin, 1970; Penney and Shemerdiak, 1973; Lobes and Penney, 1974; Storey and Hochachka, 1974a,b; Lutz *et al.*, 1978; Simon *et al.*, 1979); 3) tolerance of a buildup of metabolic acids and of a decline in blood pH (Johlin and Moreland, 1933; Bellamy and Peterson, 1968; Clark and Miller, 1973; Jackson and Silverblatt, 1974); 4) a large bicarbonate buffering capacity (Jackson, 1969); 5) and the ability to excrete carbon dioxide into the water via extrapulmonary routes (Jackson *et al.*, 1976).

The adaptive strategies involved in anoxia tolerance include: (i) large tissue stores of fermentable fuel, (ii) a high tolerance of metabolic acidosis and compensatory ion changes that buffer and retard the development of acidosis, and (iii) metabolic rate depression to a level only 10 to 20 % of the aerobic resting rate. These combined permit homeostasis to be maintained over the long term by anaerobic glycolytic ATP production alone (Clark and Miller, 1973; Herbert and Jackson, 1985; Jackson, 1968; Penny, 1974; Robin *et al.*, 1964). In red-eared sliders, the mechanisms that mediate glycolytic rate depression (a part of the overall metabolic depression) are particularly

well characterized and include: (i) fructose-2,6-bisphosphate regulation of phosphofructokinase to control the anabolic use of carbohydrate reserves (Brooks and Storey, 1989), (ii) reversible binding of enzymes to the subcellular particulate fraction (Duncan and Storey, 1992), and (iii) covalent modification of regulatory enzymes, via reversible protein phosphorylation (Brooks and Storey, 1988; Brooks and Storey, 1989; Brooks and Storey, 1990).

The mechanisms that regulate overall metabolic suppression are not as well characterized as those of glycolytic rate depression. Recent discoveries in some related fields have suggested possible cell-specific mechanisms that may participate in metabolic reorganization during anoxia in turtles. In particular, the production of stress related proteins (srps) may be linked to anoxia tolerance in the turtle.

The Stress Proteins: A Historical Perspective

The response of organisms or cells to rapidly elevated temperatures often involves the coordinated transcription and preferential synthesis of a small suite of proteins designated as the "classical" heat shock proteins (hsps) (Ashburner and Bonner, 1979; Barnett *et al.*, 1980; Key *et al.*, 1981) of molecular sizes 104, 90, 70, 60 and 20 KDa (Sanchez and Lindquist, 1990; Welch, 1990; Hickey *et al.*, 1989; Moran *et al.*, 1982). The heat shock response was first noted by Ritossa (1962) who observed the appearance of new puffing patterns in the giant salivary gland chromosomes of the fruit fly *Drosophila busckii* in response to severe heat shock. Subsequently, it was shown (Tissieres *et al.*, 1974) that the appearance of these puffs coincided with the synthesis of a small number of proteins, the heat shock proteins.

Although first noted in *Drosophila*, the heat shock response is a universal one and has been found in all organisms surveyed, including man, plants, yeast and bacteria (Schlesinger *et al.*, 1982). In these model organisms, four major heat shock protein

(hsp) families are prominent and have been frequently referred to as hsp90, hsp70, hsp60 and the low molecular weight (lmw) heat shock proteins. The genomes encoding heat shock proteins are among the most highly conserved with representatives from distant prokaryotic and eukaryotic species having at least 50 % homology (Bardwell and Craig, 1984; Hunt and Morimoto, 1985). The ubiquitous nature and evolutionary conservation of heat shock proteins, from bacteria to man, allude to an essential role in cellular metabolism. This idea is reinforced by the finding that similar induction of these proteins is seen when cells are stressed by other suboptimal physiological conditions such as low oxygen tension, heavy metal exposure, or electron transport uncoupling compounds (Schlesinger *et al.*, 1982). Subsequently, another group of proteins, the glucose regulated proteins (grps), were found to be homologous to the heat shock proteins (Welch, 1990; Hightower, 1991).

Although the colloquialism "heat shock" has rather specific and restrictive connotations, it does provide a good historical reference. As the diversity of conditions that result in the elevated synthesis of heat shock proteins has expanded the paradigm has become more generally known as the "stress response" and the proteins as "stress-related proteins (srps)."

The Protective Role of Heat Shock Proteins

Initial studies in this area concentrated on the role of heat shock proteins in thermotolerance. In these early experiments, hsp synthesis was pre-induced by exposing whole organisms or cells to elevated temperatures. Such pre-treatment conferred thermotolerance when these whole organisms or cells were exposed to extreme temperatures. For example, it was shown that such pre-treatment protected embryos from developmental abnormalities which would normally result from heat

treatment (Mitchell *et al.*, 1979). Subsequently in experiments involving a wide variety of cell types and species, it was demonstrated that cells in which hsp synthesis had been pre-induced also survived extreme temperatures better than the control cells which had not previously been exposed to elevated temperatures (Roccheri *et al.*, 1981; Li and Werb, 1982; Tobe *et al.*, 1984). Moreover, the kinetics with which such cells acquired thermotolerance, at mildly elevated temperatures, were identical to the kinetics of hsp accumulation (Subjeck *et al.*, 1982); while the decay in acquired thermotolerance when cells were returned to normal temperatures for various periods prior to application of extreme temperature was identical to the decay of hsp levels under these conditions (Landry *et al.*, 1982). In addition, cells in which the synthesis of hsps is blocked using the protein synthesis inhibitor, cycloheximide, fail to acquire thermotolerance, indicating that this effect requires hsp synthesis (McAlister and Finkelstein, 1980). Most importantly, the increased thermotolerance produced in these experiments, by prior heat shock, is also accompanied by increase tolerance of other stressful treatments. Prior exposure to other physiological stresses results not only in hsp synthesis but also confers thermotolerance exactly as if hsp synthesis had been induced by elevated temperatures (Li, 1983; Velazquez and Lindquist, 1984).

Although some studies have failed to find a correlation between hsp levels and enhanced stress resistance (see for example Watson *et al.*, 1984; Caviccholi and Watson, 1986), most experiments indicate that increased synthesis of the hsps, whether produced by heat or other stresses, ensures increased tolerance during episodes of stress, which if left unchecked, would otherwise lead to irreversible damage and ultimately cell death. Interestingly, one exception to this correlation actually reinforces the rule that hsp synthesis protects cells from stress. Thus, when cells are treated with amino acid analogues, they synthesize the hsps but do not exhibit enhanced

thermotolerance (Li and Laszlo, 1985). However, since amino acid analogues were incorporated into the newly synthesized proteins, the hsp's made under these conditions were abnormal and thus unable to fulfil their protective function (De Domenico *et al.*, 1982).

The Role of Heat Shock Proteins in Normal Cells

Interestingly, the role of hsp's does not appear to be confined solely to cells under stress since some members of the hsp90, hsp70, hsp60 and lmw families are synthesized under normal conditions (Craig *et al.*, 1983). These constitutively synthesized stress proteins, often referred to as cognates (hsc), play a pivotal role in regulating protein homeostasis by acting as so called chaperones. Molecular chaperones are a ubiquitous group of proteins which direct the folding and assembly of other cellular proteins (Rothman, 1989; Welch, 1991; Ellis, 1990; Martin *et al.*, 1991; Braakman *et al.*, 1992; Gething and Sambrook, 1992; Hartl *et al.*, 1992; Seckler and Jaenicke, 1992). Molecular chaperones are also involved in regulating the kinetic partitioning between protein folding, translocation reactions and protein aggregation. Several classes of chaperones are known, defined primarily by their molecular weight and sequence.

The majority of studies have focused on the eukaryotic 70 kDa protein heat shock genes, which encode a large multigene family of proteins including: 1) the constitutively expressed and primarily cytoplasmic hsc70 (p72), 2) mitochondrial p75, 3) lumen-localized and glucose-responsive grp78/BiP, 4) the inducible hsp72, and 5) the major constitutive and inducible hsp70 (Cheng *et al.*, 1985; Craig *et al.* 1987; Sorger and Pelham, 1987; Voellmy *et al.*, 1985; Watowich and Morimoto, 1988; Wu *et al.*, 1985; Dworniczak *et al.*, 1987; Welch *et al.*, 1983; Drummond *et al.*, 1987). The

major heat-inducible 70 KDa protein, hsp70, is not synthesized in unstressed mammalian cells, being detectable only following stress. In this case however, other members of the hsp70 family are present in the unstressed cells, particularly when they are growing rapidly, and is only slightly heat-inducible.

It is likely, therefore, that the stress proteins fulfill similar functions in normal and stressed cells, with such functions being required at higher levels in the stressed cells. In some cases, such as the 70 KDa proteins, the need for these proteins in both normal and stressed cells is fulfilled by the synthesis of constitutive and inducible proteins, while in other cases, this is achieved by a single protein that is both constitutively synthesized and inducible. For example, hsp90, although accumulating to five-fold higher levels following thermal stress, actually constitutes approximately 1 % of total cellular protein in normal mammalian cells (Lai *et al.*, 1984).

These ideas focus attention of the role of the hsps both in normal and stressed cells. A major step in understanding the role of the hsp70 family came from the identification of the immunoglobulin heavy chain binding protein BiP as a member of the hsp70 family identical to the glucose-regulated grp78 (Munroe and Pelham, 1986). This protein was known to bind to newly synthesized immunoglobulin heavy chain molecules in the endoplasmic reticulum prior to their association with light chains to form the antibody molecule (Bole *et al.*, 1986). Hence, BiP prevents the premature folding of the heavy chain in isolation prior to light chain synthesis, and once the light chain is available promotes the assembly of the heavy and light chain complex. By extension, this finding suggested that the hsp70 family might act as so-called chaperone proteins, having essential roles in protein biosynthesis; particularly in the folding, translocation and assembly of proteins that have not yet reached their final state of assembly in cells. In so doing they would prevent inappropriate interactions from

occurring in the complex and highly concentrated cellular environment, thereby allowing these proteins to assemble into the correct multi-protein complexes. In this way they would catalyze the correct folding and assembly of multi-protein complexes both in the cytoplasm and in the endoplasmic reticulum [Figure 1(a)i; Gething *et al.*, 1991].

Interestingly, most inducers of the stress response create abnormal proteins within the cell (Hightower, 1980). Such abnormal, partially denatured proteins will have exposed hydrophobic regions promoting inappropriate interactions with other proteins and thereby resulting in potentially dangerous insoluble aggregates. To prevent this, hsp70 binds to these dangerous insoluble proteins and prevents aggregate formation. Hence, hsp70 is required in larger quantities following stress and protects cells from the consequence of stress-induced protein denaturation. Moreover, this role for members of the hsp70 family following stress simply represents an extension of its role in normal cells in preventing inappropriate interactions between newly synthesized proteins [Figure 1(b)i]. Indeed, under adverse environmental conditions the synthesis of hsp90, hsp70 and chaperonins increase. Each of these proteins then takes on additional distinct functions, which in concert act to repair denatured proteins and protect cellular proteins from environmentally induced damage.

A similar role in protein-protein interaction in other situations within the cell is fulfilled by other heat shock proteins such as hsp65 and hsp90. Thus, the hsp65 protein, which is located in mitochondria and chloroplasts, appears to be involved in the unfolding of cytoplasmic proteins and their subsequent translocation into the subcellular organelles where they are refolded into an active form [Hemmingway *et al.*, 1988; Cheng *et al.*, 1989, Figure 1(a)ii]. Interestingly a similar role in translocation of proteins into mitochondria and their subsequent refolding has recently been

demonstrated for a constitutively expressed member of the hsp70 family in yeast which is located in the mitochondrial matrix (Kang *et al.*, 1990).

The hsp90 protein, which is predominantly cytoplasmic, likewise interacts with other proteins. In this case, however, the interaction does not facilitate transport into an organelle, but rather maintains these proteins in an inactive form until they need to be activated [Figure 1(a)iii]. For example, hsp90 is found associated with the receptors for several different steroid hormones prior to steroid treatment (Joab *et al.*, 1984; Sanchez *et al.*, 1986). In this case the hsp90 prevents the inappropriate activation of the receptor which only occurs following steroid treatment resulting in the release of the receptor from hsp90 (Sanchez *et al.*, 1987).

The role of hsp90, hsp70 and hsp65 in interacting with other proteins and maintaining appropriate states of protein folding prior to the synthesis of other proteins, intracellular transport, or signal-specific activation readily provides an explanation both for their synthesis in unstressed cells and for their enhanced synthesis following thermal stress when the need to inhibit inappropriate associations of partially denatured proteins is enhanced.

Although such abnormal or denatured proteins can be rendered harmless by association with hsps, ultimately they need to be removed from the cell by degradation. It is not surprising, therefore, that heat shock or other stresses also result in the induction of at least one component of the cellular machinery for protein degradation. This component, ubiquitin, is a highly conserved 76-amino acid protein which is found in all eukaryotic cells prior to stress, and is also induced to accumulate by heat shock (Bond and Schlesinger, 1985, 1986). This small polypeptide is linked to proteins which are targeted for degradation, and thus plays an essential role both in normal

protein turnover in unstressed cells [Figure 1(a)iv] and in the enhanced turnover of denatured proteins following heat shock [Parang *et al.*, 1987; Figure 1(b)ii].

Finally, it should be noted that while the role of the small hsps such as hsp27 has not yet been defined, they do play a role in thermotolerance, at least in mammalian cells (Landry *et al.*, 1989).

The Regulation of Gene Expression

There are two primary levels at which the phenotypic expression of a gene product can be regulated. Transcription is the first step in gene expression and the principal step at which it is controlled. The initial (and sometimes the only) step in regulation is the decision on whether or not to transcribe a gene. Transcription involves synthesis of an RNA chain complementary to a single strand of DNA by a process of base pairing that is catalyzed and scrutinized by the enzyme RNA polymerase. The reaction can be divided into the three stages illustrated in Figure 2: (1) Initiation begins with the binding of RNA polymerase to the double-stranded DNA. To make the template strand available for base pairing with ribonucleotides, the strands of DNA must be separated. Unwinding is a local event that begins at the site bound by RNA polymerase. The initiation stage is protracted by the occurrence of abortive initiation, when the enzyme makes short transcripts (approximately 10 bases), and aborts them. The aborted transcripts are released and initiation recommences; this occurs repeatedly until the enzyme succeeds in moving to the next phase. The sequence of DNA needed for the initiation reaction defines the promoter. Transcription factors appear to activate transcription by binding to their respective promoter sites in a variety of combinations. When multiple factors bind, the binding may be cooperative. Once bound, these factors somehow signal, or perhaps even assist, RNA polymerase to

the correct transcription site at which the first nucleotide is incorporated is called the start site; (2) Elongation describes the phase during which the enzyme moves along the DNA and extends the growing RNA chain. As the enzyme moves, it unwinds the DNA helix to expose a new segment of the template in single-stranded condition. Nucleotides are covalently added to the 3' end of the growing RNA by the formation of phosphodiester bonds, forming an RNA-DNA hybrid in the unwound region. As the enzyme moves, the RNA that was made previously is displaced from the DNA template strand, which pairs with its original partner to reform the double helix. Thus elongation involves the disruption of DNA structure, in which a transiently unwound region exists as a hybrid RNA-DNA duplex and a displaced single strand of DNA; (3) Termination involves recognition of the point at which no further bases should be added to the chain. To terminate transcription, the formation of phosphodiester bonds must cease, and the transcription complex must come apart. When the last base is added to the RNA chain, the RNA-DNA hybrid is disrupted, the DNA reforms in duplex state, and the enzyme and RNA are both released from it. The sequence of DNA required for these reactions is called the terminator.

Transcription most often is controlled at the stage of initiation, with the advantage that energy is not wasted producing unnecessary transcripts. As such the initiation of transcription is a critical point for controlling gene expression. Often the decision on whether or not to initiate at a particular promoter is the major or sole step in determining whether a gene should be expressed.

Transcription is not usually controlled at elongation, but may be controlled at termination. For example termination may be used to prevent transcription from proceeding past a terminator to the gene(s) beyond it.

The information-bearing RNA is appropriately called messenger RNA (mRNA) and is the template directing protein synthesis. Translation of the mRNA to its corresponding protein product is the second level of regulation of gene expression. The sequence of mRNA, in triplets 5' - 3' is related to the amino acid sequence of protein, N- to C- terminal. Of the 64 triplets (codons), 61 code for amino acids and 3 provide termination signals. A codon on mRNA is recognized by an aminoacyl-transfer RNA (tRNA), which has an anticodon complementary to the codon and carries the amino acid corresponding to the codon.

The essence of protein synthesis is the formation of peptide bonds which is catalyzed by ribosomes. In a manner similar to other enzymes, ribosomes bring the reactants involved in protein synthesis (mRNA and tRNA) into close proximity and into the correct orientation for formation of a peptide bond. Both the structure and function of ribosomes depend upon the cooperative association and interaction of proteins and ribosomal RNA (rRNA). The rRNA provides the structure around which the proteins of the ribosome assemble. However, rather than acting as scaffolding, the rRNA appears to be the catalytic core of the ribosome, whereas the proteins mostly stabilize the structure and allow tRNA molecules and mRNA to interact by neutralizing the negative charges of their phosphodiester backbones.

Like RNA synthesis, the reactions involving ribosomes can be divided into three stages: (1) Initiation requires that the mRNA be aligned so that protein synthesis begins with the correct codon. In prokaryotes, this is accomplished through the dissociation of the 70S ribosome into 30S and 50S subunits, followed by binding of the 30S subunit to the mRNA. The 30S subunit then reassociates with the 50S subunit to produce an active 70S ribosome. The 70S initiation complex is completed by the association with a particular methionyl-tRNA, called the initiator tRNA (tRNA^{Met})

which is specific for a start codon; (2) Chain elongation requires that each additional amino acid is added to the nascent polypeptide chain by positioning the correct aminoacyl-tRNA in the ribosome complex, forming the peptide bond, and shifting the ribosome relative to the mRNA by one codon; (3) Termination is marked by termination codons which are not recognized by tRNAs. Instead they are recognized by release factors. The final polypeptide is then released from the ribosome complex.

Translation may be regulated, usually at the stages of initiation and termination (like transcription). Regulation of initiation has a pleasing formal analogy to the regulation of transcription: a regulator molecule determines, directly or indirectly, whether an initiation site for a coding region is available to the ribosomes. Regulators of initiation of translation may be proteins or RNA. Regulation of termination is concerned with the relationship between successive genes or with constructing variants of the same protein that have different C-terminal regions.

The Regulation of SRP Gene Expression

The regulation of the stress induced accumulation of products of normally quiescent or weakly expressed genes has been shown to be primarily transcriptional in nature, although translational controls also play an important role in some systems (Storti *et al.*, 1980; Lindquist, 1981). The repression of the synthesis of normal cellular proteins, a vital component of the heat shock response, involves the preferential and selective translation of heat shock mRNA in *Drosophila* (Storti *et al.*, 1980) and the degradation of preexisting transcripts encoding normal cellular proteins in yeast (Lindquist, 1981).

General Features of Heat Shock Gene Expression

Studies on the activation of stress protein genes have brought to light an intriguing relationship between the mechanisms of gene activation and the function of stress proteins (Nover, 1991). In eukaryotes, heat-inducible genes include a conserved sequence referred to as the heat-shock element (HSE), often present in multiple copies, in their upstream non-coding region (Bienz and Pelham, 1987). Transcription is activated when a protein called the heat-shock factor (HSF) binds the HSE, and the subsequent phosphorylation of the HSF through a multistep process occurs (Jurivich *et al.*, 1992). Interestingly, the activation of heat shock genes both by heat shock (Zimarino and Wu, 1987) and by other stresses (see for example Benjamin *et al.*, 1990) involves a similar mechanism in which HSF is activated by the stressful stimulus, and then binds to the HSE of the heat shock genes and stimulates their transcription. The list of conditions known to induce hsp70 gene expression can be subdivided into three broad categories. As shown in Figure 3, they include: (1) environmental stresses including exposure to heat shock, amino acid analogues, and heavy metals; (2) non-stress conditions including normal cell growth, development, differentiation, and activation by certain oncogenes; and (3) pathophysiology and disease states. The specific mechanisms by which heat shock or other adverse environmental conditions activate the HSF remains obscure. The current working hypothesis is the 'abnormal protein hypothesis' in which heat shock or other stressors cause an increase in damaged or abnormal proteins in the cell that in some manner activates the HSF (Ananthan *et al.*, 1986). As a consequence, induction of the stress response involves a closed regulatory loop in which denatured proteins are both the signal that activates transcription of the stress-protein genes and the substrate for the gene product. A model for the regulation of HSF is shown in Figure 4. Under non-stressful conditions

of cell growth HSF is maintained in a non-DNA binding form through interactions with hsp70. This association, which need not be as a stable complex, maintains HSF in its non-DNA binding state, possibly by stabilizing the folded conformational state of control HSF. During heat shock, the appearance of denatured, misfolded, and malformed proteins creates a large pool of new protein substrates, which compete with HSF for association with hsp70. Thus, heat shock and other stresses initiate the events that remove the negative regulatory influence of HSF-DNA binding activity. The released HSF oligomerizes, binds DNA, and acquires transcriptional activity. The activation of HSF-DNA binding leads to the elevated transcription, synthesis, and accumulation of heat shock proteins, which then associate with HSF. The association of HSF with hsp70 may be important in the regulation of its transcriptional activity and/or conversion back to the control form. Several lines of evidence support this hypothesis: (1) injection of denatured, but not native, bovine serum albumin results in transcription of heat shock genes in *Xenopus oocytes* (Ananthan *et al.*, 1986); (2) amino-acid analogues induce stress-protein synthesis (Hightower, 1980); (3) the response is inhibited by conditions that stabilize potential protein targets (Edington *et al.*, 1989); and (4) biochemical conditions that alter protein conformation also activate the HSF *in vitro* (Mosser *et al.*, 1990).

Several types of studies suggest that other stressors that induce stress proteins are also mediated through activation of the HSF (Nover, 1991). This suggests, therefore, that a common pathway exists, in which a wide variety of stresses produce increased synthesis of the heat shock proteins (Hammond *et al.*, 1982). For example, the entire suite of heat-shock proteins, not just a subset, is induced by each type of stressor. Further, deletion of the HSE eliminates the response. Gel-shift and footprint assays have demonstrated that different types of chemicals can increase the binding of

the HSF to the HSE (Mosser *et al.*, 1990). When this evidence is taken together, it supports the premise that any environmental variable that causes an increase in denatured proteins would be expected to induce the stress response through this induction pathway. Consequently, any environmental condition that increases protein denaturation would be expected to induce the classic stress response.

The Induction of Srps is Tissue Specific

Another important feature of the stress response is that the induction of stress-protein synthesis is highly tissue specific in both vertebrates and invertebrates. The specific stress proteins induced and the levels of hsp70 and chaperonin appear to depend upon tissue type. Since, according to the 'abnormal protein hypothesis,' regulation of stress protein synthesis is closely linked to the extent of protein damage at the tissue level, it follows that the intensity of the stress response and relative concentrations of chaperones among the various tissues can help identify tissues that are most vulnerable to damage caused by a particular environmental stressor. It is these tissues that might represent the "weakest link" or rate-limiting physiological process responsible for lethality.

Srp Synthesis: A Possible Role in Natural Tolerance

It is clear that in a wide variety of different organisms and cell types, the hsps play a critical role both in normal function and in the response to stress. In the studies that have been done to date, heat shock protein synthesis has generally been induced by exposing animals (or cells) to chemical or environmental stresses that are not normally encountered by these animals, and to which they do not normally adapt. In contrast to these animal models, freshwater turtles can withstand long periods of oxygen

deprivation naturally by implementing specific biochemical adaptations. It would be interesting to see whether or not animals with a natural tolerance for such stresses also exhibit the classic stress response. The regulatory controls for the classic stress response meet the criteria associated with metabolic reorganization during anoxia in turtles by being: 1) rapidly initiated; 2) easily reversed; 3) applicable to many cellular processes; 4) readily coordinated; and 5) responsive to external stimuli. As such, Red-eared sliders (*Trachemys scripta elegans*) present an ideal model system with which to examine the functional significance and universality of the stress response in animals which are stress tolerant by nature.

Recently, a study investigating the importance of *de novo* protein synthesis in *T. s. elegans* during entry into, and recovery from, environmental anoxia demonstrated that turtles recovering from exposure to anoxic environments synthesize srps (Storey and Brooks, 1993). Specifically, a 72 kDa protein was identified in liver and skeletal muscle of turtles during the aerobic recovery period after anoxia exposure.

Statement of Hypothesis

This thesis examines the hypothesis that anoxia exposure alters the transcriptional activity of turtle cells and that both qualitative and quantitative changes in messenger RNA populations are key adaptations that support natural anoxia tolerance. Since hsps are highly conserved along phylogenetic lines, the proposal that newly-synthesized proteins produced in response to anoxia exposure could be immunologically related to the hsps of other systems was also tested.

Experimental Design

To experimentally test this hypothesis, messenger RNA was purified from organs of both aerobic and anoxia-exposed turtles. This mRNA was then subjected to *in vitro* translation to identify differences in protein profiles. Homology of the total protein translated *in vivo* with the *Drosophila* hsp70 family was also assessed using immunoblotting techniques.

A crucial step in poly(A)⁺ RNA purification is the isolation of intact total RNA from tissue samples. Techniques for extracting RNA have been evolving for over 30 years. Today, they fall into two broad categories: (1) those that make use of guanidinium salts and (2) those that make use of phenol. A third subcategory uses Sodium dodecylsulfate to disrupt cells and organelles and proteinase K to digest partly and fully denatured proteins. The guanidinium salt/CsCl density gradient ultracentrifugation procedure of Chirgwin *et al.* (1979) is currently the most widely used technique for the isolation of RNA, yielding high quality, protein-free RNA. But the procedure requires an ultracentrifuge and utilizes a large amount of tissue, and rotor capacity limits the number of samples that can be processed. This thesis describes a much shorter extraction procedure [a modification of the original hot phenol procedure of Soeiro and Darrell (1969)] involving hot phenol/chloroform extractions in the presence of sodium dodecylsulfate. This technique allowed more samples to be processed more quickly than with CsCl ultracentrifugation, and with similar yields and purity. The total RNA isolated by this procedure was subjected to affinity chromatography on oligo(dT) cellulose. In contrast to rRNA and tRNA, the vast majority of mRNAs are polyadenylated at their 3' termini. The mRNA [poly(A)⁺ RNA] can therefore be separated from the bulk of cellular RNA [poly(A)⁻ RNA] by affinity chromatography on oligo(dT) cellulose by virtue of their poly A tracts

(Edmonds *et al.*, 1971; Aviv and Leder, 1972). Subsequently, poly(A)⁺ RNAs were used for *in vitro* translation in a wheat germ cell-free system. The results of this investigation indicate that the transition of *T. s. elegans* into anoxia induces both quantitative and qualitative changes in the population of mRNA available for translation *in vitro*. In addition, immunoblot analysis revealed that *T. s. elegans* do express proteins *in vivo* that are immunologically related to the *Drosophila* cognate hsp70. However, the transition into anoxia did not result in the synthesis of an immunologically related inducible isoform of hsp70.

Figure 1. Functions of the hsps in normal and stressed cells.

For each function of the hsps, a representative family with this function is illustrated. In some cases, the function in normal and stressed cells will be fulfilled by the same heat-inducible protein which is present in unstressed cells, while in others such as hsp70, a constitutively synthesized protein is active in normal cells where the heat-inducible protein is absent. similarly, several different hsps may play an important role in a particular process. For example, a member of the hsp70 family in yeast, like hsp65, plays a role in the transport of proteins into the mitochondria (Yellon and Latchman, 1991).

(a) Normal Cells

(i) Preventing inappropriate associations

(ii) Intracellular transport

(iii) Maintaining proteins in an active form

(iv) Protein degradation

(b) Stressed Cells

(i) Inhibiting abnormal interactions

(ii) Degradation of abnormal proteins

Figure 2. Transcription.

RNA is synthesized by base pairing with one strand of DNA in a region that is transiently unwound. As the region of unwinding moves, the DNA duplex reforms behind it, displacing the RNA in the form of a single polynucleotide chain.

<p>Initiation RNA polymerase binds to duplex DNA</p>
<p>RNA synthesis starts with unwinding of DNA</p>
<p>Chains of 2–9 bases are synthesized and released during abortive initiation</p>
<p>Elongation RNA is synthesized by base pairing with one strand of DNA</p>
<p>Unwound region moves along DNA with RNA polymerase</p>
<p>RNA polymerase reaches end of gene</p>
<p>Termination RNA polymerase and RNA are released, and DNA duplex reforms</p>

Figure 3. Inducers of the cellular stress response.

Representation of three general classes of conditions known to result in the elevated expression of stress proteins including: 1) environmental/physiological stress; 2) pathophysiological states including conditions of disease; and 3) non-stressful conditions such as cell growth and development. Each condition acts on the cell as diagrammed in this figure, and in the case of environmental stress and certain pathophysiological states leads to the activation of HSF, which enters the nucleus and binds to HSE in the promoters of heat shock genes. This leads to the activation of heat shock gene expression and the synthesis of heat shock proteins (Morimoto *et al.*, 1992).

Figure 4. The heat shock factor cycle.

A proposal for a model of HSF regulation: 1) in the unstressed cell, HSF is maintained in a non-DNA binding state through interactions with hsp70; 2) heat shock and other forms of stress activate HSF to an oligomeric state; 3) HSF binds to HSEs in heat shock gene promoters; 4) HSF can acquire post-translational modifications by phosphorylation; 5) the levels of hsp70 increase, which leads to the formation of a HSF-hsp70 complex; 6) dissociation of HSF from the DNA and eventual conversion to the control non-DNA binding form of HSF (Morimoto *et al.*, 1992).

METHODS AND MATERIALS

Animal Care and Tissue Sampling

Live *Trachemys scripta elegans* (8 - 10 inches) were obtained from Boreal, St. Catharines, Ontario.

The turtles were held in large tanks with running, dechlorinated water at 15°C for at least three weeks before use. The animals were fed *ad lib* on a diet of egg shells, lettuce and trout food pellets. Normoxic turtles were sampled directly from the holding tank (15°C). Anoxia was imposed by submerging turtles for 16 hours in a sealed tank of water at 15°C that had been previously bubbled with gas (95 % N₂/5% CO₂) for 12 hours. Some animals were permitted to recover from the imposed anoxia for 24 hours prior to killing. All individuals were killed by decapitation. Liver, red muscle, white muscle, heart and kidney were removed and frozen in liquid nitrogen within 60 to 90 seconds after decapitation, then stored at -70°C.

Methods of Total RNA Isolation

Preparation of Reagents: All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated and were molecular biology grade or of the highest purity available. Sterile plastic labware was used whenever possible. All reagent bottles were autoclaved before used. Gloves were worn at all times. All buffers were made in diethylpyrocarbonate (DEPC) treated double distilled water (ddH₂O containing 0.1 %, v/v, DEPC was incubated for 24 hours at 37°C and then autoclaved for 30 minutes prior to use), or were made in ddH₂O and then autoclaved if permitted.

For the guanidine hydrochloride/cesium chloride ultra- centrifugation method: Extraction buffer was made fresh before use, and consisted of 200 mM Tris-HCl (pH 8.5) containing 50 mM magnesium chloride, 20 mM potassium chloride, 10 mM dithiothreitol (DTT), and 2 % (v/v) Triton X-100. A 6 M guanidine hydrochloride solution containing 100 mM sodium acetate (pH 5.5) was prepared, filter sterilized, and stored at -20°C; 1 % (v/v) 2-mercaptoethanol was added fresh to the guanidine hydrochloride solution prior to use. A 5.7 M cesium chloride solution containing 100 mM sodium chloride, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA was prepared and stored at -20°C until ready to use.

For the modified hot phenol method: Extraction buffer was made fresh before use, and consisted of 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 9.0) containing 0.5 % (w/v) sodium dodecylsulfate (SDS) and 5 mM ethylenediaminetetraacetic acid (EDTA).

Buffered phenol was prepared as described by Maniatis *et al.* (1982). Redistilled phenol (chromatography grade, BDH Inc., Mississauga, Ont.) was melted at 60°C, 8-hydroxyquinoline was added to 0.5 % (w/v), and the melted phenol was extracted three times (in dim light) at 4°C with an equal volume of 1.0 M Tris-HCl (pH 9.0), and then with 0.1 M Tris-HCl (pH 9.0) until the pH of the phenol phase was greater than 7.8. The buffer-saturated phenol was then stored under 0.1 M Tris-HCl (pH 9.0) at 4°C in a brown bottle and used within one month. Phenol:chloroform:isoamyl alcohol (PCI), 25:24:1, was prepared by combining 25 ml of the buffer-saturated phenol with 24 ml of chloroform and 1 ml of isoamyl alcohol. Chloroform:isoamyl alcohol (CI), 24:1, was prepared by combining 1 ml of isoamyl alcohol with 24 ml of chloroform.

Tissue Preparation

Frozen tissue was pulverized to a fine powder in a mortar containing liquid nitrogen. The frozen tissue powders were then immediately processed for RNA isolation.

Guanidine hydrochloride/CsCl Ultracentrifugation Method of RNA Isolation

One gram of frozen tissue powder was placed in a 50 ml sterile polypropylene centrifuge tube and homogenized at room temperature in 5.5 volumes (w/v) extraction buffer using a Polytron PT-10 (Brinkman Instruments, Rexdale, Ont.) at high speed for 10 seconds. An equal volume of the guanidine hydrochloride solution was added and the solution was further homogenized for an additional 10 seconds at high speed. The homogenate was centrifuged at 3,000 g for 5 minutes using a table top clinical centrifuge in order to sediment any insoluble debris. The supernatant was divided into two 5.5 ml aliquots. Each 5.5 ml aliquot was layered onto a 6 ml cushion of 5.7 M CsCl in sterile polypropylene ultracentrifuge tubes (Beckman, SW 41 ultracentrifuge tubes). Each one gram tissue sample therefore occupied two buckets of an SW 41 ultracentrifuge swinging bucket rotor. The samples were then ultracentrifuged at 28,600 rpm (Beckman 8-M Ultracentrifuge) for 15 hours at 15°C. After the spin, the supernatant was carefully removed using a sterile Pasteur pipet. The RNA pellet was air dried for 2 - 4 minutes while on ice, resuspended in 500 μ l of TE buffer, transferred to a sterile 1.5 ml centrifuge tube, and extracted with an equal volume of PCI. All solutions containing RNA were kept on ice between manipulations and whenever possible. The phases were separated by centrifugation at high speed using an Eppendorf centrifuge. The PCI extraction was repeated twice, or until the interphase had completely disappeared. The final aqueous phase was extracted with an equal volume of CI.

Modified Hot Phenol Extraction of Total RNA

One gram of frozen tissue powder was placed in a 50 ml sterile polypropylene centrifuge tube and homogenized at room temperature in 11 volumes (w/v) of extraction buffer with a Polytron PT-10 (Brinkman Instruments, Rexdale, Ont.) for 10 seconds at high speed. An equal volume of PCI was then added to the homogenate, with thorough mixing by shaking vigorously for 20 seconds. The tubes were incubated in a 65°C water bath for 15 minutes (with intermittent shaking), removed, vortexed and placed on ice for 5 minutes. All solutions containing RNA were kept on ice between manipulations and whenever possible. Separation of the phases was achieved by centrifugation for 20 minutes at 15,000 g (Beckman model J2-21). This centrifugation speed resulted in a sharp interphase. Occasionally, the organic/aqueous interphase was fairly large (interphase size varied with tissue type and amount), but usually was sharpened by additional centrifugation. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and organic phase. The aqueous phase was transferred to a fresh sterile tube and the hot PCI extraction was repeated two more times. As well, one 0.5 volume of fresh TE buffer (10 mM Tris-HCl, pH 9.0, containing 1 mM EDTA) was used to re-extract each of the three organic phases. Finally the aqueous phases were pooled together and extracted with 2 - 3 volumes of CI. The CI extraction was repeated until the interphase disappeared (usually twice). In order to minimize DNA contamination of the RNA sample, the pH of the aqueous phase was adjusted to 5.5 by the addition of a 10 % (v/v) solution of acetic acid, and the RNA was selectively precipitated by the addition of an equal volume of 5 M NaCl (2.5 M NaCl final). The sample was placed on ice and the high salt precipitation proceeded for a minimum of 4 hours. The precipitate was then collected by centrifugation at 10 000 g (Beckman model J2-21) for 25 minutes

at 4°C. The supernatant was carefully removed using a sterile Pasteur pipet. The RNA pellet was air dried for 2 - 3 minutes, resuspended in 500 μ l TE buffer, transferred to a sterile 1.5 ml Eppendorf centrifuge tube, and extracted with an equal volume of PCI. Separation of the phases was achieved by centrifugation at high speed in an Eppendorf centrifuge for two minutes. The supernatant was transferred to a new sterile 1.5 ml Eppendorf centrifuge tube and was further extracted with an equal volume of CI to remove any traces of phenol.

Precipitation of Nucleic Acids

The final aqueous phase was removed to a sterile tube and the nucleic acids were precipitated out by the addition of 2.5 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate at pH 5.5. Precipitation proceeded at -20°C for a minimum of 2 hours. Alternatively, precipitation was performed with 0.6 volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.5) for a minimum of 20 minutes while on ice. The precipitated nucleic acids were collected by centrifugation at high speed in an Eppendorf centrifuge for 30 minutes at 4°C. The supernatant was removed using a sterile Pasteur pipet fitted to a vacuum aspirator. The Pasteur pipet had been previously flame heated and stretched so that the opening at the tip was very narrow thus ensuring that the tiny RNA pellet was left undisturbed while aspirating off the supernatant. The RNA pellet was washed by the addition of 70 % ethanol and gently mixed by flicking. This removes any residual salt associated with the RNA pellet. The RNA was collected again by centrifugation and the supernatant was removed by aspiration as previously described. This washing process was repeated at least two more times. The final RNA pellet was resuspended in 500 μ l TE buffer and subjected to spectrophotometric determination of nucleic acid concentration and agarose

gel electrophoresis after which the RNA was stored at -20°C under 2.5 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5.5).

Spectrophotometric Quantification of Nucleic Acids

The concentration and purity of each sample was evaluated spectrophotometrically using the absorbances at 260 and 280 nm (Maniatis *et al.*, 1982). Nucleic acids were quantified in units of mass by measuring A₂₆₀. An A₂₆₀ of 1 was taken as 40 µg of nucleic acid per ml. The ratios of A₂₆₀/A₂₈₀ measurements routinely ranged between 1.65 and 1.85, an indication that the RNA was relatively free from protein contamination. In the few cases where the ratio did not fall within this range, additional PCI extractions shifted the ratio back within range. Ratios > 2 were always obtained between absorbances measured at 260 and 230 nm, indicating little phenol contamination.

Agarose Gel Electrophoresis of Nucleic Acids

Buffers and Solutions: The agarose gel electrophoresis running buffer contained 50 mM boric acid, 5 mM disodium tetraborate, and 10 mM sodium sulfate, adjusted to a final pH of 8.3. A 4x stock solution of the loading buffer was prepared for convenience and contained 40 mM methylmercury (II) hydroxide (Johnson Mathey Catalog Company, Brampton, Ont.), 200 mM boric acid, 20 mM disodium tetraborate, 40 mM sodium sulfate, 0.4 % (w/v) bromophenol blue, 0.4 % (w/v) xylene cyanol and 20 % (v/v) glycerol. The 4x sample loading buffer was stored in 1 ml aliquots at -20°C and was thawed just prior to use. A nucleic acid staining solution was prepared by adding 10 to 20 µl of a 10 mg/ml ethidium bromide solution to 200 ml

of running buffer. The staining solution was stored in a dark bottle and was recycled after each use.

The RNA was analyzed by electrophoresis in a 0.1 % agarose denaturing gel which was prepared by dissolving 0.5 g of solid agarose (type 1, Sigma) in 50 ml of running buffer (pH 8.3) by boiling. The dissolved agarose was cooled to 60°C and cast as horizontal slabs of dimensions 10 x 6 x 0.5 cm. The 4x loading buffer was added to the RNA samples (the RNA samples ranged from 1-2 µg according to A₂₆₀) so that the final concentration of the sample was 1x. The samples were then mixed thoroughly, heated to 65°C for 3 minutes to destroy any secondary structure of the RNA, and cold snapped on ice prior to loading into the wells. The gels were run at 50 volts (approximately 4.5 V/cm for 60 minutes).

During the period of electrophoresis, the gels were submerged in the running buffer (pH 8.3) and the completion of each run was monitored by the migration of the dyes in the loading buffer. After the completion of the electrophoretic run, the gel was stained in the ethidium bromide staining solution for 20 minutes, destained in ddH₂O for 10 minutes, and then photographed on a UV light transilluminator at a wavelength of 310 nm to evaluate the quality of the RNA obtained.

Selection of Polyadenylated RNA

To select polyadenylated RNA (poly(A)⁺ RNA), total RNA isolated by the modified hot phenol procedure was subjected to affinity chromatography on oligo(dT) cellulose (Bethesda Research Laboratory, Burlington, Ont.) according to the protocol of Maniatis *et al.* (1982) (a modification of Aviv and Leder, 1972).

Buffers and Solutions: A 2x binding buffer was prepared by combining 20 mM Tris-HCl (pH 7.5) with 1 M lithium acetate. The solution was autoclaved and

then 0.2 % (w/v) *N*-lauroyl sarcosine was added. The 2x binding buffer was stored at 4°C when not in use. DEPC-treated water served as a suitable elution buffer.

Oligo(dT) cellulose (0.5 g) was suspended in 5 ml of DEPC-treated water and allowed to settle so that the fines could be poured off. This was repeated several times until there were no more fines. The final 5 ml suspension was poured into a sterile 10 ml disposable column with a pre-packed Whatman GF/A filter. The column was then equilibrated with one column volume of 1x binding buffer. RNA stored under ethanol precipitation at -20°C was collected by centrifugation, resuspended in 500 µl DEPC-treated water, heated to 65°C for 5 minutes to destroy any secondary structure and cold snapped on ice for 5 minutes. The sample volume was adjusted to 3 ml by the addition of 1 ml of ice cold DEPC-treated water and 1.5 ml of 2x binding buffer (final 1x binding buffer). The 3 ml sample was then loaded onto the column and mixed with the oligo(dT) cellulose gently by aspiration thus allowing binding of the poly(A)⁺ RNA to the column. After the oligo(dT) cellulose had settled, the flow through (primarily poly(A)⁻ RNA) was collected at a rate of 1 drop every four seconds and then was reapplied to the column two more times without disturbing the oligo(dT) cellulose. The column was then washed with one column volume of binding buffer to further elute any unbound poly(A)⁻ RNA.

The bound poly (A)⁺ RNA was eluted off the column into 12 fractions of 400 µl with DEPC-treated water (elution profile was assayed spectrophotometrically). The RNA in the eluate was recovered by the addition of 2.5 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5.5). Precipitation of the poly(A)⁺ RNA proceeded at -20°C for at least two hours before collecting by centrifugation. The poly(A)⁺ RNA collected in the 12 fractions were pooled, rebound to and re-eluted from the column and recollected by precipitation as described previously.

The quantity and the purity of the twice enriched poly(A)⁺ RNA was assessed by monitoring A₂₆₀ and agarose gel electrophoresis as done for total RNA. The poly(A)⁺ RNA was stored under 2.5 volumes of absolute ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.5).

The column was regenerated between uses by washing with 2 to 3 column volumes of DEPC-treated water followed by washing with one column volume of 0.1 M KOH containing 5 mM EDTA. The column was then washed with at least 4 column volumes of DEPC-treated water (or until the pH of the effluent was less than 8.0). The column was stored at 4°C in binding buffer containing 0.2 % sodium azide.

In vitro Translation of Poly(A)⁺ RNA

In vitro translations of poly(A)⁺ RNA were done using a wheat germ lysate translation kit purchased from Boehringer Mannheim, Laval, Que. Each 25 µl assay contained: 20% translation reaction mixture containing 25 µmol/l of each of the 19 amino acids except methionine, 1 mM ATP, 0.02 mM GTP, 8 mM creatin phosphate, 18 µg/ml spermine, 2.3 mM dithiothreitol, 20 mM Hepes (pH 7.6), 40 µg/ml creatine kinase, 0.6 mM EGTA; 40 units of RNasin ribonucleas inhibitor (Promega, 40 000 U/ml); 20 µCi of [³⁵S]methionine; and 30 % wheat herm extract supplemented with 37.5 µg/ml calf liver tRNA. Also included in each 25 µl assay were the following optimal amounts determined by RNA titrations: 1 µg of twice enriched poly(A)⁺ RNA; 136 mM potassium acetate; and 1.5 mM magnesium acetate. The mixtures were incubated for 90 minutes at 30°C. Incorporation of [³⁵S]methionine was measured as hot trichloroacetic acid (TCA) insoluble radioactivity as described by R.J. Mans *et al.*, (1961). In order to assay for acid-insoluble radioactivity, duplicate 2 µl aliquots of each reaction were diluted in 125 µl of distilled water. An equal volume of 2 M NaOH

was added, and the samples were incubated at room temperature for 10 minutes. Samples were then precipitated on ice for one hour by the addition of 1 ml of a 25 % (w/v) TCA solution containing 1 % (w/v) casein hydrolysate. The TCA- precipitable [³⁵S]methionine-incorporated translation products were collected on Whatman GFC filter paper disks (2.5 cm diameter) and washed three times with 5 % (w/v) TCA. The filter disks were dried and dissolved in scintillation cocktail (BIOFLUOR, New England Nuclear, Mississauga, Ont.). Scintillation counting was carried out on a programmable LKB 1215 Rack Beta scintillation counter equipped with automatic quench correction and a disintegrations per minute converter. The translation products were then analyzed by SDS-PAGE and fluorography.

Gel Electrophoresis and Fluorography of Newly Synthesized Proteins

Buffers and Solutions: Electrophoresis-grade chemicals were used where appropriate.

Aliquots containing equal amounts of acid-precipitable radioactivity (5 000 - 10 000 cpm) of the *in vitro* translated products were solubilized in 50 mM Tris-HCl (pH 6.8) containing 5 % (v/v) 2-mercaptoethanol, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue and 10 % (v/v) glycerol by heating at 95°C for 5 minutes. The newly synthesized proteins were then resolved by polyacrylamide slab gel electrophoresis in the presence of SDS (SDS-PAGE) in which the separating gel (16 x 18 cm x 0.75mm) was a linear gradient of 8 - 15 % (w/v) polyacrylamide with 5 % (w/v) stacking gels (ratio of acrylamide to bisacrylamide was 30:0.8) and the discontinuous buffer system of Laemmli (1970). Prestained low molecular weight protein standards (Bio-Rad, Mississauga, Ont.) were run in parallel with the samples and were used to determine the molecular weight of the translation products for each gel. These were rabbit muscle phosphorylase B, 106,000 D; bovine serum albumin,

80,000 D; hen egg white ovalbumin, 49,500; bovine carbonic anhydrase, 32,500 D; soybean trypsin inhibitor, 27,500 D; and hen egg white lysozyme, 18,500 D. The gels were electrophoresed under constant current, 20 mA, until the tracking dye in the loading buffer just ran off the bottom of the gel (approximately 4 hours). After the electrophoretic run the gel was fixed for 1 hour in a 30 % (v/v) methanol solution containing 10 % (v/v) glacial acetic acid and impregnated with EN³HANCE (New England Nuclear, Mississauga, Ont.) for 0.5 hours. To precipitate the fluorescent materials inside the gel, the EN³HANCE was replaced with cold water. The gel remained in the cold water for 0.5 hours. After the precipitation step had been completed, the gel was carefully placed onto a piece of Whatman filter paper and dried under heat (60 - 70°C) and vacuum on a slab gel drying apparatus for 3 hours. The dry gel was then autoradiographed with Kodak X-Omat RP film (Picker, Brampton, Ont.) for a suitable length of time at -70°C.

Molecular masses of the sample polypeptides for each fluorogram were determined using a standard curve that was generated by plotting the reflectance (R_f) value of each standard protein against the log of its molecular mass. Sample band molecular masses were then determined by finding their R_f values on the standard curve and reading the molecular mass from the ordinate.

Densitometry

Relative intensities of bands on the film were quantified using a Molecular Dynamics Laser Densitometer. Peaks from the scans were integrated and intensities were calculated using Image Quant software installed on an IBM compatible computer interfaced with the densitometer. The intensities were determined at a scan speed of 100 mm/min and the values were measured at an absorbance of 2. The actual

intensities are given in arbitrary units; a full-scale peak height of 2 has an intensity of 1000 units (Griffin and spalding, 1988).

SDS-Polyacrylamide Gel Electrophoresis of Total Protein

Buffers and Solutions: Tissue homogenization buffer consisted of 10 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$ and 2 mg PMSF. Electrophoresis-grade chemicals were used where appropriate. The protein fixing solution consisted of 30% (v/v) methanol containing 10% (v/v) acetic acid. The staining solution consisted of 30% methanol containing 10% acetic acid and 0.1% (w/v) Coomassie brilliant blue *R*. The destaining solution was 30% methanol containing 10% (v/v) acetic acid.

Frozen tissue (0.2 g) was homogenized in 1 ml homogenization buffer and centrifuged to remove insoluble debris. Equal amounts of total protein (90 μ g) as determined by the Biorad standard protein assay (Bradford, 1976) were solubilized, in 50 mM Tris-HCl (pH 6.8) containing 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue and 10% (v/v) glycerol, by heating at 95°C for 5 minutes. SDS-PAGE was carried out using a 8 - 15% linear gradient separating gel (16 x 18 cm x 0.75 mm) with 5% polyacrylamide stacking gels (ratio of acrylamide to bisacrylamide was 30:0.8) and the discontinuous buffer system of Laemmli (1970). The proteins were electrophoresed at 20 mA for 4 hours. After completion of the electrophoretic run, the gels were fixed for 1 hour in fixing solution, placed in the staining solution, placed in the staining solution for 2 hours, destained overnight, and photographed. electrophoresed at 20 mA constant current for 4 hours. After completion of the electrophoretic run, the gels were fixed for one hour in a 30% (v/v) methanol solution containing 10% (v/v) glacial acetic acid, stained with 0.1% (w/v) Coomassie Blue dye, destained and photographed.

Western Transfer

Buffers and Solutions: Tissue homogenization buffer consisted of 10 mM Tris-HCl (pH 7.5) containing 5 mM MgCl₂ and 2 mg PMSF. Electrophoresis-grade chemicals were used where appropriate. Western transfer buffer consisted of 20 mM Tris-HCl (pH 8.0) containing 0.18 M glycine, and 20 % (v/v) methanol. Phosphate buffered saline (PBS) contained 8 mM disodium phosphate, 1.5 mM potassium phosphate, 3 mM potassium chloride and 137 mM sodium chloride. Tris buffered saline (TBS) consisted of 50 mM Tris-HCl (pH 7.0) containing 150 mM sodium chloride. The primary antibody (Affinity BioReagents Inc., NJ., cat# MA3-001), monoclonal antibody clone 7.10 (Kurtz *et al.*, 1986), recognizes several members of the hsp70 gene family including hsp70, 72, 68, and cognate proteins (Wu *et al.*, 1988), as well as the glucose responsive protein (grp) 78, in many families of many eukaryotes, including *Drosophila*, yeast, trypanosomes, mice, soybeans, and man (Watowich and Morimoto, 1988). The secondary antibody used was a sheep antimouse SDL2513 alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories, Inc. BIO/CAN Scientific). For development of the alkaline phosphatase reaction, the following solutions were prepared within one hour prior to use: Nitro blue tetrazolium (NBT) buffer contained 100 mM Tris-HCl (pH 9.55), 25 mM diethenolamine, 100 mM sodium chloride, 2 mM magnesium chloride, 0.001 mM zinc chloride. solution A was prepared by dissolving 6.6 mg NBT (Amresco,) in 20 ml of NBT buffer. Solution B was prepared by dissolving 2 mg phenazine methyl sulfate in 1 ml of ddH₂O. Solution C was prepared by dissolving 6 mg bromochloroindolyl phosphate in 150 μ l dimethylformamide. The alkaline phosphatase developing solution was prepared just before use and consisted of solution A plus 134 μ l of solution B and 68 μ l of solution C.

Frozen tissue (0.2 g) was homogenized in 1 ml homogenization buffer and centrifuged to remove insoluble debris. The Biorad Mini Protean II Gel System was used to resolve total protein by SDS-PAGE for Western transfer. Aliquots containing equal amounts of protein (20 μ g), as determined by the standard Biorad protein assay (Bradford, 1976), were solubilized in 50 mM Tris-HCl (pH 6.8) containing 5 % (v/v) 2-mercaptoethanol, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue and 10 % (v/v) glycerol by heating at 95°C for 5 minutes. SDS-PAGE was carried out using 10 % (w/v) acrylamide separating gels with 5 % (w/v) stacking gels (ratio of acrylamide to bisacrylamide, 30:0.8) and the discontinuous buffer system of Laemmli (1970). The gels were run at 200 V for 49 minutes. Proteins were then transferred for 2 hours at 200 V and 4°C onto a nitro-cellulose sheet (Mandel Scientific Co., 0.20 μ m pore size) in transfer buffer (Towbin *et al.*, 1979). After complete transfer, the nitrocellulose sheet was stained with 0.2% (w/v) Ponceau S red (Serva) in 3% (w/v) trichloroacetic acid for about 2 minutes (until the protein bands became obvious), and then placed in water for about 30 seconds to rinse off any residual stain. The blot was sliced transversely between 97 kDa and 45 kDa as indicated by low range molecular weight markers (Biorad). This blot slice was then destained in PBS, and incubated at 37°C for 1 hour in PBS containing 5% (w/v) nonfat dry milk (Johnsone *et al.*, 1984) then washed twice for five minutes each with PBS containing 0.05% (v/v) Tween. The blot was then incubated at 37°C for one hour with the primary antibody solution (1:1000 dilution of the primary antibody in PBS containing 5% w/v nonfat dry milk) in a heat sealed hybridization membrane. The blot was then washed once with PBS, three times with TBS, then incubated with the secondary antibody in TBS containing 5% (w/v) nonfat dry milk) for 1 hour at 37°C. The blot was then washed 3 times with TBS containing 0.05% (v/v) Tween. After the final wash the blot was developed in the dark

at 37°C by the addition of the 5 ml alkaline phosphatase developing solution. When the blots were developed sufficiently (approximately 5 minutes) as evidenced by the appearance of blue spots, the developing reaction was stopped by placing blots in water. All figures represent duplicated or triplicated results.

Statistical Treatment of Data

All treatment groups were compared statistically to corresponding control counterparts. Group-group variances were determined using Normal data ANOVA (Model I). *A posteriori* determinations of exactly where the differences lie were defined by the two-tailed Dunnett's test.

RESULTS

Table 1 demonstrates the high yield and purity (based on OD₂₆₀/OD₂₈₀) of total RNA isolated by the modified hot phenol extraction procedure from liver and red muscle from *T. s. elegans*. Both the yield and purity are similar to those obtained by the guanidinium hydrochloride/CsCl ultracentrifugation procedure. Both isolation procedures yield similar patterns on agarose (0.1 %) gel electrophoresis, visualized with ethidium bromide. **Figure 5** illustrates the ethidium bromide-stained gels showing the quality of the total RNA extracted from liver and red muscle using the guanidine hydrochloride/CsCl ultracentrifugation method (**Fig. 5a**) and the modified hot phenol method (**Fig. 5b**). The nucleic acids above the 28s ribosomal band (**Fig. 5b**) is an indication of DNA contamination. The typical 28s and 18s ribosomal RNA bands routinely seen in the samples prepared by the modified hot phenol method were occasionally absent in the samples prepared by the guanidine HCl/CsCl ultracentrifugation procedure.

The modified hot phenol extraction procedure was the preferred method of choice for all total RNA extractions. The total RNA isolated by this procedure was subjected to affinity chromatography on oligo(dT) cellulose to yield poly(A)⁺ RNA. **Figure 6** illustrates the purity of the poly(A)⁺ RNA isolated from control liver and red muscle as examined by agarose (0.1 %) gel electrophoresis. The absence of the 28s and 18s ribosomal bands coupled with the presence of abundant high molecular weight RNAs is representative of pure and undegraded poly(A)⁺ RNA. Similar results were obtained for all tissues sampled by this procedure.

To determine whether anoxia resulted in altered transcriptional activity in *T. s. elegans*, the amount of total translatable mRNA (measured as the amount of acid precipitable counts per minute) in tissues from control, aerobic turtles were compared

with those from turtles that had been exposed to 16 h of anoxic submergence in N₂-bubbled water. In the case of heart and kidney, a third condition was also examined; turtles allowed to recover from anoxia for 24 h.

Total RNA was extracted from 1g of liver, red muscle, white muscle, heart and kidney sampled from control and anoxic turtles. Total RNA was also extracted from 1g of heart and kidney sampled from recovered animals. The amounts of total extracted RNA were not significantly different between tissues sampled from control animals and those sampled from treatment animals (Fig. 7). To remove any RNAs of low relative molecular mass that could have potentially inhibited translation (Piot *et al.*, 1984; Slegers *et al.*, 1989), polyadenylated mRNA [poly(A)⁺ RNA] was isolated from the total RNA using oligo(dT) cellulose affinity chromatography. The amounts of poly(A)⁺ RNA were not significantly different between tissues sampled from control animals and treatment animals except in the case of white muscle where the amount of poly(A)⁺ RNA in anoxic tissue was significantly greater than control (Fig. 8a).

Equal amounts of poly(A)⁺ RNA were used for *in vitro* translation experiments. There were significant differences between control and treatment groups with respect to the amounts of translatable mRNAs per 1.0 µg poly(A)⁺ RNA preparation. The poly(A)⁺ RNA isolated from liver (Fig. 9a) and kidney (Fig. 9b) sampled from anoxic turtles had 38.3 % and 24 % (respectively) greater levels of translatable mRNA than corresponding controls. By contrast, the poly(A)⁺ RNA from anoxic red muscle (Fig. 9a) had significantly lower levels of translatable mRNA (58 % of control). There were no differences observed between control and anoxic white muscle (Fig. 9a) and heart (Fig. 9b). The poly(A)⁺ RNA isolated from heart (Fig. 9b) of recovered turtles had slightly lower levels of translatable mRNAs than control (90 % of control). By contrast, the poly(A)⁺ RNA isolated from kidney (Fig. 9b) of

recovered turves had 16.8 % greater levels of translatable mRNAs than control (Fig. 9b). These data suggest that the transition into anoxia leads to quantitative changes *in vivo* in the levels of mRNA that can act as functional templates *in vitro*.

In addition to these quantitative analyses of mRNA pools, qualitative changes in the messenger population were also assessed. Messenger RNA was subjected to *in vitro* translation and the protein products were resolved by SDS-polyacrylamide gel electrophoresis, visualized by fluorography, and then analyzed by densitometry. It could be argued that, since there are significant differences between the levels of translatable mRNA in control and treatment tissues, there may also be differences in mRNA profiles. The transition from normoxic to anoxic or from anoxic to recovered may result in the synthesis of a different set of mRNAs that code for a markedly different suite of proteins. Figure 10 shows a fluorograph of [³⁵S]methionine-labeled proteins synthesized *in vitro* from mRNA isolated from control (lane 2) and anoxic (lane 3) liver. A polypeptide of approximately 19.5 kDa appeared to be synthesized only from the mRNA isolated from anoxic liver. The corresponding laser densitometry trace (Fig. 11) clearly shows that the 19.5 kDa polypeptide corresponds to a novel mRNA species present only in anoxic liver. The densitometry trace also showed that polypeptides of approximately 28.6 and 79.9 kDa are greatly enhanced in anoxia. Figure 12 shows a fluorograph of [³⁵S]methionine-labeled proteins synthesized *in vitro* from mRNA isolated from anoxic (lane 1) and control (lane 2) white muscle and anoxic (lane 3) and control (lane 4) red muscle. There were no major differences in the banding patterns between the two sets of proteins for white muscle. The corresponding laser densitometry trace for white muscle (Fig. 13b) showed the same result. Three bands corresponding to mRNAs coding for polypeptides of approximately 40.0, 32.0 and 28.2 kDa, were seen only in control red muscle. The

corresponding laser densitometry trace (Fig. 13a) revealed similar results as well as a new polypeptide of approximately 37.5 kDa present only in anoxic red muscle. Figure 14 shows a fluorograph of [³⁵S]methionine-labeled proteins synthesized *in vitro* from mRNA isolated from control (lane 1), anoxic (lane 2) and recovered (lane 3) heart. There is an extra band at approximately 26.8 kDa for the anoxic heart that was not obvious in control and recovered heart. The corresponding laser densitometry traces (Fig. 15a,b) suggested that this 26.8 kDa polypeptide represents a new mRNA species. Figure 16 shows a fluorograph of [³⁵S]methionine-labeled proteins synthesized *in vitro* from mRNA isolated from control (lane 3), anoxic (lane 2) and recovered (lane 1) kidney. There was an obvious new band at approximately 32.8 kDa present only in recovered kidney. The corresponding densitometry traces (Fig. 17b,c) suggested that this 32.8 kDa polypeptide represents a novel mRNA species present only in recovered kidney. A polypeptide of approximately 75.8 kDa was present in control but disappeared in anoxic and recovered kidney (Fig. 17a,c). These results show that the transition into anoxia leads to qualitative changes *in vivo* in mRNA populations in terms of their *in vitro* translational profiles.

Immunoblot analysis of total protein (Fig. 19) showed that all tissues of all treatment groups express proteins immunologically related to the *Drosophila* constitutive hsp70. The transition into anoxia did not result in the expression of a protein immunologically related to the inducible isoform of *Drosophila* hsp70, as this band was not observed in any of the tissues. All tissues with the exception of red and white muscle express proteins that are immunologically related to *Drosophila* grp70.

Figure 5. Analysis of total RNA by agarose gel electrophoresis.

(a) Total RNA isolated by the guanidine hydrochloride/CsCl ultracentrifugation method. Total RNA (1.5 μg) extracted from liver (*lane 1*) and red muscle (*lane 2*) sampled from control turtles was subjected to agarose (0.1 %) gel electrophoresis, visualized by ethidium bromide, and photographed.

(b) Total RNA isolated by the modified hot phenol method. Total RNA (1.5 μg) extracted from liver (*lane 1*) and red muscle (*lane 2*) sampled from control turtles was subjected to agarose (0.1 %) gel electrophoresis, visualized by ethidium bromide, and photographed.

(a) **(b)**

TABLE 1

Yield and Purity of Total RNA Isolated From Control Liver and Red Muscle

Tissue	Modified Hot Phenol Extraction		Guanidine HCl/CsCl Ultra-centrifugation Method	
	Yield (ug/g)	Purity (OD ₂₆₀ /OD ₂₈₀)	Yield (ug/g)	Purity (OD ₂₆₀ /OD ₂₈₀)
liver	1810.3 ± 142.4	1.90 ± 0.010	1690.0 ± 71.5	1.76 ± 0.012
red muscle	976.3 ± 52.3	1.81 ± 0.009	847.7 ± 84.0	1.79 ± 0.010

Total RNA was isolated from control liver and red muscle as described under Methods and Materials for the modified hot phenol extraction procedure and the guanidine HCl/CsCl ultra-centrifugation method. The yield was calculated from OD₂₆₀, taking an OD of 1.0 equal to 40 ug/ml. The values shown are the mean ± S.E.M for three separate determinations.

Figure 6. Analysis of poly(A)⁺ RNA by agarose gel electrophoresis.

Total RNA isolated by the modified hot phenol method was subjected to two passes of affinity chromatography on oligo(dT) cellulose for enrichment of poly(A)⁺ RNA. Twice enriched poly(A)⁺ RNA (1.5 μg) purified from liver (*lane 1*) and red muscle (*lane 2*) sampled from control animals was subjected to agarose (0.1 %) gel electrophoresis, visualized by ethidium bromide and photographed.

1 2

Figure 7. Levels of total extracted RNA

(a) Levels of total RNA extracted by the modified hot phenol method from liver, red muscle and white muscle of control and anoxic turtles. Each bar represents the mean $\pm$ S.E.M for three independent phenol extractions of 1.0 g samples of liver, red muscle and white muscle. There were no significant differences from the control values.

(b) Levels of total RNA extracted by the modified hot phenol method from heart and kidney of control, anoxic and recovered animals. Each bar represents the mean $\pm$ S.E.M. for three independent phenol extractions of 1.0 g samples of heart and kidney. There were no significant differences from the control values.

(a)

(b)

Figure 8. Levels of twice enriched poly(A)⁺ RNA

(a) Total RNA isolated by the modified hot phenol extraction method from liver, red muscle and white muscle of control and anoxic turtles was subjected to affinity chromatography on oligo(dT) cellulose. Each bar represents the mean $\pm$ S.E.M for three independent purifications from 1 g of tissue as starting material. The asterisk indicates a significant difference from the control value ($P < 0.05$).

(b) Total RNA isolated by the modified hot phenol extraction method from heart and kidney sampled from control, anoxic and recovered animals was subjected to affinity chromatography on oligo(dT) cellulose. Each bar represents the mean $\pm$ S.E.M for three independent purifications from 1 g of tissue as starting material. There were no differences from the control values.

(a)

(b)

Figure 9. Levels of total translatable mRNA.

Total translatable mRNA expressed as total acid precipitable counts per minute per μg poly(A)⁺ RNA. Equivilant amounts of twice enriched poly(A)⁺ RNA (1.0 μg) purified from the total RNA extracted from liver, red muscle and white muscle of control and anoxic turtles (Fig. 9a) and from heart and kidney of control, anoxic and recovered turtles (Fig. 9b) were translated in a cell free system without methionine. Incorporation of [³⁵S]methionine into acid precipitable proteins was measured by scintillation counting. Each bar represents the mean $\pm$ S.E.M. of triplicate determinations on one batch of isolated poly(A)⁺ RNA. The asterisk indicates a significant difference from the control value ($P < 0.01$).

(a)

(b)

Figure 10. SDS-PAGE analysis of turtle liver translational products.

Autoradiography showing protein profiles on an SDS-polyacrylamide (8 - 15 %) gel of *in vitro* translation, in a wheat germ lysate, of 1.0 μg of poly(A)⁺ RNA isolated from liver of control (*lane 2*) and anoxic (*lane 3*) turtles. *Lane 1* represents the control cell-free system without added RNA. The sample load for all lanes was approximately 1.0×10^4 acid precipitable counts per minute. Fluorography time was 120 hours. Molecular weights are indicated on the left. The arrowhead indicates a band of approximately 19.5 kDa present only during anoxia.

Figure 11. Densitometry trace of liver fluorogram (Fig. 10).

Units on the intensity scale are arbitrary. Both traces were scanned from the same gel and under identical conditions. Peaks corresponding to optically dense bands are shown. LC: control liver; LA: anoxic liver.

Peaks representing new and enhanced polypeptides present in the anoxic turtle liver (*lane 3, Fig. 10*) but not the control turtle liver (*lane 2, Fig. 10*) are indicated by arrowheads with their corresponding molecular weights.

Figure 12. SDS-PAGE analysis of turtle muscle translational products.

Autoradiograph showing protein profiles on an SDS-polyacrylamide (8 - 15 %) gel of *in vitro* translation, in a wheat germ lysate, of 1.0 μg of poly(A)⁺ RNA samples isolated from white muscle of anoxic (*lane 1*) and control (*lane 2*) turtles and from red muscle of anoxic (*lane 3*) and control (*lane 4*) turtles. The sample load for all lanes was approximately 1×10^4 acid precipitable counts per minute. Fluorography time was 120 hours. Molecular weights are indicated on the left. Arrowheads indicate bands of approximately 40.0, 32.0 and 28.2 kDa which are absent in the anoxic red muscle.

Figure 13. Densitometry traces of muscle fluorogram (Fig. 12).

Units on the intensity scale are arbitrary. All traces were scanned from the same gel and under identical conditions. Peaks corresponding to optically dense bands are shown. WC: control white muscle; WA: anoxic white muscle; RC: control red muscle; RA: anoxic red muscle.

(a) Peaks representing polypeptides that are absent or enhanced in anoxic turtle red muscle (*lane 3, Fig. 12*) are indicated by arrowheads with their corresponding molecular weights.

(b) Optical density traces between control (*lane 2, Fig. 12*) and anoxic (*lane 1, Fig. 12*) turtle white muscle.

(a)

(b)

Figure 14. SDS-PAGE analysis of turtle heart translational products.

Autoradiograph showing protein profiles on an SDS-polyacrylamide (8 - 15 %) gel of *in vitro* translation, in a wheat germ lysate, of 1.0 μg poly(A)⁺ RNA isolated from heart of control (*lane 1*), anoxic (*lane 2*) and recovered (*lane 3*) turtles. The sample load for all lanes was approximately 1×10^4 acid precipitable counts per minute. Fluorography time was 120 hours. Molecular weights are indicated on the left. The Arrowhead indicates a new or enhanced band at approximately 26.8 kDa present in the anoxic turtle heart.

Figure 15. Densitometry traces of turtle heart fluorogram (Fig. 14).

Units on the intensity scale are arbitrary. All traces were scanned from the same gel and under identical conditions. Peaks corresponding to optically dense bands are shown. **HC:** control heart; **HA:** anoxic heart; **HR:** recovered heart.

(a) Optical density traces of control (*lane 1, Fig. 14*) and anoxic (*lane 2, Fig. 14*) heart. The arrowhead indicates a new or enhanced band at approximately 26.8 kDa present in the anoxic turtle heart.

(b) Optical density traces of control, anoxic and recovered (*lane 3, Fig. 14*) turtle heart. The arrowhead indicates a new or enhanced band at approximately 26.8 kDa in the anoxic turtle heart.

(b)

Figure 16. SDS-PAGE analysis of turtle kidney translational products.

Autoradiograph showing protein profiles on an SDS-polyacrylamide (8 - 15 %) gel of *in vitro* translation, in a wheat germ lysate, of 1.0 μg poly(A)⁺ RNA isolated from kidney of control (*lane 3*), anoxic (*lane 2*), and recovered (*lane 1*) turtles. The sample load for all lanes was approximately 1×10^4 acid precipitable counts per minute. Fluorography time was 120 hours. Molecular weights are indicated on the left. The arrowhead indicates a band at approximately 32.8 kDa only present in the recovered turtle kidney.

Figure 17. Densitometry traces of turtle kidney fluorogram (Fig. 16).

Units on the intensity scale are arbitrary. All traces were scanned from the same gel and under the same conditions. Peaks corresponding to optically dense bands are shown. **KC:** control kidney; **KA:** anoxic kidney; **KR:** recovered kidney.

(a) Optical density traces of kidney from control (*lane 3, Fig. 16*) and anoxic (*lane 2, Fig. 16*) turtles. The arrowhead indicates a band at approximately 75.8 kDa absent in the anoxic turtle kidney and a band of 104.6 kDa significantly reduced in the anoxic kidney.

(b) Optical density traces of kidney from anoxic (*lane 2, Fig. 16*) and recovered (*lane 1, Fig. 16*) turtles. The arrowhead indicates a band at approximately 32.8 kDa that is only seen in the recovered turtle kidney, and a band of approximately 104.5 kDa that is significantly reduced in the anoxic kidney.

(c) Optical density traces of kidney from control (*lane 3, Fig. 16*) and recovered (*lane 1, Fig. 16*) turtles. Arrowheads indicate a band at approximately 32.8 kDa that is only present in the recovered turtle kidney and a band at approximately 75.8 kDa that is present only in the control turtle kidney.

(a)

(b)

(c)

Figure 18. SDS-PAGE analysis of total proteins.

Analysis of total protein in crude homogenates by SDS-polyacrylamide (8 - 15 %) gels. Total protein (90 μ g, as determined by the BioRad standard protein assay) from control, anoxic and recovered turtle liver (*lanes 1 - 3*, respectively); control, anoxic and recovered turtle red muscle (*lanes 4 - 6*, respectively); and control, anoxic and recovered turtle white muscle (*lanes 7 - 8*, respectively) are shown. The gel was stained with Coomassie blue as described in methods and materials, then photographed.

Figure 19. Western transfer of total proteins.

Total proteins (20 μ g, according to the BioRad standard protein assay) were separated on 10 % SDS-polyacrylamide gel using the BioRad Miniprotean II system. Nitrocellulose filters were screened with monoclonal antibody MA3-001 which recognizes several members of the hsp70 gene family including hsp70, 72, 68, and cognate proteins, as well as the glucose responsive protein (grp) 78.

(a) *Lanes 1 - 3*: control, anoxic and recovered liver, respectively; *lanes 4 - 6*: control, anoxic and recovered red muscle, respectively; *lanes 7 - 9*: control, anoxic and recovered white muscle, respectively; *lane 10*: control lane of total protein from *E. coli* cells which express the inducible, cognate and glucose responsive proteins.

(b) *Lanes 1 - 3*: control, anoxic and recovered heart, respectively; *lanes 4 - 6*: control, anoxic and recovered kidney, respectively; *lane 7*: control lane of total protein from *E. coli* cells which express the inducible, cognate and glucose responsive proteins.

(a)

(b)

DISCUSSION AND CONCLUSIONS

Methods of total RNA isolation

A fundamental step in the investigation of the induction or repression of specific mRNA sequences is a suitable method for the isolation of total RNA. Because of the abundance of *T. s. elegans*' liver and red muscle, these tissues were sampled from control animals and processed for RNA isolation using both the guanidine HCl/CsCl ultracentrifugation procedure and the modified hot phenol procedure as outlined in the methods and materials.

Table 1 demonstrates the high yield and purity (based on OD₂₆₀/OD₂₈₀ ratios) of total RNA isolated by the modified hot phenol procedure from various liver and red muscle samples. Both the yield and purity were similar to those obtained by the guanidine hydrochloride/CsCl ultracentrifugation procedure. Both isolation procedures yielded similar patterns on agarose (0.1 %) gel electrophoresis, visualized with ethidium bromide (Fig. 5a,b). The modified hot phenol procedure used in this study relies on well established principles of RNA isolation (Socero and Darnell, 1969). Its success depended on the complete pulverization of the frozen tissue at the temperature of liquid nitrogen followed by rapid homogenization and organic extraction, the effectiveness of which may be related to minimizing the exposure of nuclear-derived RNA with cytosolic RNases before complete enzymatic destruction. Of additional importance is the use of phenol, a partial ribonuclease inhibitor as well as a protein denaturant, to successfully minimize protein contamination and dissociate nucleoproteins into nucleic acid and protein moieties. Unlike the guanidine HCl/CsCl ultracentrifugation procedure, the modified hot phenol method did not escape DNA contamination as evidenced by the presence of visible high molecular weight DNA above the ribosomal bands (Fig. 5b). Because the purine bases in DNA also absorb at

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260 nm, this effect was often coupled with high ratios of A_{260}/A_{280} (data not shown). DNA contamination by this method was a consequence of the nuclear rupture caused by the homogenization performed in the presence of SDS. However, contaminating DNA was readily removed by additional high salt precipitation or by reducing the pH of the aqueous phase to pH 5.0 thus driving the DNA into the organic phase.

Compared with CsCl ultracentrifugation, the modified hot phenol procedure enabled the isolation of total RNA from many more samples in less time and with similar yield and purity. The isolation procedure (up to ethanol precipitation) may be completed in approximately two hours. The CsCl ultracentrifugation procedure typically required at least 16 hours of processing. In addition, the modified hot phenol procedure does not require the use of an ultracentrifuge. The technique described here is rapid, reproducible, and more importantly, results in relatively undegraded and pure RNA. As such, the modified hot phenol procedure was the method of choice for the isolation of intact total RNA.

Levels of total RNA

High-quality, undegraded, and uncontaminated total RNA was prepared using the modified hot phenol procedure. The levels of total RNA extracted from 1g of liver, red muscle, white muscle, heart and kidney from control, aerobic turtles were compared with those from turtles that had been previously exposed to 16 h of anoxic submergence in N_2 -bubbled water. In the case of heart and kidney, a third condition was also examined; turtles allowed to recover from anoxia for 20 h. The amounts of total extracted RNA were not significantly different between tissues sampled from control animals and those sampled from treatment groups (Fig. 3). However, the biological significance, if any, of this is unclear. It should be noted that all cellular

RNAs (e.g. rRNA, tRNA, mRNA) are present in the phenol-extraction preparation. Ribosomal RNA and transfer RNA comprise 80 % and 15 %, respectively, of total cellular RNA with half-lives on the order of days. Messenger RNA, on the other hand, claims less than 5 % of total cellular RNA and is considerably less stable with a half-life on the order of minutes. As such, it is likely that any changes in the levels of mRNA, in response to a stress, would be masked by the much larger and more stable tRNA and rRNA populations. Hence, it is not surprising that no changes in total tissue RNA were found in control versus anoxic turtles.

Levels of mRNA

Most eukaryotic mRNAs have a sequence of polyadenylic acid at the 3' end. This terminal stretch of A residues is often described as the poly(A) tail; and mRNA with this feature is denoted poly(A)⁺ RNA. The poly(A) sequence is not coded in the DNA, but is added to the RNA in the nucleus after transcription. By virtue of their poly(A) tails, mRNA was purified from total RNA by affinity chromatography on oligo(dT) cellulose, thus allowing for a more direct measurement of the levels of mRNA between normoxic and treatment groups. **Figure 6** illustrates the purity of nondegraded poly(A)⁺ RNA isolated by this technique. There were no significant differences in the levels of poly(A)⁺ RNA between normoxic and treatment groups (**Fig. 8**) with the exception of white muscle. The amount of poly(A)⁺ RNA purified from 1g of white muscle from anoxic turtles was significantly greater than the level purified from its control counterpart. White muscle exhibits low metabolic activity under normoxic conditions, however, during times of oxidative stress, white muscle is called upon for the generation of ATP via anaerobic glycolysis. The higher glycolytic potential of white muscle is a consequence of the unusually high titre of glycolytic

enzymes in this tissue (Hochachka, 1976). Because white muscle is metabolically active during episodes of oxygen limitation, it is not surprising that anoxia induced elevated transcription and hence greater levels of mRNA in this tissue. The greater level of mRNA observed in this tissue as a result of the transition into anoxia is evidence for an element of control at the level of transcription *in vivo*.

Levels of translatable mRNA

The ability to translate mRNA *in vitro* provides a crucial assay in defining the sites on mRNA that are recognized by ribosomes. *In vitro* systems provide the only approach for defining the mechanism of either translation or transcription. In both cases, the critical step in expression occurs at initiation. The levels of *in vitro* translatable mRNA in turtle tissues from control compared with those from treatment groups was evaluated using the translational machinery provided by a wheat germ cell free system. This method is useful because, while it does not reveal amounts of specific mRNA species, it does provide a relative index that can be used to compare messenger pools in the various tissues experiencing different physiological conditions (Hershey *et al.*, 1986). The results presented here are quite interesting in that the tissues that did not show altered levels of mRNA pools as a consequence of the transition into anoxia, did exhibit changes within the mRNA pools in terms of levels of mRNA capable of acting as functional templates to direct protein synthesis *in vitro*. For example, the transition into anoxia resulted in higher levels of *in vitro* translatable mRNA in liver and kidney while lower levels of translatable mRNA were observed in red muscle. Translational control must take the form of preventing mRNAs from reaching the protein-synthesizing apparatus. This may be achieved by sequestering them in a form in which they are physically unavailable. The inhibition of mRNA

translation *in vivo* by their complex formation with messenger ribonucleoproteins (mRNPs) is a common mechanism by which mRNAs are stored and stabilized during quiescent states for later use (Rosenthal *et al*, 1980). However, the method of RNA isolation used in this study liberated mRNP particles from the nucleic acid fractions. Thus any mRNA masking by association with mRNPs could not be observed *in vitro* since removal of mRNPs would render these mRNAs active *in vitro*. The results of this particular study do, however, provide evidence that the transition into anoxia alters the conditions *in vivo* that act on an mRNA template. These changes can render an mRNA more or less efficient in terms of its availability as a functional template *in vitro*. The more efficient mRNAs would be preferentially translated *in vitro* at the expense of less efficient messengers.

It is interesting to note that the transition into anoxia did not alter the levels of translatable mRNA *in vitro* for white muscle (Fig.9a) despite the increased levels of the total mRNA pool. Although this result seems unusual, it can be argued that oxidative stress to white muscle is generally in the form of burst exercise, where there is a demand for a large ATP reserve to support working muscle. It is tempting to speculate that oxidative stress in the form of anoxia imposed by submergence is not stressful to white muscle. Under such circumstances, it is not surprising that the mRNA of white muscle from anoxic turtles did not exhibit changes in terms of levels of translatable mRNA. These results support the idea that the increased levels of mRNA in white muscle resulting from the transition to anoxia by submergence may indeed be an early molecular response to oxygen deprivation preparing white muscle for burst exercise if the need should arise.

Qualitative Analysis of mRNA

In addition to the quantitative results obtained in this investigation, *in vitro* translations demonstrated that the mRNAs from anoxic tissues produce a polypeptide pattern which is different from that produced by translating mRNAs from control tissues (except in the case of white muscle). The transition into anoxia induced mRNA in liver, heart and kidney that translated *in vitro* into protein products of approximately 27 kDa. As well, a novel mRNA found in anoxic liver, translated into a 20 kDa protein product *in vitro*. Very little is known about the low molecular weight stress proteins in terms of their roles in stressed and normal cells. However, hsp27 has been shown to be rapidly phosphorylated in response to heat or treatment of cultured cells with phorbol esters or calcium ionophores (Welch, 1985) and it has been suggested that such phosphorylation of hsp27 may represent a rapid protective response to stress which precedes enhanced synthesis of all the heat shock proteins, including hsp27.

In this study, the transition into anoxia also induced higher levels of mRNA coding for a 79.9 kDa protein product *in vitro* for liver. It is highly possible that the higher expression of certain mRNAs as a result of the transition to anoxia is related to their capacity to tolerate anoxia. This expression may reflect the initial reactions necessary to activate the specific cellular mechanisms required in conferring anoxia tolerance. Recently, a study investigating *de novo* protein synthesis in *T. s. elegans* identified a 72 kDa protein in liver and skeletal muscle which appeared only during recovery. This mRNA may represent the *in vivo* 72 kDa protein found in recovered liver. Although anoxia induced this mRNA, the mRNA could be masked from the translational machinery *in vivo* until it is called upon during aerobic recovery where the protein product may be involved in preventing or repairing cellular damage during re-oxygenation. Studies of protein degradation after re-oxygenation of oxygen-deprived

tissues have shown that the production of oxygen radicals during re-oxygenation can damage (Stadtman, 1990) and degrade proteins (Narasimham *et al.*, 1991). Although this effect should be short-lived, lasting only for as long as it takes for oxygen radicals to form and be degraded, the effects of oxygen radical damage may be long lasting, especially in poikilotherms which have low metabolic rates. Thus, the *in vivo* protein product of this particular mRNA may play a role similar to the srp70 proteins of mammals by stabilizing protein structure and preventing loss of activity in stressed cells.

In red muscle, the transition into anoxia resulted in the disappearance of certain mRNAs. This suggests that during anoxic exposure, some of the mRNAs normal present in this tissue are degraded, inactivated, or in a nontranslatable form. The transition into anoxia also resulted in a novel mRNA coding for a 37.5 kDa protein product *in vitro*. This mRNA may be represented by the chaperonins. Chaperonins are largely mitochondrial and are known to be induced by various stresses. They are one of the major stress protein families known to participate in the proper folding and translocation of proteins. Red muscle exhibits the classic stress response in that certain (normal) mRNAs are prohibited from the translational apparatus while others are preferentially translated.

The transition into anoxia resulted in no changes in the *in vitro* protein profile of mRNAs from white muscle. This further supports the idea that anoxia may not be a stress *per se* for white muscle.

From these results we conclude that anoxic exposure in *T. s. elegans* triggers a rapid cellular response by modifying the translatable mRNA population. *In vitro* translation of the mRNA pools from tissues of anoxic turtles produced a polypeptide pattern which was quite different from that produced by translating mRNAs from

tissues of normoxic turtles. The occurrence of novel or enhanced transcripts in an *in vitro* translational assay is evidence for an element of transcription, although control at the level of mRNA stability cannot be ruled out.

Due to the evolutionary conservation and the ubiquitous nature of stress proteins, it was of interest to examine the universality of the stress response in *T. s. elegans*. Immunoblot analysis revealed that *T. s. elegans* express proteins immunologically related to the *Drosophila* hsc70 and grp70. The transition into anoxia did not lead to the expression of proteins immunologically related to the inducible isoform of *Drosophila* hsp70. This may be an effect related to the animal's natural tolerance to anoxia. Since anoxia is not a stress *per se* for this animal, it is not unusual that the inducible isoform of hsp70 was not induced by anoxia. From, this it can be concluded that the induction of this stress protein does not play a role in natural tolerance to anoxia in *T. s. elegans*.

Overall, the turtle must have complex cellular control which enables it to detect limited oxygen supply and adjust its metabolism accordingly. The elucidation at the molecular level beginning with signal recognition and ending with the selective activation of genes will enhance our understanding of gene regulation during anoxia in turtles.

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