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TRANSLATIONAL CONTROL OF PROTEIN SYNTHESIS IN HEAT-SHOCKED MAIZE SEEDLINGS

by

Nora Catherine Hogan

Department of Zoology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario

July, 1989

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ABSTRACT

Translational control of protein synthesis in the plumules of heat-shocked maize seedlings was investigated. Maize plumules respond to heat shock by synthesizing nuclear-encoded hsp's with M_r's of 108, 89, 84, 76, 73, and 17-29kDa. Synthesis of some, but not all, 25°C (control) proteins is repressed in vivo. RNA blot and in vitro translation analyses of non-polyribosomal and polyribosomal RNP RNA suggest that repression ensues from either a decrease in mRNA levels or from the inefficient translation of control messages in heat-shocked cells (relative to heat shock mRNAs) due to changes in the rates of both initiation and elongation.

Hsp70 and hsp18 mRNAs (or heat shock-like mRNAs) are synthesized at low levels in nonstressed cells. During heat shock, these messages accumulate on polyribosomes within 5 to 10 minutes, are maximal within 1 to 2h and decline therafter (ie. maize plumules acclimate). The recovery profile is similar. Dissociation of message from the polyribosomes under these conditions is not accompanied by a re-association of heat shock mRNAs with non-polyribosomal RNPs suggesting that 1) the messages released from the polyribosomes are degraded and 2) the mechanism(s) governing these responses is independent of the incubation temperature. Acclimation and recovery are not characterized by a complete return to the control state as low molecular weight heat shock mRNAs continue to associate with the polyribosomal and non-polyribosomal fractions.

Heat shock and control mRNAs are differentially distributed in the non-polyribosomal RNP of control cells. However, messages active in translation at 25°C are primarily associated with cytoskeletally-associated polyribosomes. Heat shock results in a change in both the distribution of heat shock mRNAs on the ribosomes of polyribosomes and their non-polyribosomal to polyribosomal ratios. Heat shock mRNAs accumulate primarily in the free-cytoplasmic non-polyribosomal and polyribosomal fractions suggesting that translation and possibly transport of these messages during heat shock is

largely independent of the cytoskeleton. The negligible changes in the equilibrium of membrane-derived non-polyribosomal fractions together with the accumulation of mRNAs destined for the endoplasmic reticulum in free-cytoplasmic non-polyribosomal RNP are consistent with this interpretation. Neither the distribution of 25°C mRNAs on polyribosomes nor their equilibrium between non-polyribosomal and polyribosomal RNP's is affected by the temperature shift.

The kinetics of association/dissociation of mRNAs with the non-polyribosomal and polyribosomal RNP indicate that heat shock messages associate with non-polyribosomal RNP prior to their integration into polyribosomes.

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LIST OF ABBREVIATIONS

ATP	adenosine triphospate
ie.	(id est) that is to say
et al	(et alii) and others
hsp's	heat shock proteins
hsc	heat shock cognate
eIF-2	eucaryotic initiation factor 2
r-protein	ribosomal protein
p72	protein 72
grp	glucose regulated protein
htp	high temperature production
tk	thymidine kinase
HSE	heat shock element
HSTF	heat shock transcription factor
CTF	CCAAT-binding transcription factor
DNA	deoxyribonucleic acid
ssDNA	salmon sperm deoxyribonucleic acid
RNA	ribonucleic acid
pre-mRNA	precursor messenger ribonucleic acid
mRNA	messenger ribonucleic acid
RNP	ribonucleoprotein
hnRNP	heterogeneous nuclear ribonucleoprotein
snRNP	small nuclear ribonucleoprotein
HSG's	heat shock granules
Gmhsp 26-A	Glycine max heat shock protein 26-A
E. coli	Escherichia coli
S. cerevisiae	Saccharomyces cerevisiae
C. elegans	Caenorhabditus elegans
CEF	chicken embryo fibroblast
cv	cultivar
LTM	low temperature melting
mAP	messenger affinity paper
kDa	kilodalton
g	grams
cpm	counts per minute
rpm	revolutions per minute
xg	acceleration of gravity
UV	ultraviolet
M_r	relative molecular mass
ER	endoplasmic reticulum
FC	free-cytoplasmic
MA	membrane-associated
CSK	cytoskeleton
PSII	photosystem II
SDB	sample dilution buffer
RB	resuspension buffer
RSB	reticulocyte standard buffer
FSB	freezing salt buffer
1-D	one-dimensional

2-D	two-dimensional
IEF	isoelectric focusing
pI	isoelectric point
As	arsenic
Cd	cadmium
BSA	bovine serum albumin
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDTA	ethylene dinitilo-tetraacetic acid, disodium salt
EtBr	ethidium bromide
HEPES	4-2-(hydroxyethyl)-1-piperazine ethanesulphonic acid
Mg-acetate	magnesium acetate
Na-acetate	sodium acetate
NP-40	nonidet-40
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PPO	2, 5 diphenyloxazole
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TE	tris ethylene dinitilo-tetraacetic acid

CHAPTER 1

THE HEAT SHOCK RESPONSE

1.1 General Introduction

Eucaryotic cells respond to a transient stress such as heat shock by inducing the synthesis of a specific set of proteins, the heat shock proteins (hsp's). This physiological response to environmental extremes has been found in all animals, plants, and bacteria. Its ubiquity suggests that the response is an important homeostatic mechanism that enables the cell to survive a variety of stresses (Craig, 1985; Lindquist, 1986).

The heat shock response is essentially a single cell response but is mediated by several factors including the organisms environment. In addition to hsp synthesis, the cellular response to heat shock comprises several reversible changes in the pattern of gene expression and other cellular metabolic activities/structures (Schlesinger et al, 1982; Nover, 1984; Atkinson and Walden, 1985; Pardue et al, 1989). These include: 1) reprogramming of gene transcription by RNA polymerase II (Spradling et al, 1977; Findly and Pederson, 1981), 2) cessation of synthesis of most other mRNAs, RNAs encoding histones and mitochondrial proteins being exceptions (Ritossa, 1964; Berendes, 1968; Bonner and Pardue, 1976), 3) disruption of RNA processing (Ellgaard and Clever, 1971; Lengyel and Pardue, 1975; Rubin and Hogness, 1975; Yost and Lindquist, 1986), 4) induction of noncoding RNAs (Hallberg and Hallberg, 1989), 5) reprogramming of the translational machinery (Storti et al, 1980; Kruger and Benecke, 1981; Scharf and Nover, 1982; Howe and Hershey, 1984; Panniers et al, 1985), 6) rearrangements of the cytoskeleton and other cellular components (Biessmann et al, 1982; Tanguay and Vincent, 1982; Colliers and Schlesinger, 1986; Sherwood et al, 1989), and 7) changes in protein turnover (Schlesinger et al, 1989; Pratt et al, 1989).

The hsp's can generally be classified into three families based upon structural homologies: 1) hsp90, a consititutively synthesized, heat-inducible hsp, 2) hsp70, the

most highly conserved hsp, and 3) the low molecular weight hsp's (Craig, 1985; Lindquist, 1986). Several additional proteins are known to be heat-inducible, the more important of which include a nuclear-encoded mitochondrial protein required for the assembly of mitochondrially-imported proteins into oligomeric complexes (McMullin and Hallberg, 1987; McMullin and Hallberg, 1988; Hemmingsen et al, 1988; Cheng et al, 1989) and ubiquitin (Bond and Schlesinger, 1985; Bond and Schlesinger, 1986; Finley et al, 1987; Atkinson et al, 1989). It is now apparent that many of the hsp's or related proteins are also expressed in nonstressed cells/tissues (Welch, 1985; Catelli et al, 1985; Sanchez et al, 1985; Dechaies et al, 1988; Chirico et al, 1988; Arrigo et al, 1988a).

A variety of other chemical, physical, and biological stresses can also induce hsp synthesis. The synthesis of similar proteins in response to widely divergent stimuli suggests that some common cellular metabolic signal is responsible for the induction of hsp's (Hightower, 1980). Several mechanisms have been proposed including oxidative stress (Varshavsky, 1983; Lee et al, 1983) and the accumulation and/or catabolism of abnormal intracellular proteins (Hightower, 1980; Hiromi and Hotta, 1985; Hightower et al, 1985; Ananthan et al, 1986). The latter mechanism has been recently subsumed in a ubiquitination-mediated induction pathway (Munro and Pelham, 1985).

1.2 Transcription and Translation of Heat Shock Proteins

Transcriptional activation of the heat shock genes requires both cis- and trans-activating elements. Early studies using various gene constructs that linked a *Drosophila* hsp70 promoter to the transcription units of other proteins demonstrated that this promoter sequence functioned efficiently in heterologous systems suggesting that the factor(s) involved in the regulation of heat shock transcription was(were) highly conserved. Deletion analysis mapped the activation site to a consensus region located (on the *Drosophila* hsp70 genes) between 47 and 66 base pairs (bp) upstream of transcription initiation (Pelham, 1982; Pelham and Bienz, 1982). A single conserved sequence within

this region -- CTGGAAT-TTCTAGA, later simplified to C--GAA--TTC--G and referred to as the heat shock element (HSE) -- was found to confer '·.at inducibility on the herpes simplex virus thymidine kinase (tk) gene (Pelham and Bienz, 1982).

Although a single consensus sequence can confer heat-inducible transcription on exogenous genes in heterologous systems, many of the heat shock genes, including those in plants, contain multiple HSE's (Schoffl et al, 1984; Czarnecka et al, 1985; Nagao et al, 1985; Bienz and Pelham, 1987). The most proximal HSE is located 1.5 helical turns (15-18bp) upstream of the TATA box (Bienz and Pelham, 1987). In general, the remaining HSE's are located at variable distances 5' to the proximal HSE and are spatially arranged so that the centers of symmetry are located on the same side of the DNA helix.

Expression assays have established that DNA sequences matching the core HSE in seven of the eight nucleotides constitute transcriptionally functional HSE's and bind a heat shock activated factor (heat shock transcription factor (HSTF)-see below)(Bienz and Pelham, 1987). However, a 6 of 10 match can function when additional copies are present (Pelham and Bienz, 1982; Ayme et al, 1985; Bienz, 1985). Moreover, the proximity of the HSE to the TATA box, the accessibility of HSTF to the HSE, and the affinity of HSTF for the HSE also determine the degree of transcriptional activation (Pelham, 1985; Bienz, 1985; Gurley et al, 1986; Pauli et al, 1986). In more recent studies, contiguous arrays of a 5bp unit sequence (-GAA-) arranged in alternate orientations have been shown to constitute functional HSE's (Amin et al, 1988; Lis et al, 1989).

Other cis-acting regulatory elements that are required for transcriptional activation of the heat shock genes are the TATA motif and/or the CCAAT motif (regulatory elements commonly associated with RNA polymerase II-activated promoters). CCAAT boxes or SP1 binding sites are often found along with multiple HSE's in vertebrate genes when the proximal HSE is located far upstream from the TATA box (Bienz and Pelham, 1982; Wu et al, 1986). The presence of these promoter elements allows the HSE's to act as an enhancer (Bienz and Pelham, 1987).

The heat shock transcription factor (HSTF) that interacts with the HSE has been identified in yeast (Sorger et al, 1987; Sorger and Pelham, 1987a; Weiderrecht et al, 1987), Drosophila melanogaster (Parker and Topol, 1984; Topol et al, 1985; Wu, 1984a; Wu et al, 1987; Zimarino and Wu, 1987), and human HeLa (Kingston et al, 1987; Sorger et al, 1987) cells. The factor has been purified recently from Drosophila and yeast and has a relative molecular mass (M_r) of 70 and/or 110kDa (Wu et al, 1987; Wiederrecht et al, 1987). HSE-binding activity, which appears rapidly in heat-shocked cells, is not blocked by the addition of protein synthesis inhibitors (Ashburner and Bonner, 1979; Kingston et al, 1987; Zimarino and Wu, 1987) suggesting that HSTF is present in nonstressed cells in an inactivated form and/or sequestered in a subcellular compartment. Factors exhibiting HSE-binding activity have been detected in nonstressed yeast (Sorger et al, 1987; Sorger and Pelham, 1987a) and Drosophila (Wiederrecht et al, 1987; Wu et al, 1987) cells. In yeast, the HSTF extracted from heat-shocked cells appears to be more extensively phosphorylated than the HSTF extracted from control cells. It has been suggested that transcriptional activation is a consequence of heat-induced phosphorylation of HSTF (Sorger et al, 1987; Jakobsen and Pelham, 1988).

Mosser et al (1988) have shown that this activity is also present in nonstressed human cells. In these cells, the kinetics of heat shock-induced HSE-binding activity during heat shock and recovery from heat shock parallel the kinetics of hsp70 gene transcription. Concomittant with the decline in heat-induced HSE-binding activity during recovery is an increase in control HSE-binding activity (Mosser et al, 1988). Also of interest in these studies is the observation that differential induction of hsp70 genes by different stressors is reflected in the differential levels of stress-induced HSTF.

DNase I protection assays have demonstrated that a second protein factor binds constitutively to the TATA box region in Drosophila (Wu, 1984a) and human (Wu et al, 1987) cells. This factor protects a region extending from the TATA box to position +30 (Parker and Topol, 1984; Wu, 1984a; Wu, 1985) and may, therefore, interact with

sequences downstream from the transcription start site (5' untranslated leader sequence?). The factor alone is not sufficient to activate transcription of the heat shock genes (Wu, 1984a).

In vertebrates, a third factor -- the CCAAT-binding transcription factor (CTF)(Jones et al, 1985) -- is required for the heat-induced activation of vertebrate promoters containing multiple distance HSE's (Hickey et al, 1986; Bienz and Pelham, 1987; Morgan et al, 1987).

Chromatin mapping studies demonstrate that the 5' ends of Drosophila heat shock genes are hypersensitive to DNase I (Wu, 1980) while the body of the gene exists in an ordered nucleosome array (Dietz et al, 1989). Induction of the heat shock genes is correlated with a change in the chromatin structure. The transcribed region becomes accessible to DNase I and the regular nucleosome pattern becomes indistinct (Wu, 1980; Dietz et al, 1989). Binding of HSTF correlates positively with these changes as regions centered around the HSE become refractory to digestion (Wu, 1984a). It has been suggested that binding of the TATA box factor and/or a specifically positioned nucleosome generate the upstream DNase I hypersensitive sites leaving the HSE's accessible. The folding of the DNA in the nucleosome would juxtapose distant HSE's thereby facilitating gene activation (Dietz et al, 1989). Alternatively, CTF may serve to anchor distantly bound HSTF dimers next to the TATA factor (Bienz and Pelham, 1987).

Although transcription of most RNA species is repressed during heat shock (Findly and Pederson, 1981), some transcripts continue to be synthesized at near normal rates including ribosomal, histone, and viral RNAs in Drosophila and plants (Spradling et al, 1977; Scott et al, 1980; Scharf and Nover, 1987). At temperatures above 34°C, the processing of ribosomal RNA precursors is interrupted resulting in the cessation of ribosome synthesis in Drosophila (Ellgaard and Clever, 1971; Lengyel and Pardue, 1975; Rubin and Hogness, 1975; Spradling et al, 1977). Heat shock has since been shown to disrupt mRNA splicing in yeast (Yost and Lindquist, 1988), Drosophila (Yost and

Lindquist, 1986; Yost and Lindquist, 1988), avian (Bond and Schlesinger, 1986), and mammalian (Kay et al, 1987) cells. In Drosophila, the RNAs accumulating (hsp83 and adh transcripts) after a severe heat shock have normal transcription start sites and are uncut at both their 5' and 3' splice junctions indicating that the heat-induced block occurs prior to the first catalytic reaction in the splicing pathway (Yost and Lindquist, 1986; Yost and Lindquist, 1988). Some of these unspliced transcripts exit the nucleus and enter the pool of cytoplasmic mRNAs. Translation of these mRNAs yields truncated proteins. Pretreatment at temperatures that induce hsp's obviates the block in processing. Moreover, the degree of protection varies with the duration of the pretreatment and, therefore, with the level of hsp accumulation (Yost and Lindquist, 1986). In contrast, heat shock has little effect on RNA processing in plants (Czarnecka et al, 1988). However, both cadmium and copper, heavy metals which induce hsp's in soybean, disrupt processing of Glycine max hsp 26-A (Gmhsp 26-A) RNAs in a manner analogous to that observed in Drosophila. Whether inhibition of splicing in plants is restricted to a specific class of heat shock transcripts or whether, like Drosophila, it is a general phenomenon is not known (Czarnecka et al, 1988).

Although some aberrant transcripts escape the nucleo-cytoplasmic block, inhibition of splicing during heat shock is thought to prevent the accumulation of high levels of intron-containing transcripts in the cytoplasm and the subsequent synthesis of aberrant proteins that may be detrimental to the cell. In Drosophila, carboxy terminal deletions of hsp70 have the dominant phenotype of prolonging the time required for the cell to recover from heat shock (Yost and Lindquist, 1988). The transcripts of most heat-inducible genes escape this block by virtue of the fact that they lack introns.

Although the precise nature of the disruption is unknown, Mayrand and Pederson (1983) have suggested that heat shock disrupts heterogeneous nuclear ribonucleoprotein (hnRNP) particle assembly thus leading to abortive processing of precursor mRNAs. However, other reports indicate that RNP particles are not disrupted (Kloetzel and Bautz,

1983). In a more recent study, heat shock has been shown to disrupt a subset of small nuclear ribonucleoproteins (snRNP's)(Bond, 1986). Alterations in these particles are accompanied by the assembly of labeled pre-mRNA transcripts into aberrant splicing complexes in vitro (Bond, 1986).

In addition to the alterations in transcription and RNA processing, heat shock also induces a major change in the pattern of translation. In Drosophila, translation of normal cellular messages ceases within minutes of a shift from 25°C to 37°C. That this transition does not involve a general inhibition of protein synthesis is demonstrated by the fact that heat shock messages, when they appear, are translated at very high rates (Linquist, 1980). Furthermore, pre-existing messages appear to be sequestered as a group in the cell and can be recruited into active protein synthesis following a relief from the stress condition (Mirault et al, 1978; Storti et al, 1980; Lindquist, 1981; DiDomenico et al, 1982a; DiDomenico et al, 1982b; Kruger and Benecke, 1981). In contrast, heat shock results in the rapid degradation of pre-existing mRNAs in yeast (Lindquist, 1981). In plant cells, the majority of control mRNAs are sequestered during heat shock as in Drosophila (Nover and Scharf, 1984; Key et al, 1985). However, some mRNAs continue to be synthesized during heat shock (Cooper and Ho, 1983; Key et al, 1983; Scharf and Nover, 1987) while others are degraded as in yeast (Nover and Scharf, 1984; Belanger et al, 1986).

The molecular mechanisms governing translational control remain to be established. In Drosophila (Falkner et al, 1981; Biessmann et al, 1982; Walter and Biessmann, 1987) and vertebrate (Collier and Schlesinger, 1986; Welch and Suhan, 1986) cells, heat shock results in the collapse of the intermediate-sized filament system in the perinuclear region. Disruption and fragmentation of both the Golgi complex and endoplasmic reticulum (ER) have also been reported in plant and animal cells (Welch and Suhan, 1986; Belanger et al, 1986). It has, therefore, been suggested that translation of heat shock messages proceeds largely independent of the cytoskeleton on free polyribosomes (Falkner et al, 1981; Nover, 1984; Welch and Feramisco, 1985)

After heat shock, there is a preferential utilization of heat shock mRNAs. Cell free lysates prepared from heat-shocked Drosophila cells translate only heat shock mRNAs while lysates from nonheat-shocked cells translate both control and heat shock mRNAs suggesting discrimination is based on a signal contained within the RNA sequence and/or RNA conformation (Storti et al, 1980; Kruger and Benecke, 1981; Scott and Pardue, 1981). More recent studies have demonstrated that the signals effecting this change are contained within the 5' untranslated leader sequence of heat shock mRNAs (McGarry and Lindquist, 1985; Klemenz et al, 1985; Hultmark et al, 1986).

The mechanism by which heat shock alters the protein synthetic apparatus is unclear. However, the restoration of translation of 25°C messages in heat-shocked cell lysates by a crude ribosomal preparation isolated from nonheat-shocked cells suggests that components normally associated with the ribosomes are inactivated by heat shock (Scott and Pardue, 1981). In many systems, inhibition of protein synthesis during heat shock has been attributed to changes in the rate of polypeptide chain initiation (McKenzie et al, 1975; Panniers and Henshaw, 1984). However, the subcellular distribution of translationally inhibited messages in Drosophila suggests blocks at both initiation and elongation (Kruger and Benecke, 1981; Ballinger and Pardue, 1983). Whatever the step in protein synthesis, modifications and/or changes in the ribosome-protein/RNA composition have been reported in heat-shocked HeLa (Duncan and Hershey, 1984), Ehrlich (Panniers and Henshaw, 1984), Drosophila (Glover, 1982), plant (Scharf and Nover, 1982), and Tetrahymena (McMullin and Hallberg, 1986; Kraus et al, 1987) cells.

In HeLa cells, eucaryotic initiation factor-2 (eIF-2), eIF-4 and either one or both eIF-3 and eIF-4F are inhibited by heat shock. Furthermore, phosphorylation of eIF-2 increases while that of eIF-4F decreases following heat shock (Duncan and Hershey, 1984).

In heat-shocked Ehrlich ascites tumor cells, a reduction in the level of 40S initiation complexes is accompanied by an inhibition of polypeptide chain initiation (Panniers and

Henshaw, 1984). Addition of highly purified eIF-4F, an initiation factor which specifically binds mRNA cap structures, to lysates prepared from heat-shocked Ehrlich cells rescues translation of nonheat-shocked mRNAs (Panniers et al, 1985).

In Drosophila (Glover, 1982) and tomato (Scharf and Nover, 1982) cells, there is a rapid but reversible decline in the phosphorylation level of an S6-like ribosomal protein (r-protein) of the small subunit during heat shock. Decreased phosphorylation coincides with the onset of hsp synthesis. In plant cells, Scharf and Nover (1982) have suggested that S6 phosphorylation level plays a role in recruitment of new mRNA into the polyribosomes.

In Tetrahymena, heat-induced functional alterations in the protein synthetic machinery have been correlated with both an increased affinity for a r-protein of 22kDa (McMullin and Hallberg, 1986) and the accumulation and association of a small RNA polymerase III transcript (G8 RNA) with the ribosomes and/or polyribosomes (Kraus et al, 1987). The kinetics of accumulation and association of G8 RNA in a mutant strain (ie. a strain unable to thermostabilize protein synthesis but able to alter its translational discriminatory properties)(Kraus et al, 1986) is similar to that observed in the wild type strain suggesting that this RNA plays some role in the selectivity of mRNA utilization. Sequence analysis demonstrates homology with the eucaryotic 7SL RNA (a structural and functional component of the signal recognition particle) and the Escherichia coli (E. coli) 4.5S RNA (interacts via elongation factor G) both of which are known to associate with ribosomes and to be involved in the modification or regulation of protein synthesis (Hallberg and Hallberg, 1989).

In Drosophila, cells maintained at high temperature continue to synthesize hsp's to the virtual exclusion of other proteins. When these cells are returned to 25°C, hsp synthesis continues for a time proportional to the severity of the stress. This is followed by a repression of hsp synthesis and the restoration of translation of pre-existing mRNAs. Treatments that interfere with the synthesis or function of hsp's result in extended translation during recovery (DiDomenico et al, 1982a; DiDomenico et al, 1982b). The

decline in hsp70 message is not due to a gradual decline in translational efficiency of hsp70 mRNAs but rather to the withdrawal of hsp70 messages from translation. Translational repression occurs only after a return to normal temperature where it is coupled to mRNA degradation. Degradation is highly selective since pre-existing messages are stable during both heat shock and recovery.

With prolonged high temperature treatment, hsp mRNAs, particularly hsp70 mRNAs are stable and continue to be translated (Lindquist and DiDomenico, 1985; Banerji et al, 1986). Heat shock itself is responsible for the stabilization of hsp70 mRNA (Theodorakis and Morimoto, 1987; Peterson and Lindquist, 1988). The instability of this message is not a feature peculiar to cells recovering from heat shock but is a normal feature of the message in nonheat-shocked cells. Theodorakis and Morimoto (1987) have shown that hsp70 stability increases in the presence of protein synthesis inhibitors suggesting that a heat shock-sensitive labile protein regulates its turnover. This message also contains one copy of a sequence, AUUUA (Hunt and Morimoto, 1985), commonly found in the 3' noncoding regions of mRNAs with short half lives (Shaw and Kamen, 1986). Simcox et al (1985) demonstrated that the half life of a Drosophila hsp70 mRNA truncated at the 3' end (hsp45) is increased during recovery from heat shock. Therefore, sequences at both the 5' and 3' end of hsp70 mRNA, together with modifications and/or changes in the translational machinery and cytoskeleton, may regulate the turnover of this message.

Translational control in plants, like animals, is dependent upon the intensity, duration, and nature of the stress (Altschuler and Mascaranhas, 1982; Altschuler and Mascaranhas, 1985; Key et al, 1981; Cooper and Ho, 1983; Czarnecka et al, 1984; Baszczynski, 1984; Baszczynski et al, 1985; Edelman et al, 1986; Wu et al, 1988; Rees et al, 1989) and is tissue and/or species specific (Altschuler and Mascaranhas, 1982; Cooper et al, 1984; Mascaranhas and Altschuler, 1985; Baszczynski et al, 1985; Belanger et al, 1986). However, in soybean seedlings, prolonged heat shock results in a gradual decline in heat shock specific poly(A)$^+$ RNAs suggesting that a return to control temperatures is

not required for translational repression (Schoffl and Key, 1982; Altschuler and Mascarenhas, 1982). Furthermore, prolonged exposure to low concentrations of arsenic (As) and cadmium (Cd) mimic this response (Edelman et al, 1986). Depletion of heat shock mRNAs is rapid in seedlings following a relief from the stress (Schoffl and Key, 1982).

1.3 The Heat Shock Proteins: Intracellular Location and Function

There has been a tremendous increase in our understanding of the structure and function of hsp's within the last few years. Much of this information has resulted from the fact that most of the stress proteins are present in appreciable levels in cells grown under normal conditions. In addition to providing new insight into function, these studies illustrate the importance of the stress proteins in mediating a number of processes essential to the activity of nonstressed cells. However, the relevance of these proteins during or after heat shock remains to be established.

The proceeding paragraphs summarize some of the current knowledge concerning the hsp's. Due to the broad scope of the current heat shock literature, this discussion will be limited to those proteins or groups of proteins relevant to the subsequent study.

1.3.1 Hsp90

The hsp90 gene family encodes constitutively synthesized heat-inducible proteins ranging in molecular mass from 83 to 108kDa (with one exception, a 62.5kDa protein in E. coli). Sequence analysis of cloned hsp90 genes from a variety of organisms demonstrates that these proteins are highly conserved. The proteins of distantly related eucaryotes share 50% amino acid identity while all hsp90 proteins share at least 40% identity with the E. coli protein. A region of high negative charge density, although poorly conserved, is located at the same relative position in all eucaryotic proteins comprising this family. A second smaller region of high negative charge density is located near the carboxy terminus. Although the carboxy terminal regions of these proteins are generally divergent, the four

most terminal amino acids, glu-glu-val-asp, are highly conserved and are also found at the carboxy terminus of eucaryotic hsp70 proteins (Craig, 1985; Lindquist and Craig, 1988).

Drosophila contain a single hsp90 gene, hsp83 which is induced only 5 to 6 fold by heat shock (Arrigo et al, 1980). This gene is also developmentally induced during oogenesis (Zimmerman et al, 1983). Hsp83 is unique in that it is one of only two Drosophila hsp genes possessing an intron (Holmgren et al, 1981).

Two proteins, hsp83 and heat shock cognate 83 (hsc83), comprise the hsp90 gene family in Saccharomyces cerevisiae (S. cerevisiae). Hsp83 is also developmentally regulated and has been shown to accumulate as cells transit into stationary phase or begin to sporulate (Lindquist and Craig, 1988). That the proteins synthesized by the hsp90 genes are essential is demonstrated by the fact that, although mutations in either gene are viable, double mutations are lethal (Lindquist and Craig, 1988).

The related E. coli protein encoded by the high temperature production G (htp G) gene has an apparent molecular mass of 62.5kDa. Interestingly, deletion of the htp G gene is not lethal (Bardwell and Craig, 1987).

In vertebrate cells, the hsp90 gene family constitutively encodes soluble cytoplasmic and ER-destined proteins. There is some deposition of the cytosolic protein in nuclei during heat shock. Biochemical analysis of cytosolic hsp90 in mammalian and chicken cells demonstrates a transient interaction with a number of tyrosine kinases, such as the transforming protein of Rous Sarcoma Virus, $pp60^{src}$ (Welch et al, 1989; Lindquist and Craig, 1988). $Pp60^{src}$ is quantitatively associated with hsp90 and a 50kDa phosphoprotein (Lindquist, 1986; Brugge et al, 1981). The src protein is inactive (ie. displays neither tyrosine kinase activity nor phosphorylated tyrosine residues) in this complex. However, when released from association with hsp90 at the plasma membrane, src is deposited at the inner side of the membrane and exhibits both kinase activity and autophosphorylated tyrosine (Brugge et al, 1983).

Hsp90 also associates with another cellular kinase, the heme-regulated eIF-2 alpha kinase (Welch et al, 1989). In contrast to its role in tyrosine kinase activity, hsp90 apparently stimulates eIF-2 alpha activity. As increased phosphorylation of eIF-2 alpha is correlated with a decrease in ribosomal initiation complexes and the subsequent inhibition of protein synthesis (Duncan and Hershey, 1984), it has been suggested that hsp90 may indirectly function in translational regulation (Welch et al, 1989).

Molecular cloning and immunocharacterization have shown that hsp90 is also a component of the steroid hormone receptor complex (Lindquist and Craig, 1988). In the native, heterooligomeric, nonactive 8S receptor, hsp90 caps the receptor DNA binding site (Baulieu et al, 1989). Hormone 'transforms' the 8S form, releasing hsp90 and active (4S-) receptor which can bind DNA and trigger the hormonal response (Catelli et al, 1985; Baulieu, 1987).

At least one hsp90 gene encodes a protein destined for translocation into the ER (Welch et al, 1989; Lindquist and Craig, 1988; Mazzarella and Green, 1987; Sorger and Pelham, 1987b). This protein, which is induced by glucose starvation (Welch et al, 1989; Sorger and Pelham, 1987b), has a molecular mass ranging between 94 and 108kDa. Hsp90 ER proteins, like their cytosolic counterparts, contain the amino acid sequence glu-glu-val-asp. However, these proteins contain an additional 24 C-terminal amino acids with the four most terminal, lys-asp-glu-leu, being identical to those comprising the hsp70 ER protein (Sorger and Pelham, 1987b). This sequence has been shown to prevent secretion of luminal ER proteins (Munro and Pelham, 1987).

Analogous proteins in plant cells may be hsp83, a constitutively synthesized hsp in maize seedlings (Baszczynski et al, 1985) and/or hsp89.

Both the high degree of sequence homology between the hsp90 genes and the similarity in their patterns of expression suggest that these proteins perform similar roles in their respective cellular compartments in regulating the activity of other macromolecules in the cell.

1.3.2 Hsp70

Hsp70 of most, if not all, eucaryotes is a member of a multigene family whose genes are expressed under a variety of physiological conditions (Lindquist and Craig, 1988). The proteins encoded by these genes are highly conserved. Polyclonal antibodies prepared against chicken hsp70 react with 70kDa proteins from a variety of organisms including plants (Kelley and Schlesinger, 1982; Schlesinger et al, 1982). Homologies ranging from 60 to 78% have been found among eucaryotic proteins. The human protein is 73% identical to the Drosophila protein and 50% identical to E. coli dnaK (Lindquist, 1986) while the plant protein, more specifically maize hsp70, is 63% homologous to Drosophila hsp70 and 75% homologous to the Drosophila hsc1 protein (Rochester et al, 1986).

The Drosophila hsp70 multigene family contains 5-6 copies of the hsp70 gene, one copy of the heat-inducible hsp68 gene and seven genes, denoted hsc1-7, that are expressed during normal development (Lindquist and Craig, 1988). Hsp70 localizes primarily within the nucleus and secondarily at cell membranes during heat shock. Upon recovery, hsp70 exits the nucleus and accumulates in the cytoplasm (Arrigo et al, 1980; Sinibaldi and Morris, 1981; Velazquez and Lindquist, 1984). Two of the hsc gene products, hsc70 and hsc72, have been shown to accumulate around the nucleus and within the ER respectively (Palter et al, 1986). After heat shock, hsc72 can be found within the nucleus (Lindquist and Craig, 1988).

In S. cerevisiae, the hsp70 genes can be divided into four groups, denoted SSA, SSB, SSC, and SSD (Craig et al, 1987). An additional member, KAR2 has recently been identified (Lindquist and Craig, 1988). The sequence relationships among these genes are complex with nucleotide identities ranging from 50-96%. Furthermore, the level of expression of these genes is regulated differentially during heat shock. SSA3 and SSA4 are synthesized at low levels at 23°C but are strongly induced by heat shock (Werner-Ashburne et al, 1987). The converse is true for SSB1 and SSB2 (Craig and Jacobsen,

1985). Changes in the level of SSA2 synthesis after heat shock are negligible while the levels of SSA1, SSC1, SSD1, and KAR2 increase 3-10 fold after heat shock (Craig, 1985; Lindquist, 1986; Lindquist and Craig, 1988).

Analyses of strains containing mutations in members of the hsp70 family indicate that the KAR2 gene encodes an ER-destined protein that is essential for karogamy (Lindquist and Craig, 1988) while the SSC1 gene encodes a protein destined for translocation into the mitochondria (Craig et al, 1989) and apparently essential for growth (Craig et al, 1987). The SSB gene products are either functionally or compartmentally distinct from those encoded by the SSA genes (ie. SSA1-4). However, each of the proteins encoded by SSA1-4 can substitute at least partially for the absence of the other three (Lindquist, 1986; Lindquist and Craig, 1988).

Biochemical and genetic evidence indicates that Ssa proteins are involved in the post-translational translocation of proteins into the ER and mitochondria (Chirico et al, 1988; Deschaies et al, 1988). Furthermore, Chirico et al (1988) suggest that hsp70 is responsible for ATP-dependent unfolding or disaggregation of the prepro-alpha-factor before translocation. That the unfolding step is rate limiting is shown by the fact that the rate of translocation increases when denatured substrate is used in the translocation assay (Chirico et al, 1988).

E. coli have only a single hsp70 gene which encodes DnaK, a protein required for replication of lambda DNA (Craig, 1985) and shown recently to be involved in host DNA replication (Lindquist and Craig, 1988). The purified protein has a weak ATPase activity and self phosphorylates at a threonine residue (Zylicz et al, 1983). DnaK also possesses a 5' nucleotidase activity that is inhibited by AppppA (Bochner et al, 1986). Recently, purified dnaK has been shown to stimulate post-translational translocation of prepro-alpha-factor into yeast microsomes (Waters et al, 1989).

The nomenclature for the mammalian hsp70 family is extremely confusing and therefore, for the purposes of this discussion, the members of this family will be addressed

using the nomenclature of Watowich and Morimoto (1988). The 70kDa stress protein family consists of at least five members: the major heat-inducible protein, hsp70 which is also induced during cell growth by serum stimulation (Milarski and Morimoto, 1986) and after infection of human cells (but not rodent cells) with either of two cooperating oncogenes, E1A or myc (Kingston et al, 1984; Wu et al, 1986); protein 72 (p72), often referred to as hsp73 (Welch et al, 1989), which exhibits a high level of basal expression and is only slightly heat-inducible; hsp72, a protein expressed only after heat shock; glucose regulated protein 78/80 (grp78/80), an ER protein; and grp75, a mitochondrial protein.

Biochemical fractionation and immunological studies have shown that newly synthesized hsp70 and p72 localize within the nucleoplasm, nucleolar matrix, and nucleoli during heat shock (Welch and Feramisco, 1984). The nucleolar deposition correlates with marked alterations in the integrity of the nucleoli (Pelham, 1984) and a concomitant decrease in nucleolar function (Rubin and Hogness, 1975; Spradling et al, 1977). Welch and Suhan (1986) have shown that hsp70 and p72 are primarily deposited in the granular region (ie. that area of the nucleolus involved in small ribonucleoprotein and pre-ribosome assembly). Recovery of normal nucleolar morphology after heat shock is rapid in the presence of high levels of hsp70 (Pelham, 1984). During recovery, the protein exits the nucleus and associates primarily with ribosomes and polyribosomes where it may function in the recovery of the translational machinery (Welch and Suhan, 1986).

Hsp70 and p72 have been shown to interact with a variety of macromolecules including cellular tumor antigens (Lindquist and Craig, 1988) and non-sterified fatty acids (Hightower et al, 1985). More recently, p72 has been shown to facilitate the uncoating of clathrin triskelions from clathrin coated vesicles in vitro (Chappell et al, 1986).

Grp78/80 is a constitutively synthesized ER protein that is induced following glucose/calcium deprivation (Welch et al, 1989). The protein is homologous to BiP, an ER protein that interacts transiently with maturing immunoglobulin heavy chains (Haas and

Wabl, 1983; Munro and Pelham, 1986). Grp78/80 binds both ATP and aberrant proteins as they traffic through the ER (Welch and Feramisco, 1985; Kassenbrock et al, 1988).

Recently, a grp of 75kDa has been detected in the mitochondria of mammalian cells. The precursor is processed during translocation into the mitochondria. Whether grp75 functions in the assembly/disassembly of mitochondrial proteins is unknown (Welch et al, 1989).

In plant cells, at least two hsp70 genes have been reported (Rochester et al, 1986). Both genes are heat-inducible and share 88% nucleotide sequence homology. Suprisingly, each gene contains an intron. Each intron is located in the same position as that of the Drosophila hsc1 gene. The proteins encoded by these genes are 90% identical in the first exon (Rochester et al, 1986). The proteins share considerable homology with the Drosophila hsp70 and hsc1 protein as mentioned.

Expression of at least some tomato hsp70 family members is developmentally regulated (Duck et al, 1989). RNA transcripts have been localized to the vascular system of the ovary, dividing cells of the lateral root tips, and the inner integument of developing seeds. The transmitting tissue, immature anthers, and embryo also express 70kDa RNA (Duck et al, 1989). It is interesting to note that many of these tissues are highly secretory while many of the organs contain zones of rapidly dividing cells.

In maize roots, hsp70 cosediments with plasma membrane fractions during heat shock while hsp72 apparently localizes to both the ER and Golgi (Cooper and Ho, 1987). A 76kDa protein, absent from cell free translation products, has been shown to accumulate in vivo in maize seedlings (Baszczynski et al, 1985) and may be analogous to grp78 (Lindquist, 1986).

In summary, the proteins encoded by the hsp70 gene family appear to be involved in the disruption of either intramolecular or intermolecular protein-protein interactions. Pelham (1988) has suggested that hsp70 binds to incorrectly or incompletely folded proteins, preventing self-aggregation and possibly unfolds them using the energy of ATP

hydrolysis. In the case of assembly of multi-subunit structures, this function could be complemented by those of other proteins.

1.3.3 The low molecular weight hsp's

Unlike the hsp70 gene family, the low molecular weight hsp's constitute a considerably heterogeneous group. The proteins of any given species show greater homology to each other than to proteins of other species. Members of the soybean low molecular weight hsp family have 99% identity (Nagao et al, 1985) while members of the Drosophila low molecular weight hsp's share 50% identity (Ingolia and Craig, 1982; Southgate et al, 1983). However, the soybean low molecular weight hsp's have only 20% identity with the low molecular weight hsp's of Drosophila, Caenorhabditus elegans (C. elegans), and Xenopus (Nagao et al, 1985). Hydropathy profiles for Drosophila, Xenopus, C. elegans, and soybean hsp's do show a similarity in major hydrophilic and hydrophobic regions (Nagao et al, 1985). All low molecular weight hsp's contain a relatively conserved hydrophobic domain, (asn or asp)-gly-val-leu-thr, near their carboxy terminus. Moreover, the low molecular weight hsp's of all organisms share both sequence and structural homology with the alpha crystallin lens protein (Ingolia and Craig, 1982; Rassnak and Candido, 1985; Hickey et al, 1986).

Both Drosophila (Tissieres et al, 1974; Corces et al, 1980; Arrigo et al, 1980) and plant (Barnett et al, 1980; Schoffl and Key, 1982; Baszczynski et al, 1982; Mansfield et al, 1987) cells contain multiple low molecular weight hsp's while yeast (Lindquist et al, 1982), avian (Kelley and Schlesinger, 1978), and mammalian (Hickey and Weber, 1982; Hickey et al, 1986; Arrigo and Welch, 1987) cells contain only a single low molecular weight hsp. Until recently, it was thought that these hsp's may be unique to eucaryotes. However, a protein of 18kDa, shown to have homology with eucaryotic low molecular weight hsp's, has been detected in the mycobacterium, M. leprae (Lindquist and Craig, 1988).

under normal growth conditions and after exposure to a heat shock in order to discriminate between changes in cytoplasmic and organellar protein synthesis. Moreover, comparative analysis of the proteins synthesized under the above conditions with those synthesized by polyribosomal mRNAs in vitro provides a more precise characterization of the heat shock response in maize seedlings.

HSG). In mammalian cells, hsp28 is distributed in a polarized fashion within the perinuclear region of the cell in close proximity to the Golgi complex (Arrigo et al, 1988a). That these particles are distinct from the prosome, a ubiquitous cytoplasmic RNP to which they have been likened, is evident from their different sedimentation behaviour in sucrose gradients, the reduced synthesis of the prosomal proteins during heat shock, and the absence of any hsp's within the prosome (Arrigo et al, 1985; Arrigo et al, 1988b; Falkenburg et al, 1988; Nover et al, 1989; Martins de Sa et al, 1989). However, a single prosomal protein, p29K (p27K in most vertebrates) is a stable and basic component of HSG's (Martins de Sa et al, 1989).

During heat shock, a reversible aggregation of HSG from the 16S precursor particles occurs (Arrigo, 1987; Arrigo et al, 1988a; Nover et al, 1989; Haass et al, 1989). A similar dynamic is observed in sea urchin after fertilization and during development (Akhayat et al, 1987b). When mammalian cells are made thermotolerant (ie. given a prior mild heat shock), significantly less hsp28 is found in the insoluble fraction and recovery is accelerated (Arrigo et al, 1988a). Based on these studies, it has been suggested that the distribution of the low molecular weight hsp's within the cell can be used to assess the cells thermotolerance (Arrigo et al, 1988a).

Although the role of the HSG's is unknown, it has been suggested that these aggregates preserve inactive (ie. untranslated) control mRNAs during heat shock (Nover et al, 1989). Alternatively, the low molecular weight hsp's may be enzymes required for cellular recovery from stress. During heat shock, the enzymes polymerize to form stable complexes. Recovery would lead to dissociation and activation of the enzyme (Collier et al, 1988).

In plants, low molecular weight hsp's have been shown to localize to chloroplasts (Kloppstech et al, 1985; Vierling et al, 1986; Vierling et al, 1989). These proteins are synthesized as precursor proteins that are cleaved to yield a mature form during import into the chloroplast (Vierling et al, 1988). Two nuclear encoded hsp's, hsp22 and hsp29, have

under normal growth conditions and after exposure to a heat shock in order to discriminate between changes in cytoplasmic and organellar protein synthesis. Moreover, comparative analysis of the proteins synthesized under the above conditions with those synthesized by polyribosomal mRNAs in vitro provides a more precise characterization of the heat shock response in maize seedlings.

nonpolyribosomal to polyribosomal ratio can change for specific mRNAs in response to specific signals and for some or all mRNAs under a variety of environmental (such as heat shock) and developmental circumstances (Dreyfuss, 1986). Thus, the translation repertoire of the eucaryotic cell can vary for a given set of mRNAs due to differential selection of mRNAs for translation. This qualitative suppression of the expression of certain genes is referred to as translational regulation or control (Imaizumi-Scherrer et al, 1982; Dreyfuss, 1986).

Translational control during heat shock has been shown in a variety of systems including plants. In Drosophila, the translational machinery is modified to selectively translate heat shock mRNAs and pre-existing mRNAs are sequestered (as discussed previously). Sequestration may be due to changes in the rates of both initiation and elongation (Ballinger and Pardue, 1983; Ballinger and Pardue, 1985). In HeLa cells, heat-induced lesions in initiation account for the observed translational inhibition of control messages (Hickey and Weber, 1982) while in yeast, control messages are degraded during heat shock (Lindquist, 1981). Thus the mechanism of regulation differs in different organisms. Subtle differences may also exist between species.

At the time when this research began, heat-induced translational control in plants had been most extensively studied in soybean seedlings (Key et al, 1981; Schoffl and Key, 1982; Altschuler and Mascarenhas, 1982). Alterations in the pattern of protein synthesis correlated with dramatic changes in the distribution of ribosomes on polyribosomes (Key et al, 1981). Recruitment of 25°C mRNAs into active protein synthesis following relief from the stress, together with the isolation and efficient in vitro translation of high levels of 25°C mRNAs from heat-shocked cells, indicated that these messages were preserved during the stress (Key et al, 1981). However, the cellular distribution of these mRNAs and constitutive hsp mRNAs during heat shock was unknown. This thesis investigates the nature of translational regulation in plants, and more specifically in the plumules of maize seedlings, under heat shock conditions.

Maize seedlings were chosen for several reasons. First, prior characterization of the heat shock response at the protein level in a variety of tissues and genotypes suggested that the degree of translational control was dependent on a number of factors including temperature. Second, isogenic strains of maize were available thus eliminating the possibility of any genetic variability within the response. Third, maize is an agronomically important cereal crop. An understanding of the heat shock response will provide a mean for extending the growth range and subsequent yield of such crops.

The initial growing temperature, shift increment and temperature regime over which the tissues were shifted were chosen based on previous reports which indicated that a shift from 25°C to 42.5^{o}C resulted in the induction of the full spectrum of hsp's in maize plumules (Baszczynski et al, 1985). Concomitant with the induction of hsp's was a repression in the synthesis of control proteins at this temperature (Baszczynski et al, 1982; Baszczynski et al, 1985).

This study begins by examining the heat shock response in maize plumules. The question of translational regulation is addressed in a broad sense by comparing the in vivo and in vitro protein synthetic profiles. The step in protein synthesis affected by heat shock is assessed by examining changes in both the level and subcellular distribution on control and heat shock mRNAs in seedlings maintained at a normal growing temperature (25C) or subjected to a brief temperature shift (42.5C for 1h). Further fractionation of the subcellular fractions details the distribution of these messages within the soluble cytoplasmic, membrane, and cytoskeletal components of the cell during brief or prolonged heat shock. Whether recovery of the translational machinery requires a return to the normal growth temperature, as in Drosophila, is determined by examining the effects of both prolonged heat shock and recovery from a brief heat shock on the subcellular accumulation and localization of heat shock messages. An indirect measure of message stability is provided by comparing the kinetics of mRNA accumulation in the non-polyribosomal and polyribosomal RNP during this time period.

In summary, the major objectives of this thesis can be defined by the following questions:

1) Does translational regulation occur in the plumules of heat-shocked maize seedlings?
2) Does heat shock affect the distribution of message (either control or heat shock) between or within the non-polyribosomal and polyribosomal RNP particles?
3) What effect does a brief or prolonged heat shock have on the distribution of mRNA in free-cytoplasmic and membrane-associated polyribosomal RNP particles?
4) What effect does a brief or prolonged heat shock have on the distribution of mRNA in free-cytoplasmic and membrane-associated non-polyribosomal RNP particles?

Further clarification of these objectives is provided in the introductory paragraphs accompanying each chapter.

CHAPTER 2

THE RESPONSE OF MAIZE SEEDLINGS TO A BRIEF HEAT SHOCK

2.1 Introduction

The response of plants to heat shock exhibits many of the characteristics of the heat shock response in other organisms (Kimpel and Key, 1985). However, in contrast to other eucaryotes in which the high molecular weight hsp's represent the majority of hsp synthesis (Atkinson and Walden, 1985), the most abundant hsp's in plants are a complex group of 10-30 low molecular weight hsp's. Although the profile and complexity of the low molecular weight hsp's vary considerably among plant species, a relatively large proportion of these hsp's have been shown to accumulate in all plant species (Kimpel and Key, 1985; Baszczynski et al, 1982; Nover and Scharf, 1983). Furthermore, the low molecular weight hsp's in several plant systems have been shown to be antigenically related (Baszczynski, 1986; Baszczynski, 1989).

In higher plants, the hsp's localize in various subcellular fractions including the nucleus, ribosomes, ER, Golgi, mitochondria (Lin et al, 1984; Kimpel and Key, 1985; Cooper and Ho, 1987), and chloroplasts (Kloppstech et al, 1985; Vierling et al, 1986) during heat shock. In maize seedlings, one of these hsp's is apparently encoded by the mitochondria (Nebiolo and White, 1985; Sinibaldi and Turpen, 1985).

Although various respiratory inhibitors/uncouplers induce hsp synthesis (Ashburner and Bonner, 1979; Lin et al, 1984; Czarnecka et al, 1984) and high temperature has been shown to affect photosynthetic activity (Berry and Bjorkman, 1980), the role of these subcellular organelles during heat shock is not fully understood. Ribosomal inhibitors are often used to determine which metabolic changes in higher plants are linked directly to protein synthesis. In this chapter, I examine the effect of the antibiotics cycloheximide and chloramphenicol on protein synthesis in the plumules of maize seedlings

under normal growth conditions and after exposure to a heat shock in order to discriminate between changes in cytoplasmic and organellar protein synthesis. Moreover, comparative analysis of the proteins synthesized under the above conditions with those synthesized by polyribosomal mRNAs in vitro provides a more precise characterization of the heat shock response in maize seedlings.

2.2 Materials and Methods

2.2.1 Germination of seeds and growth of seedlings

Seeds of the inbred cultivar 'Oh43' of Zea mays, L. were treated with the commercial anti-fungal powder 'Vitoflo' (Uniroyal Chemical) and placed between two sheets of moistened 3MM Whatman filter paper, embryo down and 1-2cm apart, in 15 x 26cm flats. The flats were covered with aluminium foil and the seeds were allowed to germinate in the dark at 25°C until the plumules were 1-2cm in length (approximately 5-6 days).

2.2.2 Treatment of seedlings

All seedlings were either maintained at 25°C or subjected to a 1h incubation at 42.5^{o}C (heat shock) prior to protein or RNA extractions. Cycloheximide (Sigma Chemical Co., St. Louis, MO) and chloramphenicol (Sigma Chemical Co.) were prepared as concentrated stock solutions in water. The terminal 0.5-1.0cm of the plumules of intact maize seedlings were excised and duplicate groups of 20 plumules were pre-incubated for 15 minutes at 25°C in petri dishes lacking antibiotic (controls) or containing increasing concentrations of cycloheximide or chloramphenicol diluted in water. After the pre-treatment, 300uCi/ml of L-[^{35}S] methionine (New England Nuclear; NEN; Boston, MA; specific activity=1100 Ci/mmol.) was added to each petri dish and one member of each set was maintained at 25°C for 1h prior to protein extraction while its duplicate was incubated at 42.5^{o}C for 1h prior to protein extraction. Lysates isolated from three replicates were analyzed.

2.2.3 Extraction of proteins from maize plumules

Following treatment, the excised plumules were rinsed with water and homogenized in 10 volumes of extraction buffer (200mM Tris-HCl, pH7.5, 5% sodium

dodecyl sulphate (SDS), 7.5% 2-mercaptoethanol, and 1mM phenylmethylsulphonylfluoride (PMSF)) (Baszczynski et al. 1982) in a glass homogenizer fitted with a teflon pestle as described by Baszczynski (1984). The extracts were transferred to 30ml corex tubes and centrifuged at 2000 rpm for 10 minutes. The supernatants were transferred to 15ml corex tubes, boiled for one minute, and stored until needed at -20°C.

For immunoprecipitation experiments, plumules were homogenized in immunoprecipitation buffer A (50mM Tris-HCl, pH7.2, 150mM sodium chloride (NaCl), 1.0% sodium deoxycholate (Na-DOC), 1.0% Triton X-100, and 0.1% SDS) as described by Baszczynski (1986).

2.2.4 Incorporation of ^{35}S-methionine into soluble proteins

Incorporation of ^{35}S-methionine into the soluble proteins was measured by both the filter paper disc method of Mans and Novelli (1960) and the method of Robash and Ford (1974) with some modifications. In the former case, 5ul aliquots of lysate were spotted onto individual glass fibre filter discs (Whatman) which were then pre-soaked for 30 minutes in 10% trichloroacetic acid (TCA), followed sequentially by two washes each with 5% TCA, 3:1 ethanol:anhydrous ether, and ether. The filters were dried for 1h at 50°C and transferred to scintillation vials containing 10ml of scintillation cocktail (8.0g omnifluor; NEN; per 1000ml of 'scintanalyzed' toluene; Fisher Scientific). Incorporated radioactivity was measured using a Beckman LS-230 scintillation counter.

Incorporated ^{35}S-methionine was also determined by precipitating 100ul aliquots of lysate in 1.0ml of 10% TCA on ice for 30 minutes. The precipitates were then vortexed and poured onto Whatman glass filter discs pre-soaked with 10% TCA, washed twice with 5% TCA, 3:1 ethanol:anhydrous ether, and ether, and air-dried in uncovered glass petri dishes. The dry filters were each placed in a scintillation counting vial and 0.5ml of NCS tissue solubilizer (Amersham Canada Limited, Oakville, Ontario) was added to each vial

which was then loosely capped and incubated at $50^{o}C$ for 30 minutes. The vials were cooled to room temperature, 17ul of glacial acetic acid and 10ml of scintillation cocktail were added to each vial and the radioactivity determined by scintillation counting.

2.2.5 Isolation of total polyribosomal RNP mRNAs

Total polyribosomal RNP mRNAs were isolated from the plumules of intact maize seedlings according to the procedure of Lerner et al (1971) for mouse brain tissue with some minor modifications (Venkatesan and Steele, 1972). Briefly, plumules of intact seedlings maintained at $25^{o}C$ or subjected to a 1h heat shock at $42.5^{o}C$ were excised, weighed, ground in a mortar, and homogenized on ice in 16 volumes (per 1.0g of tissue) of 0.25M sucrose-TKMD-A buffer (50mM Tris-HCl, pH7.4, 250mM KCl, 5mM $MgCl_2$, 2mM dithiothreitol (DTT), 0.5mM PMSF, 0.2mg/ml emetine dihydrochloride, and 0.1mg/ml heparin)(Lerner et al, 1971; Gupta and Siminovitch, 1978; Ramsey and Steele, 1977; Heikkila et al, 1981) in a glass homogenizer fitted with a teflon pestle (10 strokes). One-nineth volume of 10% (w/w) Triton X-100 (in H_2O) was added and the mixture was homogenized as above with 3 strokes. The homogenate was centrifuged at 3500 rpm (1470xg) for 20 minutes at $4^{o}C$ (SS-34 rotor, Sorvall) to sediment crude nuclei and non-ionic detergent insoluble material. The supernatant was decanted and mixed with one-nineth volume of 13% (w/w) sodium deoxycholate (in H_2O) and centrifuged at 14000 rpm (24000xg) for 30 minutes at $4^{o}C$ (SS-34 rotor, Sorvall) to remove the deoxycholate insoluble material (Pearson, 1969). The supernatant was carefully layered over 6ml of 1.65M sucrose-TKMD-A buffer lacking emetine dihydrochloride (Larkins and Davies, 1975) and the polyribosomes pelleted by centrifugation at 38000 rpm (162000xg) for 4h at $4^{o}C$ (Ti 60 rotor, Beckman Instruments, Inc., Palo Alto, CA). The supernatants were decanted and the tubes were buried in ice for 15 minutes. The tube walls were wiped clean and the pellets resuspended in a small volume of digestion buffer (0.01M Tris-HCl, pH7.8, 5mM ethylene dinitrilo-tetraacetic acid, disodium salt (EDTA), 0.5% SDS, 1mg/ml

proteinase K (self-digested for 1h at 50°C), and 0.1mg/ml heparin)(Brawerman et al, 1972; Maniatis et al, 1982). The polyribosomal RNPs were incubated for 30 minutes at 37°C and the RNA was extracted with phenol and/or chloroform (Maniatis et al, 1982). The RNA was precipitated overnight at -70°C from 2.5 volumes of 95% EtOH-0.3M sodium acetate (Na-acetate), pH 5.2 and pelleted by centrifugation at 11500 rpm (16000xg) for 1h at 4°C. Deproteinized polyribosomal RNA was resuspended in 100ul of translation buffer (50mM Hepes, pH7.6, 25mM Mg-acetate, 1mM DTT, and 0.1mg/ml heparin) (Maniatis et al, 1982) and stored at -70°C until required. A small aliquot (10ul) was removed for determination of RNA concentration by spectrophotometry.

2.2.6 Translation of polyribosomal mRNA in a cell-free rabbit reticulocyte lysate system

Polyribosomal RNP RNA was used to direct the in vitro synthesis of polypeptides in a heterologous in vitro translation system from NEN. Rabbit reticulocyte lysate was programmed with 0.5-100.0ug of polyribosomal mRNA in the presence of L-[^{35}S] methionine (NEN; specific activity=1100Ci/mmol.) at 37°C for 1h. The reaction was terminated by placing the tubes in an ice-water bath. Incorporation of ^{35}S-methionine into polypeptides during the in vitro reaction was measured by TCA precipitation. Briefly, duplicate 1.0ul aliquots of lysate were spotted onto Whatman filter paper and air-dried. The filters were boiled for 10 minutes in 10% TCA containing cold L-methionine and washed sequentially with water, 95% ethanol, and acetone. Air-dried filters were placed in scintillation vials and treated as in Section 2.2.4.

2.2.7 Immunoprecipitation of proteins synthesized in vivo and in vitro by total polyribosomal mRNAs

Polyclonal antibodies raised against the 18kDa hsp's (provided by C. A. B. Rees, Dept. of Plant Sciences, University of Western Ontario, London, Ontario) were used to immunoprecipitate both the proteins synthesized in vivo (repeated at least three times) and

those synthesized in vitro (repeated more than three times) by total polyribosomal mRNAs according to the procedures of Baszczynski(1986) and Rees et al (1986) respectively. Approximately 30ul of protein lysate (containing 100,000 cpm) was mixed with an equal volume of antiserum and incubated at room temperature for 20 to 30 minutes. In vitro translation products were pre-incubated with 0.5% RNase A (1ug/ul; Boehringer Mannheim Canada, Dorval, Que.) and 0.5% RNase T1 (1U/ul; Boehringer Mannheim Canada, Dorval, Que.)(Stephan and van Huystee, 1980) for 15 minutes at 30°C before the addition of 50ul of antiserum and 12.5ul of immunoprecipitation buffer B (10mM sodium phosphate, pH7.5, 150mM NaCl, 10% sodium deoxycholate, and 10% Triton X-100). The samples were incubated at room temperature as described for in vivo samples. Approximately 100ul aliquots of formalin-treated Staphylococcus aureas cells of the Cowan 1 strain (Calbiochem-Behring, La Jolla, CA; standardized to bind 2.0 +/- 0.1mg IgG/ml) were added to each of the tubes containing antibody-treated in vivo or in vitro protein lysates. After 20 minutes at room temperature, the samples were centrifuged for three minutes in a Beckman microfuge (Model B). The supernatants were removed and the pellets washed three times in immunoprecipitation buffer B. After the final wash, the pellets were resuspended in 50ul of extraction buffer, boiled for 1 minute and pelleted (three minutes in a Beckman microfuge) (Baszczynski, 1986; Rees et al, 1986). The supernatants were analyzed by two-dimensional polyacrylamide gel electrophoresis.

2.2.8 Polyacrylamide gel electrophoresis

2.2.8.1 One-dimensional (SDS) polyacrylamide gel electrophoresis

One-dimensional (1-D) polyacrylamide gel electrophoresis in the presence of SDS was carried out according to the method described by Laemmli (1970) except that the separating gel consisted of a 7.5-17.5% linear polyacrylamide gradient. Samples were diluted with four volumes of extraction buffer (Baszczynski et al, 1982) and boiled for 1

minute prior to loading into preformed wells in a 3% polyacrylamide stacking gel. Standard proteins from a low molecular weight calibration kit (phosphorylase-b, 94000; albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; trypsin inhibitor, 20100; lactalbumin, 14400; Pharmacia Fine Chemical, Piscataway, NJ) were co-electrophoresed in the polyacrylamide slab gels for use in M_r determinations. The slab gels were electrophoresed at 5mA per gel (constant current) overnight. The current was increased to 15mA per gel and maintained there for 45 minutes after the dye front had run off to increase protein separation.

2.2.8.2 Two-dimensional (IEF-SDS) polyacrylamide gel electrophoresis

Two-dimensional (2-D) analysis of the proteins synthesized in vivo and in vitro followed the gel electrophoresis method developed by O'Farrell (1975) with minor modifications (Atkinson, 1981; Baszczynski et al, 1983; Rees et al, 1986). The first dimension isoelectric focusing (IEF) tube gels consisted of two parts 40% ampholines pH range 3.5-10.0 and three parts 40% ampholines pH range 5.0-8.0 (LKB Instruments Inc., Rockville, MD) while the second dimension polyacrylamide slab gels consisted of 7.5-17.5% linear polyacrylamide gradients overlayed with 3% stacking gels. Samples were mixed with four volumes of sample dilution buffer (SDB; 9.0M urea, 5% 2-mercaptoethanol, 2% ampholines, 8% NP-40) prior to loading on the IEF tube gels. The gels were electrophoresed for 12h at 400V followed by 2h at 800V (constant voltage). The IEF tube gels were extracted, equilibrated for 10 minutes in extraction buffer and layered onto the preformed 3% polyacrylamide stacking gels using 1% agar in 1% SDS. Electrophoresis in the second dimension was the same as that described above for one dimensional slab gels. The pH gradients established in the electrophoretically focused gels were determined by slicing companion gels and determining the pH of the water extract (Saleem and Atkinson, 1976).

2.2.9 Visualization of electrophoretically separated proteins

2.2.9.1 Staining polypeptides with coomassie brilliant blue

Following electrophoresis, the slab gels were removed and stained in 0.2% Coomassie brilliant blue R-250 (Sigma), 50% methanol, and 10% acetic acid overnight. The gels were then destained sequentially in 50% methanol and 10% acetic acid, 25% methanol and 10% acetic acid and finally in 10% methanol and 10% acetic acid. The gels were transferred to 7% acetic acid for storage prior to photography and/or fluorography.

2.2.9.2 Fluorography

Destained slab gels were dehydrated in dimethyl sulfoxide (DMSO), impregnated with 2, 5 diphenyloxazole (PPO; Bonner and Laskey, 1974) and dried down on Whatman 3MM filter paper. Fluorograms were prepared by apposing dried gels at -70°C to Kodak X-Omat film (XR-1) that had been preflashed to an optical density of 0.15 (Laskey and Mills, 1975).

Fluorograms were scanned using a laser densitometer (Ultro Scan^{TM} XL Densitometer; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to quantitate the relative amount of protein.

2.2.9.3 Photography of stained gels and fluorograms

Stained gels were photographed with a 35mm camera by transillumination using Kodak 2415 Technical Pan film with a red or green filter to maximize contrast and resolution. Fluorograms were photographed in the same manner without a filter. Films were developed for 10 minutes at 20°C in Kodak HC-110 developer and fixed in Kodak fixer (Craig, personal communication). Following washing and drying, the negatives were printed on Ilford Multigrade paper.

2.3 Results

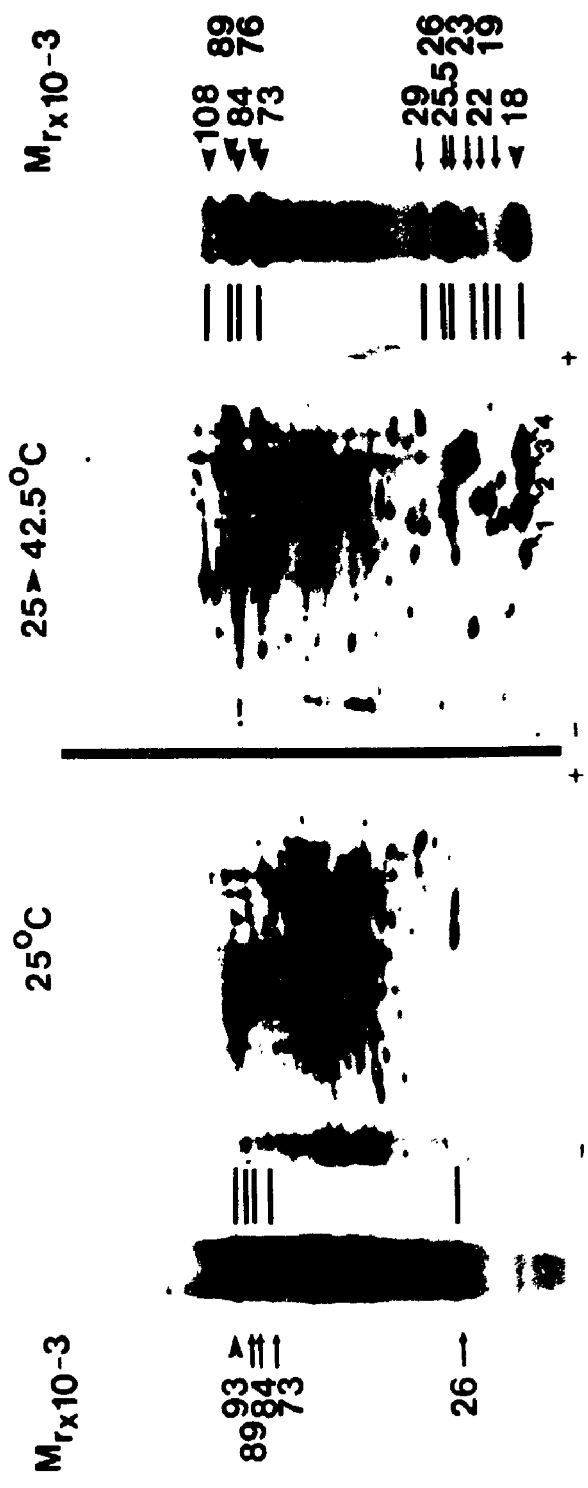
2.3.1 Response of maize seedlings to heat shock

Proteins synthesized by the plumules of intact maize seedlings maintained at 25°C (control) or subjected to a 1h heat shock at 42.5°C were analyzed by 1- and 2-D polyacrylamide gel electrophoresis (Figure 1). In addition to the six major hsp classes identified in earlier studies (Baszczynski et al, 1982; Cooper and Ho, 1984), proteins with M_r's ranging between 19kDa and 29kDa also exhibited new and/or enhanced synthesis in response to a 1h heat shock (Figure 1). Although not evident in this electrophoretic separation, a group of proteins with M_r's of 17kDa also comprise plumule hsp's (see Figure 4).

2.3.2 The effects of antibiotics on protein synthesis in control and heat-shocked maize seedlings

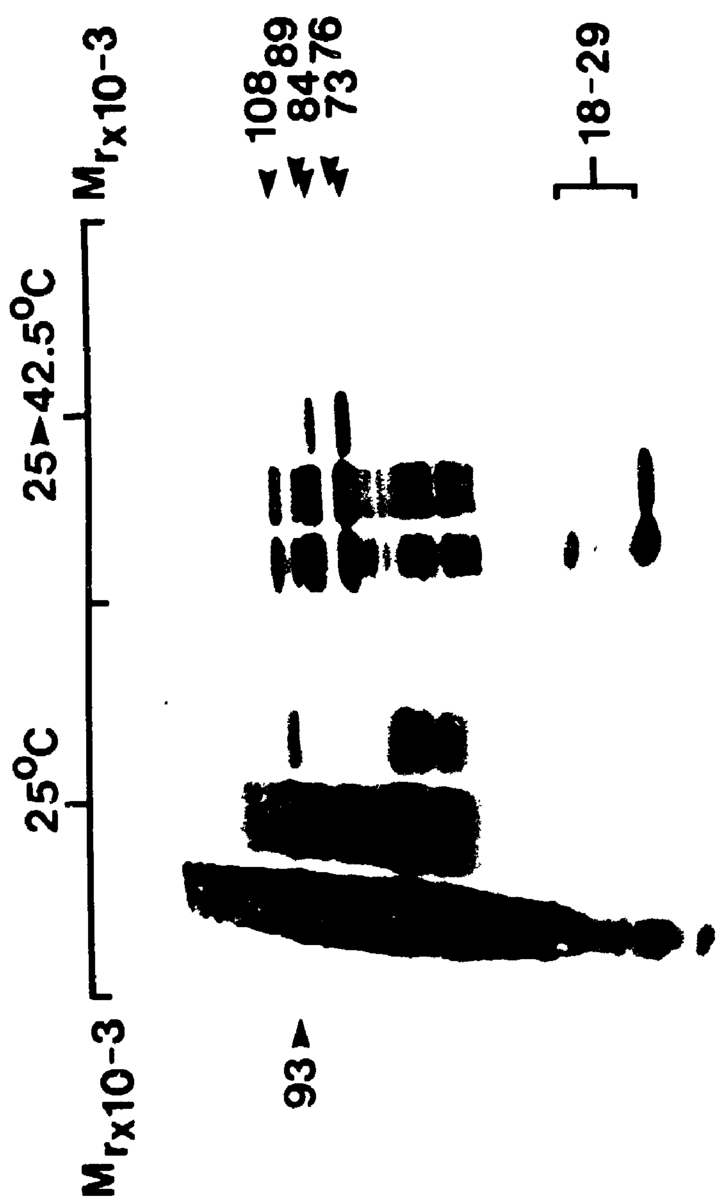
The nuclear origin of maize hsp's has been inferred by the synthesis of these hsp's from poly $(A)^+$ RNA in vitro (Baszczynski et al, 1983). However, in order to further demonstrate that these hsp's are in fact the products of nuclear genes, I examined the effect of the antibiotic, cycloheximide, on their biosynthesis. Intact maize seedlings were treated with varying concentrations of cycloheximide prior to protein extraction and the cell lysates were analyzed by 1- and 2-D polyacrylamide gel electrophoresis. Increasing concentrations of cycloheximide progressively inhibited the synthesis of both control proteins and hsp's (Figure 2). The incorporation of ^{35}S-methionine into proteins extracted from 25°C and 42.5°C plumules is shown in Figure 3. Total protein synthesis in control and heat-shocked plumules decreased to 23% and 24% of the maximum respectively after treatment with 0.5ug/ml cycloheximide. Synthesis continued to decline with increasing cycloheximide concentrations reaching approximately 2.3% of the maximum in both control and heat-shocked cells at 50ug/ml cycloheximide.

Figure 1: Fluorograms of 1-D and 2-D polyacrylamide gel electrophoretic separations of the polypeptides synthesized in vivo by plumules of intact maize seedlings maintained at 25°C or subjected to a brief heat shock (1h) at 42.5^{o}C. Lysates from at least three independent experiments were analyzed and a representative separation photographed. Arrowheads on the right mark the positions and M_r's of the six prominent hsp classes. The four prominent 18kDa hsp variants synthesized during a heat shock are numbered 1-4. Additional hsp's synthesized by the plumules of heat-shocked maize seedlings are identified by arrows on the right. The arrowhead on the left marks the position of a 93kDa protein that exhibits repressed synthesis during heat shock while the arrows on the left mark the positions of 25°C proteins with hsp-like M_r's. Approximately 50,000 cpm of acid-precipitable lysate was loaded onto each IEF gel and into the side wells of each second dimension slab gel.



Mrx10-3
108 84 73
89 76
29 25.5 22 18
26 23 19
25→42.5°C
1 2 3 4
25°C
Mrx10-3
93 84 73
89
26

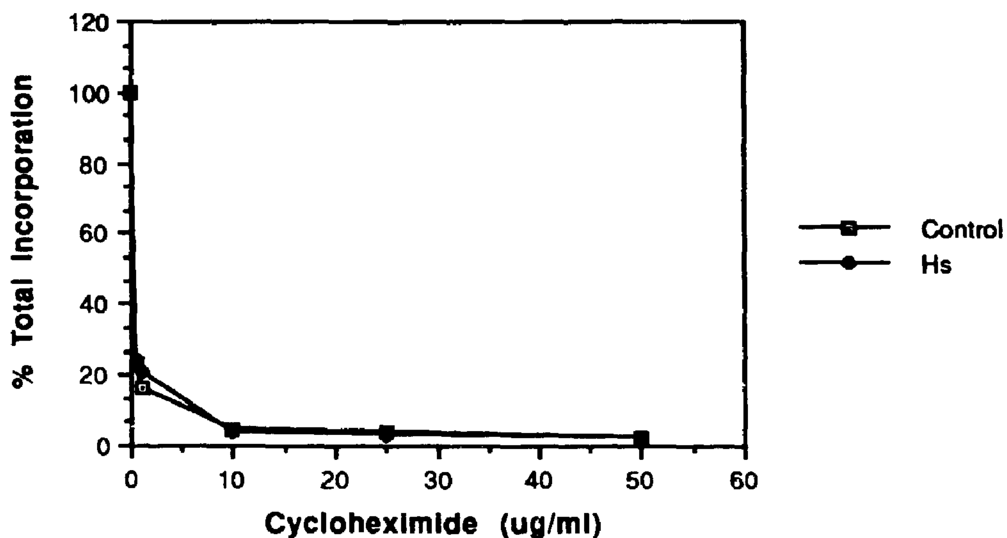
Figure 2: Fluorogram of a representative (lysates from one of three experiments) 1-D polyacrylamide gel electrophoretic separation of proteins synthesized in vivo by the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock in the presence of increasing concentrations of cycloheximide. M_r's of prominent hsp's are indicated by arrowheads and bars on the right. The arrowhead on the left marks the position of a 93kDa protein exhibiting repressed synthesis during heat shock. Approximately 50,000 cpm of acid-precipitable lysate was loaded into each well of the gel.



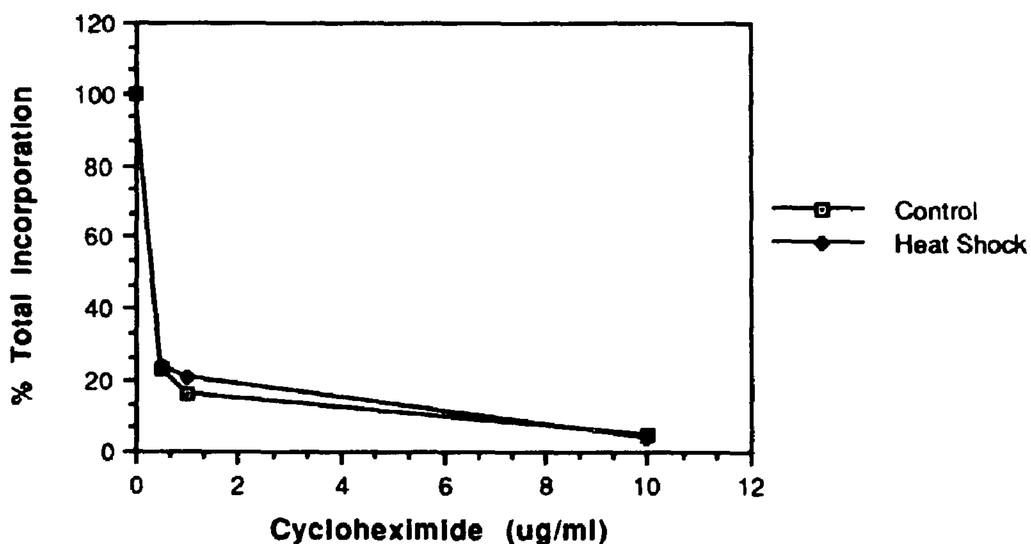
M_rx10-3
25°C
25▸42.5°C
M_rx10-3
93
108
89
84
76
73
18-29
0 0.5 1.0 10.0 0 0.5 1.0 10.0
CYCLOHEXIMIDE (ug/ml)

Figure 3: Effect of cycloheximide concentration on protein synthesis in the plumules of maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C. The relative incorporation of ^{35}S methionine into newly synthesized, acid-precipitable, products is plotted as a function of cycloheximide concentration in both (A) and (B). The values represent mean cpm calculated from three independent determinations expressed as a percentage of the total. The graph in (B) represents that portion of graph (A) corresponding to 0-10.0ug/ml cycloheximide.

A. Effect of cycloheximide on total protein synthesis



B. Effect of cycloheximide on total protein synthesis

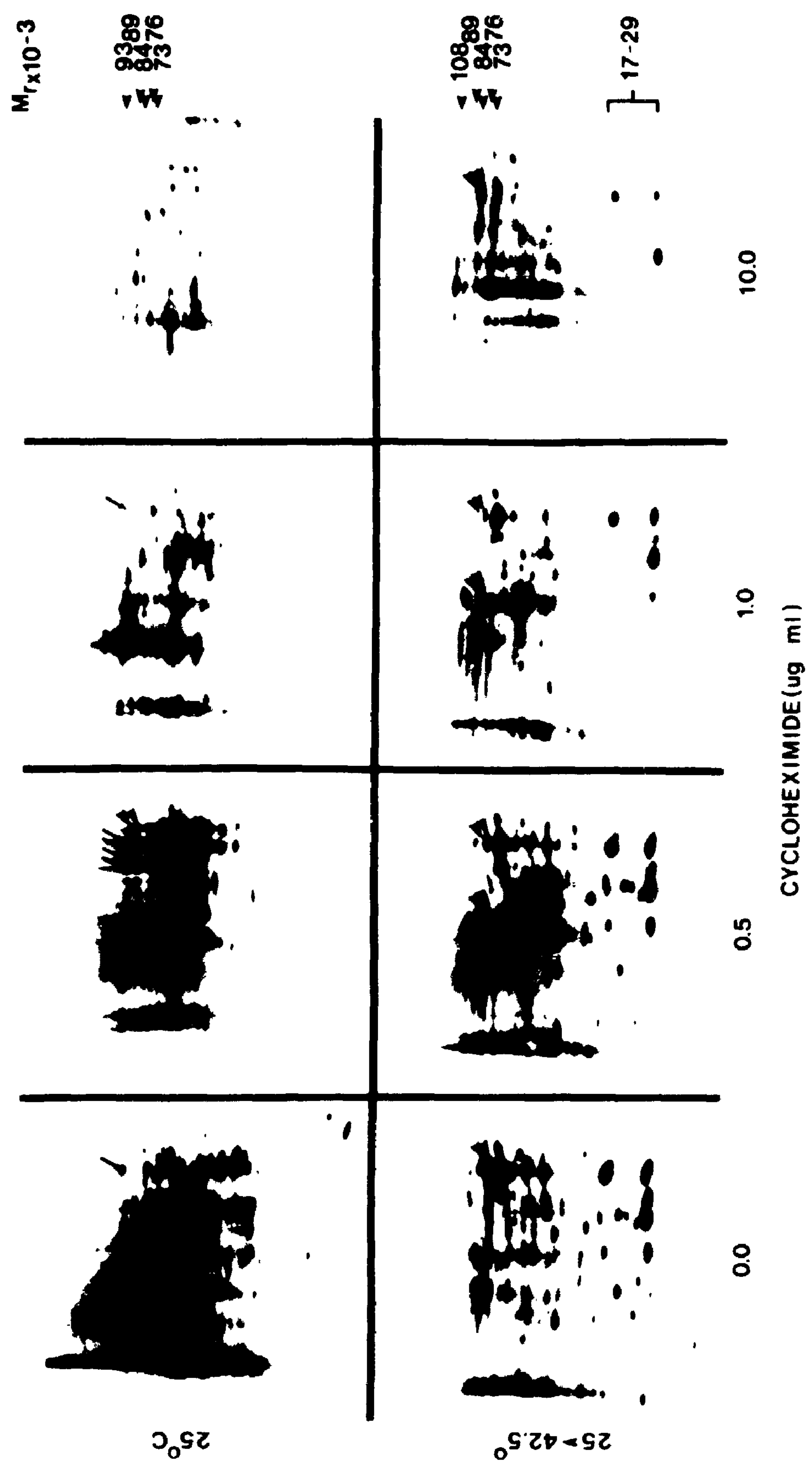


Analysis of these same cell lysates by 2-D polyacrylamide gel electrophoresis reveals that treatment with increasing concentrations of cycloheximide differentially affects the synthesis of some proteins in both control and heat-shocked plumules (Figure 4). Low levels (0.5ug/ml) of cycloheximide induce the synthesis of more basic isoelectric variants of the 84 and 89kDa proteins in heat-shocked cells while reducing the synthesis of the more acidic variants observed in cell lysates not treated with the antibiotic (Figure 4, arrowheads in lower panels). Synthesis of these variants persists, although at reduced levels, following a 1h heat shock in the presence of 1.0ug/ml cycloheximide. However, treatment with 10.0ug/ml cycloheximide results in an increase in the synthesis of the original more acidic variants. Increased synthesis of proteins with similar M_r's and both similar and more acidic isoelectric points (pI) is also observed in control plumules treated for 1h with 0.5ug/ml cycloheximide (Figure 4, arrowheads in upper panels). Furthermore, new and/or enhanced synthesis of several 93kDa variants is observed following a 1h treatment with 0.5ug/ml cycloheximide (Figure 4, arrows). The 84kDa and 89kDa variants are not detected among the proteins synthesized by untreated 25°C plumules or 25°C plumules subjected to 1.0ug/ml or 10.0ug/ml cycloheximide treatments while only one of the four 93kDa variants is detected in untreated plumules or plumules treated with 1.0ug/ml cycloheximide.

Synthesis of some of the more basic 93kDa variants, a 73/76kDa hsp variant and several proteins ranging in M_r between 50kDa and 68kDa is enhanced after a 1h heat shock in the presence of 0.5ug/ml cycloheximide (Figure 4). At 1.0ug/ml, synthesis of these same proteins is drastically reduced. However, synthesis of a even more basic 73/76kDa protein is observed. Synthesis of this latter variant, together with the acidic variant observed in cells that have not been treated with antibiotic, persists even after a 1h heat shock in the presence of 10.0ug/ml cycloheximide (Figure 4, circles).

While cycloheximide treatment induces protein synthesis both in control and heat-shocked plumules, it does not induce the synthesis of major hsp's in seedlings maintained

Figure 4: Fluorograms of representative (analysis of three independent experiments) 2-D polyacrylamide gel electrophoretic separations of the proteins synthesized in vivo by plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock in the presence of increasing concentrations of cycloheximide. The M_r's of prominent hsp's are indicated by arrowheads and bars on the right. Arrowheads in the panels mark the positions of isoelectric variants of the 84 and 89kDa hsp's. The arrows identify 93kDa variants exhibiting enhanced synthesis after a 1h treatment with 0.5ug/ml cycloheximide while the circles mark the position of a more basic 73/76kDa variant. Approximately 50,000 cpm of acid-precipitable lysate was loaded onto each tube gel.

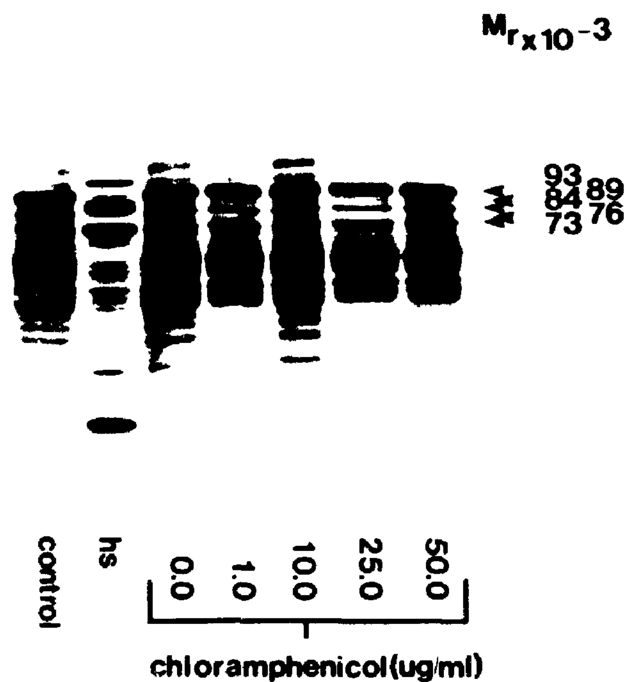

M_rx10-3
93 89 84 76 73
108 89 84 76 73
17-29
10.0
1.0
0.5
0.0
CYCLOHEXIMIDE (ug ml)
25°C
25→42.5°

at 25°C. Furthermore, there is an overall inhibition of protein synthesis in the presence of increasing concentrations of the antibiotic as would be expected for proteins translated by cytoplasmic ribosomes.

The cycloheximide experiments strongly suggest that the major plumule hsp's are the products of nuclear genes. To further demonstrate their nuclear origin and to determine if any of the proteins synthesized by maize plumules are organellar in origin, the plumules of maize seedlings were treated with increasing concentrations of chloramphenicol at 25°C or 42.5^{o}C prior to protein extraction as described in the Materials and Methods and the cell lysates were analyzed by 1- and 2-D gel electrophoresis. Treatment of plumules with increasing concentrations of chloramphenicol appeared to have no effect on the synthesis of plumule proteins extracted from maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C (Figure 5). However, analysis of these same cell lysates by 2-D gel electrophoresis revealed that, as with cycloheximide, treatment with chloramphenicol did in fact affect protein synthesis. Synthesis of several high molecular weight 25°C proteins was inhibited by treatment with chloramphenicol (Figure 6, arrows) while synthesis of other proteins was enhanced in the presence of the antibiotic (Figure 6, arrowheads). The increased synthesis of proteins with similar M_r's is observed in heat-shocked plumules treated with chloramphenicol. Different concentrations of antibiotic also resulted in variable synthesis of 89, 84, and 73/76kDa hsp variants in both control and heat-shocked plumules (Figure 6, arrowheads). Some of these variants had similar pI's to those observed in plumules treated with cycloheximide (compare Figures 4 and 6). It is of interest to note that the accumulation of most of the 89, 84, and 73/76kDa variants in 25°C plumules was similar in both treated and untreated plumules while the accumulation of similar variants in heat-shocked plumules increased with increasing chloramphenicol concentrations. Furthermore, chloramphenicol concentrations of 10.0ug/ml and 50.0ug/ml resulted in the synthesis of an extremely acidic 73/76kDa protein in heat-shocked plumules (Figure 6, circle). These results clearly indicate that chloramphenicol is affecting the synthesis of high

Figure 5: Fluorograms of 1-D polyacrylamide gel electrophoretic separations of the proteins synthesized in vivo by the plumules of intact maize seedlings maintained at 25°C (A) or subjected to a 1h heat shock at 42.5°C (B) in the presence of increasing concentrations of chloramphenicol. The separation is representative of that obtained in three independent experiments. Lanes 1 and 2 in A and B contain proteins synthesized in vivo by 25°C or 42.5°C plumules respectively in the absence of any drug (ie. experimental controls). M_r's of prominent control proteins and M_r's of hsp's are indicated by arrowheads and/or bars on the right in A and B. Approximately 50,000 cpm of acid-precipitable lysate was loaded into each well.

A. Effect of chloramphenicol on 25°C protein synthesis



B. Effect of chloramphenicol on 42.5°C protein synthesis

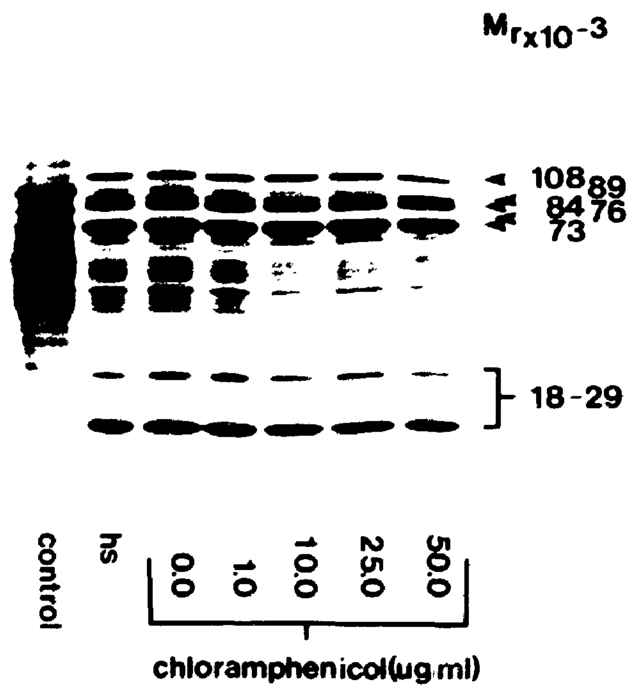
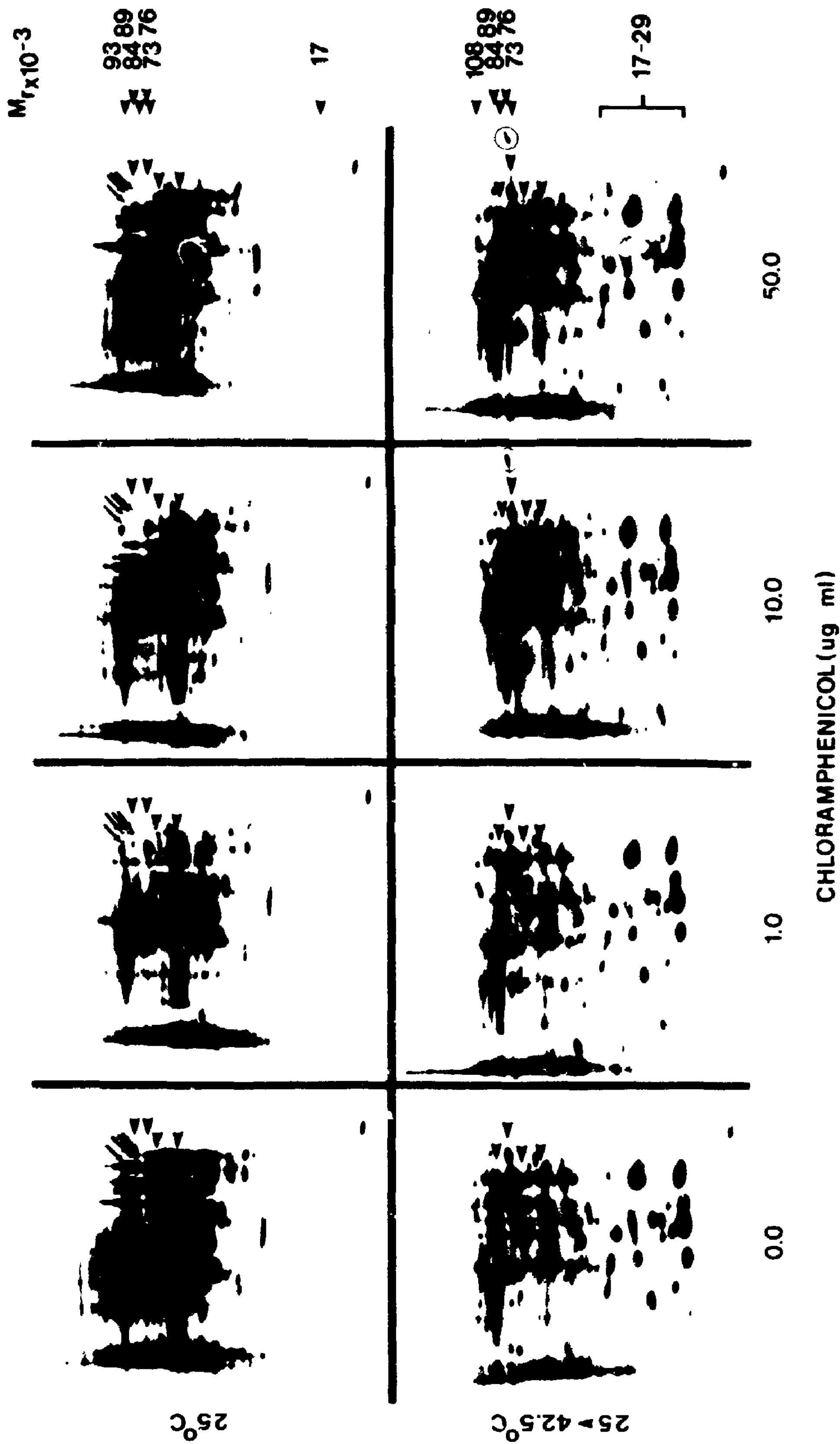


Figure 6: Fluorograms of representative (from three independent experiments) 2-D polyacrylamide gel electrophoretic separations of the proteins synthesized *in vivo* by the plumules of intact maize seedlings maintained at 25°C or exposed to a 1h heat shock at 42.5^{O}C in the presence of increasing chloramphenicol concentrations. M_r's of prominent control proteins and hsp's are indicated on the right by arrowheads and bars. Arrows in the panels identify protein variants exhibiting repressed synthesis in the presence of chloramphenicol. Arrowheads in the panels mark the relative positions of proteins exhibiting enhanced synthesis with increasing chloramphenicol concentrations. The circle identifies a 73kDa variant synthesized in heat-shocked cells in the presence of high chloramphenicol concentrations. Approximately 50,000 cpm of acid-precipitable lysate was loaded into each tube gel.



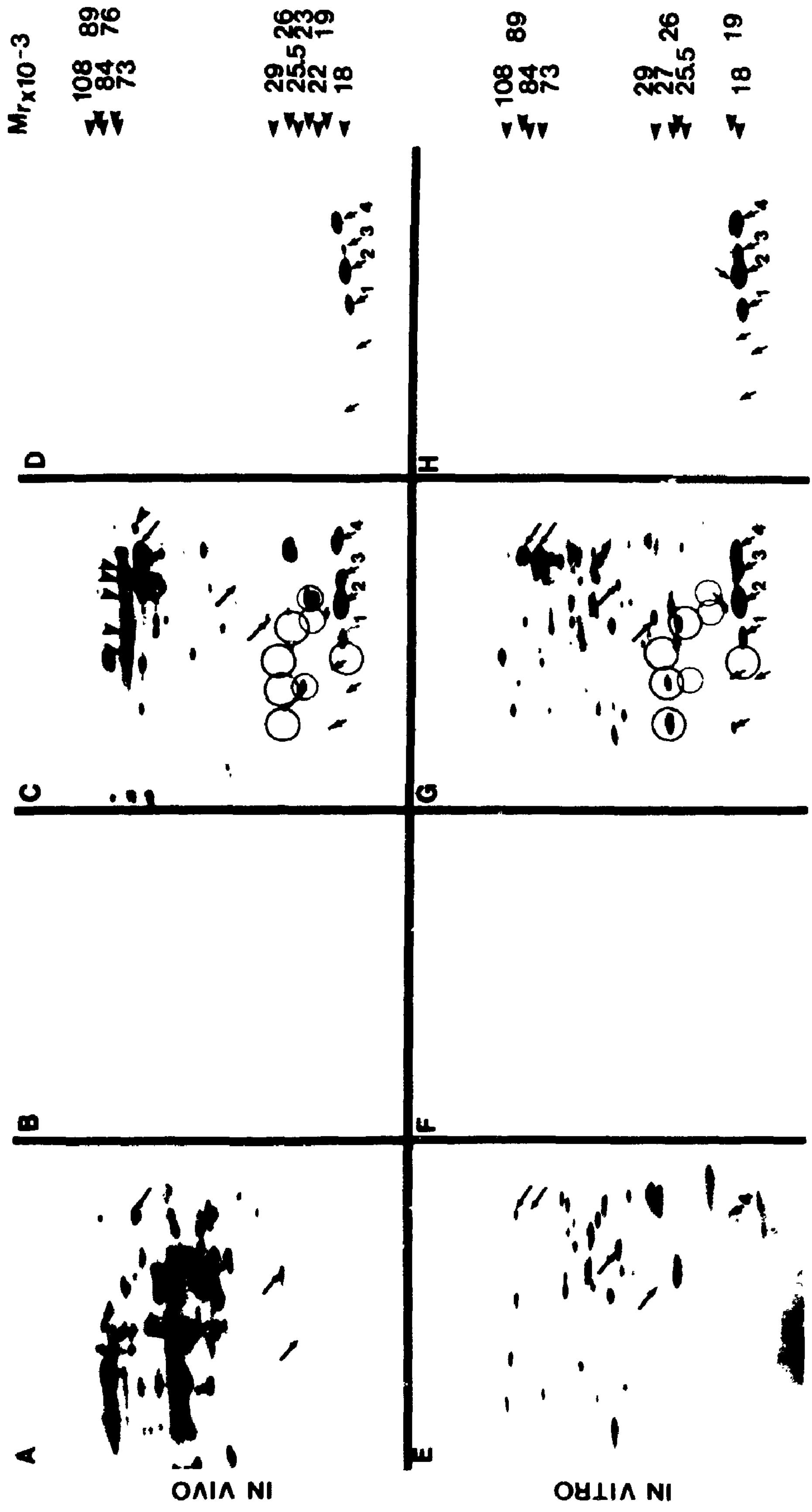
M_rx10-3
93 89
84 76
73
17
108 89
84 76
73
17-29
0.0
1.0
10.0
50.0
CHLORAMPHENICOL (ug ml)
25°C
25→42.5°C

molecular weight proteins in both control and heat-shocked plumules. However, within the limits of detection of these experiments, the antibiotic appears to have no effect on the synthesis of low molecular weight proteins extracted from plumules maintained at 25°C or subjected to a 1h heat shock at 42.5°C.

2.3.3 A comparison of the proteins synthesized in vivo with those synthesized in vitro

In order to determine whether the hsp's synthesized in vivo were similar to those synthesized in vitro by total polyribosomal RNA, both the proteins synthesized in vivo by total cell lysates and in vitro by total polyribosomal RNAs and the proteins precipitated from aliquots of the respective samples by 18kDa hsp antiserum were analyzed by 2-D gel electrophoresis. In general, proteins with similar M_r's and isoelectric points (pI's) to those synthesized in vivo are synthesized in vitro by total polyribosomal RNA (Figure 7, panels A and C and panels E and G). However, several minor quantitative (arrows) and qualitative (circles and arrowheads) differences exist between the proteins synthesized in vivo by total cell lysates (small circles, arrowheads) and those synthesized in vitro by total polyribosomal RNA (large circles). Heat shock results in a decrease in the synthesis of some, but not all, normal cellular (control) proteins in vivo. However, many of these proteins are synthesized in essentially unchanged amounts by total polyribosomal mRNAs extracted from the plumules of heat-shocked seedlings (Figure 7; compare panels A and C with E and G). Additional isoelectric variants of the 73, 84, and 89kDa hsp's synthesized in vivo by total cell lysates are not detected among the proteins synthesized by total polyribosomal RNA (Figure 7, arrowheads). Furthermore, a 76kDa protein synthesized in vivo is not detected among the in vitro translation products (Figure 7, arrowhead). Additional (27kDa) and/or different (18, 25.5, and 26kDa) isoeletric variants of the more complex group of low molecular weight hsp's are observed among the in vitro translation products (Figure 7, large circles) while two 22kDa and two 23kDa variants appear to be synthesized only in vivo (Figure 7, small circles). An 18kDa protein with similar pI to one

Figure 7: Fluorograms of representative (from more than three experiments) 2-D polyacrylamide gel electrophoretic separations of the proteins synthesized in vivo (panels A and C) and in vitro by total polyribosomal RNA (panels E and G) together with the proteins immunoprecipitated by 18kDa antiserum from total protein lysates (panels B and D) and cell-free rabbit reticulocyte lysates after the addition of polyribosomal mRNA (panels F and H). In vivo proteins and polyribosomal mRNAs used for in vitro translations were extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5°C. The four prominent 18kDa hsp variants are numbered 1-4. The small arrows without numbers identify additional proteins (17kDa and 19kDa) that are immunoprecipitated by 18kDa antiserum. Arrows in the panels mark the positions of proteins which exhibit enhanced or repressed synthesis in vivo or in vitro following a heat shock. Large circles identify low molecular weight proteins synthesized in vitro by total polyribosomal RNA while small circles identify proteins synthesized in vivo two of which may be isoelectric variants of the in vitro proteins. Arrowheads in the panels mark the positions of high molecular weight isoelectric variants that are synthesized in vivo only. M_r's of prominent hsp's are indicated by arrowheads on the right. Approximately 50,000 cpm of acid-precipitable counts were loaded onto each tube gel.



IN VIVO
IN VITRO
A
B
C
D
E
F
G
H
Mr x10-3
108
84
73
89
76
29
25.5
22
18
26
23
19
108
84
73
89
29
27
25.5
26
18
19

of the four prominent (Figures 1 and 7, #'s 1-4) 18kDa hsp's synthesized both in vivo and in vitro following a heat shock is synthesized in vitro by total polyribosomal RNAs extracted from 25°C plumules. A similar protein is not detected among the proteins synthesized in vivo by 25°C plumules. Furthermore, antiserum to the 18kDa hsp's did not react with any of the proteins synthesized in vivo by 25°C plumules (Figure 7, panel B). Surprisingly, this antiserum did not precipitate an 18kDa protein from the translation products synthesized by 25°C total polyribosomal RNAs (Figure 7, panel F). However, proteins with similar M_r's and pI's to those synthesized in vivo by 42.5^{O}C plumules and in vitro by 42.5^{O}C plumule mRNAs were immunoprecipitated from aliquots of the above total cell lysates and in vitro translation products respectively (Figure 7, panels D and H).

2.4 Discussion

Maize seedlings respond to a 1h heat shock by synthesizing hsp's analogous to those reported in animal systems. Previous studies characterizing the heat shock response in maize plumules identified six molecular weight hsp classes, one of which included a group of low molecular weight proteins with M_r's of approximately 18kDa. These studies indicate that this grouping may be too narrow as proteins ranging in M_r from 19 to 29kDa also exhibit enhanced synthesis in response to heat shock. I would, therefore, like to extend the low molecular weight hsp group to include these 16-20 proteins. More recent investigations concur with this re-definition (Mansfield and Key, 1987).

As expected for nuclear encoded proteins, increasing concentrations of cycloheximide inhibited protein synthesis. Although inhibitory to protein synthesis in vivo in higher plants (MacDonald and Ellis, 1969), the variable effect of cycloheximide on both isolated plant ribosomes (Marcus and Feeley, 1966; Ellis and MacDonald, 1967; Jachymczyk and Cherry, 1968; Ellis, 1969) and different plant tissues (Ellis, 1969; Ellis and MacDonald, 1970) demonstrates that the antibiotic can disrupt cellular metabolism other than by inhibiting protein synthesis and that, in some instances, the latter may be due to interference with energy transfer. Disruption of energy transfer and/or a conformational change in the ribosome elicited by cycloheximide binding may account for the observed differential accumulation/synthesis of high molecular weight isoelectric variants in cycloheximide-treated control and heat-shocked maize plumules. Enhanced synthesis of cytoplasmically translated proteins in response to low (0.1ug/ml) levels of cycloheximide has been observed in Neurospora crassa. It has been suggested that synthesis of these proteins on cycloheximide-sensitive ribosomes may represent an adaptive response of the organism (Perlman and Feldman, 1982).

Adaptation or resistance (ie. differential susceptibility) to cycloheximide or other kinds of environmental stress is thought to involve structural modifications of the ribosomes and/or associated protein factors (Frankel, 1970; Ellis and MacDonald, 1970;

Davies and Exworth, 1973; Hallberg et al. 1985). That cycloheximide resistance can be mediated through either ribosomal subunit supports this contention (Sutton et al. 1978). Furthermore, changes in ribosome conformation elicited by various pretreatments, including heat shock, starvation, and treatment with protein synthesis inhibitors such as cycloheximide have been observed (Hallberg and Wilson, 1981; Hallberg and Hallberg, 1983; Hallberg et al, 1984; Hallberg et al, 1985). The decreased sensitivity of Tetrahymena to the inhibitory effects of both cycloheximide and emetine following a heat shock corroborate these findings (Hallberg and Hallberg, 1983; McMullin and Hallberg, 1986). Heat-induced functional alterations in protein synthetic machinery have been correlated with changes in the association and/or structure of initiation factors and/or ribosomal proteins (Glover, 1982; Scharf and Nover, 1982; Duncan and Hershey, 1984; Panniers and Henshaw, 1984; McMullin and Hallberg, 1986). In more recent studies, changes in ribosomes conformation have been correlated with an increased affinity for a ribosomal protein (p-22) and an RNA polymerase III transcript (G8 RNA) in Tetrahymena (McMullin and Hallberg, 1986; Hallberg and Hallberg, 1989).

The variable effects of chloramphenicol on the synthesis of high molecular weight proteins in both control and heat-shocked plumules may involve a mechanism similar to that suggested for cycloheximide as high concentrations (0.3 to 0.4mg/ml) of chloramphenicol are known to impair certain energy-linked functions in the mitochondria (Hanson and Hodges, 1963; Ellis, 1969). Although inhibition of chloroplast ribosomal activity is stereospecific for the D-threo isomer of chloramphenicol (Ellis, 1969), this isomer also inhibits ion uptake (Ellis, 1969) and oxidative phosphorylation (Hanson and Krueger, 1966). Therefore, as with cycloheximide-treated plumules, the apparent effects of chloramphenicol on protein synthesis may ensue from effects on energy production.

Both cycloheximide and chloramphenicol interfere with oxidative phosphorylation in a manner analogous to the respiration suppressing phase of uncoupler action. Treatment with cycloheximide has been shown to cause an initial inhibition of oxygen uptake (Ellis

and MacDonald, 1970) while treatment with high concentrations of chloramphenicol depresses oxygen uptake (Hanson and Hodges, 1963). Furthermore, chloramphenicol concentrations in excess of 0.4mg/ml inhibit calcium binding by the mitochondria (Hanson and Hodges, 1963). In mammalian cells, glucose/calcium deprivation results in the increased synthesis of a group of proteins referred to as the grp's (Welch et al, 1989). The increased synthesis of protein variants in both control and heat-shocked plumules (ie. 84, 89, and 93kDa variants) in the presence of agents known to induce oxygen and/or calcium deprivation suggests that grp-like proteins exist in maize plumules.

Proteins exhibiting both cycloheximide-resistance and chloramphenicol-sensitivity were not detected in total cell lysates extracted from either control or heat-shocked maize plumules. However, heat-induced mitochondrially-associated proteins have been identified in a variety of organisms (McMullin and Hallberg, 1988; McMullin and Hallberg, 1987) including plants (Lin et al, 1984; Sinibaldi and Turpen, 1985; Nebiolo and White, 1985). Furthermore, in heat-shocked maize seedlings, at least one of these proteins is apparently encoded within the mitochondria (Sinibaldi and Turpen, 1985; Nebiolo and White, 1985). The inability to detect a mitochondrially-encoded protein in total cell lysates extracted from cycloheximide- or chloramphenicol-treated plumules following a heat shock may reflect the level of synthesis of this protein relative to the synthesis of total cellular protein. Alternatively, the protein may be absent as Nieto-Sotelo and Ho (1987) report that the 52/60kDa mitochondrial protein is in fact synthesized by bacteria contaminating the mitochondrial preparations.

The inhibitory effect of cycloheximide on hsp synthesis in vivo together with the in vitro synthesis of hsp's by total polyribosomal RNA with M_r's and pI's similar to those synthesized in vivo demonstrate that the major plumule hsp's are nuclear encoded. The qualitative differences observed between the proteins synthesized in vivo and in vitro may reflect post-translational modifications. Several plant species are known to synthesize low molecular weight nuclear-encoded hsp's that localize to chloroplasts (Kloppstech et al,

1985; Vierling et al, 1986). These proteins are synthesized as precursor proteins with amino terminal extensions or "transit" sequences that are removed during import into the chloroplast (Vierling et al, 1988). In maize leaf tissue, precursor proteins of 29 and 27.5kDa are cleaved to yield a mature 24kDa chloroplast hsp (Vierling et al, 1989). Analogous proteins in maize plumules may be the 27 and 25.5kDa proteins synthesized in vitro and the 22kDa protein synthesized in vivo. Alternatively, these differences and others may reflect co- or post-translational modifications involving ER-destined or secretory proteins and will be discussed elsewhere.

Synthesis of similar 18kDa hsp variants both in vivo and in vitro by total polyribosomal RNA suggests that these variants are encoded by different genes and/or gene products (ie. transcriptional and/or post-transcriptional control). Furthermore, the low molecular weight 18kDa hsp variants synthesized in vitro by maize plumules share common epitope(s) with those synthesized in vivo. The low molecular weight 18kDa hsp's in several plant systems have been shown to be antigenically related (Baszczynski, 1986; Baszczynski, 1989). In more recent studies, plant low molecular weight hsp's (both cytoplasmic and chloroplast) have been shown to share a common structural domain (ie. homologous carboxyl terminal regions) with low molecular weight hsp's synthesized in other eucaryotes (Vierling et al, 1988). However, antigenic conservation of the chloroplast-localized carboxyl terminal protein domain is restricted to chloroplast hsp's indicating a higher conservation between chloroplast hsp's of different species than within low molecular weight hsp's of the same species. The absence of an 18kDa hsp in the immunoprecipitates from in vitro 25°C lysates may, therefore, reflect the inaccessibility and/or absence or lack of conservation of a similar or different domain in 25°C proteins.

Changes in the protein synthetic profile in maize plumules following a heat shock can be explained, in part, by changes in the relative levels of the corresponding mRNAs as is inferred by the in vitro translation studies. The relative abundance of specific mRNAs is often the major factor determining protein levels. However, differential selection of

mRNAs for translation can also alter the translation repertoire of the eucaryotic cell as is indicated by comparison of the in vivo and in vitro protein synthetic profiles of heat-shocked plumules. Although translational control during heat shock has been demonstrated, both the mechanism(s) and extent of this regulation are unknown. The remaining chapters focus on changes in the relative amounts and distribution of heat shock mRNAs within subcellular compartments in an attempt to characterize translational regulatory mechanisms involved in the response of maize seedlings to heat shock.

CHAPTER 3

SUBCELLULAR DISTRIBUTION OF mRNAs IN THE PLUMULES OF CONTROL AND HEAT-SHOCKED MAIZE SEEDLINGS

3.1 Introduction

In the cytoplasm of eucaryotic cells, mRNA has been identified in two types of mRNP-protein complexes; polyribosomal and non-polyribosomal RNPs as discussed in Section 1.5 (Spirin, 1969; Morel et al, 1971; Civelli et al, 1976; Civelli et al, 1980; Vincent et al, 1980; Vincent et al, 1981; Vincent et al, 1983). Most eucaryotic cells (Spirin, 1969; Spohr et al, 1970), including plants (Ferrer et al, 1979; Kremp et al, 1986), contain non-polyribosomal RNP in the cytoplasm. Controversial views have been proposed concerning the role of this untranslated mRNA population in the 'cascade' of post-transcriptional controls of gene expression. Possible functions include the degradation of already translated 'aged' mRNA (Imaizumi-Scherrer et al, 1982), serving as carriers of post-transcriptional regulative signals (Civelli et al, 1980), acting as transport forms of mRNA from the nucleus to the cytoplasm via the cytoskeleton (Bag and Pramanik, 1986), and/or as storage forms of mRNA for later translation as is the case in many oocytes (Bienz and Gurdon, 1982; Dreyfuss, 1986).

Cytoplasmic control of mRNA translation is a well documented phenomenon. Selective translation of mRNAs has been extensively studied in avian erythroblasts (Imaizumi-Scherrer et al, 1982). In duck erythroblasts, approximately 200 mRNA species comprise the polyribosomal mRNA population while approximately 1400 different mRNAs are contained within the non-polyribosomal RNP mRNA population. Individual equilibria of activity exist for each mRNA which allow almost full translation and expression of some genes but condition the full repression at post-transcriptional and cytoplasmic levels of a majority of the activated genes. This qualitative suppression of the expression of specific genes has resulted in the definition of cytoplasmic mRNA compartments. The mRNA not

represented in the polyribosomes and therefore not active in translation is contained within an mRNA compartment that includes long-term repressed or 'masked' mRNA. Some mRNAs, however, exist simultaneously in both active and repressed forms. By definition, these short-term or partially repressed mRNAs are present in both the polyribosomal and non-polyribosomal compartment (Imaizumi-Scherrer et al, 1982). The nature of such short-term repression can be directly demonstrated in Drosophila cells exposed to a heat shock. The pre-existing mRNA is temporarily repressed but re-enters the polyribosomes some time after the return to normal temperature (Mirault et al, 1978; Lindquist, 1981; Kruger and Benecke, 1981; Ballinger and Pardue, 1982; Lindquist, 1986). A similar control mechanism has been observed in soybean seedlings following a heat shock (Key et al, 1981; Schoffl and Key, 1982; Czarnecka et al, 1984). This sequestering of pre-existing messages and preferential synthesis of hsp's is coordinated with a dramatic induction of the heat shock genes in Drosophila cells (Ashburner, 1982; Bonner, 1985) and soybean seedlings (Schoffl and Key, 1982; Czarnecka et al, 1984; Key et al, 1985). However, in chicken reticulocytes (Banerji et al, 1984) and Xenopus oocytes (Bienz and Gurdon, 1982), the preferential synthesis of at least one hsp, hsp70, after a heat shock results not from the dramatic induction of hsp70 genes but rather from the re-entry into the polyribosomes of translationally repressed hsp70 mRNA. Thus, in addition to transcriptional control mechanisms, post-transcriptional control mechanisms other than translational discrimination characterize the heat shock response.

In an attempt to assess the mechanisms governing the heat shock response in maize seedlings, I examined 1) the effect of a heat shock on mRNA levels in maize plumules, 2) the subcellular distribution of hsp mRNAs in control and heat-shocked plumules and 3) the effect, if any, heat shock has on the subcellular distribution of pre-existing nonhsp mRNAs.

3.2 Materials and Methods

3.2.1 Treatment of glassware and solutions

All glassware was either treated for 12 hours at 37°C with a solution of 0.1% diethypyrocarbonate (DEPC, Sigma) and autoclaved or baked at 250°C for 4 or more hours prior to use. Where possible, sterile, disposable plasticware was used. Solutions were prepared using pretreated glassware, DEPC-treated glass-distilled autoclaved water, and dry chemicals that were reserved for work with RNA only. Wherever possible, the solutions were treated with 0.1% DEPC for at least 12 hours and autoclaved (Anderson and Key, 1971; Maniatis et al,1982). Solutions that could not be autoclaved were sterile-filtered. All procedures were carried out at 0-4°C unless stated otherwise. Disposable gloves were worn during the preparation of materials and solutions for use in the isolation procedure, during the isolation and whenever RNA was being handled.

3.2.2 Growth and treatment of seedlings

Seeds of Zea mays L. (cv. Oh43) were germinated as described in Section 2.2.1. Intact seedlings were either maintained at 25°C or subjected to a 1h incubation at 42.5°C. Following the incubation period, proteins, total cellular RNAs, non-polyribosomal RNPs and polyribosomal RNP were extracted from the plumules of intact maize seedlings.

3.2.3 Extraction of protein from maize plumules

Individual intact seedlings were pre-incubated for 1h at 25°C or 42.5°C and labelled for an additional 1h at 25°C or 42.5°C in the presence of 100uC/ml (1Ci=37GBq) of L-[^{35}S] methionine (NEN; specific activity=1100Ci/mmol). After labelling, the plumules were excised and the proteins homogenized in extraction buffer (see Section 2.2.3).

3.2.4 Isolation of total polyribosomal RNP RNAs

Polyribosomal RNPs were isolated from the plumules of intact maize seedlings according to the procedure of Lerner et al (1971) as described in Section 2.2.5 except that the homogenization buffer contained 0.25M sucrose, 200mM Tris-Cl pH8.5, 400mM KCl, 5mM $MgCl_2$, 2mM DTT (Sigma Chemical Co., St. Louis, MO), 0.5mM PMSF, 0.2mg/ml emetine-dihydrochloride (Boehringer Mannheim Canada, Dorval, Que.)(TKMD-B buffer), and 0.1-1.0mg/ml heparin (Breen et al, 1971; Davies et al, 1972; Ramagopal and Hsiao, 1973; Jackson and Larkins, 1976; Gupta and Siminovitch, 1978; Clemens, 1986). The higher salt concentration and pH improve the yield of polyribosomal RNPs from plant tissue (integrity was examined by electron microscopy and sucrose gradient analyses). After centrifugation, the supernatants were aspirated and saved for analysis (see Section 3.2.8) or discarded. The tube walls were wiped clean and the pellets resuspended in a small volume of either translation buffer (50mM Hepes pH 7.4, 25mM KCl, 2mM Mg-acetate, 1mM DTT, 0.1mg/ml heparin)(Maniatis et al, 1982) or digestion buffer (0.01M Tris-Cl pH 7.8, 0.005M EDTA, 0.5% SDS, 1mg/ml proteinase K and 0.1mg/ml heparin)(Brawerman et al, 1972; Maniatis et al, 1982) for analysis of intact polyribosomes or deproteinized polyribosomal RNA respectively. In the latter case, the polyribosomes were incubated at 37°C for 30 minutes and the RNA was extracted with phenol and/or chloroform (Maniatis et al, 1982).

3.2.5 Isolation of free-cytoplasmic, membrane-, and cytoskeletally-associated polyribosomal RNP RNAs by differential centrifugation

Polyribosomal RNPs were isolated from several different fractions of maize plumules following the procedure of Venkatesan and Steele (1972) with some modifications (Ramsey and Steele, 1977; Heikkila et al, 1981; Cevera et al, 1981). Following treatment, plumules were excised, weighed, ground with a mortar and pestle and homogenized on ice in seven volumes of 0.25M sucrose-TKMD-B containing emetine

dihydrochloride (Breen et al, 1971; Davies et al, 1972; Ramagopal and Hsiao, 1973; Jackson and Larkins, 1976; Gupta and Siminovitch, 1978; Clemens, 1986) in a glass homogenizer with a motor-driven teflon pestle. The resulting homogenate was centrifuged at 4°C for 2 minute at 2000 rpm (740xg) and then the speed was increased to 25000 rpm (110,000xg) and maintained there for 15 minutes to pellet rough microsomes (SW41Ti rotor, Beckman). The supernatant containing the free-cytoplasmic polyribosomal RNPs was stored in an ice bath while the pellet (particulate fraction) was suspended by homogenization in 16ml of 0.25M sucrose-TKMD-B buffer lacking emetine dihydrochloride. The mixture was made 1% with 10% (w/w) Triton X-100 (in H_2O), re-homogenized with three strokes of a motor-driven teflon pestle as above, and centrifuged at 3500 rpm (1470xg) for 20 minutes at 4°C (SS-34 rotor, Sorvall). The pellet, which in animal tissue contains the cytoskeletal framework and nuclei, was stored in an ice bath for further treatment while the supernatant was mixed with one-nineth volume of 13% (w/w) deoxycholate and centrifuged at 14000 rpm (24000xg) for 30 minutes at 4°C (SS-34 rotor, Sorvall)(Pearson, 1969). The resulting supernatant containing the membrane-associated polyribosomal RNPs was placed in an ice bath. The pellet containing the nuclei and putative cytoskeletal fraction was resuspended by vortexing in 14ml of reticulocyte standard buffer (RSB; 10mM Tris-Cl pH 7.4, 10mM NaCl, 1.5mM $MgCl_2$, 1mM PMSF and 0.1mg/ml heparin) and homogenized with five strokes of a motor-driven teflon pestle in the presence of a mixture of Tween 40 and sodium deoxycholate added to a final concentration of 1% and 0.5% respectively (Cervera et al, 1981). The nuclei were pelleted by centrifugation at 3500 rpm (1470xg) for 2 minutes at 4°C (SS-34 rotor, Sorvall) and stored in an ice bath for later use (see Section 3.2.7). The supernatant, which is assumed to contain cytoskeletally-associated polyribosomal RNPs based on fractionation of animal cells, and the supernatants containing the free-cytoplasmic and membrane-associated polyribosomal RNPs were each carefully layered over 6ml of 1.65M sucrose-TKMD-B buffer (Larkins and Davies, 1975) and centrifuged at 38000 rpm (162,000xg) for 3h and

25 minutes at $4^{o}C$ to pellet the polyribosomal RNPs (Ti 55.2, Beckman). The supernatants were aspirated and saved for further analysis (see Section 3.2.8). The tube walls were wiped clean and the pellets were resuspended in a small volume of digestion buffer, incubated at $37^{o}C$ for 30 minutes, followed by extraction of the RNA with phenol and/or chloroform (Brawerman et al. 1972; Maniatis et al. 1982).

3.2.6 Isolation of total cellular RNA

Plumules were excised from intact maize seedlings and pulverized in liquid nitrogen (N_2). After the liquid N_2 evaporated the powder was resuspended in 15ml of ice cold 4M guanidine isothiocyanate, 1.65ml 3M sodium acetate, pH6.0, and 1.67ml B-mercaptoethanol (GIT buffer) and transferred to a 50ml conical centrifuge tube. The suspension was homogenized with a Polytron (2x 45s bursts; Brinkman Instruments, Westbury, NY). An additional 10ml of ice cold GIT buffer was added and the suspension re-homogenized (1x 30s burst). The debris was loosely pelleted by centrifugation at 2000 rpm for 5 minutes in a clinical table-top centrifuge. The supernatant was layered over 4.5ml of $CsCl_2$ buffer and centrifuged at 32000 rpm (174,000xg) for 27.5h at $20^{o}C$ (SW41Ti rotor, Beckman). After centrifugation, the DNA and most of the supernatant were removed with a Pasteur pipet. Any remaining supernatant was removed with a micropipet and by swabbing the walls of the tube. The pellet was resuspended in 270ul of H_2O and the RNA was precipitated overnight at $-70^{o}C$ with 2.5 volumes of 95% EtOH-0.3M Na-acetate pH 5.2. The RNA was pelleted by centrifugation at 11500 rpm (16000xg) for 1h at $4^{o}C$ (SS-34 rotor, Sorvall), washed with 80% EtOH and lyophylized to dryness (Glisin et al. 1974; Chirgwin et al. 1979; MacDonald et al. 1987; Davis et al. 1986). The pellet was resuspended in 100ul of H_2O and the quantity of RNA determined by reading the OD at 260nm.

3.2.7 Isolation of nuclear RNA

Nuclear RNA was recovered by the guanidium procedure used for the preparation of total cellular RNA (Chirgwin et al, 1979; Nevins, 1987; MacDonald et al, 1987). The integrity of the nuclei (from Section 3.2.5) was monitored by light microscopy of Giemsa-stained nuclei or by phase microscopy.

3.2.8 Isolation of non-polyribosomal RNP RNA

Post-polyribosomal supernatants (from Sections 3.2.4 and 3.2.5) were layered over 6ml of 15% sucrose-TKMD-B buffer and centrifuged at 38000 rpm (162,000xg) for 21h at 4°C (Ti 55.2 rotor, Beckman)(Spohr et al, 1972; Gander et al, 1973). After centrifugation, the supernatants were aspirated and saved for further analysis or discarded. The tubes were inverted and buried in ice for 15-20 minutes. The tube walls were wiped clean and the pellets (crude non-polyribosomal RNP) resuspended in a small volume of either translation buffer or digestion buffer for analysis of intact or deproteinized non-polyribosomal RNP respectively.

3.2.9 Isolation of poly(A)$^{\pm}$ RNA

Poly(A)$^{+}$ RNA was isolated from deproteinized RNA using Hybond messenger affinity paper (Hybond-mAP, Amersham) according to the procedure outlined by Amersham. Briefly, a known quantity of deproteinized RNA was lyophylized and resuspended in a small volume (100-200ul) of resuspension buffer (RB; 0.5M NaCl, 0.01M Tris-Cl pH6.8, 0.001M EDTA). The sample was heated for 5 minutes at 60-65°C to denature the RNA, immediately chilled on ice, and then slowly spotted to a piece of Hybond-mAP (approximately 1 cm^2 assuming that 8-12ug poly(A)$^{+}$ RNA can be isolated per cm^2 Hybond-mAP) that had been equilibrated in RB and placed on 4 layers of 3MM filter paper. The Hybond-mAP was then left in a covered petri dish for 1h or until the sample had been completely absorbed by the paper. In some cases, additional buffer was added to the samples which were then re-heated, cooled and applied to the paper.

Following the incubation period, the paper was placed in a sterile petri dish and washed in 0.5M NaCl (5ml/cm^2) for 5 minutes with continuous shaking. This wash was repeated with fresh 0.5M NaCl and followed with a final wash in 70% EtOH (5ml/cm^2) for 2 minutes with continuous shaking. The paper was blotted to filter paper and allowed to completely air dry. The Hybond-mAP was transferred to a 1.5ml eppendorf tube containing 600ul of H_2O and heated to 70^oC for 5 minutes to release the poly(A)$^+$ RNA from the paper. The H_2O (containing the RNA) was transferred to a sterile 1.5ml eppendorf tube while the initial tube was centrifuged in a microfuge at 14000 rpm for 1 minute at 4^oC to extract any remaining liquid. The latter was pooled with the former and the poly(A)$^+$ RNA was stored at -70^oC.

3.2.10 Cell-free translation of intact, deproteinized and mAP-selected polyribosomal and non-polyribosomal RNP RNAs

Intact polyribosomal and non-polyribosomal RNPs along with deproteinized and poly(A)$^+$ cellular, polyribosomal and non-polyribosomal RNP RNAs from control and heat-shocked maize plumules were used to direct the in vitro synthesis of polypeptides in a cell-free rabbit reticulocyte lysate system (New England Nuclear, NEN, Boston, MA) using L-[^{35}S] methionine (NEN; specific activity ~1100Ci/mmol) as the labelling probe. Translations were carried out as described in Section 2.2.6 using a range of RNA concentrations. To assess co-translational processing or core glycosylation, microsomal membranes (0.5ul/reaction tube) isolated from dog pancreas (NEN; Protein Processing Translation System) were added to the lysate prior to its addition to the appropriate duplicate samples (NEN Protein Processing Translation System instruction manual).

3.2.11 Gel electrophoresis

3.2.11.1 One- and two-dimensional polyacrylamide gel electrophoresis and fluorography

One-dimensional (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (1970) except that the separating gel consisted of a 7.5-17.5% polyacrylamide gradient as described in Section 2.2.8.1. Two-dimensional (IEF-SDS) analysis of the proteins followed with minor modifications (Atkinson, 1981; Baszczynski et al, 1983; Rees et al, 1986) the gel electrophoresis method developed by O' Farrell (1975) as described in Section 2.2.8.2.

Coomassie brilliant blue R-250 stained slab gels were processed for fluorography (see Section 2.2.9.2.).

3.2.11.2 Electrophoresis of RNA after denaturation with glyoxal and DMSO

RNA was denatured essentially as described by McMaster and Carmichael (1977). Briefly, RNA (up to 20ug/16ul reaction mixture) was incubated in 1.5ml eppendorf tubes for 1h at $50^{o}C$ in a buffer containing 1.0M glyoxal, 50% (v/v) dimethysulfoxide (DMSO) and 10mM NaH_2PO_4, pH7.0. The reaction mixture was cooled on ice to $20^{o}C$ and 4.0ul of sterile loading buffer (50% glycerol, 10mM NaH_2PO_4, pH7.0 and 0.4% bromophenol blue) was added to each tube. The samples were electrophoresed on horizontal 1% agarose gels in 10mM NaH_2PO_4 buffer, pH7.0 for 5-6h (until dye front was two-thirds of the way down the gel) at 70V. As glyoxal readily dissociates from RNA at pH8.0 or higher, constant recirculation of the buffer was required to maintain the pH7.0. RNA ladder (BRL Life Technologies, Inc., Burlington, Ontario, Can.), a mixture of six RNA components with each component containing sequences derived from bacteriophage T7, yeast 2u circle and bacteriophage \DNA, was denatured and co-electrophoresed with the RNA samples for use in M_r determinations. If the RNA was to be transferred from the agarose gel to membrane, the wells containing the RNA ladder were cut away from the gels following electrophoresis, stained for 15 minutes in 5ug/ml ethidium bromide (EtBr), destained for 15 minutes in H_2O and photographed by short wave UV (302nm) transillumination (Wratten #9 filter)(Spectroline, Model TR-302, Spectronics Corporation, Westbury, NY).

When transfer was not required, the gels were stained as described by Alwine et al (1983). Glyoxal adducts were removed by 4-25 minute washes in 50mM NaOH. The gel was neutralized by 2-5 minute washes in 200mM NaH_2PO_4, pH6.5 and stained with 0.5ug/ml EtBr in 20mM NaH_2PO_4, pH6.5 for 45 minutes. Destaining was for 30 minutes to 1h in 20mM NaH_2PO_4, pH6.5 and photography was as described above.

3.2.12 Transfer of glyoxylated RNA to biodyne A nylon membranes

RNA was transferred from agarose gels to biodyne A membranes (Pall Ultrafine Filtration Corporation, Glen Cove, NY) using 3M NaCl/ 0.3M trisodium citrate (20x SSC) essentially as described by Thomas (1980). Transfer was for 30h with the paper towels being changed every 30 minutes for the first 2h and every 6-12h thereafter as required. The membrane was removed from the gel surface, marked for identification, dried for 10 minutes at $60^{o}C$ and baked in a vacuum oven for 2h at $80^{o}C$. Prior to use, the RNA blots were placed in 200ml of 20mM Tris buffer pH8.0 at $100^{o}C$ and allowed to cool to room temperature. The efficiency of the transfer was assessed by staining the blotted gel for 45 minutes in 0.5ug/ml EtBr in H_20.

3.2.13 Plasmids, recovery, and labelling of DNA fragments

Plasmid MON9502 (provided by Dean E. Rochester, Monsanto Company, St. Louis, MO) contains the entire maize hsp70 coding region, 81 nucleotides of the 5' non-translated region, and 66 nucleotides of the 3' non-translated region (Shah et al, 1985). The gene encodes a 73kDa protein in maize plumules and radicles. Plasmid small 2 (provided by R. M. Sinibaldi, Sandoz Crop Protection, Zoecon Research Institute, Palo Alto, California) contains the protein-coding region of a maize hsp18 gene. This fragment selects poly(A)+ RNAs encoding an 18kDa protein from maize plumules and four 18kDa hsp variants from maize radicles (Atkinson et al, 1989). Plasmid MAc1 (provided by R. B. Meagher, Dept. of Molecular and Population Genetics, University of Georgia, Athens,

Georgia) contains a maize actin gene (Shah et al, 1983). Competent JM103 cells were transformed with pMON9502 or small 2 while competent HB101 cells were transformed with pMAc1 (Hanahan, 1983). Transformants were amplified and the plasmids re-isolated (Clewel and Helsinki, 1972). Plasmid DNA was digested with the appropriate restriction endonucleases (Maniatis et al, 1982) and electrophoresed in an EtBr-containing 0.6% low-melting-temperature (LTM) agarose gel underlaid for support with a 1.5% agarose gel (normal agarose). The bands were visualized using a UV (302nm) transilluminator (Spectroline, Model TR-302, Spectronics Corporation, Westbury, NY). The fragment encoding the gene that was to be radiolabelled with ^{32}P by random primer extension (Random Primers DNA labelling system, BRL Life Technologies, Inc., Burlington, Ont., Can.) for use as a hybridization probe was excised. The DNA was recovered from the LTM agarose using the Gene Clean procedure described in the Bio 101 Gene Clean instruction manual (Bio 101, Inc., La Jolla, California). Aliquots of the isolated DNA fragments were electrophoresed on EtBr-containing 0.8% agarose gels and sized prior to labelling.

3.2.14 Hybridization

Prehybridization, hybridization and washing were carried out in either seal-O-meal baggies or hybridease chambers (Hoeffer Scientific Instruments, San Francisco, CA) as described by Thomas (1980, 1983). RNA blots were prehybridized in buffer containing 5x Denhardt's, 5x SSC pH7.0, 50mM NaH_2PO_4, pH6.5, 0.1% SDS, 250ug/ml sheared salmon sperm DNA and 50% (v/v) formamide for 8-20h at 42°C. Hybridization buffer contained four parts of prehybridization buffer and one part 50% (v/v) dextran sulphate. The ^{32}P labelled probes (randomly primed using the Random primers DNA labelling system, BRL Life Technologies, Inc., Burlington, Ontario, Can.) used in these studies (see Section 3.2.19) were denatured at 100°C for 5 minutes, cooled and added to the hybridization buffer and the blots were hybridized for 20h at 42°C. The RNA blots were

washed with five changes of 2x SSC, 0.1% SDS for 5 minutes each at room temperature and then washed with three changes of 0.2x SSC, 0.1% SDS for 15 minutes each at $50^{o}C$. The blots were wrapped in Saran wrap and apposed at $-70^{o}C$ to Kodak X-Omat film (XR-1) using a Kodak intensifying screen (Cronex Xtra Life, Dupont, Can.).

3.2.15 Rehybridization

Removal of probe was carried out according to the procedure by Pall (1983). RNA blots were placed in seal-0-meal baggies each containing 100ml NaH_2PO_4, pH6.5, 50% (v/v) formamide and incubated for 1h at $65^{o}C$. The blots were then washed with 250ml of 2x SSC, 0.1% SDS for 15 minutes at room temperature with continuous agitation. Excess buffer was drained and the blots were wrapped in Saran wrap and apposed at $-70^{o}C$ to Kodak X-Omat film. If probe removal was satisfactory, the blots were prehybridized and hybridized with the desired probe as above.

3.2.16 Dot blot hybridization

The bio-dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA) was assembled with equilibrated biodyne A membrane according to the Bio-Rad instruction manual. The membrane was rehydrated and pretreated RNA and DNA samples (Thomas, 1980; Thomas, 1983), diluted to 500ul with H_2O (Bio-Dot microfiltration apparatus instruction manual), were applied into the appropriate wells with the vacuum off but the flow valve open. Nucleic acid was bound to the membrane by gentle vacuum pressure. The wells were washed with 500ul of 20x SSC under vacuum until completely dry. The membrane was removed from the apparatus, marked for identification, dried for 10 minutes at $60^{o}C$ and baked for 2h at $80^{o}C$ under vacuum. Prehybridization, hybridization and washing were as described above (see Section 3.2.13) except that dextran sulphate was not included in the hybridization buffer (Anderson and Young, 1986).

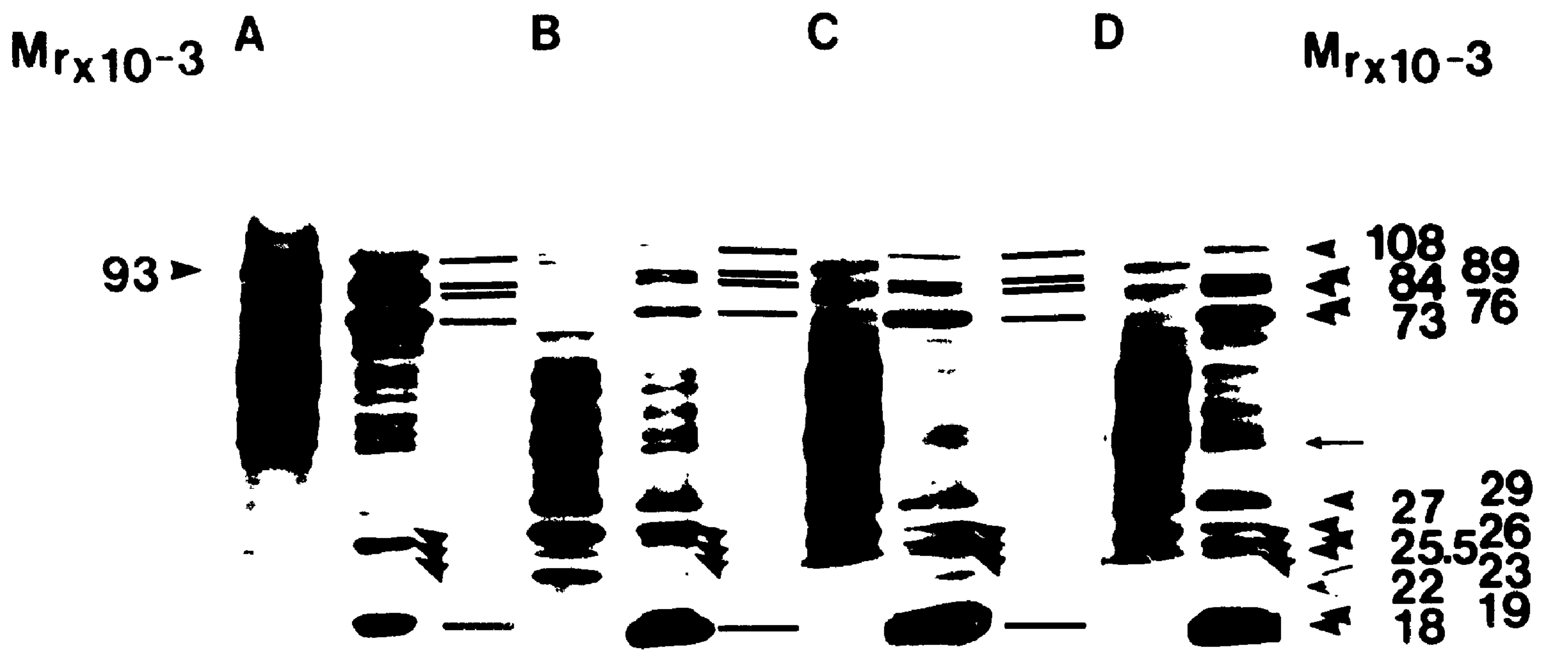
The autoradiograms were scanned using a laser densitometer and the relative amount of RNA determined.

3.3 Results

3.3.1 Characterization and intracellular distribution of hsp mRNPs

In order to determine the extent to which heat shock affected the intracellular distibution of RNPs, the products of in vitro translation of mRNAs in polyribosomal and non-polyribosomal RNPs were analyzed by one- (Figure 8) and two- (Figure 9) dimensional polyacrylamide gel electrophoresis. Cytoplasmic mRNA-protein particles were isolated by differential centrifugation (see Materials and Methods) from the plumules of intact maize seedlings maintained at 25°C (control) or subjected to a 1h heat shock at 42.5^{o}C. Since the non-polyribosomal RNPs were inactive in promoting protein synthesis in vitro (see Appendix 1), the mRNAs associated with these particles were extracted by deproteinization with phenol and/or chloroform. Optimal amounts of non-polyribosomal and polyribosomal RNP mRNAs (see Appendix 1 and Appendix 2), as well as total cellular RNAs, were translated in a cell-free reticulocyte lysate system. The products of in vitro translation of the respective mRNA populations were compared by 1-D polyacrylamide gel electrophoresis with the hsp's synthesized by plumules in vivo (Figure 8). Heat shocking the plumules of intact maize seedlings results in the new and/or enhanced synthesis of a characteristic group of plumule hsp's (Baszczynski et al, 1984; Rees et al, 1986) of 108, 89, 84, 76, 73 (referred to in the remaining text as 70kDa), and 18-29kDa in vivo (Figure 8, panel A) and the repressed synthesis of some 25°C proteins (Baszczynski et al, 1982; Cooper and Ho, 1983). Polypeptides with similar M_r's (albeit quantitatively different) are synthesized in vitro by total cellular (Figure 8, panel B), non-polyribosomal RNP (Figure 8, panel C) and polyribosomal RNP (Figure 8, panel D) mRNAs extracted from the plumules of heat-shocked seedlings. Additional proteins of 27 and 25.5kDa are differentially synthesized in vitro by mRNAs extracted from these subcellular fractions (Figure 8, lanes 4, 6 and 8). Furthermore, 25°C proteins are synthesized in vitro (albeit

Figure 8: Fluorograms of 1-D polyacrylamide gel electrophoretic separations of the polypeptides synthesized in vivo by plumules of intact maize seedlings (panel A) and in a cell-free translation system by total cellular (panel B), non-polyribosomal RNP (panel C) and polyribosomal RNP (panel D) mRNAs isolated from plumules of intact maize seedlings maintained at 25°C (control, lanes 1, 3, 5 and 7) or subjected to a 1h heat shock at 42.5°C (lanes 2, 4, 6 and 8). Protein synthetic profiles are representative of those obtained in three independent experiments. Arrowheads in the panels mark the positions of a 26kDa polypeptide synthesized both in vivo and in vitro and two proteins with M_r's of 27000 and 25500 synthesized only in vitro. M_r's of hsp's are indicated by arrowheads on the right. The arrowhead on the left marks the position of a 93kDa protein which exhibits repressed synthesis after a heat shock. The arrow on the right marks the position of a putative maize actin. Approximately 100,000 cpm of acid-precipitable lysate was loaded into each well.

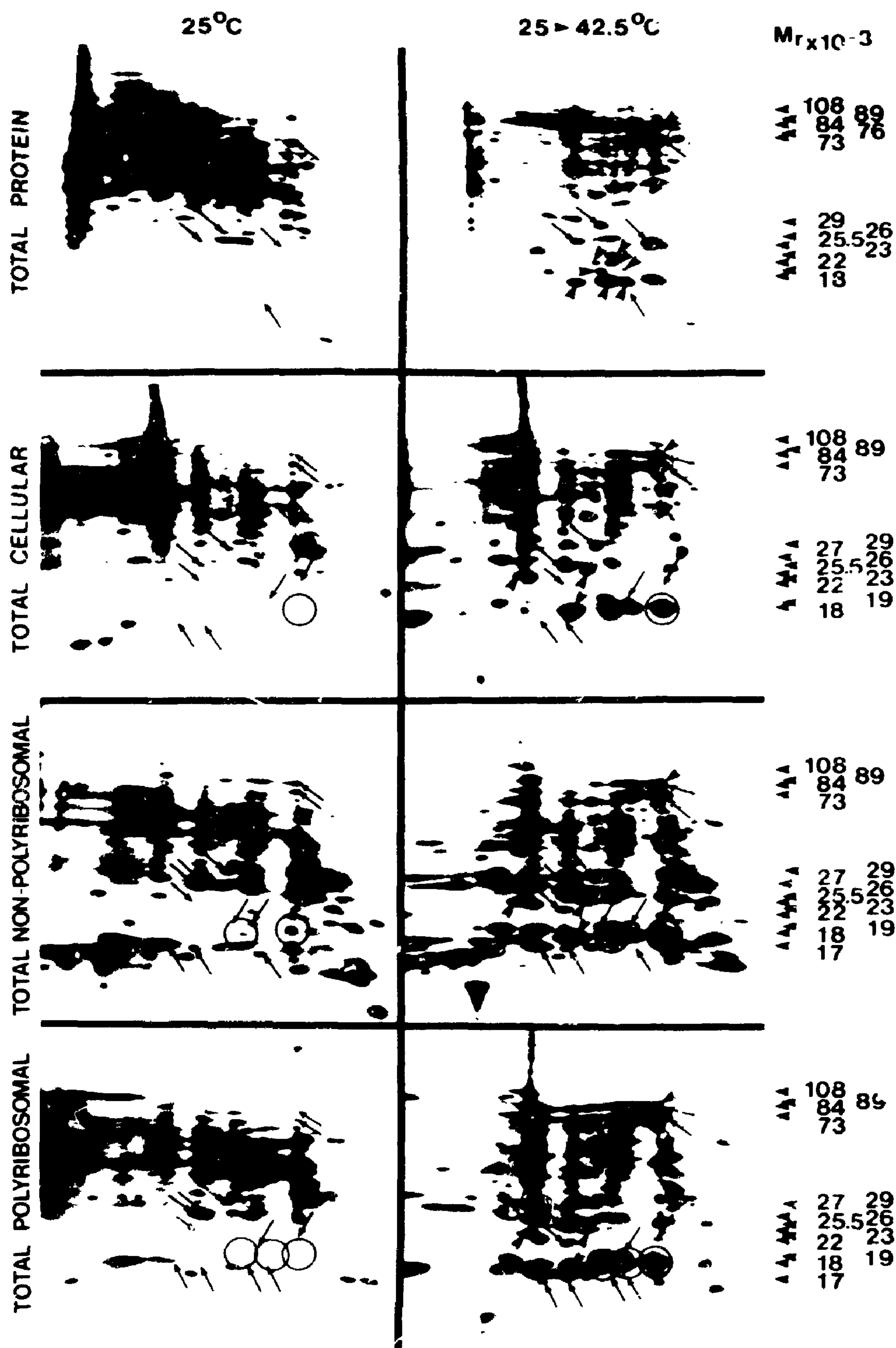


Mrx10-3
A
B
C
D
Mrx10-3
93
108
89
84
76
73
29
27
26
25.5
23
22
19
18
1 2 3 4 5 6 7 8

variably) by the mRNAs comprising these fractions in plumules subjected to a 1h heat shock.

The complexity of the protein synthetic response to heat shock becomes more apparent in Figure 9. Several minor quantitative (Figure 9, arrows and large circles) and qualitative (Figure 9, arrowheads and intermediate circles) differences are observed not only between the in vivo and in vitro protein synthetic profiles but also between the proteins synthesized by total cellular, non-polyribosomal RNP and polyribosomal RNP mRNAs. Many of these differences are observed among the more complex group of low molecular weight proteins (Figure 10). Non-polyribosomal RNP and polyribosomal RNP mRNAs extracted from heat-shocked plumules synthesize additional and/or different isoelectric variants of the 18kDa, 22kDa, 23kDa, 25.5kDa, and 26kDa proteins (Figure 10, large circles) than are synthesized in vivo (Figure 10, intermediate circles). Similar variants are synthesized in vitro by total cellular RNA in reduced amounts. Total cellular, non-polyribosomal, and polyribosomal mRNAs also encode 27kDa proteins that are not detected in vivo (Figure 10, large circles). These proteins are more prominent among the translation products synthesized by non-polyribosomal and polyribosomal RNP mRNAs. A 17kDa protein synthesized by non-polyribosomal RNP and polyribosomal RNP mRNAs is not observed among the translation products of total cellular mRNAs but is detected in low levels among the proteins synthesized in vivo (Figure 10, square). Furthermore, several additional 17kDa variants are differentially synthesized by mRNAs associated with non-polyribosomal and polyribosomal RNPs (Figure 10, arrows). Non-polyribosomal RNP mRNAs also direct the synthesis of a 26kDa variant that is not detected among the proteins synthesized by any of the other cellular fractions (Figure 10, small circle). Differences in the high molecular weight proteins synthesized in response to a 1h heat shock are limited to a 76kDa hsp that is synthesized in vivo but not in vitro by any of the mRNA populations (Figure 9, intermediate circle).

Figure 9: Fluorograms of representative 2-D gel electrophoretic separations of the polypeptides synthesized *in vivo* by plumules of intact maize seedlings and in a cell-free system by total cellular, non-polyribosomal RNP and polyribosomal RNP mRNAs isolated from the plumules of maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5°C. Protein synthetic profiles are representative of those obtained from more than three independent isolations. Arrows in the panels mark the positions of polypeptides which exhibit enhanced or repressed synthesis *in vivo* and *in vitro* following a heat shock. Arrowheads in the panels mark the positions of proteins detected only after a heat shock. Large circles identify 18kDa variants that are synthesized *in vitro* by 25°C mRNAs and have similar pI's to the 18kDa variants synthesized *in vitro* following a heat shock. Intermediate circles identify a 76kDa protein that is synthesized *in vivo* but is not detected among the proteins synthesized *in vitro* by total cellular, non-polyribosomal RNP or polyribosomal RNP mRNAs. M_r's of hsps are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable lysate was loaded onto each IEF gel.


25°C
25 ► 42.5°C
Mr x 10-3
TOTAL PROTEIN
TOTAL CELLULAR
TOTAL NON-POLYRIBOSOMAL
TOTAL POLYRIBOSOMAL
108 89
84 76
73
29 26
25.5 23
22
18
108 89
84
73
27 29
25.5 26
22 23
18 19
108 89
84
73
27 29
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22 23
18 19
17
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84
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27 29
25.5 26
22 23
18 19
17

2

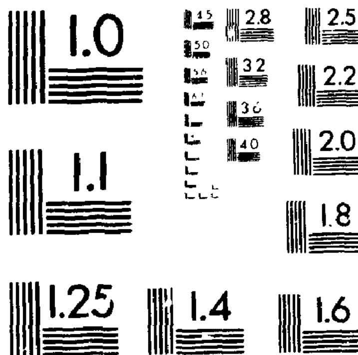
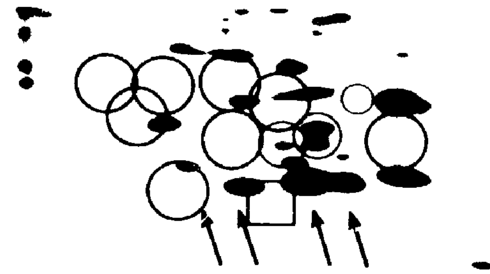
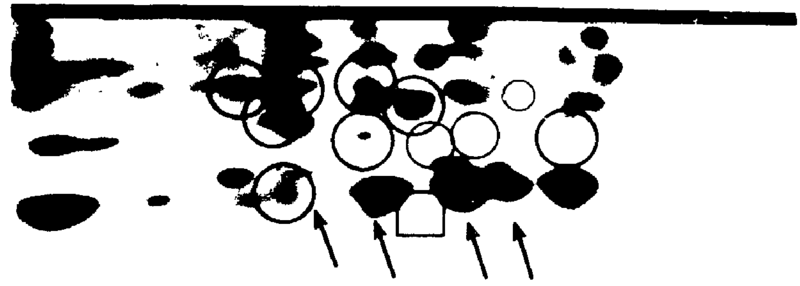
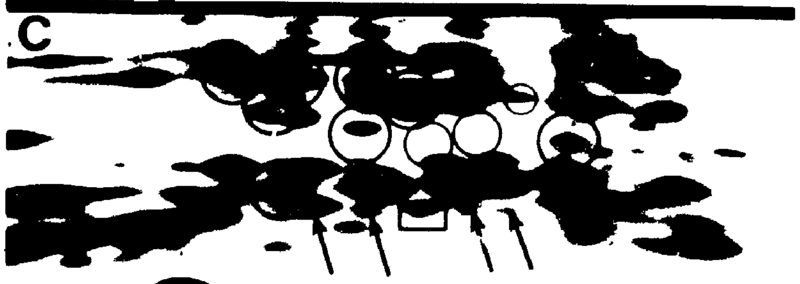


Figure 10: Fluorograms of 2-D gel electrophoretic separations of the low molecular weight proteins synthesized in vivo by heat-shocked plumules (panel A) and in vitro by total cellular (panel B), non-polyribosomal RNP (panel C), and polyribosomal RNP (panel D) mRNAs extracted from heat-shocked plumules. Arrows in the panels identify proteins that are variably synthesized by non-polyribosomal RNP and polyribosomal RNP mRNAs. Large circles mark the positions of proteins synthesized in vitro by total cellular, non-polyribosomal RNP, and polyribosomal RNP mRNAs. Intermediate circles mark the positions of putative isoelectric variants of some of these proteins that are synthesized in vivo. The square marks the position of a protein that is synthesized both in vivo and in vitro by non-polyribosomal RNP and polyribosomal RNP mRNAs while the small circle identifies a protein that is detected only among the translation products synthesized by non-polyribosomal RNP mRNAs. M_r's of hsp's are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable lysate was loaded onto each IEF gel.


A
$M_r \times 10^{-3}$
29
25.5 26
22 23
18 19
17


27 29
25.5 26
22 23
18 19
17


C
27 29
25.5 26
22 23
18 19
17

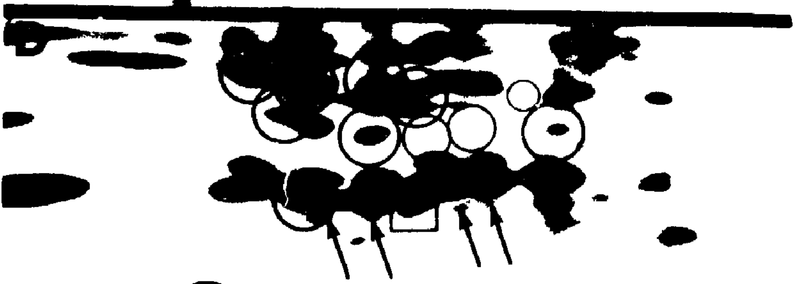

27 29
25.5 26
22 23
18 19
17

Figure 9 also demonstrates that isoelectric variants of the 70kDa (Figure 9, arrows) hsp's are synthesized in vitro by 25°C total cellular, non-polyribosomal RNP and polyribosomal RNP mRNAs. Furthermore, the cellular fractionation studies indicate that mRNAs encoding proteins with M_r's and pI's similar to all but one of the four prominent 18kDa hsp's accumulate at 25°C. Although there are some differences in the level of nonheat shock proteins synthesized after a heat shock in vivo, 25°C mRNAs extracted from control or heat-shocked tissue are translated in roughly equivalent amounts in vitro.

The intracellular distribution of specific mRNAs in control and heat-shocked maize plumules was further characterized by Northern blot analysis. Total cellular, nuclear, non-polyribosomal RNP and polyribosomal RNP mRNAs extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock were denatured and electrophoretically separated on neutral agarose gels. The RNAs were transferred to an uncharged nylon membrane and hybridized initially to a 4.0kb EcoR1-BamH1 fragment encoding a maize hsp70 gene and then to a 0.58kb Pst1-Sal1 fragment encoding a maize hsp18 gene. Both hsp70 (2.6kb) and hsp18 (0.9kb; although barely detectable) mRNAs are present in 25°C plumules (Figure 11, A and B, lanes 1-4). Moreover, the association of these mRNAs with the cytoplasmic fractions corroborates the in vitro translation results. Heat shock results in the enhanced synthesis (although to different degrees) and/or increased stability of both the 70kDa and 18kDa RNA transcripts (Figure 11, A and B, lanes 2 and 6) and the subsequent association and/or accumulation of these transcripts with the non-polyribosomal RNP (Figure 11, A and B, lanes 3 and 7) and on the ribosomes of polyribosomes (Figure 11, A and B, lanes 4 and 8) respectively.

Following a 1h heat shock, changes in the level of 70kDa RNA transcripts in the cellular and nuclear fractions were similar while changes in the level of these transcripts in the cytoplasmic fractions were more variable (Figure 11 and Table 1). The increase in hsp70 mRNA synthesis following a heat shock is small relative to that observed for hsp18 mRNA where heat shock results in the rapid accumulation of 18kDa RNA transcripts in the

Figure 11: Northern blot analysis of total cellular, nuclear, non-polyribosomal RNP and polyribosomal RNP mRNAs extracted from the plumules of intact maize seedlings. The autoradiograms are representive of those obtained following hybridization of probe to RNA from several (>3) isolations. Positions of the 25S and 18S maize rRNAs are indicated by bars between panels A and B. M_r's of standards co-electrophoresed with RNA samples are indicated on the left. Lanes 1-8 in panel A correspond to lanes 1-8 in panel B. Lane 1: 10.0ug of total cellular RNA extracted from control (25°C) plumules; Lane 2: 10.0ug of 25°C nuclear RNA; Lane 3: 10.0ug of 25°C non-polyribosomal RNP RNA; Lane 4: 10.0ug of 25°C polyribosomal RNP RNA. Lanes 5-8 correspond to lanes 1-4 except that the seedlings were subjected to a 1h heat shock at 42.5^{o}C prior to RNA extraction. A) pUC9 and a 4.0kb EcoR1-BamH1 maize hsp70 gene fragment were used as hybridization probes. The 4.0kb fragment hybridized to mRNAs of 2.6kb in both 25°C and 42.5^{o}C RNA extracts from the different cellular fractions. pUC9 failed to hybridize to lanes containing RNA samples (data not shown). B) The blot in A was washed and re-probed with a 0.58kb Pst1-Sal1 fragment containing a maize hsp18 gene. This fragment hybridized to mRNAs of 0.9kb in all heat-shocked fractions (lanes 5-8). Hybridization to 25°C RNAs of a similar size, although variable, was detected in the RNA populations from all 25°C cellular fractions. The specific activities of the ^{32}P-labelled DNA hybridization probes were similar (1.7x10^{9}cpm/ug DNA, 24h exposure).

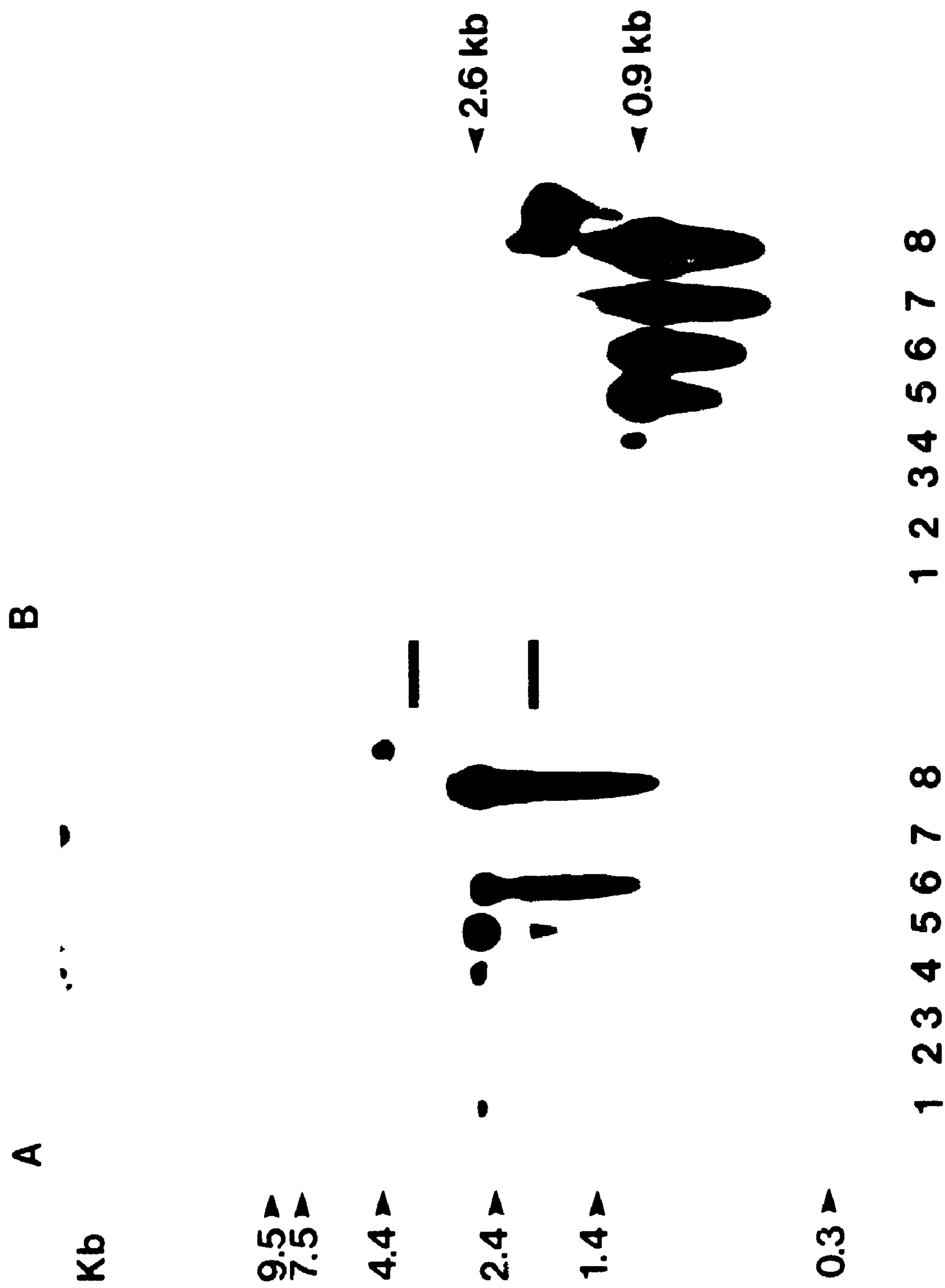

A
B
Kb
9.5
7.5
4.4
2.4
1.4
0.3
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
2.6 kb
0.9 kb

TABLE 1: Effects of heat shock on protein synthesis and mRNA levels in maize plumules[1]

Cellular Fractions	Protein Synthesis[3]			mRNA Levels[2]								
				70			18			Actin		
	70	18	Actin	TP[4]	N[5]	DB[6]	TP	N	DB	TP	N	DB
Control total extract	0.57	0.24	1.3									
Control total cellular				0.09	0.21	0.00	0.20	0.00	0.00	0.55	ND	0.10
Control total nuclear				ND	0.12	ND	ND	0.00	ND	ND	ND	ND
Control total RNP				0.20	0.10	0.23	0.12	0.00	0.18	0.95	ND	0.57
Control total polysomes				0.21	0.19	0.24	0.17	0.18	0.06	0.93	ND	0.54
HS total extract	2.50	1.53	0.33									
HS total cellular				0.47	1.78	0.48	1.82	3.16	0.28	0.22	ND	0.10
HS total nuclear				ND	0.85	ND	ND	3.05	ND	ND	ND	ND
HS total RNP				2.17	0.15	0.12	1.99	3.12	0.12	0.32	ND	0.18
HS total polysomes				2.30	2.47	1.48	2.18	3.22	1.99	0.31	ND	0.23

[1]The values listed in the table are given as mean relative absorbances from two to three independent determinations.

[2]RNA levels for hsp70, hsp18, and actin were determined by scanning densitometry of the appropriate bands of the fluorograms shown in Figure 8B, C, and D, the Northern blots in Figure 11, or the RNA dot blots in Figure 17.

[3]Hsp70, hsp18, and actin syntheses were determined by scanning densitometry of the appropriate bands of the fluorograms in Figure 8.

[4]RNA levels were determined indirectly by scanning densitometry of the appropriate translation products synthesized *in vitro* and detected by fluorography (Figure 8B, C, and D).

[5]RNA was analyzed by Northern blot (Figure 11) and the level of hybridization determined by scanning densitometry. Standard errors for values ranging from 1) 0.1-0.21 are 0.010-0.270; 2) 0.85-2.47 are 0.390-0.590 and 3) 3.05-3.22 are 0.140-0.330.

[6]RNA was analyzed by RNA dot blot (Figure 17) and the level of hybridization determined by scanning densitometry. Star ' -d errors for values ranging from 1) 0.06-0.18 are 0.000-0.110; 2) 0.23-0.57 are 0.028-0.0.110 and 3) 1.48-1.99 are 0.071-0.385.

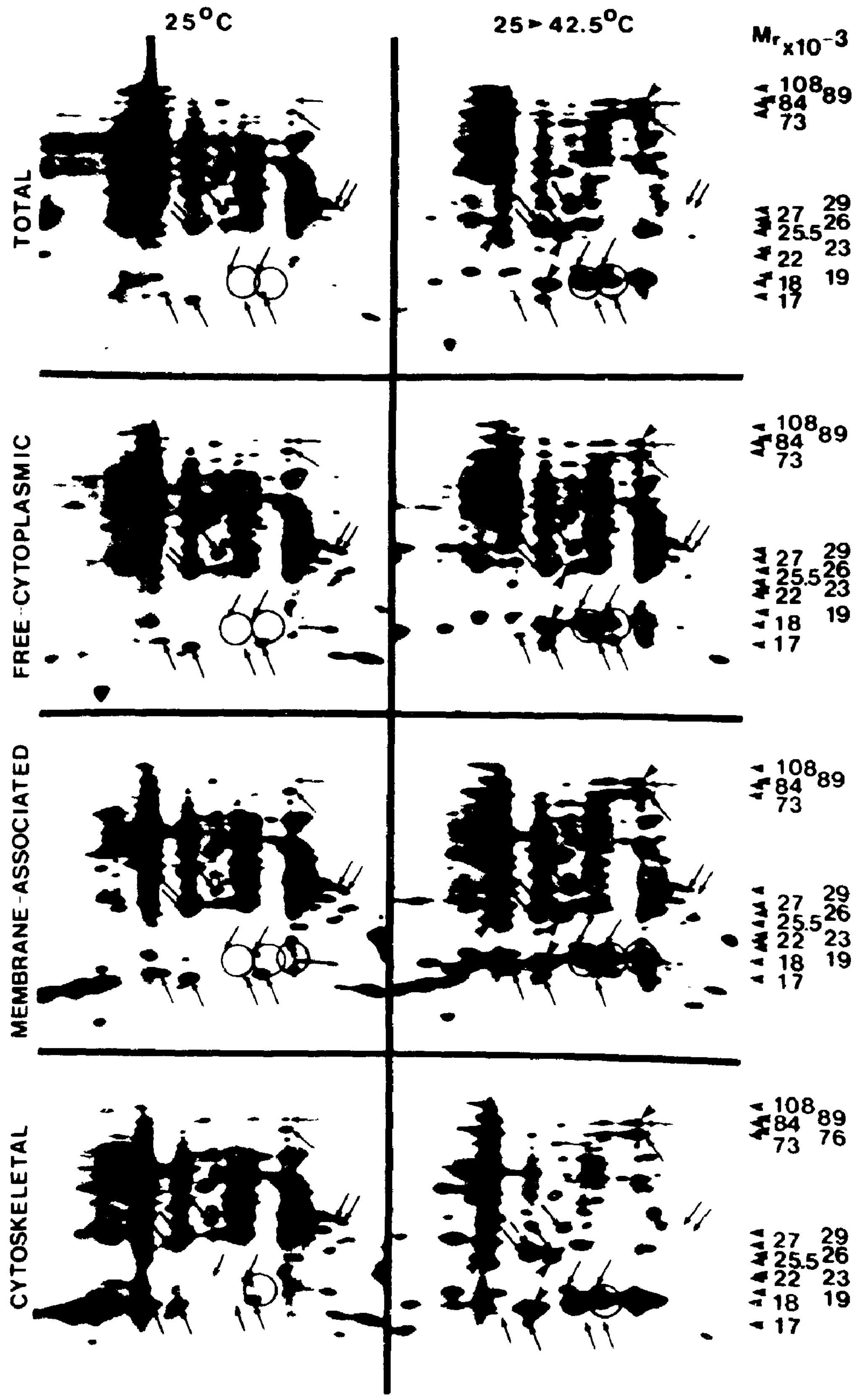
cytoplasm of heat-shocked cells both as non-polyribosomal and polyribosomal RNPs (Figure 11 and Table 1). The variability in RNA transcript levels detected in the non-polyribosomal RNP fractions before and after a 1h heat shock may reflect the inherent variability (due to synthesis and/or stability) of 70kDa and 18kDa mRNAs associated with this fraction and/or the differential sensitivity of Northern and dot blot analyses. Additional analyses (refer to Table 2) support these contentions.

Although heat shock results in an increase in 70kDa and 18kDa mRNA levels, it appears to have little effect on the general cytoplasmic distribution of these messages. Indirect quantitiation of the fluorograms in Figure 8 indicates that the increases in 70kDa and 18kDa mRNA levels correlate with increases in hsp70 and hsp18 levels synthesized in vivo and in vitro by total cellular mRNAs (Table 1). The variable changes in cytoplasmic 70kDa and 18kDa mRNA levels are reflected in the in vitro translation products. Furthermore, the decrease in actin mRNA levels during a 1h heat shock correlate with the decreased synthesis of a protein, observed both in vivo and in vitro, with a M_r similar to that predicted for a maize actin from sequencing data (Shah et al, 1983).

3.3.2 Distribution of mRNAs among polyribosomal and non-polyribosomal RNPs

To further characterize the distribution of mRNA on the ribosomes after a 1h heat shock, the total polyribosomal RNP fraction was fractionated as detailed in the Materials and Methods into free-cytoplasmic, membrane- and putative cytoskeletally-associated polyribosomal RNPs. The latter fraction was isolated using methodology developed for the isolation of cytoskeleton from animal tissues and is assumed to contain plant cytoskeleton. Poly(A)$^+$ RNA from each polyribosomal RNP fraction was analyzed indirectly by in vitro translation (Figures 12 and 13). A comparison of the polypeptides synthesized in vitro by total, free-cytoplasmic, membrane- and putative cytoskeletally-associated polyribosomal poly(A)$^+$ RNAs reveals minor quantitative and qualitative differences (Figure 12). Several proteins synthesized by 25°C poly(A)$^+$ RNAs from

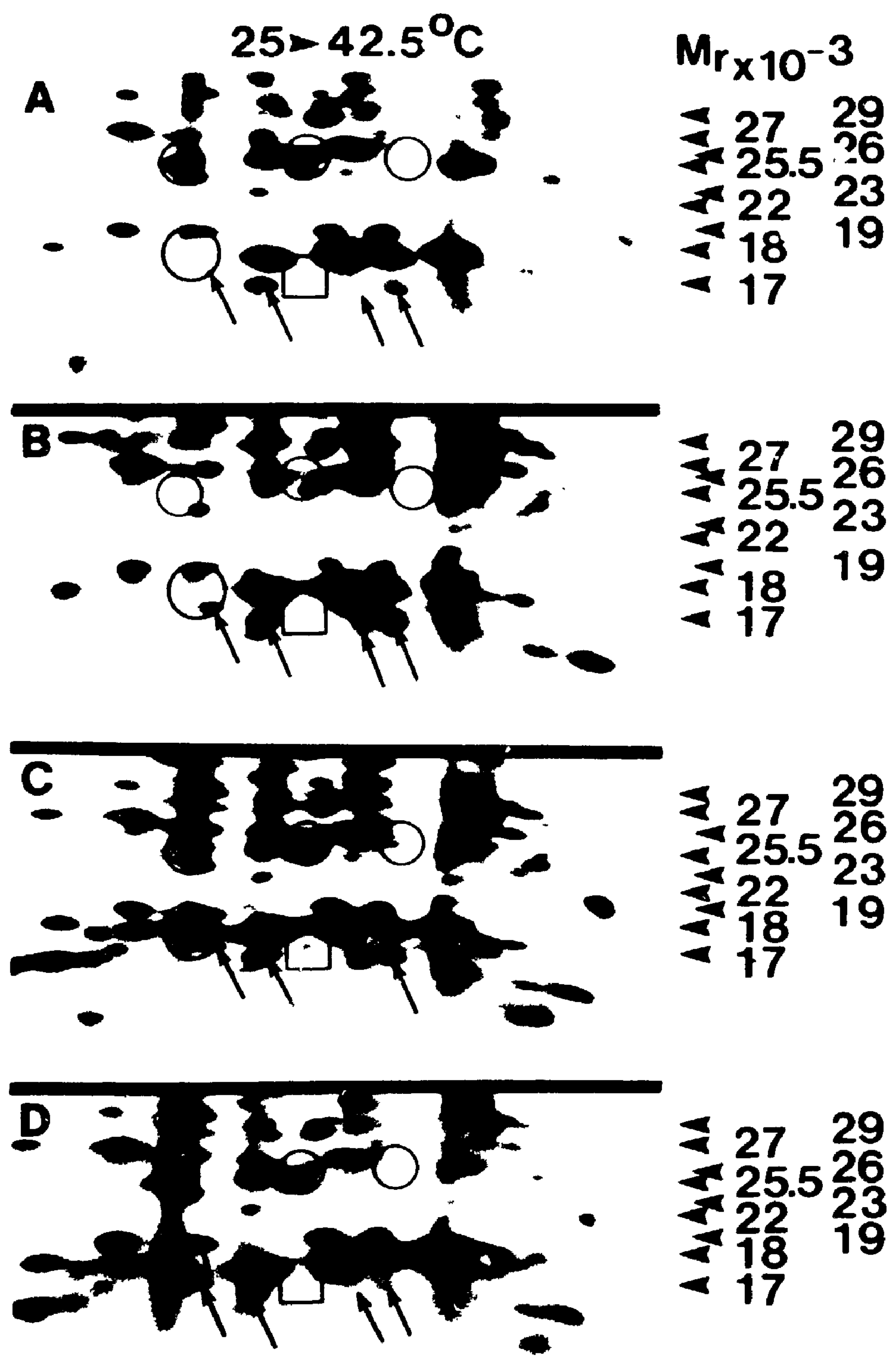
Figure 12: Fluorograms of representative (from more than three experiments) 2-D gel electrophoretic separations of the translation products synthesized by total, free-cytoplasmic, membrane- and putative cytoskeletally-associated polyribosomal poly(A)$^+$ RNAs. Arrows in the panels mark the positions of 25°C proteins synthesized by mRNAs exhibiting increased or decreased stability and/or accumulation, as reflected by the level of protein in the translation products, after a 1h heat shock. Arrowheads in the panels identify proteins that are synthesized in vitro by 42.5°C poly(A)$^+$ RNAs. Large circles mark the positions of 18kDa hsp variants that are synthesized in vitro by 25°C mRNAs and share common M_r's and pI's with proteins exhibiting enhanced synthesis in vitro following a heat shock. The intermediate circles mark the position of a 76kDa protein detected both in vivo and among the translation products of cytoskeletally-associated polyribosomal poly(A)$^+$ RNAs. M_r's of hsp's are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable products from translation mixtures was loaded onto each IEF gel.


25°C
25 ► 42.5°C
M_r x10^{-3}
TOTAL
FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
CYTOSKELETAL
108
89
84
73
76
29
27
26
25.5
23
22
19
18
17

different cellular fractions exhibit an increased or decreased synthesis after a 1h heat shock suggesting that 1) the synthesis and/or stability of these mRNAs has been altered due to heat shock and/or 2) that there is an increase or decrease in the accumulation and translation of these mRNAs on the ribosomes following a heat shock (Figure 12, arrows). Other proteins are synthesized by poly(A)$^+$ RNAs extracted from 42.5^{o}C ribosomal RNAs only (Figure 12, arrowheads). At least one and in some cases three proteins with similar pI's to the four prominent 18kDa hsp variants synthesized in vivo after a 1h heat shock are synthesized in vitro by 25°C polyribosomal poly(A)$^+$ RNAs (Figure 12, large circles). The remaining 18kDa hsp is synthesized by polyribosomal poly(A)$^+$ RNAs extracted from plumules only after a heat shock (Figure 12, arrowheads). An additional 18kDa hsp variant (Figure 13, large circle) and a 17kDa hsp variant (Figure 13, square) synthesized in vitro following a heat shock are synthesized by poly(A)$^+$ RNAs predominantly associated with membrane and cytoskeleton. Poly(A)$^+$ RNAs encoding two hsp 26kDa proteins (one of which was thought to be synthesized by non-polyribosomal RNP mRNAs only) and a 25.5kDa protein are also predominantly associated with the ribosomes of membrane and cytoskeletal elements (Figure 13, intermediate circles). Furthermore, a 76kDa protein similar to that observed in vivo is detected among the in vitro translation products of cytoskeletally-associated polyribosomal poly(A)$^+$ RNAs (Figure 12, intermediate circles). An hsp with similar M_r is observed among the in vitro translation products of total and membrane-associated polyribosomal poly(A)$^+$ RNAs when the lysate is supplemented (co-translationally) with microsomal membranes isolated from dog pancreas (Figure 14).

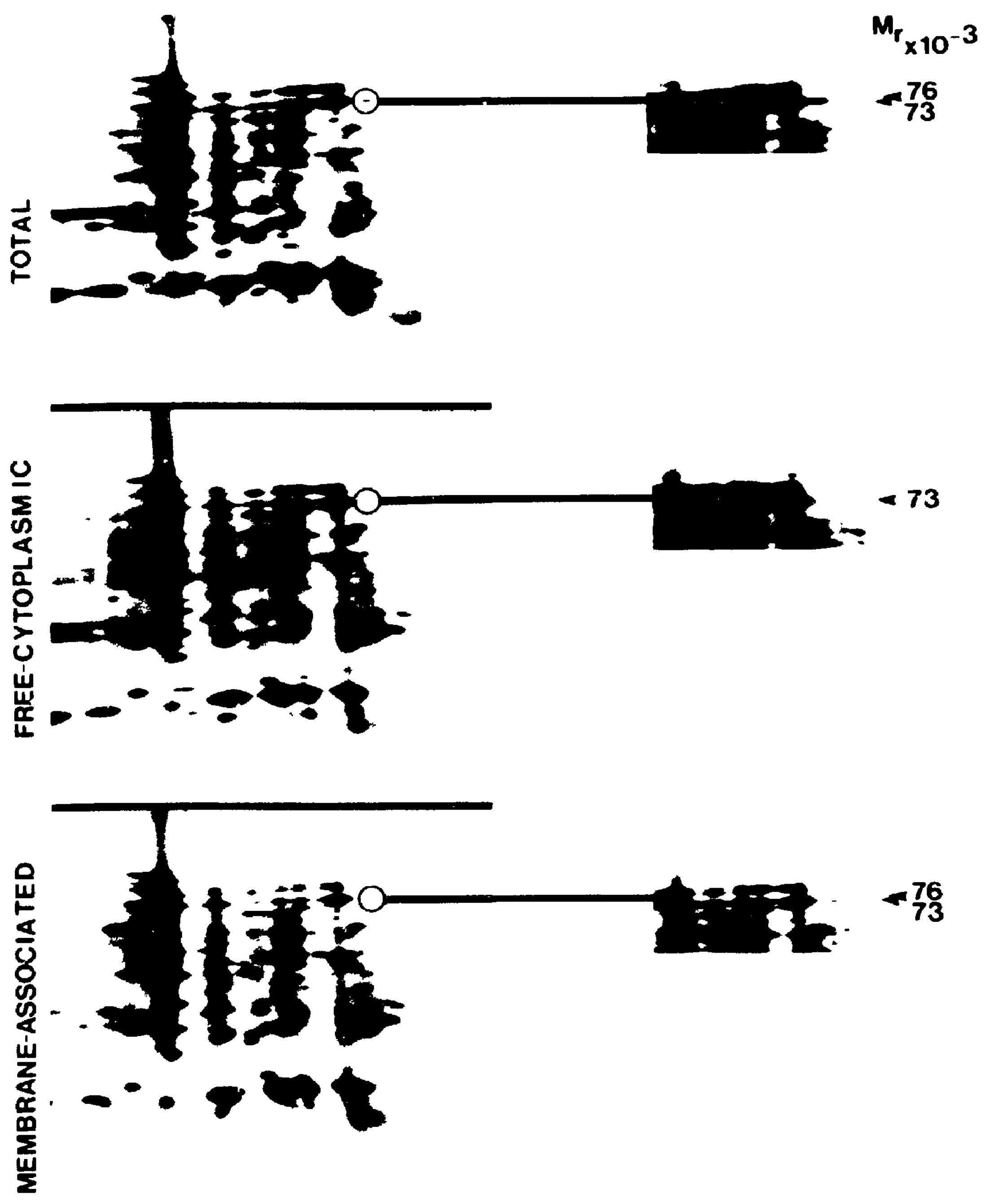
The role of non-polyribosomal RNP mRNAs in the heat shock response was characterized in a manner similar to that used for polyribosomal RNPs. RNPs were pelleted from the total, free-cytoplasmic, membrane- and putative cytoskeletally-associated post-polyribosomal supernatants as described in the Materials and Methods. Poly(A)$^+$ RNP RNA was translated in vitro and the products of translation analyzed by 2-D polyacrylamide gel electrophoresis (Figures 15 and 16). Although differences in the

Figure 13: Fluorograms of the 2-D gel electrophoretic separations of the low molecular weight translation products synthesized by total (panel A), free-cytoplasmic (panel B), membrane (panel C)- and putative cytoskeletally (panel D)-associated poly(A)$^+$ RNAs shown in Figure 12. Symbols (large and intermediate circles and squares) mark the positions of proteins that are synthesized in vitro by polyribosomal poly(A)$^+$ RNAs predominantly associated with membrane and cytoskeleton. Arrows mark the positions of 17kDa proteins that are variably synthesized in vitro by 42.5^{o}C polyribosomal poly(A)$^+$ RNAs. M_r's of the small hsp's are indicated by arrowheads on the right.



25►42.5°C
Mr x10⁻³
A
27
25.5
22
18
17
29
26
23
19
B
27
25.5
22
18
17
29
26
23
19
C
27
25.5
22
18
17
29
26
23
19
D
27
25.5
22
18
17
29
26
23
19

Figure 14: Comparison of fluorograms of 2-D gel electrophoretic separations of the co-translational cell-free reaction products obtained from the in vitro translation of 42.5°C total, free-cytoplasmic, and membrane-associated poyribosomal poly(A)$^+$ RNA in the presence of microsomal membranes isolated from dog pancreas. RNA from two independent isolations was added to the in vitro system in the presence of membrane (supplied co-translationally). The circled area in each panel on the left, which is emphasized on the right, demonstrates that 42.5°C poly(A)$^+$ RNAs derived from total and membrane-associated polyribosomal RNAs direct the cell-free synthesis of a 76kDa protein in the presence of membrane. This protein is not detected among the co-translational cell-free reaction products obtained from the in vitro translation of 42.5°C free-cytoplasmic polyribosomal poly(A)$^+$ RNAs in the presence of membrane. M_r's of hsp's are indicated by arrowheads. Approximately 50,000 cpm of acid-precipitable cell lysate was loaded onto each IEF gel.



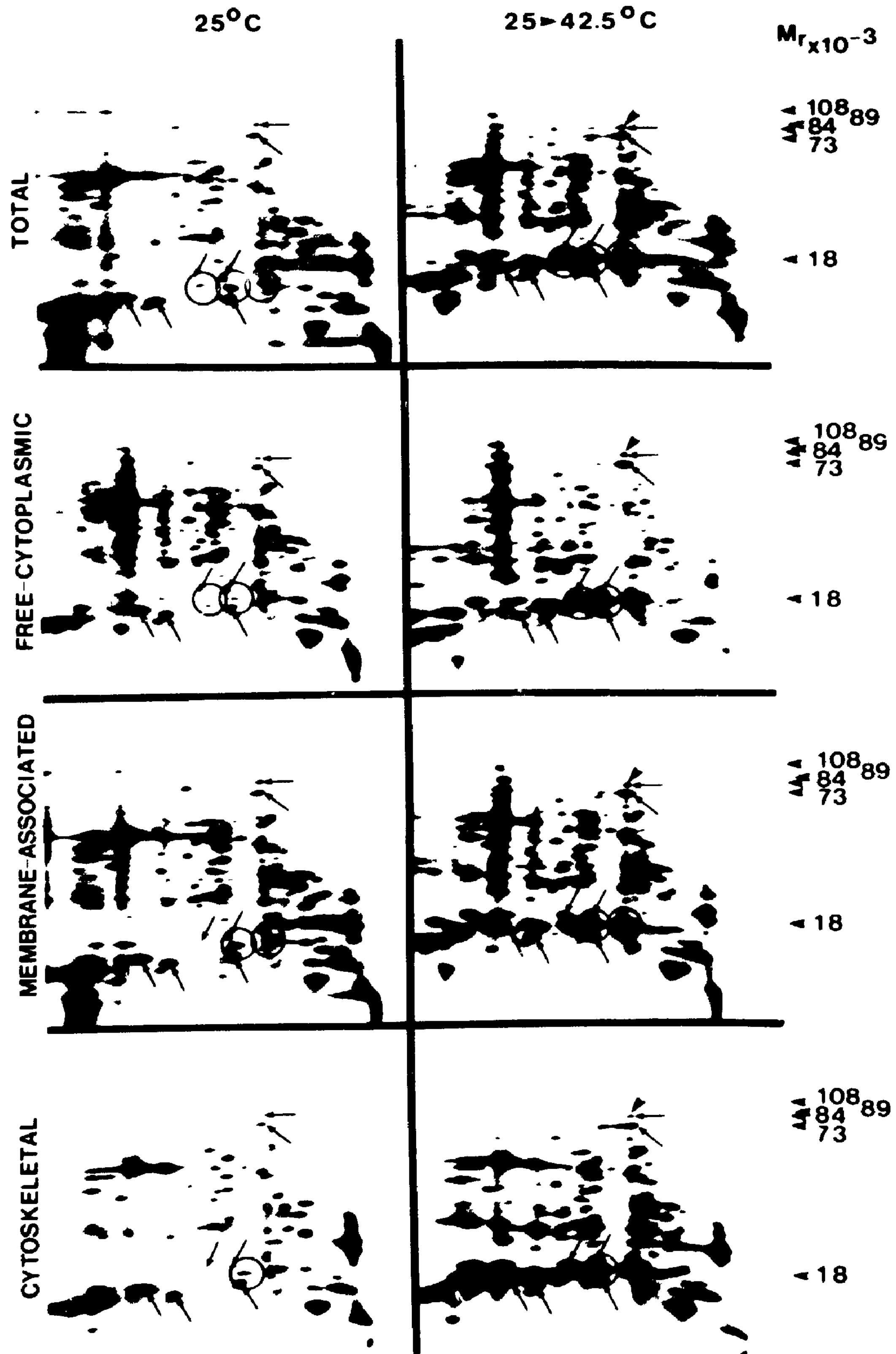
TOTAL
FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
M_r x10^-3
76
73
73
76
73

relative proportions of proteins synthesized by the different populations of mRNA can be detected, all 25°C non-polyribosomal RNP mRNAs synthesize a similar spectrum of polypeptides in vitro (Figure 15). After a heat shock, these poly(A)$^{+}$ RNAs synthesize the characteristic group of hsp's (Figure 15). Poly(A)$^{+}$ RNP RNAs active in protein synthesis in 25°C tissue are enhanced or incompletely repressed following a 1h heat shock as reflected by the in vitro translation products (Figure 15, arrows). RNAs encoding proteins with M_r's similar to the proteins synthesized in vitro by 42.5^{o}C polyribosomal poly(A)$^{+}$ RNAs comprise the population of mRNAs pelleted from the post-polyribosomal supernatants after a heat shock. Furthermore, three of the four prominent 18kDa hsp variants synthesized by 25°C polyribosomal poly(A)$^{+}$ RNAs in vitro are also synthesized in vitro by 25°C non-polyribosomal poly(A)$^{+}$ RNP RNA (Figure 15, large circles). Many of the poly(A)$^{+}$ RNAs predominantly associated with membrane- and putative cytoskeletally-associated ribosomes are variably associated with the non-polyribosomal RNP fractions (Figure 16, large circles). However, both the 26kDa and 25.5kDa proteins, that were synthesized by polyribosomal poly(A)$^{+}$ RNAs predominantly associated with membrane and cytoskeleton, are synthesized by poly(A)$^{+}$ RNAs predominantly associated with free-cytoplasmic non-polyribosomal RNP (Figure 16, intermediate circles). Furthermore, a 19kDa protein synthesized by all polyribosomal poly(A)$^{+}$ RNAs is not detected among the proteins synthesized by cytoskeletally-associated non-polyribosomal RNP (Figure 16, small circles).

3.3.3 Quantitation of intracellular polyribosomal and non-polyribosomal RNP mRNAs

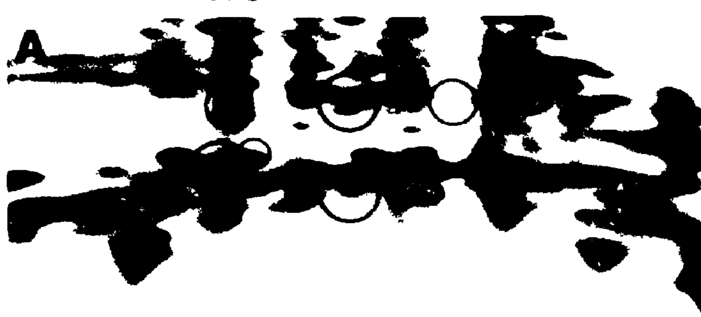
The distribution of 70kDa and 18kDa mRNAs in the cytoplasm of 25°C and/or 42.5^{o}C cells was further clarified by dot blot analyses. The autoradiograms acquired following exposure of blots probed with the hsp70 and hsp18 gene fragments were compared with those obtained following hybridization with an actin encoding gene fragment. Low levels of hsp70 mRNA were detected among the total cellular mRNAs

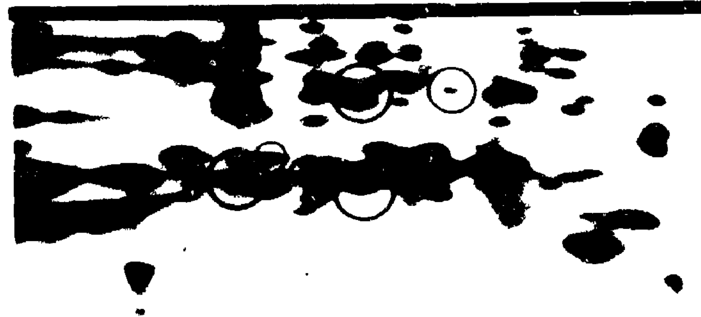
Figure 15: Fluorograms of representative 2-D gel electrophoretic separations of the translation products obtained from the in vitro translation of poly(A)$^+$ RNAs mAP-selected from non-polyribosomal RNP RNA pelleted from total, free-cytoplasmic, membrane- and putative cytoskeletally-associated post-polyribosomal supernatants. Similar protein synthetic profiles were obtained from more than three independent RNA isolations. Arrows in the panels mark the positions of proteins exhibiting enhanced or repressed synthesis in vitro after a 1h heat shock. Arrowheads in the panels identify some proteins detected after a 1h heat shock at 42.5^{O}C. Control proteins with similar M_r's and pI's to three of the four prominent 18kDa hsp variants synthesized after a heat shock are enclosed in large circles. M_r's of hsp's are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable products from translation mixtures were loaded onto each IEF gel.



25°C
25►42.5°C
Mr x10-3
TOTAL
FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
CYTOSKELETAL
108
89
84
73
18

Figure 16: Fluorograms of 2-D gel electrophoretic separations of the low molecular weight proteins synthesized by total (panel A), free-cytopiasmic (panel B), membrane (panel C)- and putative cytoskeletally (panel D)-associated non-polyribosomal poly(A)$^+$ RNP RNAs shown in Figure 15. Large circles mark the positions of some proteins that are synthesized by poly(A)$^+$ RNAs predominantly associated with specific ribosomal fractions and variably associated with non-polyribosomal RNP fractions. Proteins encoded by mRNAs exhibiting some compartmentalization are enclosed by intermediate or small circles. M_r's of low molecular weight hsp's are indicated by arrowheads on the right.


25 ► 42.5°C
Mr x10-3
A
27
25.5
22
18
17
29
26
23
19


27
25.5
22
18
17
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26
23
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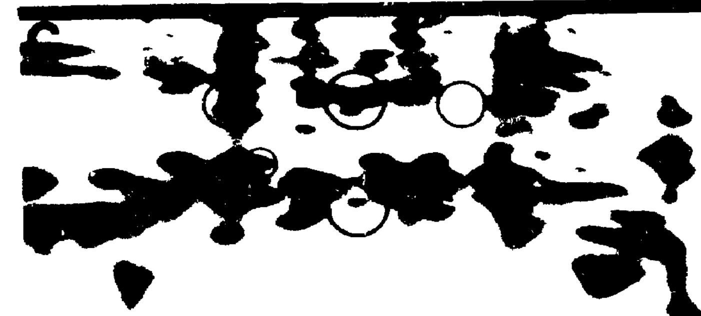
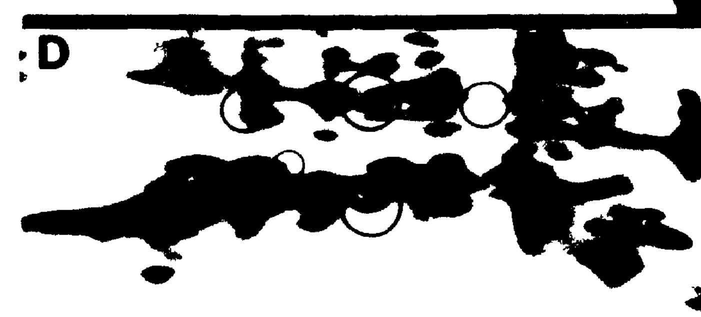

C
27
25.5
22
18
17
29
26
23
19


D
27
25.5
22
18
17
29
26
23
19

extracted from control plumules. Heat shock resulted in a significant increase in the level of 70kDa RNA transcripts in the cell (Figure 17A, wells 1 and 6). Hsp18 mRNAs, on the other hand, were detected in cells only after a heat shock (Figure 17B, wells 1 and 6). However, cytoplasmic fractionation of the cell revealed that both hsp70 and hsp18 mRNAs were associated with the population of mRNAs comprising the non-polyribosomal and polyribosomal RNP in 25°C plumules (Figure 17, A and B, wells 2 and 7). Further fractionation revealed that these mRNAs were not evenly distributed between or within the non-polyribosomal and polyribosomal RNP compartments (Figure 17, A and B, wells 3-5 and 8-10). In control cells, the 70kDa transcripts were found to be predominantly associated as RNP free of ribosomes (ie. non-polyribosomal RNP) in the free-cytoplasmic (FC) and membrane-associated (MA) fractions. In contrast, in the putative cytoskeletal (CSK) fraction, more 70kDa RNA transcripts were found to be associated with ribosomal RNP (ie. polyribosomal RNP). The distribution of 18kDa RNA transcripts between the non-polyribosomal and polyribosomal RNP compartments in 25°C plumules was found to be similar (albeit more restrictive).

The distribution of 70kDa and 18kDa RNA transcripts within the non-polyribosomal and polyribosomal RNP fractions reflected their distribution between the cytoplasmic compartments in control tissue (Figure 17, A and B, Table 2). In the non-polyribosomal RNP fractions, the greatest amount of 70kDa and 18kDa mRNAs was found in the free-cytoplasmic fraction followed by the membrane-associated fraction and lastly in the putative cytoskeletal fraction (FC>MA>/=CSK). The association of 70kDa and 18kDa RNA transcripts with the ribosomal fractions was essentially the inverse of the RNP associations (ie. CSK>FC>/=MA). Actin mRNA was found to be associated in a similar manner with both the non-polyribosomal and polyribosomal RNPs (ie. CSK>FC>/=MA) (Table 2). Heat shock had little effect on the distribution of 70kDa and 18kDa mRNAs among the non-polyribosomal RNPs (ie. FC>CSK>/=MA). Furthermore, it had no effect on the distribution of a normal mRNA (actin) among either the non-

Figure 17: RNA dot blot analysis of non-polyribosomal RNP and polyribosomal RNP mRNAs extracted from the plumules of intact maize seedlings. The autoradiograms are representative of those obtained following hybridization of probe to RNA from three independent isolations. The blot was probed initially with a maize hsp70 gene fragment (A) and re-probed after washing with a maize hsp18 gene fragment (B). Wells 1-10 in A correspond to wells 1-10 in B. Well 1: 5.0ug of total cellular RNA extracted from control ($25^{o}C$) plumules; Well 2: 5.0ug of $25^{o}C$ total non-polyribosomal RNP or polyribosomal RNP RNA; Well 3: 5.0ug of $25^{o}C$ free-cytoplasmic non-polyribosomal RNP or polyribosomal RNP RNA; Well 4: 5.0ug of $25^{o}C$ membrane-associated non-polyribosomal RNP or polyribosomal RNP RNA; Well 5: 5.0ug of $25^{o}C$ putative cytoskeletally-associated non-polyribosomal RNP or polyribosomal RNP RNA. Wells 6-10 correspond to wells 1-5 except that the RNA was fractionated from the plumules of maize seedlings following a 1h heat shock at $42.5^{o}C$. The specific activities of the ^{32}P-labelled DNA hybridization probes were similar ($1.7x10^{9}$cpm/ug DNA, 12h exposure).

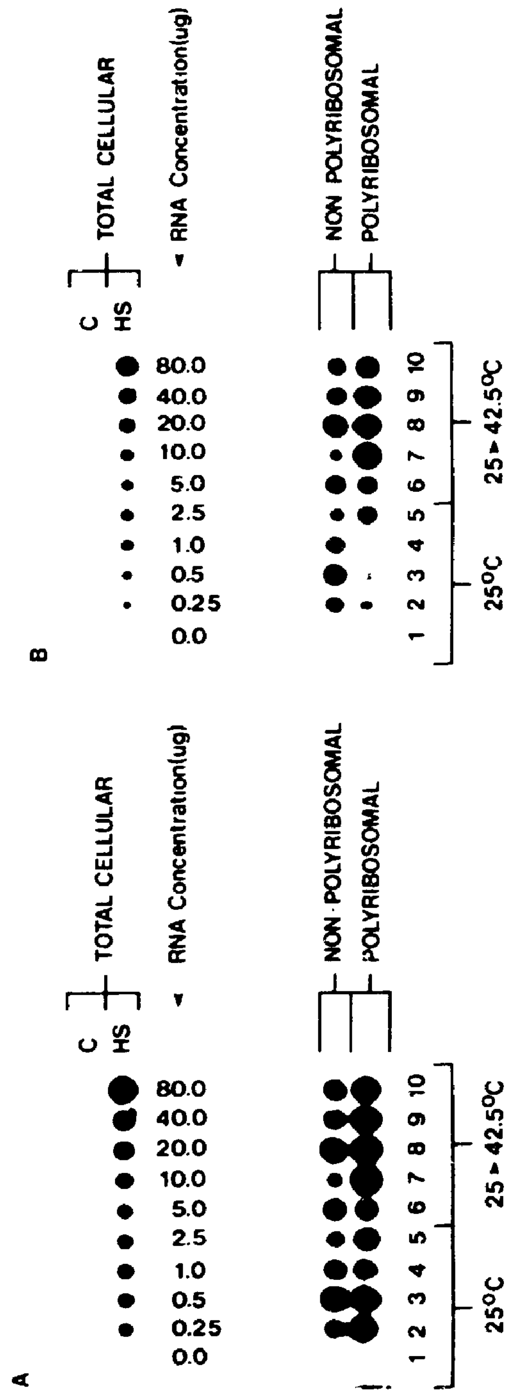

A
B
C
HS
TOTAL CELLULAR
RNA Concentration(ug)
80.0
40.0
20.0
10.0
5.0
2.5
1.0
0.5
0.25
0.0
NON-POLYRIBOSOMAL
POLYRIBOSOMAL
1 2 3 4 5 6 7 8 9 10
25°C
25 ▸ 42.5°C

TABLE 2: Effects of heat shock on the subcellular distribution of mRNAs in maize plumules[1]

Cellular Fractions	mRNA Levels[2]					
	HSP70		HSP18		Actin	
	RNP[3]	Polys[4]	RNP	Polys	RNP	Polys
Control-total	0.23	0.24	0.18	0.06	0.57	0.54
Control free-cytoplasmic	0.87	0.21	1.28	0.04	1.65	0.29
Control membrane-associated	0.40	0.14	0.37	0.00	0.93	0.22
Control cytoskeletally-associated	0.13	0.55	0.11	0.33	2.84	0.65
HS total	0.12	1.48	0.12	1.99	0.18	0.23
HS free-cytoplasmic	1.31	1.47	2.00	1.34	0.92	0.12
HS membrane-associated	0.49	1.43	0.54	1.23	0.92	0.12
HS cytoskeletally-associated	0.65	0.93	0.62	0.83	1.69	0.32

[1]The values listed in the table are given as relative absorbance values.

[2]RNA levels were determined by scanning densitometry of autoradiograms of RNA dot blots. Each value represents the mean relative absorbance from three independent determinations with standards errors given for non-polyribosomal and polyribosomal RNP RNA values separately. Representative relationships between mean relative absorbances and increasing RNA concentrations are provided in Appendices 3 and 8.

[3]RNP denotes non-polyribsomal RNP RNA levels as determined by quantitation of autoradiograms similar to those represented in Figure 17. Standard errors for values ranging from 1) 0.00-0.230 are 0.000-0.110; 2) 0.37-0.93 are 0.004-0.127 and 3) 1.28-2.00 are 0.244-0.499.

[4]Polys denotes polyribosomal RNP RNA levels as determined by quantitation of autoradiograms similar to those represented in Figure 17. Standard errors for values ranging from 1) 0.00-0.33 are 0.000-0.081; 2) 0.54-0.93 are 0.018-0.046 and 3) 1.23-1.99 are 0.011-0.385.

polyribosomal or polyribosomal RNP fractions. In contrast, the 70kDa and 18kDa RNA transcripts were found to be inversely associated with the ribosomal fractions after a heat shock (ie. FC>/=MA>CSK)(Figure 17, A and B, wells 3-5 and 8-10; Table 2).

The distribution of 70kDa and 18kDa mRNAs among the RNP fractions was inversely proportional to the increases in mRNA levels (over control levels) associated with these fractions following a heat shock. Heat shock resulted in a marginal increase (1-2 fold) in the amount of 70kDa and 18kDa RNA transcripts associating with the free-cytoplasmic and membrane-associated non-polyribosomal RNP while a 5-6 fold increase was observed in the levels of these transcripts associating with the non-polyribosomal RNP comprising the putative cytoskeletal fraction (Table 2). Similarly actin mRNA distribution inversely reflected the fold decreases in actin RNA transcripts after a heat shock among both the non-polyribosomal and polyribosomal RNP fractions. However, this relationship did not extend to the distribution of 70kDa and 18kDa mRNAs among the ribosomal fractions where changes in the mRNA levels after a heat shock (the most dramatic of which is the increase-33 fold-in 18kDa mRNA levels in the free-cytoplasmic and membrane-associated ribosomal fractions) were directly reflected in the distribution of those mRNAs among the ribosomal fractions.

3.4 Discussion

Heat shocking the plumules of inta. maize seedlings results in the new and/or enhanced synthesis of a characteristic group of plumule hsp's (Baszczynski, 1984; Rees et al, 1986) and the repression in synthesis of some 25°C proteins (Baszczynski et al, 1982; Cooper and Ho, 1983). The kinetics and rates of synthesis differ for each group of hsp's (Baszczynski,1984). Changes in the pattern of protein synthesis after a heat shock can be partially explained by changes in the relative levels of the corresponding mRNAs as increased synthesis of at least two maize hsp's, hsp70 and hsp18, correlates with the accumulation of their heat shock mRNAs in various subcellular fractions. Accumulation of 70kDa and 18kDa RNA transcripts during a heat shock may be due to increased message stability and/or new transcription. Heat shock has been shown to stabilize hsp70 mRNA in Drosophila (Peterson and Lindquist, 1988) and human (Theordorakis and Morimoto, 1987) cells. Changes in the levels of other cytoplasmic RNAs, such as maize actin mRNAs, could be due to decreased message stability resulting in degradation of pre-existing mRNA as in yeast (Lindquist, 1981) or to a block in RNA polymerase II transcription (Spradling et al, 1977; Findly and Pederson, 1981) and/or to the inability to process pre-mRNAs while in the nuclei of heat-shocked cells (Mayrand and Pederson, 1983; Yost and Lindquist, 1986; Czarnecka et al, 1988; Yost and Linquist, 1988; Bell et al, 1988).

In Drosophila, where the heat shock response is controlled at the level of transcription and translation (Lindquist, 1981), heat shock mRNAs are preferentially translated over control mRNAs while the latter are maintained in a translationally repressed state (Mirault et al, 1978; Storti et al, 1980; Kruger and Benecke, 1981; Ballinger and Pardue, 1983) and can be reactivated during recovery (Mirault et al, 1978; Storti et al, 1980, Lindquist 1981; Kruger and Benecke, 1981; DiDomenico et al, 1982a; DiDomenico et al, 1982b). Similar 'translational' control mechanisms have been reported in both

mammalian (Thomas and Mathews, 1982) and plant (Barnett et al, 1980; Altschuler and Mascarenhas, 1982; Scharf and Nover, 1982; Key et al, 1985) systems.

In maize plumules, total cellular, non-polyribosomal RNP, and polyribosomal RNP mRNAs synthesize hsp's with similar M_r's to those observed in vivo. In vitro translation studies demonstrate that heat shock (or heat shock-like) mRNAs are present in both the non-polyribosomal and polyribosomal RNPs of 25°C maize plumules and that normal (25°C) mRNAs comprise the population of mRNAs associated with these subcellular fractions in heat-shocked plumules. Although the predominant translation products after a heat shock are the hsp's, the synthesis of normal cellular proteins by mRNAs extracted from heat-shocked plumules is observed, with some exceptions, in unchanged amounts in heat-shocked plumules when compared with the in vitro translation products of non-polyribosomal RNP and polyribosomal RNP mRNAs from control cells. Moreover, although the synthesis of some 25°C proteins is selectively repressed in vivo after a heat shock, synthesis of many other 25°C proteins remains unchanged. These results suggest a differential regulation of control messages in heat-shocked maize plumules. The reduction in synthesis of some 25°C proteins in vivo may ensue from a change in the synthesis and/or stability of the corresponding mRNAs as is suggested by the decrease in actin mRNA levels or from the inefficient translation of these mRNAs (relative to heat shock mRNAs) in heat-shocked cells possibly due to changes in the rates of both initiation and elongation. The relatively equal distribution of high levels of 25°C messages (as determined by the level of in vitro translation product) in both the non-polyribosomal and polyribosomal RNP's during heat shock support this contention. Thus, control mRNAs are not sequestered as a group during a temperature shift in maize plumules as is the case in many other systems (Ballinger and Pardue, 1985; Lindquist and DiDomenico, 1985; Key et al, 1985). Translational selection and not translation discrimination appears to be the operative mechanism governing the response of maize seedlings to a 42.5^{o}C heat shock.

Similar observations have been made in other cereal grains (Key et al, 1985; Belanger et al, 1986) and in tomato cell cultures (Scharf and Nover, 1987; Nover et al, 1989).

Northern hybridization analyses corroborate some of the in vitro studies. RNAs encoding 70kDa and 18kDa proteins are detected in the nuclear and cytoplasmic fractions of 25°C plumules. Levels of the latter transcripts are quite variable. Heat shock results in the increased synthesis and subsequent accumulation of these transcripts in both non-polyribosomal and polyribosomal RNP. The simultaneous existence of these transcripts in both cytoplasmic compartments in nonheat-shocked plumules suggests that the 70kDa and 18kDa mRNAs like the 25°C mRNAs in heat-shocked Drosophila cells represent short term or partially repressed mRNAs (Vincent et al, 1981). Following a temperature shift, these mRNAs together with newly synthesized transcripts may be shunted onto the ribosomes. Alternatively, low levels of constitutively synthesized heat shock mRNAs may associate with non-polyribosomal RNP prior to integration into polyribosomes as has been shown in nonstressed immature duck erythrocytes (Spohr et al, 1972).

To further characterize the nature of this association with the polyribosomal and non-polyribosomal RNPs, the distribution of polyribosomal RNP and non-polyribosomal RNP poly(A)$^{+}$ RNAs in the free-cytoplasmic, membrane- and putative cytoskeletally-associated polyribosomal and non-polyribosomal RNP fractions was examined by in vitro translation and RNA blot analyses. The distribution of polyribosomal poly(A)$^{+}$ RNAs in the different ribosomal fractions isolated from 25°C and 42.5^{o}C plumules demonstrates that specific mRNAs are differentially distributed between the free-cytoplasmic, membrane- and putative cytoskeletally-associated polyribosomes indicating that these ribosomal fractions are distinct compartments in the cell. Poly(A)$^{+}$ RNAs encoding at least five hsp's (hsp's 17, 18, 25.5, 26, and 76) were shown to be predominantly associated with membrane- and putative cytoskeletally-associated ribosomes. Poly(A)$^{+}$ RNAs encoding proteins with similar M_r's exhibited preferential association with and differential distribution within the non-polyribosomal RNP fractions. This compartmentalization of

message suggests that the distribution of at least some mRNAs in maize plumules is non-random. Similar conclusions were drawn from observations in Drosophila (Kruger and Benecke, 1981; Ballinger and Pardue, 1983). More recently, Nover et al (1989) have shown that untranslated control mRNAs are selectively sequestered from the ribosomes as HSG's in tomato cell cultures.

The in vitro translation studies also demonstrate that poly(A)$^+$ RNAs encoding a 70kDa hsp and three 18kDa hsp's with similar pI to three of the four prominent 18kDa hsp's (in addition to lesser 18kDa hsp variants) are constitutively transcribed and distributed variably within the polyribosomal and non-polyribosomal RNP fractions. Furthermore, the co-isolation of RNA transcripts encoding a membrane-requiring 76kDa protein (which may be analogous to mammalian GRP and/or BiP) (Welch et al, 1989; Pelham, 1988; Pelham, 1986) in vitro with what are believed to be cytoskeletally-associated ribosomes suggests that this fraction contains disrupted ER. Alternatively, the 76kDa protein synthesized by cytoskeletally-associated polyribosomal RNA may be a cytoskeleton-requiring hsp70-related protein. Proteins of similar M_r have also been shown to be involved in the post-translational import of at least some proteins into the ER and into the mitochondria (Deschaies et al, 1988; Chirico et al, 1988).

The association of both 70kDa and 18kDa RNA transcripts with the non-polyribosomal RNPs isolated from 25°C plumules is most evident in the dot blot shown in Figure 18. Although heat shock resulted in an increase in the accumulation of these transcripts in the RNPs, it had little effect on the distribution of the 70kDa and 18kDa mRNAs within the non-polyribosomal RNP fractions (ie. FC>MA>/=CSK becomes FC>CSK>/=MA). The association of actin mRNA with the non-polyribosomal and polyribosomal RNP, either before or after a heat shock, is the inverse of the above (CSK>FC>/=MA). A similar distribution of 70kDa and 18kDa mRNAs was observed in the polyribosomes of plumules maintained at 25°C (ie. CSK>FC>/=MA). Moreover, mRNAs translated at the control temperature were predominantly associated with a fraction

believed to contain cytoskeleton. Heat shock, however, resulted in a dramatic change in the distribution of these mRNAs. Both the 70kDa and 18kDa mRNAs maximally accumulated in the free-cytoplasmic and membrane-associated ribosomal fractions while accumulation of these transcripts in the putative cytoskeletal fractions was marginal (FC>MA>/=CSK). Furthermore, although heat shock resulted in a general cellular increase in 70kDa and 18kDa mRNAs, the distribution of these transcripts between the cytoplasmic compartments indicated that, in contrast to the control situation, most of the 70kDa and 18kDa mRNAs accumulating after a temperature shift were associated with polyribosomes.

The apparent association of 70kDa and 18kDa mRNAs with the putative cytoskeletally-associated ribosomes in 25°C plumules together with the primary association of a normal mRNA (actin) with this fraction in 25°C and 42.5^{o}C plumules suggests that the cytoskeleton plays a role in protein synthesis. It has been shown for a number of eucaryotic cells that polyribosomal mRNAs (Lenk and Penman, 1979; Cevera et al, 1981; van Venrooij et al, 1981; Fey et al, 1986) and translation initiation factors (Howe and Hershey, 1984) are associated with the cytoskeletal framework. Furthermore, it has been proposed that attachment of mRNA to the cytoskeleton is obligatory for translation (Lenk and Penman, 1979; Cevera et al, 1981). This association with the cytoskeleton may be a normal situation in nonheat-shocked cells and may in addition serve as an early response to heat shock by shunting pre-existing 70kDa and 18kDa mRNAs onto the ribosomes. The shift in ribosome association after a heat shock, together with the decreased accumulation but greater (although marginal) degree of change in transcript levels (over control levels) associated with putative cytoskeletal components of the non-polyribosomal RNP, suggest a modification of the cytoskeletal system and/or a change in the role of the cytoskeleton after heat shock In Drosophila (Falkner et al, 1981; Biessmann et al, 1982; Leicht et al, 1986; Walter and Biessmann, 1987) and vertebrate (Collier and Schlesinger, 1986; Welch and Suhan, 1986) cells, heat shock results in the disintegration and aggregation at the cell

nucleus of the intermediate filament network. Disruption of both the Golgi complex and ER have also been reported (Welch and Suhan, 1986; Belanger et al, 1986). Furthermore, in heat-shocked Drosophila and vertebrate cells, the small hsp's have been shown to be associated with the cytoskeleton as HSG's (Leicht et al, 1986; Collier et al, 1988). In plant cells, it has been suggested that HSG's containing untranslated control messages are also cytoskeletally-associated (Nover et al, 1989). The continued association of control messages with a fraction believed to contain cytoskeleton during heat shock in maize plumules supports this contention.

In contrast, the heat shock messages in maize plumules accumulate maximally on the free-cytoplasmic ribosomes during a temperature shift indicating that translation under heat shock conditions is largely independent of this putative cytoskeletal containing fraction. In heat-shocked mammalian cells, disruption of the vimentin-containing intermediate filaments together with the simultaneous disruption of the actin microfilaments and microtubules has no effect on the transcription, translation and translocation of hsp's in these cells (Welch and Feramisco, 1985). It has been suggested that association with the cytoskeleton functions more as a means of transport for newly synthesized message to the cytoplasm in nonheat-shocked cells. Once in the cytoplasm, these cytoskeletally-associated complexes either interact with the ribosomes for translation or are preferentially released from the cytoskeleton and accumulate as non-polyribosomal RNP (Bag and Pramanik, 1986). In maize plumules, association with the cytoskeleton for translation may not be an absolute requirement in heat-shocked cells. Furthermore, although the cytoskeleton may still serve to transport mRNA in heat-shocked cells, as is suggested by the continued accumulation of 70kDa and 18kDa mRNAs in the non-polyribosomal RNP, these studies indicate that this function may not be essential to the production of hsp's.

CHAPTER 4

TRANSLATIONAL REGULATION IN MAIZE PLUMULES DURING HEAT SHOCK -- RIBOSOMAL CONTROL

4.1 Introduction

In heat-shocked cells, the transition from normal protein synthesis to hsp synthesis involves transcriptional and/or translational controls (see Section 1.2; Schlesinger et al, 1982; Atkinson and Walden, 1985; Lindquist, 1986; Sachs and Ho, 1986). The protein factors and DNA sequences involved in the induction of heat shock genes have been identified by both genetic and biochemical techniques (Pelham, 1982; Wu, 1984a; Wu, 1984b; Parker and Topol, 1984). However, the mechanism by which heat shock messages are selectively translated is less well understood.

In Drosophila, this change in translational specificity is characterized by a shift in polyribosome profile from a unimodal distribution characteristic of cells incubated at 25°C to a bimodal distribution characteristic of cells incubated at 36°C for 1h (Lindquist, 1980a; Lindquist, 1981; Ballinger and Pardue, 1985). This transition is not caused by a general inhibition of protein synthesis as heat shock mRNAs are translated with high efficiencies (Lindquist, 1980b). Furthermore, the disappearance of normal polysomes does not result in the degradation of control mRNAs (Mirault et al, 1978; Storti et al, 1980; Lindquist, 1981; Kruger and Benecke, 1981; DiDomenico et al, 1982a; DiDomenico et al, 1982b). When the cells are shifted back to normal growth conditions, an increasing number of heat shock messages are translationally inactivated and degraded (eg. hsp70 mRNA), while those remaining in the translational pool retain full ribosome loading (DiDomenico et al, 1982a).

Translational selection involves changes in the rates of protein synthesis initiation and elongation (Kruger and Benecke, 1981; Ballinger and Pardue, 1983). The role of the

cytoskeleton in this process appears to be minimal as its' disruption has no affect on the production of hsp's (see Section 3.3; Welch and Feramisco, 1985). However, modifications and/or changes in initiation factors, ribosomal proteins and/or small RNAs (Glover, 1982; Scharf and Nover, 1982; Duncan and Hershey, 1984; Panniers and Henshaw, 1984; McMullin and Hallberg, 1986; Hallberg and Hallberg, 1989) in combination with signals contained within the 5' untranslated leader sequence of heat shock messages (Klemenz et al, 1985; McGarry and Lindquist, 1985; Hultmark et al, 1986) may be responsible for this preferential translation.

In higher plants, the induction of hsp's is transient, lasting for only a few hours despite the continuous challenge of heat shock (Schoffl and Key, 1982; Altschuler and Mascarenhas, 1982; Cooper and Ho, 1983; Cooper et al, 1984; Baszczynski, 1984). A return to normal growth temperatures results in a rapid decline in hsp synthesis (Key et al, 1981; Schoffl and Key, 1982; Cooper and Ho, 1983; Baszczynski, 1984) . In soybean seedlings, these changes are coincident with a decrease in the level of heat shock messages in the cell suggesting that, as in Drosophila, these messages are degraded (Schoffl and Key, 1982; Altschuler and Mascarenhas, 1985; Key et al, 1985).

To further characterize the heat shock response in maize plumules, I have examined the protein and mRNA complement of polyribosomal RNPs in cells maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C in order to assess crude changes in the translational machinery during heat shock. The fate of 25°C mRNAs and heat shock mRNAs are also analyzed by examining the interaction of these messages with polyribosomes under conditions of prolonged heat shock or recovery from a brief heat shock.

4.2 Materials and Methods

4.2.1 Growth and treatment of seedlings

Seeds of *Zea mays* L. (cv. Oh43) were germinated in the dark on moistened filter paper at 25°C for 5-6 days as described in Section 2.2.1. Etiolated seedlings were maintained at 25°C or subjected to 1) a 1h incubation at 42.5°C, 2) a heat shock of varying duration (0-24h) or 3) a 1h heat shock at 42.5°C followed by a recovery period of varying duration at 25°C prior to RNA extraction.

4.2.2 Isolation of total, free-cytoplasmic and membrane-associated polyribosomal RNP RNAs

Polyribosomal RNPs were isolated from the plumules of intact seedlings as described previously (see Section 3.2.4 and 3.2.5) except that excised plumules were homogenized on ice in seven volumes/1.0g of tissue of either 0.25M sucrose-TKMD-A buffer (50mM Tris-Cl, pH7.4, 250mM KCl, 5mM $MgCl_2$, 2mM DTT, 0.5mM PMSF, 0.2mg/ml emetine-dihydrochloride, and 0.1-1.0mg/ml heparin) (Lerner et al, 1971; Ramsey and Steele, 1977; Gupta and Siminovitch, 1978; Heikkila et al, 1981) or 0.25M sucrose-TKMD-B buffer (200mM Tris-Cl, pH8.5, 400mM KCl, 5mM $MgCl_2$, 2mM DTT, 0.5mM PMSF, 0.2mg/ml emetine-dihydrochloride, and 0.1-1.0mg/ml heparin) (Breen et al, 1971; Lodish, 1971; Davies et al, 1972; Ramagopal and Hsiao, 1973; Jackson and Larkins, 1976; Gupta and Siminovitch, 1978; Clemens, 1986) in a glass homogenizer with 10 strokes of a motor-driven teflon pestle. The free-cytoplasmic and membrane-associated polyribosomal RNPs were pelleted by centrifugation at 4°C for 3h and 25 minutes at 38000 rpm (162000xg; Ti 55.2 rotor, Beckman). The supernatants were removed and saved for further analysis (see Section 5.2.2.1). The tube walls were wiped clean and the pellet resuspended in 100-200ul of TKMD-A buffer without sucrose. Approximately 50 A_{260} units of ribosomes were layered over an 11ml linear sucrose

gradient (15-40%, in the same buffer) and the material was centrifuged at 4°C for 2h and 30 minutes at 35000rpm (151,000xg, SW41 Ti rotor, Beckman). The gradients were analyzed with a UV monitor (ISCO model UA-5, 254mn) attached to an ISCO model 640 gradient fractionator. Alternatively, the pellets were resuspended in either translation buffer (Maniatis et al, 1982) to a final concentration of 100-200 A_{260} units or digestion buffer (Brawerman et al, 1972, Maniatis et al, 1982). Pellets resuspended in translation buffer were dissociated with 0.02umol EDTA per A_{260} unit (Gander et al, 1972). Aliquots were removed, diluted with extraction buffer (Baszczynski et al, 1982), boiled for 1 minute and analyzed by 1-D polyacrylamide gel electrophoresis. Pellets resuspended in digestion buffer were incubated for 30 minutes at 37°C and the RNA extracted with phenol and/or chloroform (Maniatis et al, 1982, Clemens, 1986). Poly(A)$^+$ RNA was isolated from deproteinized polyribosomal RNA using Hybond messenger affinity paper (mAP-selected) according to the procedure outlined in Section 3.2.9.

4.2.3 Cell-free translation of mAP-selected polyribosomal RNP RNAs

Polyribosomal poly(A)$^+$ RNA, isolated from the plumules of control and heat-shocked maize seedlings, was used to direct the in vitro synthesis of polypeptides in a heterologous in vitro translation system from New England Nuclear as described previously (see Section 2.2.6). Rabbit reticulocyte lysate was programmed with 0.5-1.0ug of total, free-cytoplasmic or membrane-associated polyribosomal poly(A)$^+$ RNA in the presence of 5.0ul of ^{35}S-methionine.

4.2.4 Gel electrophoresis

4.2.4.1 One- and two-dimensional polyacrylamide gel electrophoresis

Ribosomal proteins and polypeptides synthesized by in vitro translation of polyribosomal poly(A)$^+$ RNA were analyzed by 1-D polyacrylamide gel electrophoresis on

7.5-17.5% linear gradients as described previously (see Section 2.2.8.1). Samples were diluted with four volumes of extraction buffer and boiled for 1 minute. The samples consisting of either a constant amount of protein or a constant amount of radioactivity were loaded into preformed wells in a 3% polyacrylamide stacking gel and electrophoresed as described. The in vitro translation products were mixed with four volumes of sample dilution buffer (9.0M urea, 5% 2-mercaptoethanol, 2% ampholines, 8% NP-40) and analyzed by 2-D polyacrylamide gel electrophoresis as described previously (see Section 2.2.8.2). The slab gels were stained with Coomassie brilliant blue R-250, destained with methanol/acetic acid, and photographed. The destained gels were dehydrated with DMSO, impregnated with PPO, dried down on Whatmann 3MM filter paper and apposed at -70°C to Kodak X-Omat film (XR-1) that had been pre-flashed to an optical density of 0.15.

4.2.4.2 Gel electrophoresis of RNA after denaturation with glyoxal and DMSO

Polyribosomal poly(A)$^{+}$ RNA was denatured by treatment with glyoxal and DMSO and electrophoresed on horizontal 1% agarose gels as described in Section 3.2.11.2. RNA ladder (BRL Life Technologies, Inc., Burlington, Ont., Can.) was denatured and co-electrophoresed with the RNA samples for use in M_r determinations. The gels were either stained with EtBr or the RNA was transferred to biodyne A membrane. Prior to use, the RNA blots were placed in boiling 20mM Tris-Cl buffer, pH8.0 for 5 minutes. The efficiency of the transfer was assessed by staining the blotted gel for 45 minutes in 0.5ug/ml EtBr.

4.2.5 Hybridization

Prehybridization, hybridization and washing were carried out as described in Section 3.2.14. Maize hsp70,hsp18, and actin gene fragments were isolated, labelled with ^{32}P, and used as probes in these studies (see Section 3.2.13).

4.2.6 Dot blot hybridization

The bio dot microfiltration apparatus were assembled with either equilibrated biodyne A membrane (as described in Section 3.2.16) or pre-wetted (H_2O) zeta probe membrane according to the Bio Rad instruction manual. The biodyne A and zeta probe membranes were rehydrated with 20x SSC and 10mM Tris-Cl, pH8.0, 1mM EDTA (TE buffer) respectively. Pretreated RNA and DNA samples were diluted to 500ul with H_2O and applied to the appropriate wells as described in the bio dot microfiltration apparatus instruction manual. The wells were washed with either 20x SSC or TE buffer under vacuum until completely dry. The membranes were removed from the apparatus, marked for identification, dried for 10 minutes at 60^oC, and baked for 2h at 80^oC under vacuum. Prehybridization, hybridization, and washing of biodyne A membranes were as described by Thomas (1980; 1983)(see Section 4.2.5). The zeta probe membranes were washed with 95^oC 20mM Tris-Cl, pH8.0, 1mM EDTA and prehybridized in 5x Denhardt's, 5x Pipes (3.0M NaCl, 0.1M Pipes, pH6.8, 0.1M EDTA), 0.2% SDS, 50% formamide, and 0.1mg/ml ssDNA (prehybridization buffer) for 8-20h at 42^oC (Anderson and Young, 1986). The prehybridization buffer was removed and fresh prehybridization buffer containing the denatured ^{32}P-labeled probe was added to the chamber. Hybridization was for 20h at 42^oC. The RNA blots were washed twice with 2x SSC, 0.2% SDS for 1h/wash at 42^oC. The final wash with 0.2x SSC, 0.2% SDS was for 1h at 55^oC. Excess buffer was drained from the blot and it was wrapped in saran wrap and apposed at -70^oC to XR-1 film using a Kodak intensifying screen (Cronex Xtra Life, Dupont, Can.).

The autoradiograms were scanned using a laser densitometer to determine the relative levels of mRNA.

4.2.7 Rehybridization

Probe removal from biodyne A membrane was as described in Section 3.2.15. Probe was removed from zeta probe membrane by washing the blots with boiling 5mM

potassium phosphate buffer (1M KH_2PO_4, 1M K_2HPO_4 stock solution) two to three times (Anderson and Young, 1986). Excess buffer was drained and the blots were wrapped in Saran wrap and apposed to XR-1 film at -70°C. If probe removal was satisfactory, the blots were prehybridized and hybridized with the desired probe as above.

4.3 Results

4.3.1 Characterization of the free-cytoplasmic and membrane-associated polyribosomal RNPs

Polyribosomal RNPs were isolated from the plumules of intact seedlings and analyzed by sucrose-gradient centrifugation. The profiles of free-cytoplasmic and membrane-associated polyribosomal RNPs isolated from seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C were indistinguishable (Figure 18). There were, however, minor differences in ribosome distribution between the two fractions (Figure 18; compare A and B to C and D). In both fractions, there was an accumulation of monosomes and low proportion of large polysomes indicative of some degradation probably due to the low Tris/pH used in the isolation. None the less, these profiles demonstrate that heat shocking maize seedlings does not result in a rapid shift of polyribosomes into monoribosomes and subunits as has been observed in maize leaves stressed by pathogen infection and herbicide paraquat treatment (Wu et al, 1988) and in other systems subjected to a heat shock (Key et al, 1981; Lindquist, 1986).

To further characterize the effect of a temperature shift on polyribosomal RNPs, I examined both the protein and RNA complement of these complexes. Free-cytoplasmic and membrane-associated polyribosomal RNPs were dissociated with buffered EDTA and the suspensions analyzed by 1-D gel electrophoresis (Figure 19A). The proteins associated with each of the ribosomal fractions were similar in both control (Figure 19A, lanes 1 and 2) and heat-treated (Figure 19A, lanes 3 and 4) seedlings. Proteins of 15 to 30kDa are common to both the free-cytoplasmic and membrane-associated ribosomes and may be ribosomal and/or prosomal proteins as the latter complex co-purifies with polyribosomal RNPs (Vincent et al, 1981; Arrigo et al, 1988b). Proteins of 45, 47, and 105kDa were also detected among the polypeptides associated with these ribosomal fractions. Additional proteins with relative molecular masses ranging between 47000 and 155000 were detected

Figure 18: Polyribosome profiles from control (25°C) and heat-shocked (42.5^{o}C) maize plumules. Free-cytoplasmic (A and B) and membrane-associated (C and D) polyribosomes were isolated from the plumules of intact maize seedlings maintained at 25°C (A and C) or incubated for 1h at 42.5^{o}C (B and D) prior to RNA extraction. Polyribosomes were extracted in a low Tris/pH buffer according to the procedure of Venkatesan and Steele (1972) with some minor modifications as indicated in Materials and Methods. Subunits sedimented to the left of the monoribosome peak while polyribosomes sedimented to the right.

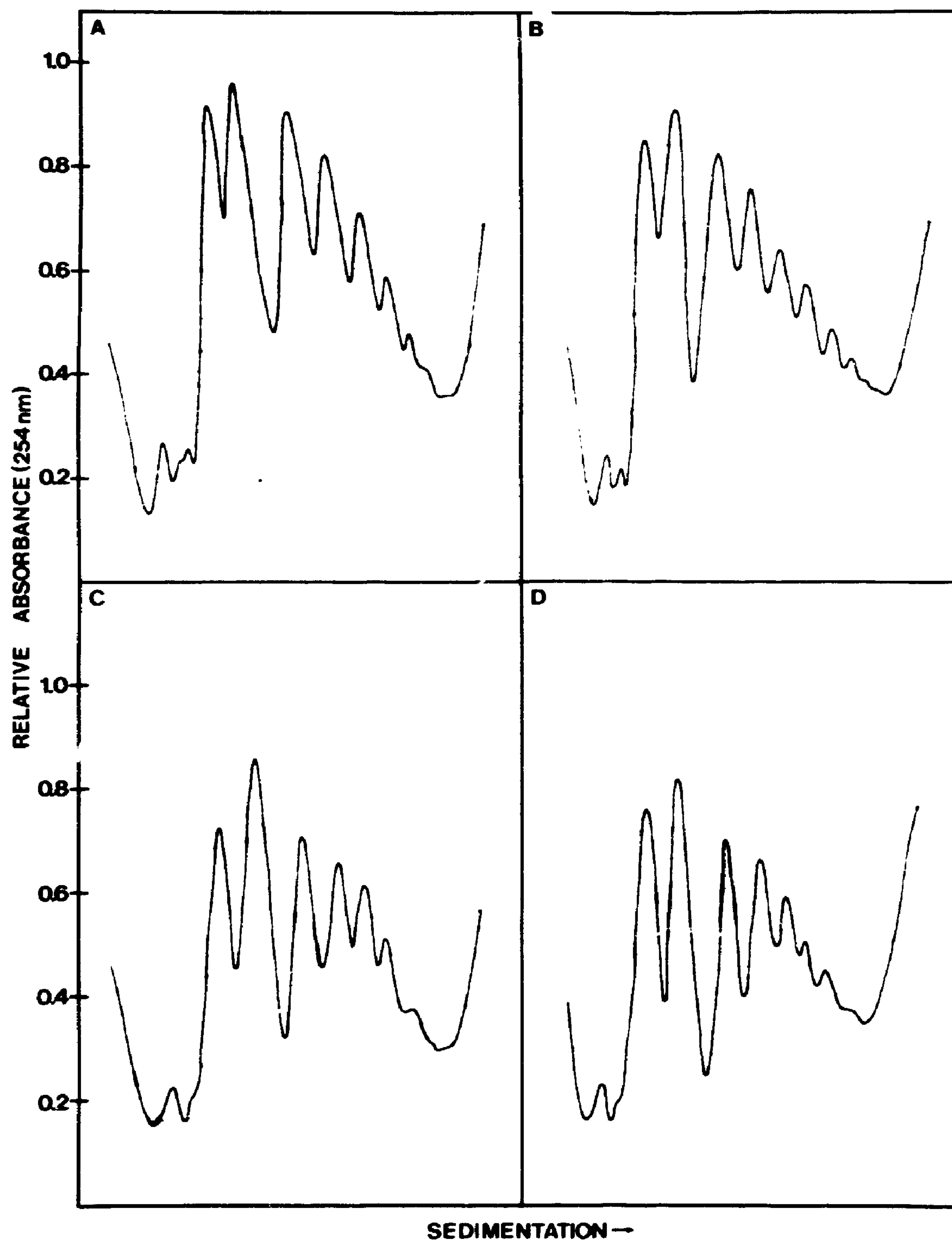
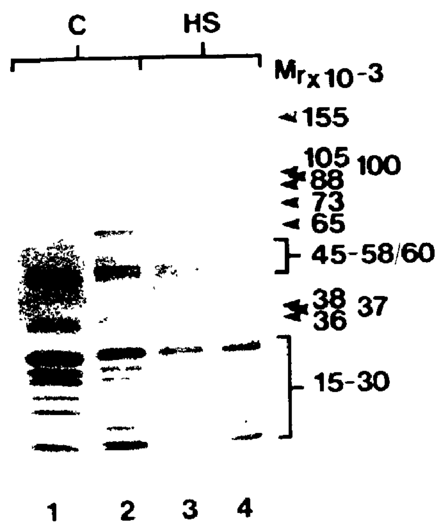

A
B
C
D
1.0
0.8
0.6
0.4
0.2
RELATIVE ABSORBANCE (254 nm)
SEDIMENTATION →

Figure 19: A comparison of the protein and mRNA complement of polyribosomal RNPs isolated from the plumules of maize seedlings.

A. Free-cytoplasmic and membrane-associated polyribosomal RNPs isolated from the plumules of intact maize seedlings maintained at 25°C (C; lanes 1 and 2) or subjected to a 1h heat shock at 42.5^{o}C (HS; lanes 3 and 4) were dissociated with buffered EDTA and 50ug of the suspension analyzed by 1-D polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue R-250, destained in methanol/acetic acid and photographed. Proteins in lanes 1 and 3 were isolated from free-cytoplasmic polyribosomes while proteins in lanes 2 and 4 were isolated from membrane-associated polyribosomes. M_r's are indicated by arrowheads and bars on the right.

B. Fluorogram of 1-D polyacrylamide gel electrophoretic separations of the polypeptides synthesized in vitro by free-cytoplasmic (lanes 1 and 3) and membrane-associated (lanes 2 and 4) polyribosomal poly(A)$^{+}$ RNAs extracted from the plumules of maize seedlings incubated at 25°C (C; lanes 1 and 2) or 42.5^{o}C (HS; lanes 3 and 4) for 1h. The protein synthetic profiles are representative of those obtained by analysis of translation products obtained from more than three RNA extractions. M_r's of hsp's are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable lysate was loaded into each well.

A.



B.

among the proteins comprising the membrane-derived ribosomal fraction (Figure 19A, lanes 2 and 4). A protein with similar M_r to the poly(A) binding protein (Schonfelder et al, 1985; Setyono and Greenberg, 1981; van Venrooij et al, 1977), although present in both ribosomal fractions, did not accumulate appreciably in either fraction. However, a 65kDa protein formed a major component of the proteins comprising the membrane-associated fraction. Many of the other proteins have M_r's similar to eucaryotic initiation and elongation factors (eg. 47kDa and 105kDa proteins)(Vincent et al, 1981).

The mRNA complement of polyribosomal RNPs was assayed indirectly by in vitro translation analyses. Free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA was extracted from plumules maintained at 25°C or subjected to a 1h heat shock. Poly (A)$^+$ RNA was translated in vitro and the products of translation analyzed by 1-D polyacrylamide gel electrophoresis (Figure 19B). Prior to a temperature shift, the mRNA distribution between the free-cytoplasmic and membrane-associated ribosomes appeared to be similar. Heat shock resulted in the new and/or enhanced accumulation of hsp encoding mRNAs on the ribosomes associated with both cytoplasmic fractions. An additional protein of 25.5kDa was synthesized by mRNAs accumulating on membrane-associated ribosomes. Furthermore, many of the 25°C mRNAs remained ribosome-bound after a heat shock.

Two dimensional gel electrophoretic separations of the proteins synthesized in vitro by free-cytoplasmic and membrane-associated polyribosomal poly(A)+ RNAs have already been shown in Section 3.3.2 (see Figure 12). These fluorograms showed that several proteins synthesized in vitro by 25°C poly(A)$^+$ RNAs from the different ribosomal fractions are variably synthesized by 42.5^{o}C poly(A)$^+$ RNAs indicating that heat shock results in changes in either the synthesis and/or stability and/or accumulation and translation of these mRNAs on the ribosomes of polyribosomes. Furthermore, poly(A)$^+$ RNAs encoding three 18kDa proteins with similar pI to three of the four prominent 18kDa hsp's synthesized by polyribosomal mRNAs after a heat shock are synthesized by 25°C mRNA.

In addition to the appearance of the major low molecular weight in vitro translation products, at least eight other mRNAs encoding polypeptides with M_r's of 89000, 27000, 26000 (at least two variants), 25500, 19000, 18000, and 17000 accumulate on the ribosomes after a heat shock. Some of these mRNAs preferentially accumulate on membrane-associated ribosomes. Interestingly, heat shock results in only a marginal decrease in the relative levels of actin (control) poly(A)$^+$ RNAs bound to the ribosomes (see Figure 21B for quantitation of actin mRNA levels).

Changes in the relative levels of polyribosomal poly(A)$^+$ RNAs were examined directly by RNA blot analyses (Figures 20 and 21A). Hsp70 RNA transcripts are associated with both free cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNAs from control plumules corroborating the in vitro translation studies. In contrast, mRNAs encoding the 18kDa hsp variants are not detected among the population of poly(A)$^+$ RNAs comprising either subcellular fraction at 25°C. However, both 70kDa and 18kDa mRNAs become abundant after a heat shock (Figures 20 and 21A). Hsp70 and hsp18 mRNA levels accumulate rapidly on the ribosomes associated with both cytoplasmic fractions to 6.3 fold and 100 fold higher levels respectively within the first hour at 42.5^{o}C whereas the levels of actin mRNA (indicative of 25°C mRNAs) decrease by approximately 1.5 fold during this period (Figure 21B).

The absence of a transition in the polyribosome profiles following a 1h heat shock at 42.5^{o}C, the absence of any obvious change in the polyribosomal protein profiles as determined by Coomassie brilliant blue staining, the dramatic accumulation of heat shock mRNAs on the polyribosomes, and the isolation and efficient translation in vitro of a number of 25°C polyribosomal poly(A)$^+$ RNAs from heat-shocked plumules suggest that competition for the translational machinery is a major factor contributing to the selectivity of the protein synthetic pattern during heat shock. To further characterize the mechanism by which seedlings respond to a temperature shift, I examined mRNA levels in plumules under conditions of prolonged heat shock or during recovery from a brief heat shock.

Figure 20: The effect of a rapid temperature shift on the accumulation of 70kDa and 18kDa mRNAs on the polyribosomes of maize seedlings. Free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA was isolated from the plumules of intact maize seedlings incubated at the normal growth temperature (25^oC) or subjected to a 1h heat shock at 42.5^oC. Poly(A)$^+$ RNA was denatured with glyoxal/DMSO and electrophoresed on 1% neutral agarose gels. The agarose gels were either stained with 0.5ug/ml EtBr (panel A) or the RNA was transferred to biodyne A membrane and hybridized to a ^{32}P-labelled maize hsp70 gene fragment (panel B) or maize hsp18 gene fragment (panel C). RNA ladder (lane a in panel A) was co-electrophoresed with the RNA samples for use in M_r determinations. The autoradiograms are representative of those obtained following hybridization of probe to RNA blots from more than three independent experiments. Positions of 25S and 18S maize rRNAs are indicated by bars between the panels. Lanes 1-4 in panel A correspond to lanes 1-4 in panels B and C. Lanes 1and 3 contain 2.5ug of 25^oC free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA respectively. Lanes 2 and 4 contain 2.5ug of 42.5^oC free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA respectively.

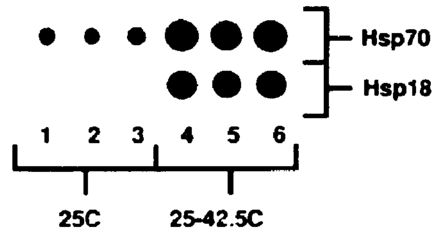
Kb
9.5
7.5
4.4
2.4
1.4
0.3
A
a 1 2 3 4
B
1 2 3 4
C
1 2 3 4
2.6 kb
0.9 kb

Figure 21: Quantitation of actin and hsp mRNA levels accumulating on the free-cytoplasmic and membrane-associated ribosomes of maize seedlings.

A. Free-cytoplasmic and membrane-associated polyribosomal poly$(A)^+$ RNA was isolated from the plumules of maize seedlings as described. Poly$(A)^+$ RNA was applied to biodyne A membrane and hybridized sequentially to ^{32}P-labelled maize gene fragments encoding actin (not shown), hsp70, and hsp18. Wells 1 and 4 contain 2.5ug of total polyribosomal poly$(A)^+$ RNA. Wells 2 and 5 contain 2.5ug of free-cytoplasmic polyribosomal poly$(A)^+$ RNA. Wells 3 and 6 contain 2.5ug of membrane-associated polyribosomal poly$(A)^+$ RNA.

B. The autoradiograms shown in A together with similar autoradiograms of RNA dot blots (made from independent RNA isolations) probed with the maize actin gene fragment were scanned using a laser densitometer and the relative levels of actin, 70kDa and 18kDa mRNAs were determined.

A. RNA dot blot



B. Quantitation of normal mRNA and hsp mRNA levels in maize plumules

TABLE 1: Effect of heat shock on mRNA levels in maize plumules[1]

Cellular fractions	mRNA Levels[2]					
	Hsp70		Hsp18		Actin	
	TP[3]	DB[4]	TP	DB	TP	DB
C-total	0 17	0 44	0 21	0 0	ND	0 10
C-free-cytoplasmic	0 18	0 38	0 26	0 0	1 14	0 11
C-membrane-associated	0 22	0 54	0 37	0 0	1 33	0 14
Hsp-total	1 11	2 81	1 66	2 62	ND	0 08
Hsp-free-cytoplasmic	1 16	2 72	2 10	2 56	0 75	0 08
Hsp-membrane-associated	0 87	2 94	1 76	2 70	0 82	0 08

[1] The values listed in the table are given as relative absorbances

[2] RNA levels for hsp70, hsp18, and actin were determined by scanning desitometry of the appropriate bands on the fluorogram shown in Figure 19B and the appropriate wells in Figure 21A

[3] RNA levels were determined indirectly by scanning densitometry of the appropriate translation products synthesized _in vitro_ and detected by fluorography (Figure 19B) Values represent mean relative absorbances from three independent determinations

[4] RNA was analyzed by RNA dot blot (Figure 21A) and the level of hybridization determined by scanning densitometry Values represent mean relative absorbances from three independent determinations with standard errors for any point not exceeding 0 113 Representative relationships between mean relative absorbances and increasing RNA concentrations are provided in Appendix 4

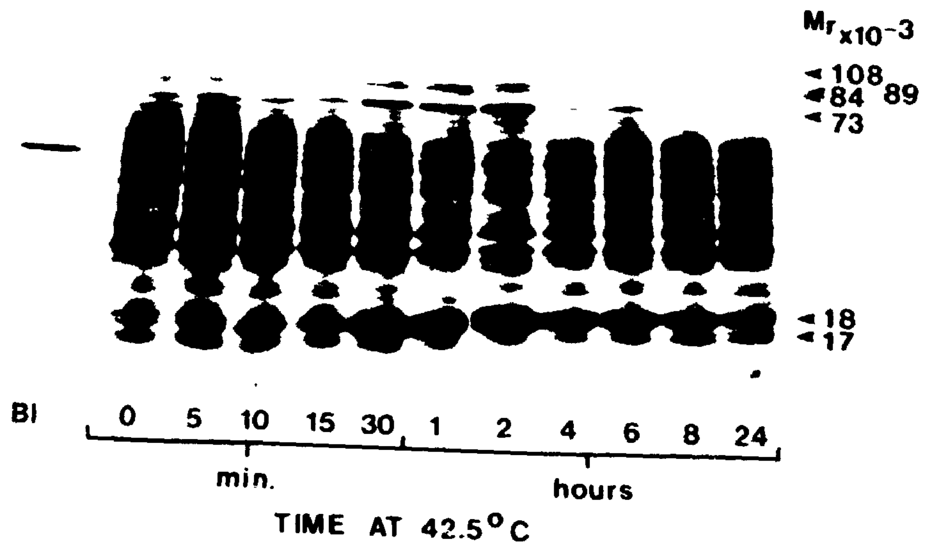
4.3.2 Effect of prolonged heat shock on maize seedlings

The effect of a prolonged heat shock on mRNA accumulation in the plumules of maize seedlings was examined indirectly by in vitro translation analyses. Free-cytoplasmic and membrane-associated polyribosomal RNA was extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a heat shock at 42.5°C of varying duration prior to RNA extraction. Poly(A)$^+$ RNA was translated in a heterologous cell-free system and the products of translation analyzed by 1- and 2-D polyacrylamide gel electrophoresis (Figures 22 and 23). The 70kDa and 18kDa mRNAs accumulate on both the free-cytoplasmic and membrane-associated ribosomes during the initial 5 to 10 minutes of a heat shock and are maximal within 2h (Figure 22, A and B). In the free-cytoplasmic fraction, the level of 70kDa mRNA (as determined by quantitation of the translation products) declines abruptly after 2h at 42.5°C, increases again at 6h and then declines to levels comparable to those observed in 25°C plumules by 24h (Figure 22A; see Appendix 5). In the membrane-associated polyribosomes, this decline is more gradual from 2h through to 24h (Figure 22B). In contrast, the amount of 18kDa mRNA associated with the free-cytoplasmic and membrane-associated ribosomes, does not return to pre-heat shock levels by 24h. This is most notable in the free-cytoplasmic fraction where a single 18kDa band is observed after 24h of heat shock (Figure 22A).

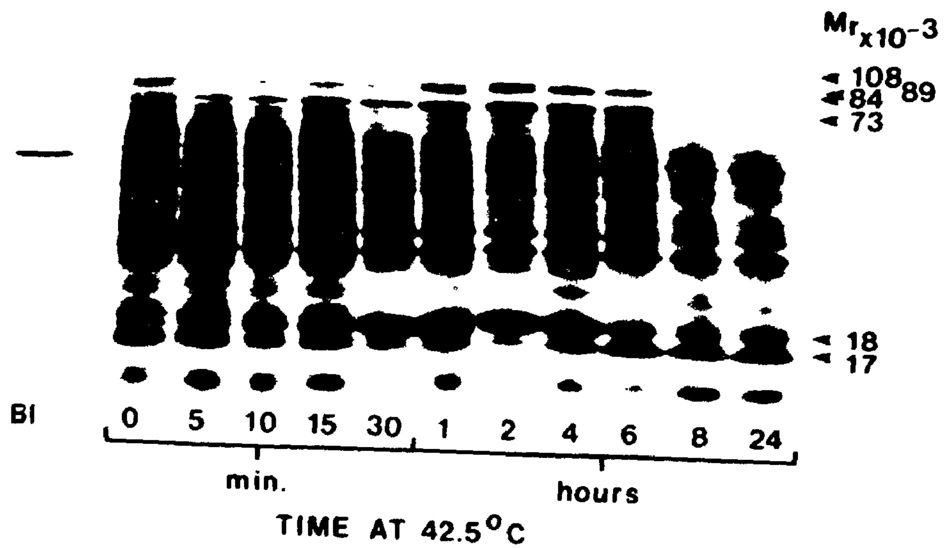
Poly(A)$^+$ RNAs encoding the remaining hsp's accumulate variably on the ribosomes of polyribosomes after 30 minutes of heat shock, but all reach maximal levels by 2h of heat shock and then either gradually dissociate from the ribosomes through to 24h as is the case for the free-cytoplasmic fraction or continue to accumulate through 4h afterwhich dissociation occurs. After 24h at 42.5°C, the heat shock mRNA levels in polyribosomes, with the exception of the 18kDa mRNAs, approximate control levels. Coincident with the decline in heat shock mRNAs in the polyribosomal fractions, is an increase in the accumulation of a 17kDa mRNA(s) in these fractions (Figure 22).

Figure 22: Fluorograms of representative (from two independent experiments) 1-D gel electrophoretic separations of the translation products derived from the in vitro translation of free-cytoplasmic (A) and membrane-associated (B) polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C (lane 2, 0 min) or subjected to a heat shock of varying duration at 42.5^{o}C prior to RNA extraction. M_r's of hsp's are indicated on the right. Bl marks the position of the translation blank. Approximately 100,000 cpm of acid-precipitable products from translation mixtures were loaded into each well.

A. FREE-CYTOPLASMIC



B. MEMBRANE-ASSOCIATED



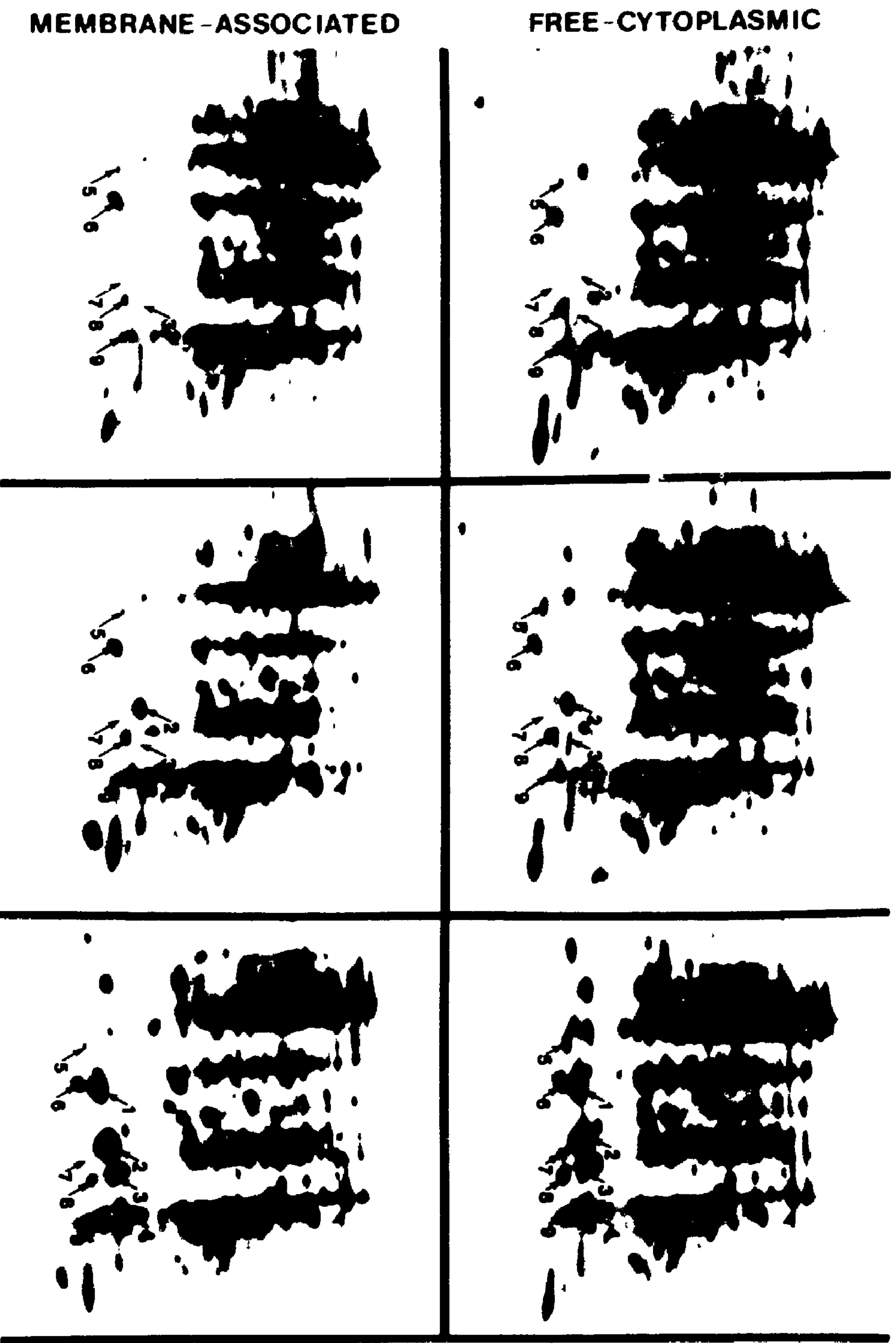
Furthermore, a marginal increase in 25°C mRNA accumulation is observed after 6-8 hours of heat shock (see Appendix 5). The level of control message either plateaus, as in the free-cytoplasmic fractions, or continues to decline, as in the membrane-associated fractions, through to 24h.

The kinetics of mRNA synthesis during a prolonged heat shock are more clearly illustrated in Figure 23. During the first 5 minutes of heat shock, a single 18kDa hsp variant accumulates to significant levels in the translation products synthesized by both free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA (Figure 23, panel 2, #2; refer to Section 2.3.1 for numbering system). After 30 minutes, the increased synthesis in vitro of additional 18kDa variants is observed (Figure 23, panel 3, #'s 1-4). Coincident with this accumulation, is the appearance among the in vitro translation products of mRNAs encoding the high molecular weight hsp's and other low molecular weight hsp's. Over the next 3-4h at 42.5°C, there are subtle quantitative and qualitative changes in the mRNA accumulating on the ribosomes. For instance, the appearance and accumulation of 26kDa, 18kDa and 17kDa isoelectric variants among the in vitro translation products of both ribosomal fractions vary over this period (Figure 23, panels 3 and 4). These differences are indicative of the differential expression of individual hsp's. After 6h at 42.5°C, there is a significant decline in hsp encoding mRNA levels, the most obvious of which is for mRNAs encoding the low molecular weight hsp's (Figure 23, panel 5). Coincident with this decline, is a maximal accumulation on both the free-cytoplasmic and membrane-associated ribosomes of mRNAs encoding proteins of 17kDa (Figure 23, panel 5, #'s 5-9). These mRNAs, which are active in protein synthesis in 25°C plumules, retain a high level of association with the ribosomes after 24h of heat shock. Poly(A)$^+$ RNAs encoding proteins of 18kDa also remain associated with the ribosomal fractions after 24h at 42.5°C although at greatly diminished levels.

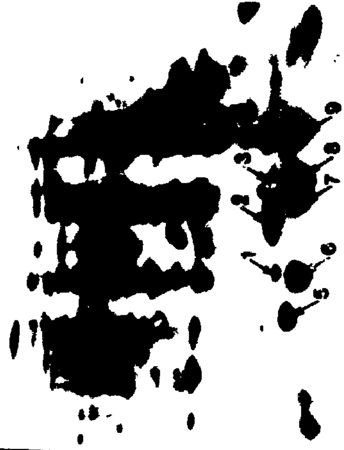
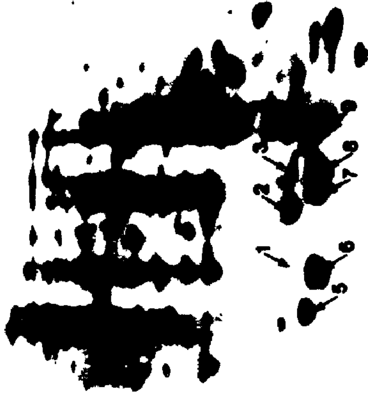
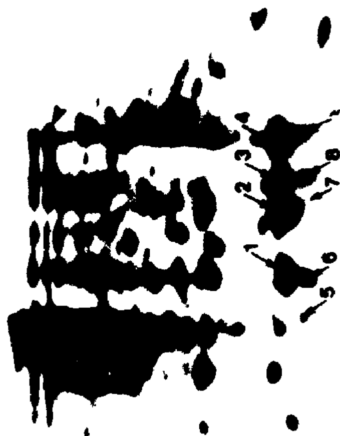
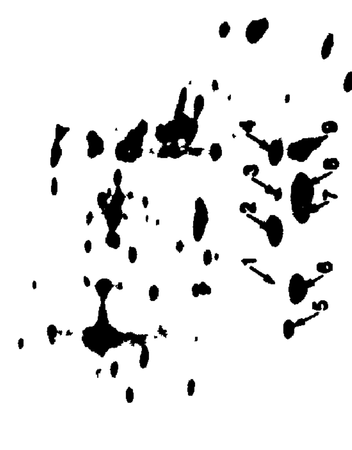
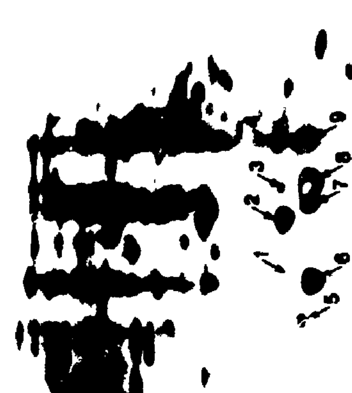
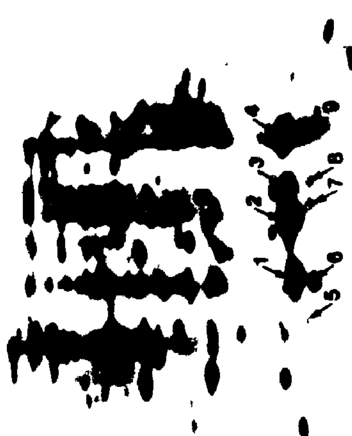
Northern blot hybridizations confirm the in vitro translation studies (Figure 24). Both the 70kDa and 18kDa mRNAs accumulate on the free-cytoplasmic and membrane-

Figure 23: The photographic plates for Figure 23 can be found on the following two pages.

Fluorograms of representative 2-D gel electrophoretic separations of the translation products obtained from the in vitro translation of free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C (panel 1) or subjected to a 5 minute heat shock at 42.5°C (panel 2), a 30 minute heat shock at 42.5°C (panel 3), a 2h heat shock at 42.5°C (panel 4), a 6h heat shock at 42.5°C (panel 5), or a 24h heat shock at 42.5°C (panel 6) prior to RNA extraction. Numbers and arrows in the panels mark the positions of 18kDa (#'s 1-4) and 17kDa (#'s 5-9) protein variants with similar pI's that are synthesized in vitro by 25°C and/or 42.5°C polyribosomal RNP mRNAs. Arrowheads mark the position of 70kDa proteins synthesized in both control and heat-shocked maize plumules. M_r's of hsp's are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable products from translation mixtures were loaded onto each IEF gel. Protein synthetic profiles are representative of those obtained from more than three independent RNA isolations.

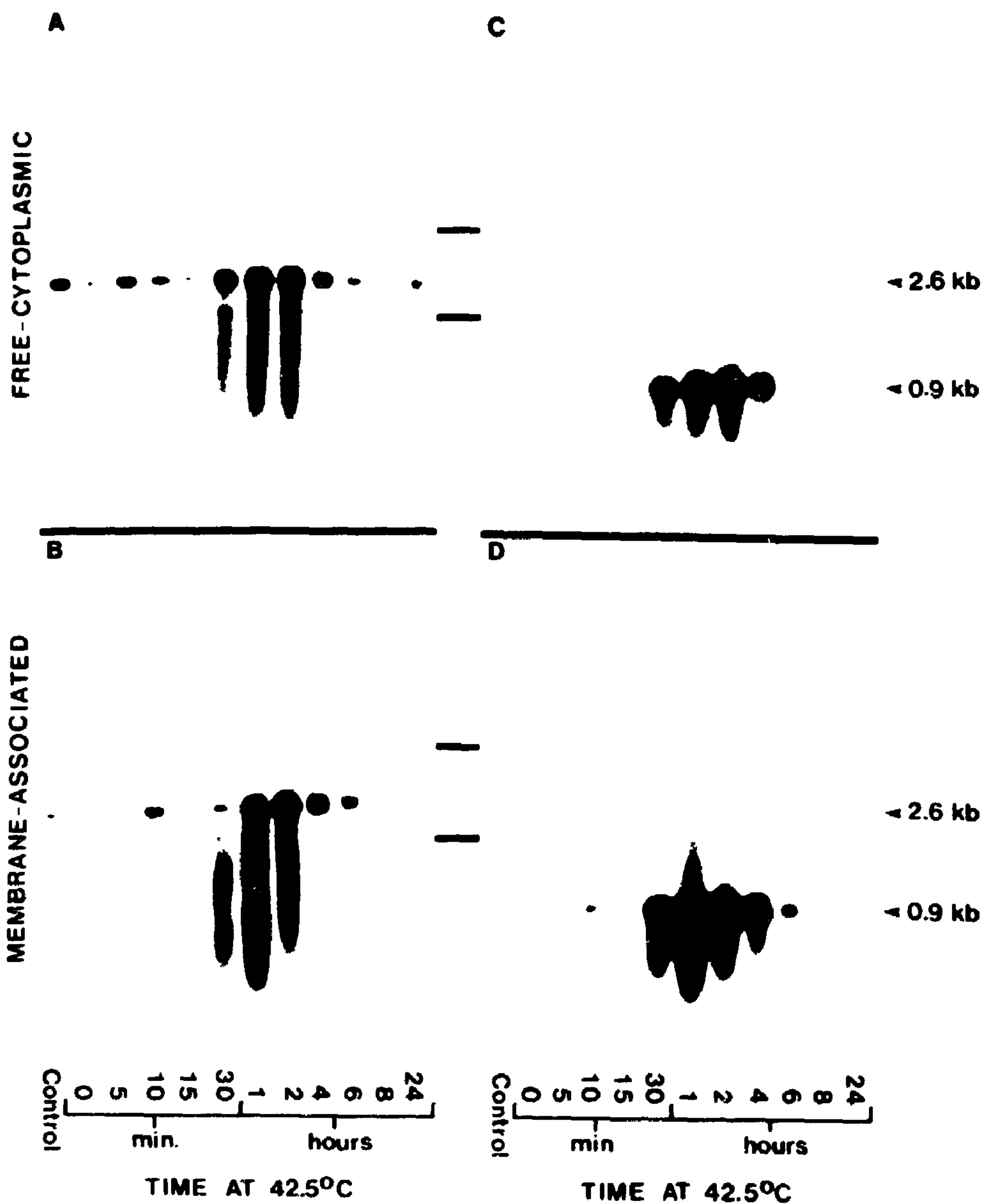


MEMBRANE-ASSOCIATED
FREE-CYTOPLASMIC
1
2
3

M_r x10 -3
108 89
84
73
27 26
25.5
18
17
4
5
6

Figure 24: RNA blot analysis of free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a heat shock of varying duration at 42.5^{o}C prior to RNA extraction. In each case (A-D), lane 1 contains 5.0ug of 25°C free-cytoplasmic polyribosomal poly(A)$^+$ RNA (control; internal standard). Lane 2 contains 5.0ug of 25°C free-cytoplasmic or membrane-associated polyribosomal poly(A)$^+$ RNA (0 min). The remaining lanes contain 5.0ug of free-cytoplasmic (A and C) or membrane-associated (B and D) polyribosomal poly(A)$^+$ RNA extracted from the plumules of seedlings heat-shocked at 42.5^{o}C for the indicated time periods prior to RNA extraction. The hsp70 and hsp18 gene fragments were used as hybridization probes in A and B, and C and D respectively. The autoradiograms are representative of those obtained following hybridization of probe to RNA blots from more than three independent experiments.


A
C
FREE-CYTOPLASMIC
2.6 kb
0.9 kb
B
D
MEMBRANE-ASSOCIATED
2.6 kb
0.9 kb
Control 0 5 10 15 30 1 2 4 6 8 24
min.
hours
TIME AT 42.5°C
Control 0 5 10 15 30 1 2 4 6 8 24
min
hours
TIME AT 42.5°C

associated ribosomes during the initial 5 to 10 minutes of heat shock. Maximal accumulation of these transcripts varies between the fractions with maximal levels being attained on the free-cytoplasmic ribosomes after 2h at 42.5^{O}C and after 1h at 42.5^{O}C on the membrane-associated ribosomes. Over the next 24h at 42.5^{O}C, a gradual decline in the mRNAs associated with the ribosomes is observed in both fractions. Hsp70 mRNA levels are comparable to control levels after 24h while hsp18 mRNA levels are similar to those observed following a 5 minute heat shock. A similar induction profile is observed by dot blot analyses of the polyribosomal poly(A)$^{+}$ RNAs (Figure 25). The levels of hsp70 mRNA accumulate rapidly to 12- and 15-fold higher levels in the free-cytoplasmic and membrane-associated ribosomes respectively over a 1h incubation at 42.5^{O}C while hsp18 mRNA levels accumulate to 30+-fold higher levels in both polyribosomal fractions over the same time period. In both cases, mRNA levels decline, for the most part, through to 24h as in the Northern blot studies. A slight rise in transcript levels is observed after 8h at 42.5^{O}C in the free-cytoplasmic fraction. This is followed by an immediate decline to near normal levels by 24h.

The in vitro translation studies and RNA blot analyses suggest that prolonged exposure of maize seedlings to 42.5^{O}C results in acclimation. The rapid accumulation of 70kDa and 18kDa RNA transcripts within the initial 1-2h of heat shock is followed by a return to near normal levels after 24h at the elevated temperature (ie. 42.5^{O}C). However, the situation is not identical to that in control tissue as 17kDa and 18kDa proteins continue to be synthesized in vitro by polyribosomal poly(A)$^{+}$ RNAs. Furthermore, the relative contributions of change in rate of synthesis and turn-over rate to this depletion of heat shock mRNAs is not known.

4.3.3 Recovery of maize seedlings from a 1h heat shock

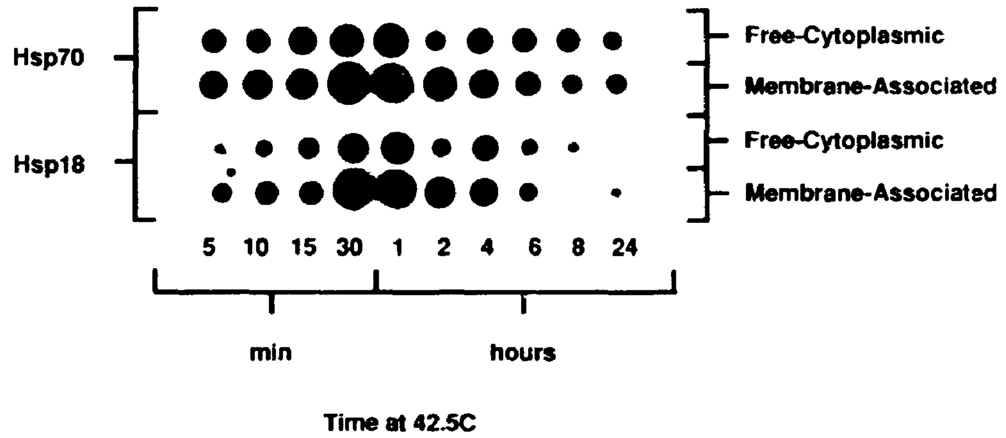
The mechanism(s) by which maize seedlings recover from a heat shock was(were) also examined by in vitro translation and RNA blot analyses. Free-cytoplasmic and

Figure 25: RNA dot blot analyses and quantitation of free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a heat shock of varying duration at 42.5^{o}C prior to RNA extraction.

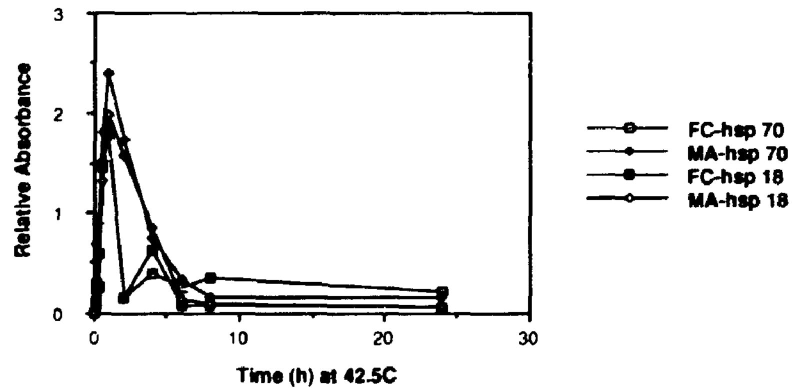
A. RNA dot hybridization of free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA to the hsp70 or hsp18 gene fragments.

B. Quantitation of the relative levels of 70kDa and 18kDa mRNAs accumulating on the free-cytoplasmic and membrane-associated ribosomes during prolonged heat shock. Values represent mean relative absorbances from two independent determinations.

A. RNA blot



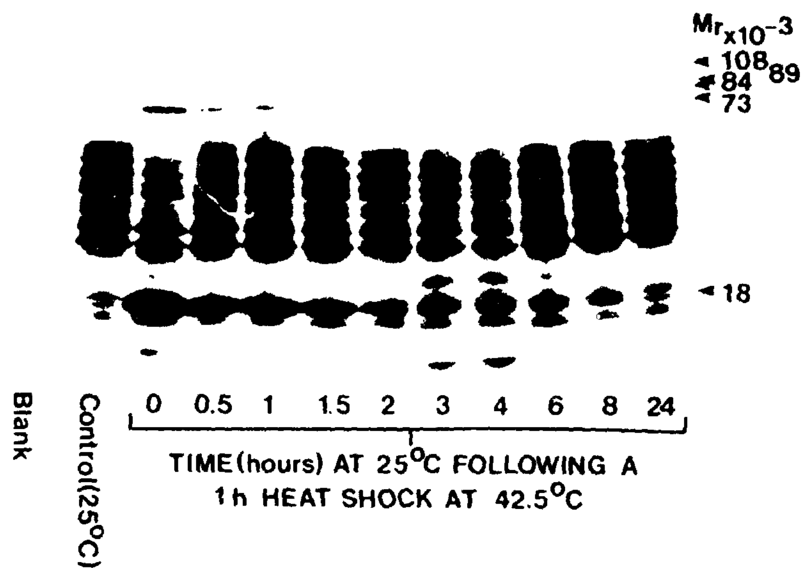
B. Relative levels of hsp mRNAs



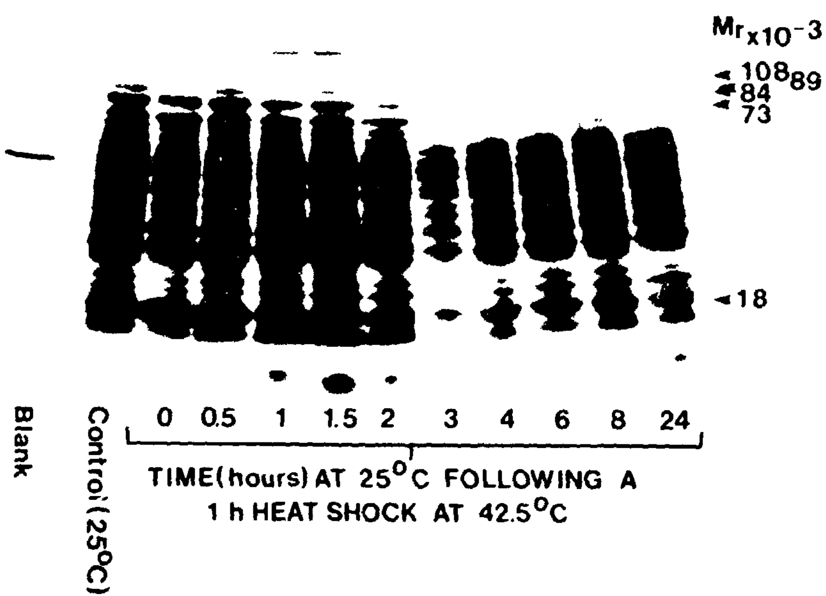
membrane-associated polyribosomal poly(A)$^+$ RNAs were isolated from the plumules of maize seedlings that were heat-shocked for 1h at 42.5^{o}C and allowed to recover for varying periods of time at 25°C prior to RNA extraction. Poly(A)$^+$ RNA was translated in vitro and the products of translation examined by 1- and 2-D polyacrylamide gel electrophoresis (Figures 26 and 27). A 1h heat shock results in the rapid accumulation of hsp mRNAs on both the free-cytoplasmic and membrane-associated ribosomes. When seedlings are returned to the normal growth temperature of 25°C, the level of heat shock mRNAs associated with the ribosomal fractions declines differentially (Figure 26). Poly(A)$^+$ RNAs encoding the high molecular weight hsp's are dissociated from the free-cytoplasmic polyribosomes more rapidly than from the membrane-associated ribosomes, while mRNAs encoding the low molecular weight hsp's (more specifically the 18kDa hsp's) are dissociated from the membrane-associated ribosomes more rapidly than from the free-cytoplasmic ribosomes. After 30 minutes at 25°C, there appears to be a rapid depletion of 70kDa and 18kDa mRNAs (as determined by the levels of in vitro translation products) from the free-cytoplasmic fraction (see Appendix 6). After 2h at 25°C, the level of 70kDa mRNA accumulating as free-cytoplasmic polyribosome is comparable to that observed in control tissue. The level of 18kDa mRNA associated with the free-cytoplasmic ribosomes does not approximate control levels until hour 3 at 25°C. In the membrane-associated fraction, within the first hour after a return to 25°C, there is a significant decline in the level of hsp70 synthesized in vitro relative to the levels observed after a 1h heat shock (see Appendix 6). After 3h at 25°C, the amount of hsp70 accumulating in the in vitro translation products is comparable to the amount synthesized in vitro by 25°C membrane-associated polyribosomal poly(A)$^+$ RNA. The level of hsp18 synthesized in vitro declines rapidly within the initial 30 minutes at 25°C and continues to decline (albeit more gradually) through to 24h. In both fractions, an apparent return to the normal protein synthetic profile occurs within 24h.

Figure 26: Fluorograms of representative (from two experiments) 1-D gel electrophoretic separations of the polypeptides synthesized in vitro by free-cytoplasmic (A) and membrane-associated (B) polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C and allowed to recover for various time periods at 25°C prior to RNA extraction. M_r's of hsp's are indicated by arrowheads on the far right. Approximately 100,000 cpm of acid-precipitable products from the translation mixtures were loaded into each well.

A. FREE-CYTOPLASMIC



B. MEMBRANE-ASSOCIATED

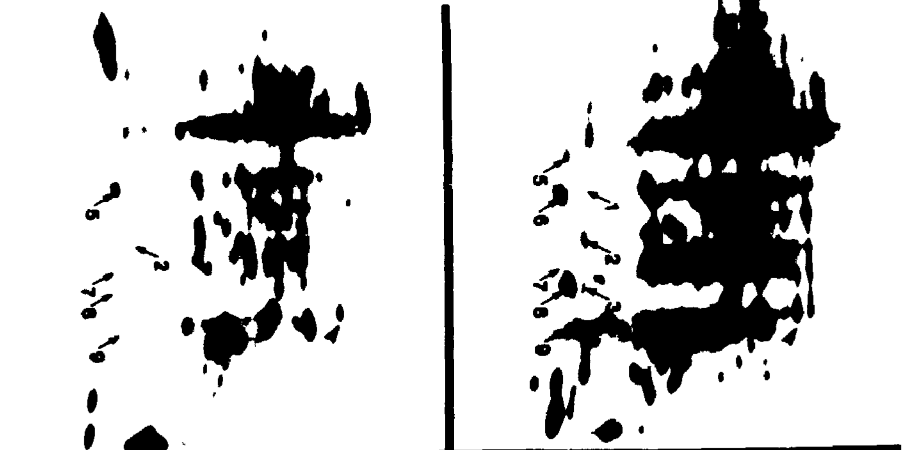


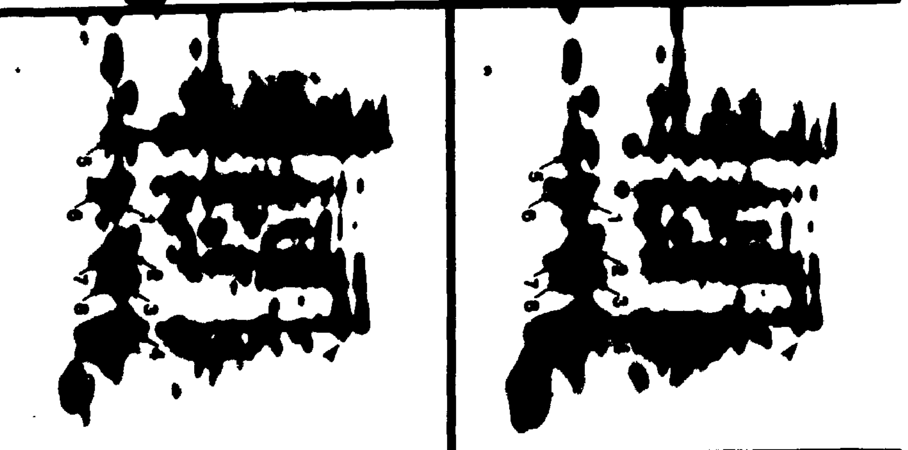
When these same translation products are analyzed by 2-dimensional polyacrylamide gel electrophoresis, it is apparent that, although hsp synthesis is drastically reduced by 24h, recovery is incomplete as synthesis of at least two 18kDa hsp variants by both free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA has not returned to control levels (Figure 27, panel 6, #'s 2 and 3). Maximal accumulation of 17kDa mRNAs on the free-cytoplasmic and membrane-associated ribosomes is coincident with the decreased accumulation of other heat shock mRNAs, more specifically the 70kDa and 18kDa mRNAs, on the ribosomes (Figure 27, panel 3). The 17kDa RNA transcripts continue to associate with the ribosomes through to 24h. However, in contrast to the induction profile, after 24h at 25°C, the level of 17kDa mRNAs bound to the ribosomes approximates that observed in control tissues. These studies indicate that when maize plumules are shifted back to 25°C, after a 1h heat shock, there is a rapid but incomplete depletion of heat shock messages from the polyribosomes.

A more direct measure of the mRNA levels in plumules recovering from a 1h heat shock was obtained by RNA blot analyses. Free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA from control, heat-shocked, and recovered plumules was isolated, transferred to membrane, and hybridized to the maize hsp70 and hsp18 gene fragments (Figure 28). The Northern blot hybridizations confirmed that the rapid decline in hsp synthesis in vitro is due, in part, to the dissociation of these mRNAs from the free-cytoplasmic and membrane-associated ribosomes. These studies also indicated that the levels of 70kDa and 18kDa mRNAs associated with the ribosomes varied between 4-6h at 25°C. The relative levels of 70kDa and 18kDa mRNAs associated with the ribosomes during recovery were determined by quantifying autoradiograms of dot blot hybridizations (Figure 29A) using a laser densitometer (Figure 29B). Within the first 30 minutes after the shift back to 25°C, the levels of 70kDa and 18kDa mRNAs associated with the free-cytoplasmic and membrane-associated ribosomes had declined significantly. After 6h at 25°C, the 70kDa mRNA levels approximated control levels. However, the mRNA levels

Figure 27: The photographic plates for Figure 27 can be found on the following two pages.

Fluorograms of representative 2-D gel electrophoretic separations of the translation products obtained from the in vitro translation of free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C (panel 1) or subjected to a 1h heat shock at 42.5^{o}C and allowed to recover at 25°C for 0h (panel 2), 1.5h (panel 3), 3h (panel 4), 6h (panel 5), or 24h (panel 6) prior to RNA extraction. Numbers and arrows in the panels mark the positions of 18kDa (#'s 1-4) and 17kDa (#'s 5-9) protein variants with similar pI's that are synthesized in vitro by 25°C and/or 42.5^{o}C polyribosomal RNP mRNAs. M_r's of hsp's are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable products from translation mixtures were loaded onto each IEF gel. Protein synthetic profiles are representative of those obtained from more than three independent RNA isolations.


MEMBRANE-ASSOCIATED
FREE-CYTOPLASMIC
1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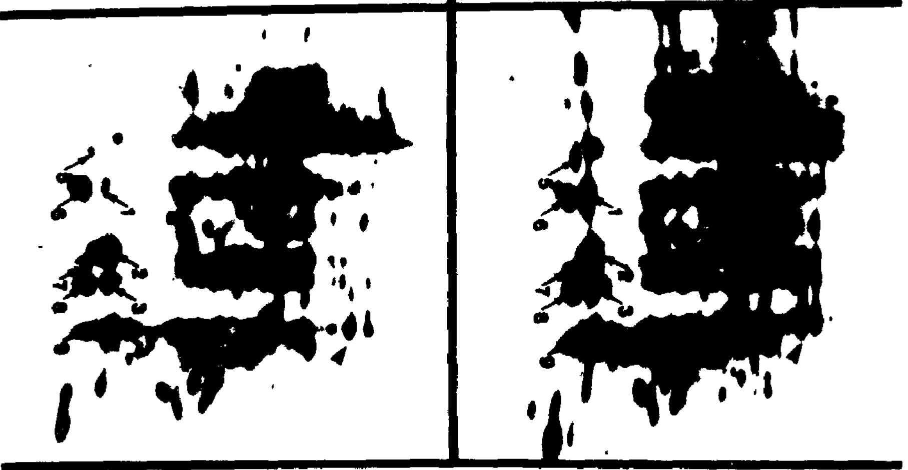
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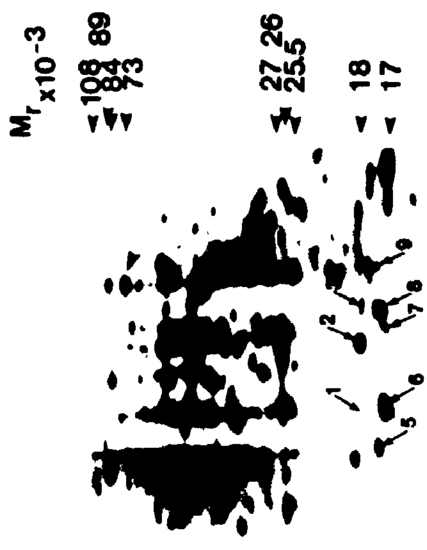

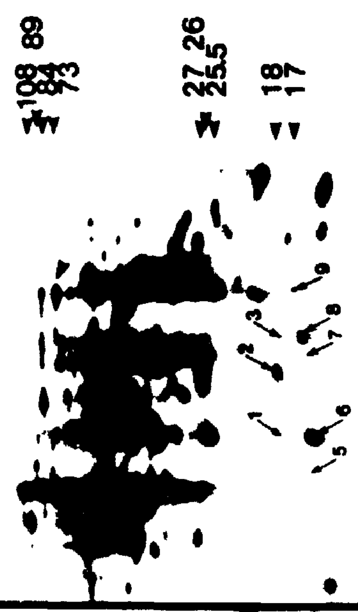
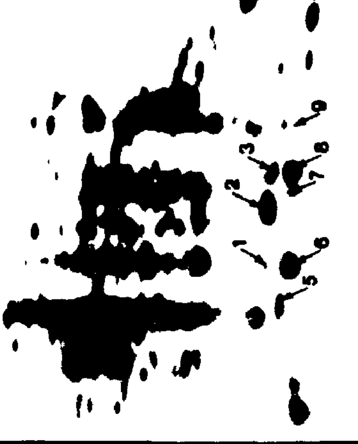

M_r x10-3
108 89
84
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27 26
25.5
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17
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4

Figure 28: RNA blot analysis of free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C and allowed to recover at 25°C for various time periods prior to RNA extraction. In each case (A-D), lane 1 contains 5.0ug of 25°C free-cytoplasmic polyribosomal poly(A)$^+$ RNA (control-1; internal standard). Lane 2 contains 5.0ug of 25°C free-cytoplasmic or membrane-associated polyribosomal poly(A)$^+$ RNA (control). The remaining lanes contain 5.0ug of free-cytoplasmic (A and C) or membrane-associated (B and D) polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings subjected to a 1h heat shock at 42.5^{o}C and allowed to recover at 25°C for various time periods prior to RNA extraction. A 4.0kb fragment containing a hsp70 gene and a 0.58kb fragment containing a hsp18 gene were used as hybridizations probes in A and B, and C and D respectively. The autoradiograms are representative of those obtained following hybridization of probe to RNA blots from more than three independent experiments.

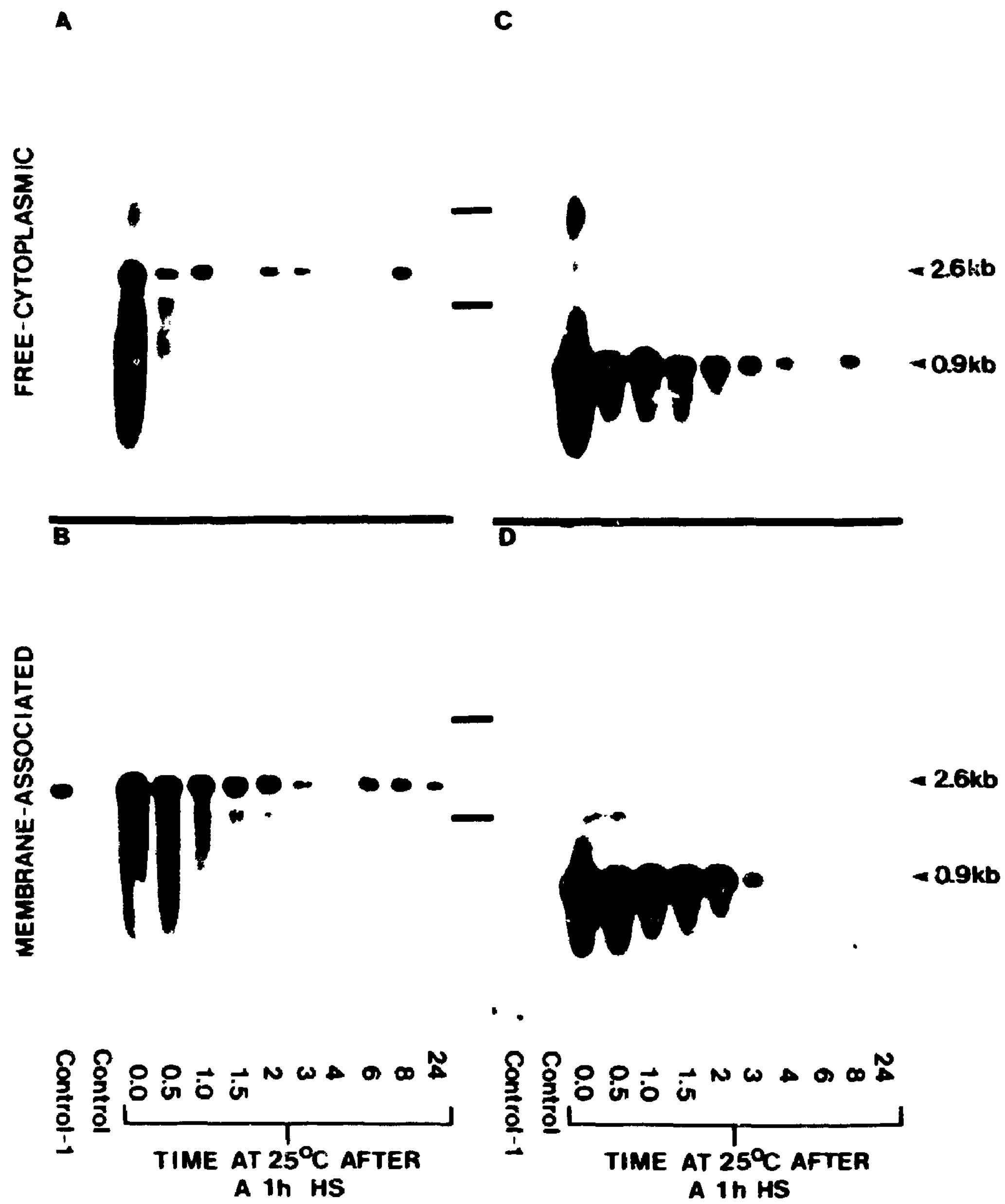
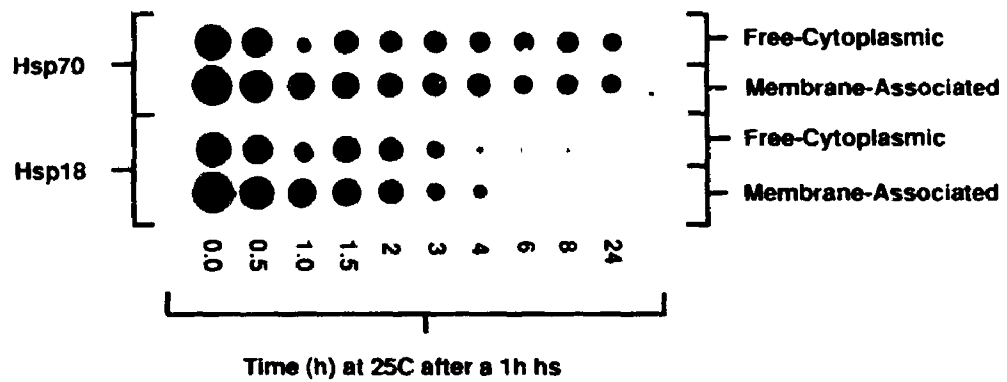

A
C
B
D
FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
2.6kb
0.9kb
2.6kb
0.9kb
Control-1
Control
0.0
0.5
1.0
1.5
2
3
4
6
8
24
TIME AT 25°C AFTER
A 1h HS
Control-1
Control
0.0
0.5
1.0
1.5
2
3
4
6
8
24
TIME AT 25°C AFTER
A 1h HS

Figure 29: RNA blot analysis of the free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA extracted from the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{O}C and allowed to recover for various periods of time at 25°C prior to RNA extraction.

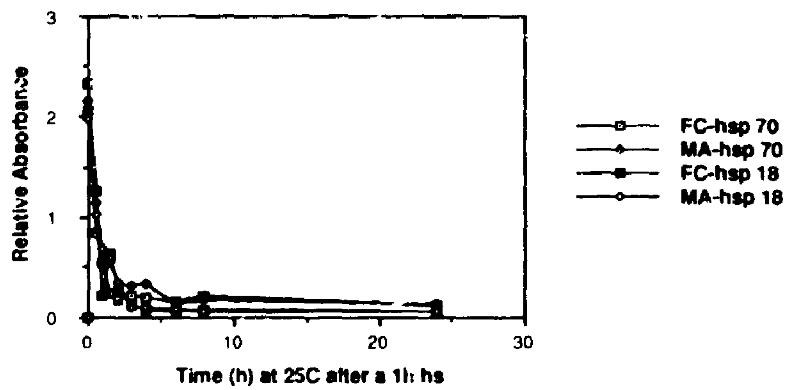
A. RNA dot hybridization of free-cytoplasmic and membrane-associated polyribosomal poly(A)$^+$ RNA to a maize hsp70 or hsp18 gene fragment.

B. Quantitation of the relative levels of 70kDa and 18kDa mRNAs associated with the free-cytoplasmic and membrane-associated ribosomes. Values represent mean relative absorbances from four independent determinations.

A. RNA blot



B. Relative levels of hsp mRNAs



increased marginally after 8h at 25°C and then declined to control levels by 24h. Similar kinetics were observed in the in vitro translation and Northern blot analyses. The levels of 18kDa mRNAs associated with the free-cytoplasmic and membrane-associated ribosomes gradually declined such that by 24h, the level of 18kDa mRNAs approximated those observed by Northern analysis after a 5 minute heat shock. These results corroborate the in vitro translation studies and suggest that while recovery from a temperature shift is rapid, some low molecular weight mRNAs continue to associate with the ribosomes after 24h at 25°C.

4.4 Discussion

In higher plants, translational specificity is variable depending on the tissue and/or species being examined (Barnett, 1980; Altschuler and Mascarenhas, 1982; Baszczynski et al, 1982; Baszczynski et al, 1983; Key et al, 1983; Cooper and Ho, 1983; Cooper et al, 1984; Mascarenhas and Altschuler, 1985; Altschuler and Mascarenhas, 1985; Belanger et al, 1986). In maize seedlings, many 25°C proteins continue to be synthesized during heat shock (refer to Sections 2.3 and 3.3; Baszczynski et al, 1982; Baszczynski et al,1983; Cooper and Ho, 1983; Cooper et al,1984). However, because the rate of total protein synthesis is unchanged, there appears to be a suppression of the synthesis of some of the normal proteins to compensate for the increase in hsp synthesis (Cooper and Ho, 1983; Baszczynski, 1984). These in vivo results together with the absence of a transition in the polyribosome profiles following a 1h heat shock at 42.5^{o}C, the absence of any obvious change (as determined within the limits of sensitivity) in the polyribosomal protein profiles eventhough there is a concomittant accumulation of heat shock mRNAs on the ribosomes of polyribosomes, and the efficient translation in vitro of polyribosome-bound 25°C poly (A)$^{+}$ RNAs during heat shock suggest that competition for the translational machinery is a major determinant in translational regulation of maize plumules. Some sequence-specific and/or structural feature of heat shock messages may confer on them a high intrinsic translational efficiency relative to control messages. The continued association of 25°C messages with polyribosomes during heat shock has been reported in Drosophila (Storti et al, 1980; Kruger and Benecke, 1981; DiDomenico et al, 1982a; Ballinger and Pardue, 1983) and HeLa (Hickey and Weber, 1982; Thomas and Mathews, 1982) cells. More recent studies in tomato cell cultures suggest that 25°C mRNAs are incorporated into HSG's. The sedimentation behaviour of these complexes is similar to that of polyribosomes (Nover et al, 1989).

A marginal decrease in the level of some control messages, such as actin mRNAs, was also observed during heat shock and may reflect a change in the synthesis, stability,

and/or translational state of these mRNAs. Degradation of cellular messages during heat shock has been reported in tomato cell cultures (Nover and Scharf, 1984) and barley aleurone layers (Belanger et al, 1986).

The induction of hsp's is transient (Schoffl and Key, 1982; Altschuler and Mascarenhas, 1982; Cooper and Ho, 1983; Cooper et al, 1984; Baszczynski, 1984). In maize seedlings, hsp's can be detected within 15 to 20 minutes of a temperature shift. With prolonged high temperature treatment, the synthesis of hsp's declines gradually such that by 24h, a new, stable protein synthetic profile is established. The rate of hsp disappearance appears to be asynchronous (Cooper and Ho, 1983; Baszczynski, 1984). Coincident with the changes in the pattern of protein synthesis are changes in the heat shock mRNA levels. In vitro translation analyses and RNA blot hybridizations confirm that the rapid labelling of hsp's during the initial 15 to 20 minutes of heat shock results in part from the rapid accumulation of heat shock mRNAs on the ribosomes of polyribosomes. Heat shock mRNAs accumulate on both the free-cytoplasmic and membrane-associated ribosomes during the initial 5 to 10 minutes of a temperature shift, are maximal within approximately 1-2h and decline thereafter concomittantly with hsp synthesis in vivo. Similar observations have been made in soybean seedlings (Altschuler and Mascarenhas, 1982; Schoffl and Key, 1982; Key et al, 1985; Altschuler and Mascarenhas, 1985; Mascarenhas and Altschuler, 1985).

Coincident with the decline in hsp synthesis in maize roots is the gradual increase in synthesis of three new polypeptides of 62, 49.5, and 19kDa. These proteins begin to appear about the time that synthesis of the other hsp's becomes maximal (Cooper and Ho, 1983). This biphasic induction of new proteins during a heat shock is apparently similar to that observed under conditions of anaerobiosis (Sachs and Ho, 1986). Proteins of similar M_r are not observed among the in vivo or in vitro protein synthetic profiles of maize plumules. However, the in vitro studies do show that the depletion of heat shock mRNAs from the ribosomes of polyribosomes after 6h at 42.5^{o}C is accompanied by a maximal

accumulation of 25°C mRNAs encoding proteins of 17kDa on the ribosomes of free-cytoplasmic and membrane-associated polyribosomes. Furthermore, these mRNAs retain a high level of association with the polyribosomes after 24h of heat shock. In addition, poly(A)$^+$ RNA encoding proteins of 18kDa also remain associated with the polyribosomes although at greatly reduced levels. The rapid accumulation of heat shock mRNAs on the ribosomes of polyribosomes followed by the gradual dissociation of these mRNAs from the polyribosomes and the return to a near normal pattern of protein synthesis both in vivo (Cooper and Ho, 1983; Baszczynski, 1984) and in vitro during prolonged heat shock suggest that maize seedlings acclimate. The stability and kinetics of association of mRNAs encoding some of the low molecular weight hsp's suggest that these proteins could play an integral role in the acclimation process.

When maize seedlings are returned to 25°C after a 1h heat shock at 42.5°C, there is a rapid depletion of heat shock messages from the ribosomes of polyribosomes. This dissociation coincides with the loss of hsp synthesis in vivo over this time period (Cooper and Ho, 1983; Baszczynski, 1984). Within the first 30 minutes after the shift back to 25°C, the levels of 70kDa and 18kDa mRNAs associated with the free-cytoplasmic and membrane-associated ribosomes have declined significantly. After 6h at 25°C, the 70kDa and 18kDa mRNA levels approximate those observed in control tissue and in tissue subjected to a 5 minute heat shock respectively. Similar transcript levels are detected after 24h at 25°C. However, between 6 and 24h, there is a marginal increase in the level of 70kDa mRNA associated with the polyribosomes. This increase may reflect the re-association with the polyribosomes of constitutively synthesized (as distinct from heat shock) short-term repressed 70kDa mRNAs. Alternatively, changes in RNA transcription and/or other post-transcriptional events may account for this increase as the probe used in these studies is a heat-inducible constitutively synthesized gene. In HeLa cells, a HSE-binding activity distinct from the stress-induced HSE-binding activity has been identified. During recovery from heat shock, both the rate of hsp70 gene transcription and stress-

induced HSE-binding activity decline while the control HSE-binding activity reappears (Mosser et al, 1988). In other systems, where mRNA processing is affected by either heat shock (Yost and Lindquist, 1988) or other stressors (Czarnecka et al, 1988), unspliced mRNAs have been shown to accumulate in the cytoplasm for translation. During recovery from the stress, these truncated mRNAs presumably turnover and full length, mature transcripts accumulate. Coincident with the depletion of heat shock mRNAs from the ribosomes is the maximal accumulation of 17kDa mRNAs with the ribosomes of polyribosomes. The 17kDa RNA transcripts together with some 18kDa RNA transcripts continue to associate (albeit at much lower levels) with the ribosomes of polyribosomes after 24h at 25°C.

Both the stability and kinetics of association of some of the low molecular weight mRNAs with the ribosomes of polyribosomes during conditions of prolonged heat shock and recovery from a 1h heat shock may indicate that the proteins encoded by these mRNAs play a role in the mechanisms governing these responses which appear to be similar in profile. In Drosophila (Arrigo and Amad-Zadeh, 1981; Arrigo et al, 1985; Arrigo, 1987; Arrigo and Pauli, 1988; Pauli et al, 1988; Haass et al, 1989) and tomato cell suspension cultures (Nover et al, 1983; Nover and Scharf, 1984), the low molecular weight proteins associate as cytoplasmic RNPs (pre-HSG's). Pre-HSG's exhibit a temperature dependent reversible precursor-product relationship with the large HSG aggregates (see Section 1.3.3). It has been suggested that pre-HSG's may serve to segregate distinct mRNA species from the translation apparatus (inactivate heat shock mRNAs) thereby aiding in the restoration of the cells synthetic pattern to a near normal state (Nover and Scharf, 1984; Collier et al, 1988). Low molecular weight hsp's can be recovered from the post-ribosomal fraction after recovery from heat shock (Nover, personal communication). Furthermore, in Drosophila, there appears to be a correlation between pupae survival after a heat shock and the recovery of the low molecular weight proteins in the soluble lysate fractions as 10- to 20-S structures (Kloetzel and Bautz, 1983; Arrigo, 1987; Arrigo and

Pauli, 1988; Haass et al, 1989). Although the cytoplasmic aggregates formed by the small hsp's co-purify with and share several structural properties of the Drosophila prosome (Arrigo et al, 1985; Pauli et al, 1988), it has been shown in mammalian cells that such aggregates may be separated from the prosome after further purification (Falkenburg et al, 1988; Arrigo et al, 1988b). More recent studies in Drosophila (Martins de Sa et al, 1989), plant (Nover et al, 1989), and vertebrate (Arrigo and Welch, 1987) cells have shown that the prosomal proteins are not only distinct from the hsp's but are not synthesized de novo during heat shock. The newly synthesized small hsp's are, however, found associated with pre-HSG's in these cells (Martins de Sa et al, 1989; Nover et al, 1989).

The depletion of heat shock mRNAs from the ribosomes of polyribosomes under conditions of prolonged heat shock and recovery from heat shock suggests that repression of hsp synthesis involves a temperature-independent translational inactivation of heat shock messages. Inactivation as a result of destabilization of the polyribosomes is unlikely as heat shock causes no change in the distribution of ribosomes in the polyribosomes. Furthermore, many 25°C mRNAs remain ribosome-bound during heat shock. In Drosophila, the repression of hsp synthesis and the restoration of normal protein synthesis are controlled both transcriptionally and post-transcriptionally as discussed in Section 1.2 (Lindquist, 1981; DiDomenico et al, 1982a; DiDomenico et al, 1982b; Lindquist and DiDomenico, 1985; Peterson and Lindquist, 1988). Accumulation of hsp's promotes the repression of hsp mRNA synthesis whether the cells are maintained at high temperature or returned to 25°C. However, at the post-transcriptional level, repression only occurs after a return to normal temperatures where it is tightly coupled to mRNA degradation (DiDomenico et al, 1982a; DiDomenico et al, 1982b). Hsp mRNAs, particularly hsp70 mRNA, are stable and continue to be translated with prolonged high temperature treatment (Lindquist and DiDomenico, 1985; Peterson and Lindquist, 1988). Furthermore, sequences in the 3' end of the hsp70 message in Drosophila appear to be important in directing its' turnover (Simcox et al, 1985). In human cell lines, heat shock has also been

shown to have a marked effect on the stability of hsp70 mRNA (Banerji et al, 1986; Theodorakis and Morimoto, 1987). However, unlike Drosophila cells, hsp70 gene transcription in human cells is independent of protein synthesis (Mosser et al, 1988). In maize seedlings, the effects of other post-transcriptional events, such as changes in mRNA stability and/or activation state after dissociation from the ribosomes, on the repression of hsp synthesis and the return to a near normal protein synthetic profile remain to be determined. Furthermore, the role of transcriptional controls in the induction and recovery process(es) are not known.

CHAPTER 5

TRANSLATIONAL REGULATION IN MAIZE PLUMULES DURING HEAT SHOCK -- NON-POLYRIBOSOMAL CONTROL

5.1 Introduction

In preceding chapters, the mRNA complement of the non-polyribosomal RNP was assayed by in vitro translation and hybridization analyses. These studies demonstrated that 1) although the spectrum of mRNA sequences was similar in the polyribosomal and non-polyribosomal RNPs, it was not identical and 2) specific mRNA sequences were common to both fractions. That the proteins associated with the RNP play a role in the translational state or activity of the particles was inferred by the fact that 1) only deproteinized non-polyribosomal RNP were translated in vitro and 2) particles deproteinized using proteinase K treatment followed by chloroform extraction (conditions which avoid denaturation of RNA hybrids and reduce the risk of loss of small RNA components into the phenol phase by association with proteins) were actively translated in vitro.

In this chapter, both the protein complement and mRNA complement of free-cytoplasmic and membrane-associated non-polyribosomal RNPs are examined in control and heat-shocked maize plumules to assess alterations ensuing from a temperature shift. The distribution of heat shock mRNAs within the non-polyribosomal RNPs and the metabolism of non-polyribosomal RNPs during prolonged heat shock and recovery from heat shock are also examined to further characterize the dynamic of this response in maize seedlings.

5.2 Materials and Methods

5.2.1 Treatment of seedlings

Etiolated seedlings were maintained at 25°C or subjected to 1) a 1h incubation at 42.5°C, 2) a heat shock of varying duration (0-24h) or 3) a brief heat shock (1h) followed by a recovery of varying duration (0-24h) at 25°C prior to RNP RNA extraction.

5.2.2 Isolation of free-cytoplasmic and membrane-associated non-polyribosomal RNPs

5.2.2.1 Isolation of non-polyribosomal RNPs

Polyribosomal RNPs were isolated from the plumules of intact maize seedlings following treatment as described in section 4.2.2. Non-polyribosomal RNPs were pelleted from the post-polyribosomal supernatants (from Section ~.2.2) as outlined in Section 3.2.8. After centrifugation, the supernatants were aspirated and saved for further analysis (see Section 5.2.2.2). The tubes were inverted and buried in ice for 15-20 minutes. The tube walls were wiped clean and the pellets resuspended in 20mM Tris-Cl, pH7.4, 150mM KCl, 3mM $MgCl_2$, and 7mM 2-mercaptoethanol. Cytoplasmic RNP complexes were fractionated by sedimentation of the resuspended post-ribosomal pellets in 10-25% (w/w) sucrose gradients, made up in the same buffer, at 4°C for 20h at 16000 rpm in a Beckman SW28 rotor (Kremp et al, 1986; Schmid et al, 1984). Fractions were monitored at 254 nm and collected using an ISCO Model 640 fraction collector attached to an ISCO Model UA-4 UV monitor and recorder. Peak fractions were pooled separately, made 0.3M with 3.0M Na-acetate, pH5.2 and precipitated with 2.5 volumes of 95% EtOH at -70°C (Maniatis et al, 1982).

Alternatively, non-polyribosomal RNPs were resuspended in a small volume of either modified translation buffer (MTB; 50mM Hepes, pH7.4, 25mM KCl, 1.0mM

EDTA, 0.5mM PMSF, and 0.1mg/ml heparin)(Nichols and Welder, 1983) or digestion buffer for analysis of intact or deproteinized non-polyribosomal RNP respectively.

5.2.2.2 Isolation of proteins associated with polyribosomal RNPs, non-polyribosomal RNPs, and post-RNP supernatants

Polyribosomal RNP pellets resuspended in TB and non-polyribosomal RNP pellets resuspended in MTB were incubated on ice with 2.0 umole EDTA per A_{260} unit of polyribosome or non-polyribosomal RNP (Gander et al, 1972). Aliquots were removed, diluted with extraction buffer, boiled for 1 minute and analyzed by 1-D polyacrylamide gel electrophoresis. Post-RNP supernatants (from Section 5.2.2.1) were made 1% with 20% SDS, 5% with 2-mercaptoethanol, and 1mM with 1.0M DTT and boiled for 1 minute. Aliquots were removed for analysis by 1-D gel electrophoresis. In all cases, protein concentrations were determined prior to loading by the turbidimetric assay described by Comings and Tack (1972) using bovine serum albumin (BSA) as a standard.

5.2.2.3 Isolation of RNA from non-polyribosomal RNP fractions

Non-polyribosomal RNP pellets resuspended in digestion buffer were incubated at 37°C for 1h and the RNA extracted with phenol and/or chloroform (Maniatis et al, 1982; Clemens, 1986). Poly (A)$^{+}$ RNP RNA was isolated from deproteinized non-polyribosomal RNP RNA using Hybond mAP. Rabbit reticulocyte lysate was programmed with 0.5-40.0ug of deproteinized or mAP-selected non-polyribosomal RNP RNA in the presence of L-[^{35}S] methionine. Reactions were carried out at 37°C and the incorporation of label into protein was determined by TCA precipitation (see Section 2.2.6).

5.2.3 Electrophoresis, transfer, and hybridization of RNA

Native RNA or RNA denatured by treatment with glyoxal and DMSO was electrophoresed on neutral 1% agarose gels as described previously (see Section 3.2.11.2). If transfer was not to follow, the gels were stained, destained, and photographed as in Section 3.2.11.2. RNA was either transferred from agarose gels or applied directly (using a Bio-Rad bio-dot microfiltration apparatus) to biodyne A or zeta probe membranes as described in Sections 3.2.12 and 3.2.16. Hybridization and washing were as outlined in Sections 3.2.14 and 4.2.6. Rehybridization followed the procedures given in Sections 3.2.15, 3.2.16, and 4.2.6.

5.2.4 Electrophoresis of proteins synthesized in vivo or in vitro by non-polyribosomal RNP RNA

One-dimensional gel electrophoresis was carried out according to the method of Laemmli (1970) with the modifications indicated in Section 2.2.8.1. Two-dimensional gel electrophoresis followed the method developed by O'Farrell (1975) with the modifications outlined in Section 2.2.8.2. Coomassie brilliant blue stained gels were either destained and photographed immediately or prepared for fluorography (see Section 2.2.9.2).

5.3 Results

5.3.1 Characterization of the free-cytoplasmic and membrane-associated non-polyribosomal RNPs

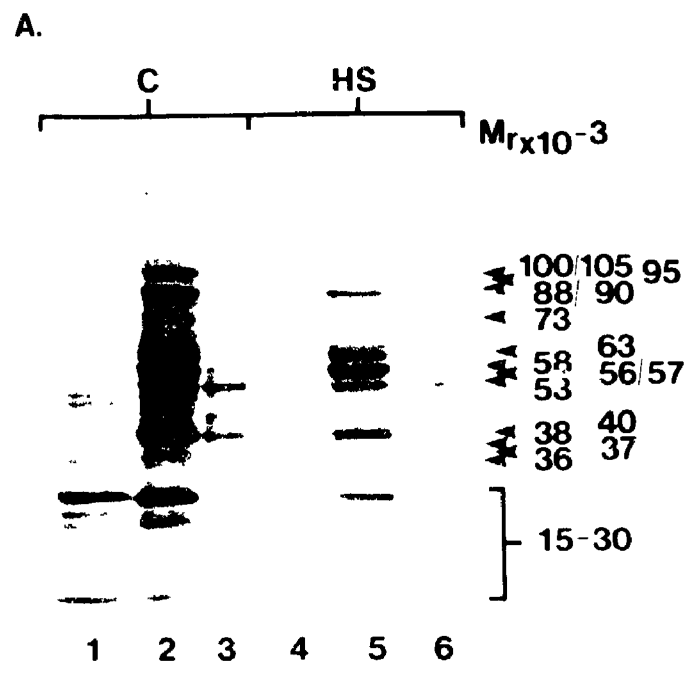
In order to determine whether heat shock affected the protein complement of non-polyribosomal RNP, the proteins associated with both non-polyribosomal RNP and post-RNP supernatants were compared by 1-D gel electrophoresis with those comprising polyribosomal RNP. Free-cytoplasmic and membrane-associated polyribosomal RNP, non-polyribosomal RNP, and post-RNP supernatants were isolated from the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C as described in Materials and Methods. The tendency of ribosomes to run off during their isolation causing polyribosomal mRNA and therefore polyribosomal RNPs to end up in the non-polyribosomal RNP population was prevented by the inclusion of emetine (1mg/ml) in the initial homogenization buffers. Analysis of the protein moieties associated with cellular fractions revealed both quantitative and qualitative differences in composition (Figure 30). The free-cytoplasmic and membrane-associated non-polyribosomal RNP fractions contained major proteins with M_r's of 38, 40, 53, 56/57, and 58kDa and minor proteins ranging in M_r between 66 and 175kDa. Additional proteins of 63, 88/90, 95, and 100/105kDa formed major components of the free-cytoplasmic non-polyribosomal RNP. A protein with similar M_r to the putative poly(A) binding protein that forms a minor component of the membrane-associated polyribosomal RNP appears to be a minor component of both the non-polyribosomal RNP and post-RNP supernatant fractions. However, a 65kDa protein which accumulates in membrane-associated polyribosomal RNP is not detected among the proteins comprising the non-polyribosomal RNP. Although distinct differences in protein composition were detected among the polyribosomal, non-polyribosomal, and post-RNP supernatant fractions, some proteins were observed to be common components of all three fractions. Co-sedimentation of ribosomal and/or

Figure 30: A comparison of the protein complement of free-cytoplasmic (A) and membrane-associated (B) polyribosomal RNP, non-polyribosomal RNP, and post-RNP supernatant fractions (from two experiments).

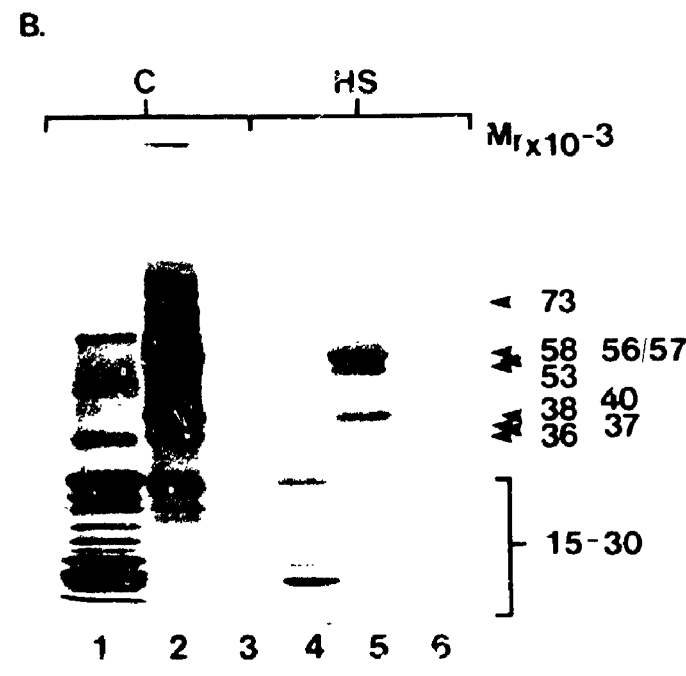
A. Free-cytoplasmic polyribosomal (lanes 1 and 4), non-polyribosomal (lanes 2 and 5), and post-RNP supernatant (lanes 3 and 6) proteins were isolated from the plumules of maize seedlings maintained at 25°C (C; lanes 1-3) or subjected to a 1h heat shock at 42.5^{o}C (HS; lanes 4-6) and analyzed by 1-D polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue R-250, destained, and photographed. M_r's of some of the more prominent proteins or groups of proteins are indicated by arrowheads or bars on the right respectively.

B. Membrane-associated polyribosomal RNP lanes 1 and 4), non-polyribosomal RNP (lanes 2 and 5), and post-RNP supernatant (lanes 3 and 6) proteins were analyzed as in A.

A.



B.



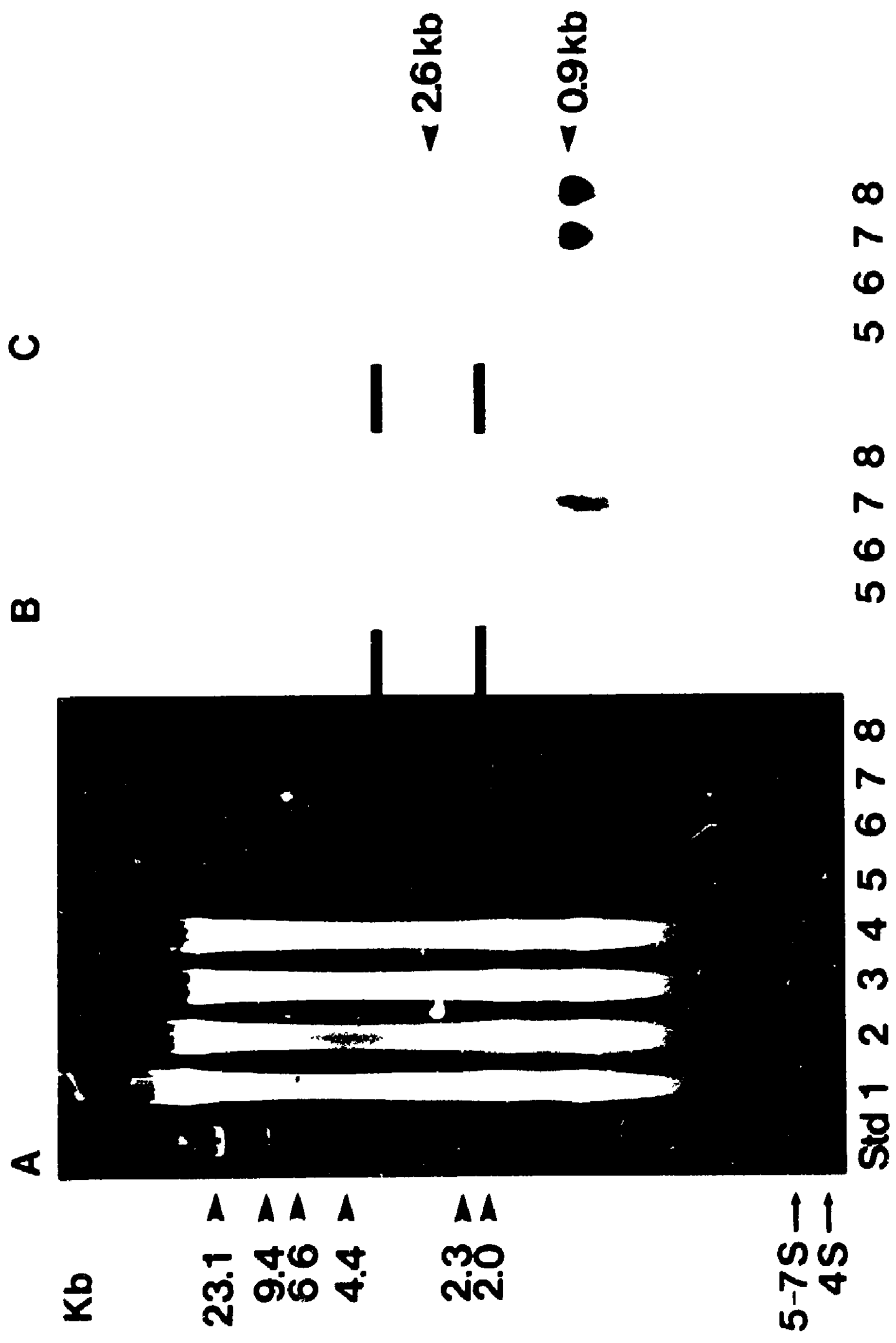
prosomal proteins with the membrane-associated non-polyribosomal RNP is marginal when compared to the free-cytoplasmic non-polyribosomal RNP. Furthermore, differential accumulation of these proteins in the free-cytoplasmic and membrane-associated post-RNP supernatants is observed. Proteins with M_r's reminiscent of translation initiation factors and associated with both free-cytoplasmic (albeit not very distinct) and membrane-associated polyribosomal RNPs, although present, are not prevalent among the proteins associated with non-polyribosomal RNP. Proteins of similar M_r were also detected among the proteins comprising the post-RNP supernatants.

The poly(A)$^+$ RNA complement of non-polyribosomal RNP was assayed indirectly by in vitro translation analyses as shown in Section 3.3.2 (see Figure 15). Although the mRNAs comprising the free-cytoplasmic and membrane-derived non-polyribosomal RNP synthesize a similar spectrum of control proteins, additional 25°C mRNAs are detected among the free-cytoplasmic fraction. A characteristic group of hsp's, some of which share M_r's and pI's with proteins synthesized by poly(A)$^+$ RNP RNAs extracted from 25°C plumules, are synthesized in response to heat shock. Most mRNAs exhibiting preferential ribosomal associations are distributed throughout the non-polyribosomal RNP fractions. However, some mRNAs appear to be predominantly associated with free-cytoplasmic non-polyribosomal RNPs.

There is an apparent decrease in control mRNAs comprising the free-cytoplasmic RNP after heat shock. Furthermore, the spectrum of 25°C mRNAs comprising the membrane-associated non-polyribosomal fraction in heat-shocked plumules shows greater similarity to that observed in the free-cytoplasmic RNP fraction of 25°C plumules.

Gel electrophoretic analysis of native free-cytoplasmic and membrane-associated poly(A)$^+$ RNP RNA corroborated the in vitro translation studies (Figure 31, panel A). Free-cytoplasmic (lanes 1 and 3) and membrane-associated (lanes 2 and 4) non-polyribosomal RNP extracted from either control (lanes 1 and 2) or heat-shocked (lanes 3 and 4) plumules contain a similar spectrum of poly(A)$^+$ RNA sequences. Furthermore,

Figure 31: The effect of heat shock on the association of RNA with non-polyribosomal RNP. Deproteinized or poly (A)$^+$ free-cytoplasmic and membrane-associated non-polyribosomal RNP RNA was isolated from the plumules of intact maize seedlings maintained at 25°C or subjected to a 1h heat shock at 42.5^{o}C (N>/=3). RNA was electrophoresed on neutral agarose gels in its' native state or after denaturation with glyoxal and DMSO. The agarose gels were either stained with 0.5ug/ml EtBr (panel A) or the RNA was transferred to biodyne A membrane and hybridized to a ^{32}P-labelled hsp70 (panel B) or hsp18 (panel C) gene fragment. DNA ladder (lane a in panel A) was co-electrophoresed with the RNA samples for use in M_r determinations. Lanes 1-4 in panel A contain native deproteinized non-polyribosomal RNP RNA. Lanes 1 and 3 contain 20.0ug of 25°C and 42.5^{o}C free-cytoplasmic non-polyribosomal RNP RNA respectively. Lanes 2 and 4 contain 20.0ug of 25°C and 42.5^{o}C membrane-associated non-polyribosomal RNP RNA respectively. Lanes 5-8 in panels A, B, and C contain denatured non-polyribosomal poly (A)$^+$ RNP RNA. Lanes 5 and 7 contain 5.0ug of 25°C and 42.5^{o}C free-cytoplasmic non-polyribosomal poly (A)$^+$ RNP RNA respectively while lanes 6 and 8 contain equivalent amounts of control and heat shock membrane-derived non-polyribosomal poly (A)$^+$ RNP RNA respectively. The autoradiograms are representative of those obtained following hybridization of RNA blots from more than three independent isolations.


A
B
C
Kb
23.1
9.4
6.6
4.4
2.3
2.0
5-7S
4S
Std 1 2 3 4 5 6 7 8
5 6 7 8
5 6 7 8
2.6kb
0.9kb

analysis of the RNA profiles revealed that the RNP fractions were not contaminated with precursor ribosomal RNA (ie. no nuclear contamination) and contained only very low levels of mature ribosomal RNA (and therefore ribosomal subunits). RNA species with a size of 4S were observed in variable amounts in both 25°C and 42.5^{o}C free-cytoplasmic and membrane-associated non-polyribosomal RNPs. Following a heat shock, 5-7S RNA was detected among the population of RNAs comprising the membrane-derived fraction. These small RNAs may represent rRNA, tRNA, and/or small cytoplasmic RNAs co-purifying with the non-polyribosomal RNPs.

The association of two specific poly(A)$^{+}$ RNAs, hsp70 and hsp18, with non-polyribosomal RNP was determined directly by Northern blot analyses (Figure 31, panel B and C). Low levels of 70kDa RNA were detected among the population of poly(A)$^{+}$ RNAs comprising both 25°C and 42.5^{o}C free-cytoplasmic and membrane-associated RNP (Figure 31, panel B, lanes 5 and 7 and lanes 6 and 8 respectively). The increase in 70kDa RNA levels associated with these fractions following a 1h heat shock is marginal. In contrast, little (after longer exposure) to no 18kDa RNA was detected among the poly(A)$^{+}$ RNA species comprising either the free-cytoplasmic or membrane-associated RNP at 25°C (Figure 31, panel C, lanes 5 and 6). However, a significant increase in the association of this RNA with both fractions is observed following a heat shock (Figure 31, panel C, lanes 7 and 8).

Although the free-cytoplasmic and membrane-associated non-polyribosomal RNP contain a similar spectrum of both protein and RNA at 25°C and 42.5^{o}C, heat shock results in the accumulation of additional mRNA species in these fractions. Moreover, the association of putative initiation factors and rRNAs with these fractions suggests that they contain initiation complexes.

5.3.2 Distribution of mRNAs within non-polyribosomal RNPs

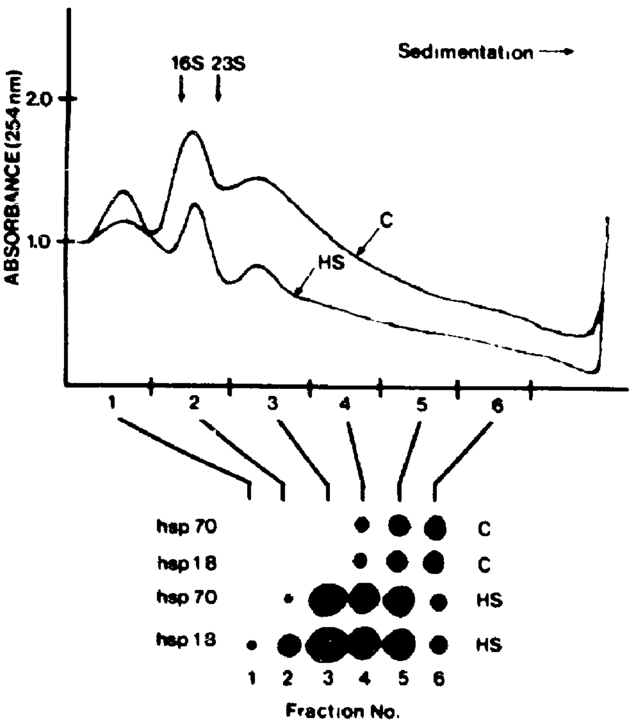
The distribution of putative RNP complexes within the post-ribosomal pellet was determined by sedimentation analyses on 10-25% sucrose gradients in 150mM KCl (salt is high enough to prevent artifactual aggregation)(Dreyfuss, 1986). Although the sedimentation profiles of free-cytoplasmic and membrane-associated post-ribosomal pellets differ, both fractions are comprised of particles sedimenting between 7 and 70/80S (Figure 32; see Appendix 7). In the free-cytoplasmic post-ribosomal pellet, three major absorbance peaks corresponding to sedimentation values of approximately 7-8S, 18-20S, and 30S were observed in 25°C extracts. Heat shock resulted in both a broadening of the 7-8S sedimentation zone and an overall decrease in the 254 absorbance of each sub-particle (Figure 32A). The membrane-associated post-ribosomal pellet consisted of a broad peak at approximately 18-20S with distinct shoulders on both the heavy (30S) and light (7-8S) sides. Although heat shock results in a decrease in the 254 absorbance of these sub-particles, it does not affect their sedimentation (Figure 32B).

The distribution of heat shock mRNAs within these sub-particles was determined by hybridization analyses of pooled gradient fractions as described in the Materials and Methods. Both the 18-20S and 30S sub-particles comprising 25°C free-cytoplasmic non-polyribosomal RNP (Figure 32A) contained low levels of 70kDa and 18kDa mRNAs. Little to no 70kDa and 18kDa RNA was detected in the 7-8S particle. However, both mRNAs were prevalent among fractions sedimenting in the 40-70/80S range. Heat shock resulted in a significant accumulation of 70kDa mRNA in both the 30S and 40-60S particles. Although less pronounced, this mRNA also accumulated in the 7-8S and 20S particles. Increased accumulation of 18kDa mRNA in the 7-70/80S sedimentation zone was also observed following a heat shock. Levels of 18kDa mRNA accumulating in the 7-8S, 30S and 40-60S particles approximated those observed for 70kDa mRNAs within these fractions. In contrast, a significant amount of 18kDa mRNA also became associated with the 18-20S sub-particle following a temperature shift. Heat shock appeared to have

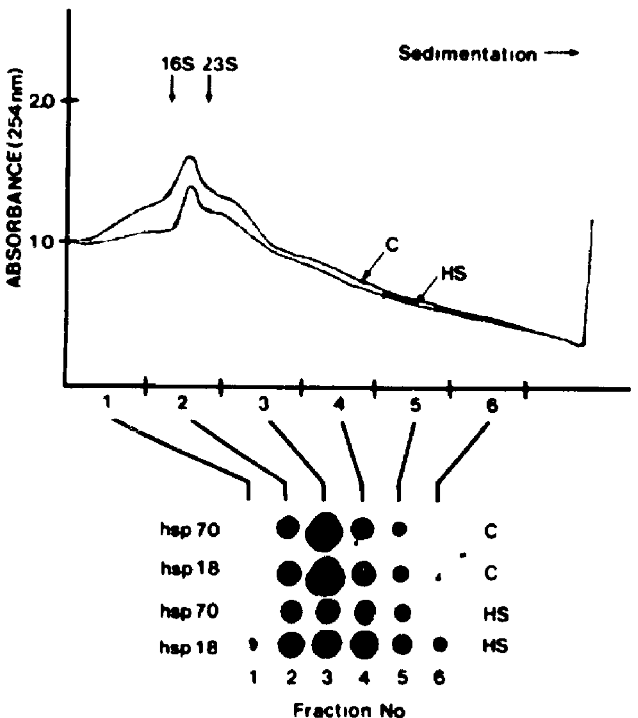
Figure 32: The effect of heat shock on RNA distribution in non-polyribosomal RNPs. Sucrose gradient profiles of free-cytoplasmic (A) and membrane-associated (B) non-polyribosomal RNPs. Non-polyribosomal RNPs were isolated from the plumules of intact maize seedlings maintained at the normal growth temperature (C) or subjected to a brief heat shock at 42.5°C (HS). E. coli 16S and 23S ribosomal RNA was fractionated on identical gradients for use in the determination of sedimentation values (see Appendix 7). RNA dot blot analyses of sucrose gradient fractions are presented below the gradient profiles. Free-cytoplasmic (A) and membrane-associated (B) RNP RNA precipitated from pooled peak gradient fractions (area pooled is bounded on either side and designated by a fraction number) was probed with an hsp70 or hsp18 gene fragment.

Representative relationships between mean relative absorbances and increasing concentrations of non-polyribosomal RNP RNA are provided in Appendix 8.

A. FREE-CYTOPLASMIC



B. MEMBRANE-ASSOCIATED



no effect, however, on the level of 70 and 18kDa mRNAs accumulating in the 70/80S zone of sedimentation.

In the membrane-associated post-ribosomal pellets (Figure 32B) extracted from control plumules, 70 and 18kDa mRNAs are primarily associated with the 30S particle but are detected in decreasing amounts in the 18-20S particle and 40S fraction, in the 60S fraction and lastly in the 7-8S particle and 70/80S fraction. Heat shock results in a decrease in the association of both mRNAs with the 30S particle. Concommitant with this decrease is a small increase in the amount of message, both 70 and 18kDa (the latter being more evident), associating with fractions sedimenting in the 40-70/80S zones.

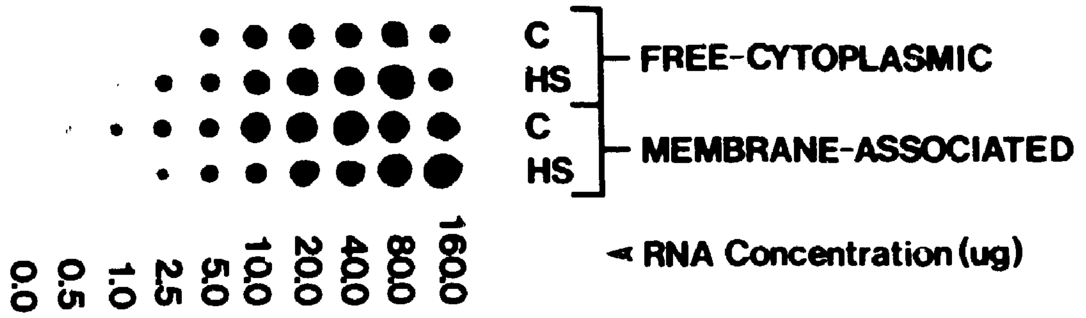
During a heat shock, the negligible increase in message accumulation in the 40-60S fractions associated with the membrane-derived non-polyribosomal RNP when compared with the significant accumulation of message in these fractions in the free-cytoplasmically-derived non-polyribosomal RNP suggests that the amount and/or turnover of heat shock message in these compartments differs both before and after a temperature shift.

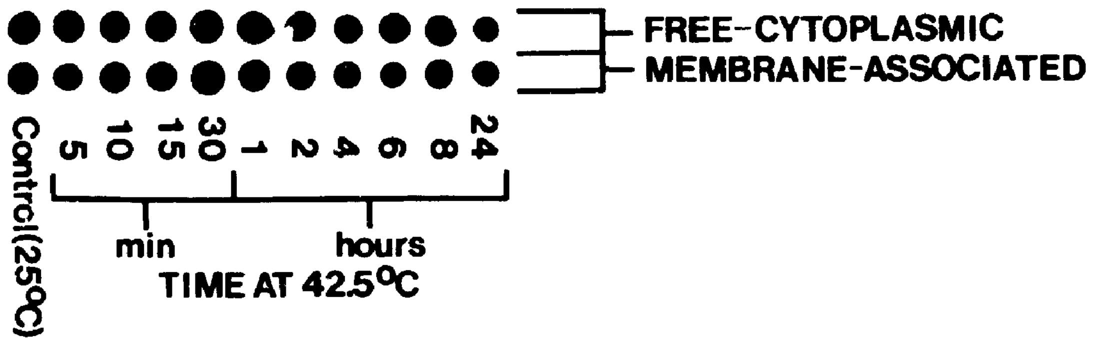
5.3.3 RNP metabolism during prolonged heat shock and recovery from a brief heat shock

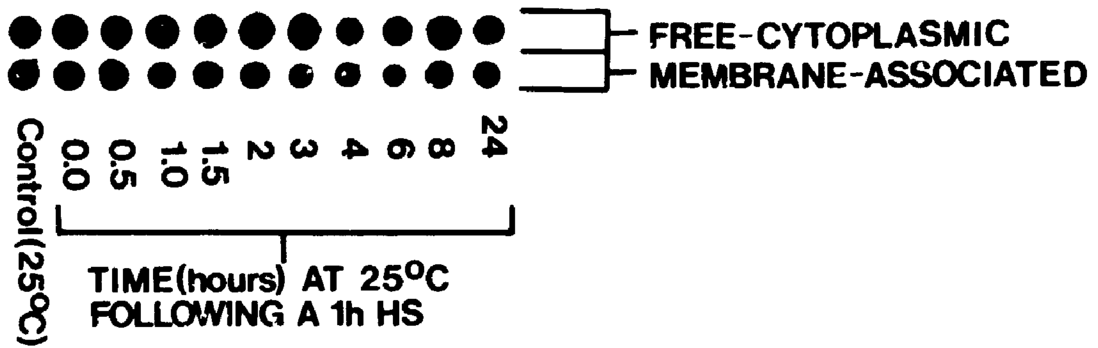
The metabolism of non-polyribosomal RNP complexes during prolonged heat shock or recovery from a brief heat shock was examined by hybridization analyses and/or in vitro translation analyses respectively. Free-cytoplasmic and membrane-associated non-polyribosomal RNP RNAs were isolated from the plumules of maize seedlings as described in section 5.2.2.1.

Prolonged heat shock had a similar effect on the association of 70kDa mRNA with both the free-cytoplasmic and membrane-associated RNP (Figure 33). Hybridization analyses and quantitation of the resultant autoradiograms by laser densitometry (see Appendix 9) indicated that 70kDa RNA transcripts accumulated to maximal levels in both free-cytoplasmic and membrane-associated RNP within the first 30 minutes of heat shock. In the free-cytoplasmic RNP, this was followed by a decline in 70kDa RNA levels through

Figure 33: RNA dot blot analyses of free-cytoplasmic and membrane-associated non-polyribosomal RNP RNA extracted from the plumules of intact maize seedlings subjected to a heat shock of varying duration at 42.5^{O}C or a heat shock of 1h at 42.5^{O}C followed by a recovery period of varying duration at 25°C prior to RNA extraction. RNA was hybridized to a maize hsp70 gene fragment. Isolations and hybridizations were repeated at least three times.


C
HS
C
HS
FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
0.0
0.5
1.0
2.5
5.0
10.0
20.0
40.0
80.0
160.0
RNA Concentration (ug)


FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
Control(25°C)
5
10
15
30
1
2
4
6
8
24
min
hours
TIME AT 42.5°C


FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
Control(25°C)
0.0
0.5
1.0
1.5
2
3
4
6
8
24
TIME(hours) AT 25°C
FOLLOWING A 1h HS

to 4h. After 6h at 42.5°C, the level of transcript increased and then declined after 24h to levels below those detected in 25°C RNP. In contrast, in membrane-associated RNP, 70kDa RNA levels declined gradually through to 24h but remained at levels above those associated with controls.

The association of 70kDa RNA with these fractions during recovery from a brief heat shock was more variable (Figure 33; see Appendix 9). Although, a significant decrease in the level of this transcript was detected in both free-cytoplasmic and membrane-associated RNP after 4-6h at 25°C, this was followed by an increase in 70kDa RNA levels after 8h and then a decline to levels approximating those associated with controls by 24h. Analysis of the translation products synthesized by free-cytoplasmic and membrane-associated RNP RNA corroborated, for the most part, the hybridization analyses (Figure 34). In the free-cytoplasmic fraction, heat shock resulted in the accumulation among the translation products of a 70kDa variant with similar pI to that synthesized by 25°C RNP RNA and four additional 70kDa variants that were not detected among the 25°C translation products. After 4h at 25°C, the level of 70kDa mRNA encoding the 25°C-like variant approximated that of the controls. Concomitant with this decline was the inability to detect the more basic 70kDa variants among the translation products. Synthesis of the single variant persisted after 8h but at reduced levels.

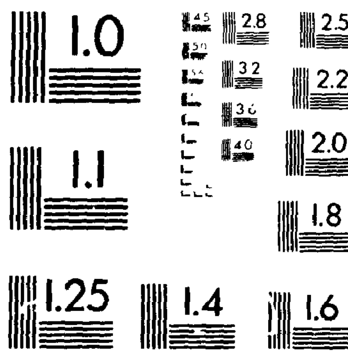
A similar protein profile (albeit less pronounced) was observed among the translation products synthesized by membrane-associated RNP. However, after 8h at 25°C, little to no 70kDa protein was detected (Figure 34, lower panels) eventhough 70kDa RNA could be detected by RNA blot analysis.

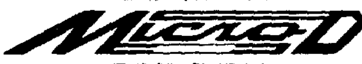
During prolonged heat shock, the kinetics of association of 18kDa mRNA with the free-cytoplasmic and membrane-associated RNP paralleled that observed for 70kDa mRNA with these fractions (Figure 35; see Appendix 10).

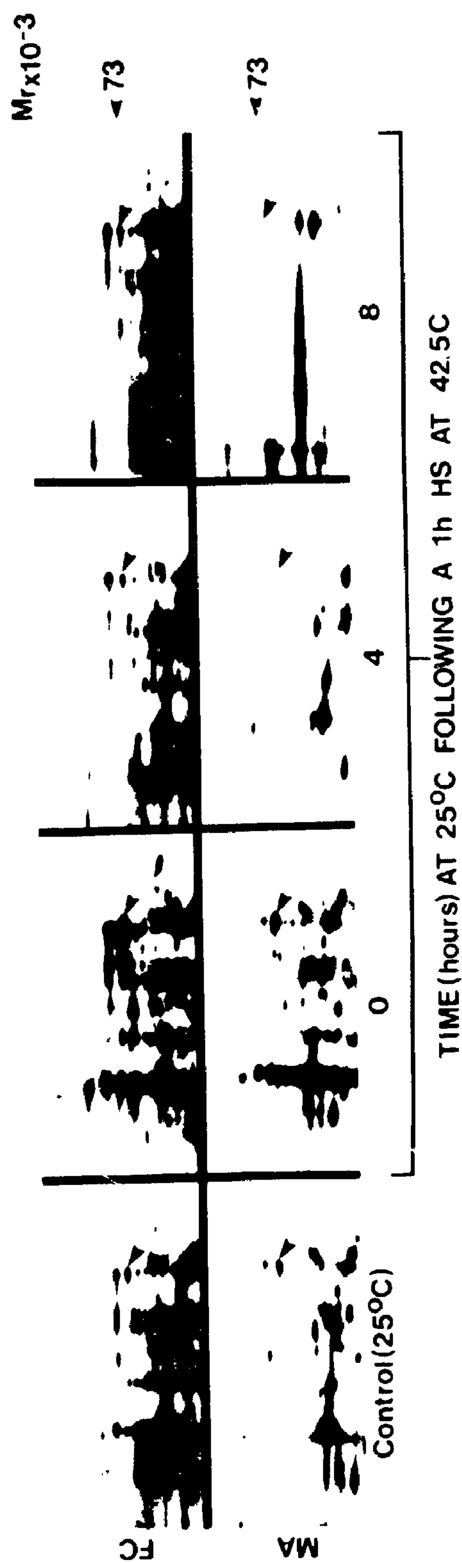
The association of 18kDa mRNA with these subcellular fractions during recovery paralleled the recovery profile for 70kDa mRNA (Figure 35; see Appendix 10). Changes

Figure 34: Fluorograms of representative (from more than three independent experiments) 2-D gel electrophoretic separations of the high molecular weight translation products obtained from the in vitro translation of free-cytoplasmic (FC) and membrane-associated (MA) non-polyribosomal RNP RNA extracted from plumules maintained at 25°C (panel 1) or subjected to a 1h heat shock at 42.5°C and allowed to recover at 25°C for 0h (panel 2), 4h (panel 3), or 8h (panel 4) prior to RNA extraction. Arrows in the panels identify 70kDa variants. M_r's of hsp's are indicated by arrowheads on the right. Approximately 100,000 cpm of acid-precipitable products from translation mixtures were loaded onto each IEF gel.

3 OF/DE 3

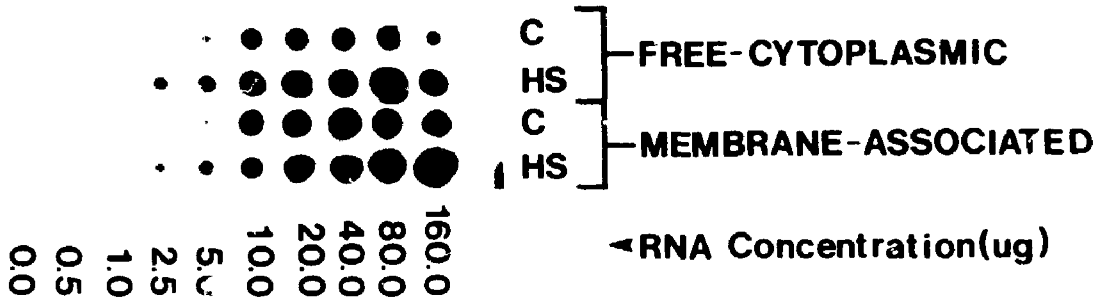


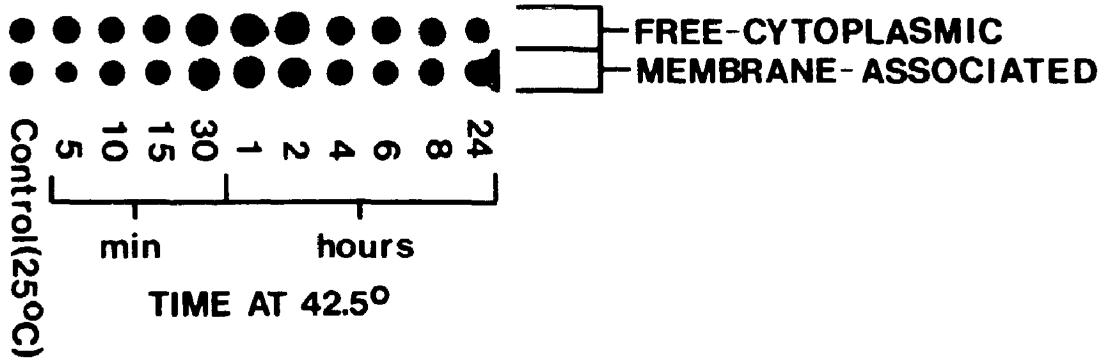


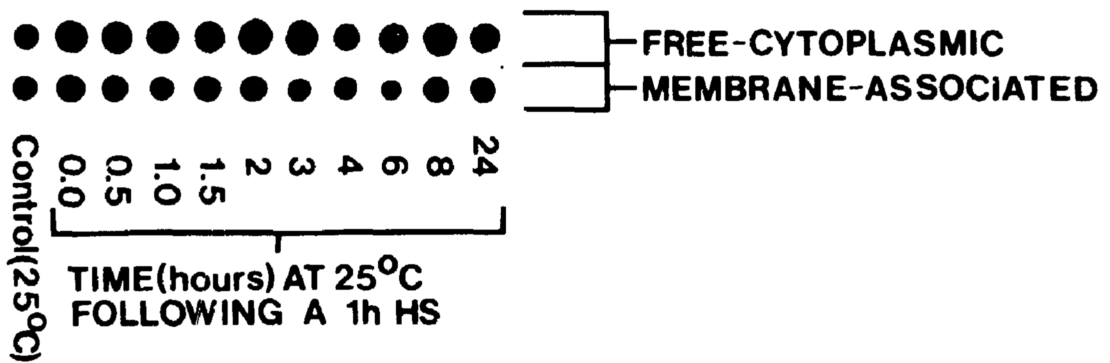


Mr x10-3
73
73
FC
MA
Control (25°C)
0
4
8
TIME (hours) AT 25°C FOLLOWING A 1h HS AT 42.5C

Figure 35: RNA dot blot analyses of free-cytoplasmic and membrane-associated non-polyribosomal RNP RNA extracted from the plumules of intact maize seedlings subjected to a heat shock of varying duration at 42.5^{O}C or a heat shock of 1h at 42.5^{O}C and allowed to recover at 25°C for a varying period of time prior to RNA extraction. RNA was hybridized with a maize hsp18 gene fragment. Isolations and hybridizations were repeated at least three times.


C
HS
C
HS
FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
0.0
0.5
1.0
2.5
5.0
10.0
20.0
40.0
80.0
160.0
RNA Concentration(ug)


FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
Control(25°C)
5
10
15
30
1
2
4
6
8
24
min
hours
TIME AT 42.5°


FREE-CYTOPLASMIC
MEMBRANE-ASSOCIATED
Control(25°C)
0.0
0.5
1.0
1.5
2
3
4
6
8
24
TIME(hours) AT 25°C
FOLLOWING A 1h HS

in 18kDa RNA levels during recovery from heat shock were also assayed indirectly by in vitro translation (Figure 36). Both quantitative and qualitative differences were observed among the proteins synthesized by free-cytoplasmic and membrane-associated RNP. In the free-cytoplasmic fraction, RNP RNA encoding at least three of the four prominent 18kDa variants was detected after 8h at 25°C indicating that recovery, at least in this fraction, is incomplete. That the same is true for membrane-associated RNP is reflected by the continued synthesis of a single 18kDa variant after 8h at 25°C and by the observation that the mRNA population comprising this fraction after 8h at 25°C in no way approximates that observed in non-polyribosomal RNP extracted from control plumules.

Figure 36: Fluorograms of representative (from more than three independent experiments) 2-D gel electrophoretic separations of the low molecular weight proteins synthesized *in vitro* by free-cytoplasmic (FC) and membrane-associated (MA) non-polyribosomal RNP RNA extracted from plumules maintained at 25°C (panel 1) or subjected to a 1h heat shock at 42.5^{o}C and allowed to recover for 0h (panel 2), 4h (panel 3), or 8h (panel 4) prior to RNA extraction. M_r's of hsp's are indicated by arrowheads on the left. Approximately 100,000 cpm of acid-precipitable products from the translation mixtures were loaded onto each IEF gel.

FC
MA
Control (25°C)
0
4
8
TIME(hours) AT 25°C FOLLOWING A 1h HS AT 42.5°C
Mr x10-3
27
25.5
22
18
17
29
26
23
19

5.4 Discussion

In the cytoplasm of animal cells, the non-polyribosomal RNA as well as the mRNA released by dissociation of polyribosomes with EDTA is found associated with protein. Major RNPs are common to different mRNAs and recognize common features such as the poly(A) tail (Vincent et al, 1981; Greenberg, 1981; Greenberg and Carroll, 1985; Adams et al, 1986), the 5' cap structure (Sonnenberg, 1981; Griffo et al, 1982; Pelletier and Sonnenberg, 1985), the polyadenylation signal (AAUAAA), and non sequence-specific ssRNA or dsRNA binding proteins (Dreyfuss, 1986). However, in addition to these proteins, there are sequence specific (eg. proteins that bind the leader sequence of heat shock mRNAs)(Bonner et al, 1984; Klemenz et al, 1985; McGarry and Lindquist, 1985; Hultmark et al, 1986) and/or compartment specific mRNA binding proteins. The latter proteins bind mRNA in a particular subcellular compartment or in a special physiological state (eg. translated, untranslated, membrane-bound or putative cytoskeletally-associated mRNAs (Dreyfuss, 1986). However, the role of these proteins in determining the translational state of mRNA is controversial (Civelli et al, 1976; Civelli et al, 1980; Greenberg, 1981; Schmid et al, 1982; Vincent et al, 1983). Although some differences have been detected in the proteins comprising polyribosomal and non-polyribosomal mRNAs (Gander et al, 1973; Vincent et al, 1983; Ovchinnikov, 1978; Jain and Sarkar, 1979; Greenberg, 1980; Nichols and Welder, 1983; Schmid et al, 1983; Bag, 1984), these differences are inconsistent and cannot alone account for the functional state of the mRNA (Setyono and Greenberg, 1981; Dreyfuss, 1986). Furthermore, there is a lack of real distinction between mRNPs and translation initiation factors.

In maize plumules, distinct differences were observed in protein composition between the polyribosomal RNP, non-polyribosomal RNP and post-RNP supernatant. Although a protein with similar M_r to the 72kDa poly(A) binding protein was detected in the subcellular fractions, it did not accumulate to significant levels. However, a prominent protein of 65kDa, which accumulated in the polyribosomal RNPs, was not detected among

the proteins comprising the non-polyribosomal RNP, and formed only a minor component of the post-RNP supernatant proteins, may be analogous to the 72kDa poly(A) binding protein. Two proteins of 68.9 and/or 66.5 and 59.4kDa have been shown to associate with the poly(A) tract in maize embryos (Nichols and Welder, 1983). Although differences in the proteins comprising each of the subcellular fractions were observed, several proteins were common to all fractions. These latter proteins probably represent translation initiation factors and ribosomal and/or prosomal proteins (Vincent et al, 1981; Schmid et al, 1984; Kremp et al, 1986; Dreyfuss, 1986).

Heat shock had no effect on either the specificity or degree of accumulation of proteins in the subcellular fractions indicating that these proteins are associated with subcellular structures that pre-exist in normal cells and remain cytoplasmic during heat shock. However, low M_r hsp's have been shown to accumulate in mRNP-like complexes during recovery from heat shock (Nover and Scharf, 1984; Arrigo et al, 1985; Arrigo et al, 1988a). Changes in the protein composition of mRNPs and/or their subcellular location have also been detected in VSV infected cells, in cells treated with other inhibitors of mRNA synthesis (Dreyfuss et al, 1984), and/or as a result of developmental cues (Akhayat et al, 1987a; Akhayat et al, 1987b; Grossi de Sa, 1988). Furthermore, Greenberg (1981) demonstrated that the mRNP complex was a dynamic structure in which bound proteins could exchange with an unbound pool. This exchange, which does not occur in proteins comprising other mRNA-containing structures such as ribosomes, may be important in converting mRNPs from one functional state to another (Greenberg, 1981).

Heat shock did effect changes in the mRNA population comprising the free-cytoplasmic and membrane-associated non-polyribosomal RNP. The most obvious changes involved the accumulation of heat shock messages within these fractions. However, subtle changes in the level and/or distribution of 25°C mRNAs were also observed during heat shock. Furthermore, small RNAs distinct from heat shock mRNA degradation products were detected among the RNAs comprising the non-polyribosomal

RNP. Heat shock in Tetrahymena induces the accumulation of a small RNA (G8 RNA; 306 nucleotides) homologous to eucaryotic 7SL RNA and E. coli. 4.5S RNA both of which are known to be associated with ribosomes and involved in the modification or regulation of protein synthesis (Hallberg and Hallberg, 1989). The accumulation of these RNAs (cytoplasmic, transfer and/or ribosomal) (Ferrer et al, 1979; Vincent et al, 1980; Schmid et al, 1984; Akhayat et al, 1987a; Akhayat et al, 1987b; Martins de Sa, 1986; Falkenburg et al, 1988) in the non-polyribosomal RNP together with the presence of putative translation initiation factors and low levels of 18S and 25S rRNAs suggest that the mRNP probably contain translation initiation complexes.

Conventional post-ribosomal fractions usually contain native ribosomal subunits (Spohr et al, 1970). Messenger RNA has been found associated with between 2 and 10% of the native 40S ribosomal subunits. In erythropoietic mouse cells induced by Friend leukemia virus, approximately 50% of non-polyribosomal globin mRNA is found in 48S initiation complexes ready to be translated. EDTA releases 15S mRNPs homologous to polyribosomal globin RNPs. A fraction of this non-polyribosomal RNA (ie. that associated with initiation complexes) can be chased into polyribosomes (Spohr et al, 1970; Spohr et al, 1972; Geoghegan et al, 1979; Imaizumi-Scherrer et al, 1982). Thus the post-ribosomal supernatant contains two different functional classes of mRNPs, one consisting of immediate precursors to mRNA functional in polyribosomes (ie. initiation complexes) and the other representing an inactive fraction of other types of potential messenger turning over in the cytoplasm (ie. 'masked' forms of mRNA)(Spohr et al, 1972; Schmid et al, 1983). It has been argued that mRNA comprising the post-ribosomal supernatant fraction is transported to the cytoplasm as a cytoskeletally-associated complex which can either be stabilized by association with ribosomes (ie. forms initiation complexes) or exist free in the cytoplasm (ie. translationally repressed mRNA)(Bag and Pramanik, 1986). Furthermore, the association of mRNA with the cytoskeletal framework is independent of both protein synthesis and the presence of ribosomes.

In maize plumules, sedimentation of non-polyribosomal free-cytoplasmic and membrane-associated RNPs revealed that 1) the non-polyribosomal RNP were comprised of sub-particles sedimenting between approximately 7S and 70/80S and 2) heat shock resulted in a change in the association of message, specifically 70kDa and 18kDa mRNA, with these sub-particles. In control cells, heat shock mRNA accumulated variably in free-cytoplasmic and membrane-associated 40S and 45-70/80S fractions. Some of these mRNA-protein complexes may represent stabilized 48S initiation complexes while the 30S mRNA-protein particles comprising the non-polyribosomal membrane-derived RNPs may represent ribosome-free complexes. Translationally repressed mRNA has been shown to be associated with mRNP particles sedimenting at 20 and 30S (25-35S) in both plant (Ferrer et al, 1979) and animal (Gander et al, 1973; Vincent et al, 1980; Vincent et al, 1983) systems.

The association of inactive mRNA with the membrane-derived fraction is consistent with both the association of mRNA with the cytoskeleton for transport (Bag and Pramanik, 1986) and the presence of non-polyribosomal RNP complexes in a readily extractable non-ionic detergent soluble form (Lenk and Penman, 1979; Cevera et al, 1981; van Venrooij et al, 1981). Cytoskeletally-associated mRNA complexes that bind ribosomes may loose the attachment to the cytoskeleton and exist in equilibrium as free- or membrane-derived polyribosomal and non-polyribosomal RNP. RNAs remaining attached to the cytoskeleton after ribosome binding may exist in a similar state. Signals within the RNA itself may determine its' distribution within the subcellular fractions in a manner analogous to that for translational selection of heat shock mRNAs. Earlier studies have demonstrated that while heat shock mRNAs and 25°C mRNAs accumulate differentially among the non-polyribosomal fractions in 25°C plumules, these mRNAs, although detected in the free-cytoplasmic and membrane-associated ribosomal fractions, accumulate predominantly in putative cytoskeletally-associated ribosomal fractions suggesting a possible and/or preferential re-association with the cytoskeleton prior to translation.

Changes in the accumulation of heat shock messages in putative initiation complexes after a heat shock suggest that the rate of message turnover, whether it be due to initiation/activation and/or degradation of heat shock mRNAs has been altered by the temperature shift. The significant accumulation of message in the putative 48S initiation complexes of free-cytoplasmic non-polyribosomal RNPs after a heat shock suggests that for heat shock mRNAs, the equilibrium between active and repressed states has been shifted by the temperature shift. Furthermore, the accumulation of heat shock mRNAs in the 18-20S and 30S sub-particles suggests an association of message with these particles prior to initiation/activation and/or a stacking of message in these particles due to saturation of intermediary 40S ribosomal subunits.

The release of mRNA from the membrane-associated 30S sub-particle together with the low level of accumulation of message in the 18-20S and 40-60S fractions suggest that either the amount and therefore the turnover of message in this fraction is little changed by heat shock or that the rate of turnover of message in this fraction has increased without shifting the equilibrium. If this fraction represents genuine 'masked' mRNA, it is possible that an early response to heat shock involves the activation of some of this message. Alternatively, the negligible change in equilibrium may reflect the cells inability to effectively recruit message from this fraction. It is interesting to note a marginal increase in the level of 25°C mRNAs associating with this fraction after heat shock (as determined by the level of in vitro translation product).

Whether recruitment of mRNA from the membrane-derived fraction is inhibited because of a masking agent or because of an intrinsically lower initiation efficiency relative to that observed for heat shock messages in the free-cytoplasmic pool is unknown. However, changes in the rate of initiation and elongation have been examined in heat-shocked Drosophila cells where 15 to 30 fold rate reductions are observed for 25°C messages (Ballinger and Pardue, 1982; Ballinger and Pardue, 1983). Although many investigators have shown that heat shock mRNAs are effectively translated in heat-shocked

cells (Lindquist, 1986), none of these studies dissect the subcellular compartmentation of these messages. Furthermore, the association of message, destined to accumulate on membrane- and cytoskeletally-associated polyribosomes, with free-cytoplasmic non-polyribosomal RNP together with the disruption of both the cytoskeleton (Biessmann et al, 1982; Collier and Schlesinger, 1986; Welch and Suhan, 1986; Walter and Biessmann, 1987) and ER (Welch and Suhan, 1986; Belanger et al, 1986) by heat shock could support either contention. Treatment of seedlings with cycloheximide prior to mRNP extraction would differentiate between these possibilities as this antibiotic, in slowing down protein chain elongation, should favour the uptake into polyribosomes of mRNAs that have a low initiation efficiency (Geoghegan et al, 1979).

The association of message with the non-polyribosomal RNPs prior to integration into polyribosomes is further supported by the kinetics of message accumulation during a prolonged heat shock. Both 70kDa and 18kDa mRNAs accumulate maximally in the non-polyribosomal RNP within 30 minutes of heat shock while maximal accumulation on the ribosomes of polyribosomes takes 1-2h. In immature duck erythrocytes, 9S and 12S RNA approached constant specific activity in the free pool at times of labelling when incorporation into polyribosomes was still increasing linearly indicating that there was a filling up of the transient free-RNA pool between nucleus and polyribosome (Spohr et al, 1972).

As for polyribosomes, prolonged heat shock results in a gradual return to near normal heat shock mRNA levels in the free-cytoplasmic non-polyribosomal RNP pool. However, mRNA levels in the membrane-derived non-polyribosomal RNP remain higher after 8h at 42.5^{O}C than those observed in control cells. Earlier work (see Chapter 4) has suggested that maize seedlings respond to prolonged heat shock by acclimation. The higher levels of heat shock mRNA associated with the membrane-derived non-polyribosomal RNP may reflect a return to a more normal non-polyribosomal to polyribosomal ratio for heat shock mRNA as the cell acclimates. Alternatively, higher

mRNA levels in this fraction may reflect a lower rate of turnover of message in the membrane-derived RNP pool.

Recovery from a brief heat shock is incomplete in both the free-cytoplasmic and membrane-associated non-polyribosomal RNP. Discrepancies between mRNA levels and their respective proteins in vitro may reflect some kind of translational inhibition and/or exaggerated mRNA levels due to non-specific hybridization to prosomal RNA (pRNA). Prosomal RNA has been shown to hybridize to poly(A) rich mRNA derived from both non-polyribosomal and polyribosomal RNPs (Martins de Sa et al. 1986; Akhayat et al. 1987b). However, the ability to synthesize at least one if not more 18kDa variants in vitro after 8h at 25°C indicates that some, if not all, of the mRNA associated with the non-polyribosomal fractions encodes protein. Furthermore, the continued association of these messages with the non-polyribosomal RNP during conditions of prolonged heat shock and recovery from heat shock is consistent with the putative role of their respective proteins in regulating these responses.

CHAPTER 6

CONCLUSIONS

Changes in gene expression can be induced by a variety of environmental (Lindquist and Craig, 1988) and/or developmental stimuli (Bond and Schlesinger, 1987). In many instances, these changes are characterized by the enhanced synthesis of a group of proteins collectively referred to as the heat shock proteins. Although the heat shock response in higher plants shares many of the characteristics of that in other eucaryotes, several differences have been observed. Some of these differences have been re-affirmed by the studies presented in this thesis on translational regulation in heat-shocked maize plumules. These differences, together with several additional findings that have emerged from these investigations, can be summarized as follows:

1) Changes in gene expression in the plumules of maize seedlings can be elicited by ribosomal inhibitors such as cycloheximide and chloramphenicol. The general inhibition of protein synthesis in vivo by cycloheximide, together with the association of mRNAs encoding the major hsp's with the ribosomes of cytoplasmic polyribosomes, indicates that these hsp's are the products of cytoplasmic ribosomes.
2) Control messages are differentially regulated in maize plumules during heat shock. In vitro translation and RNA blot analyses indicate that mRNAs encoding some 25°C proteins are preserved though not translated during heat shock while the reduced synthesis of other 25°C proteins in vivo ensues from a reduction in the level of the corresponding mRNAs. The latter may be due to a decrease in the synthesis and/or stability of these mRNAs. The continued association of 25°C messages with both the non-polyribosomal and polyribosomal RNP particles suggests that some control messages compete less effectively (relative to heat shock mRNAs) for components of

the translational apparatus in heat-shocked cells possibly due to changes in the rates of both protein synthesis initiation and elongation.

3) Hsp70 and hsp18 mRNAs (or hsp-like mRNAs) are synthesized at low levels in nonstressed cells.

4) Changes in the pattern of protein synthesis during heat shock are due, in part, to changes in the levels of corresponding mRNAs.

5) Synthesis of a similar spectrum of hsp variants both in vivo and in vitro suggests that many of these hsp variants are encoded by different genes and/or gene products.

6) Although 25°C mRNAs and heat shock mRNAs differentially accumulate among the non-polyribosomal RNP fractions in control cells, translationally active mRNAs are predominantly associated with a putative cytoskeletal containing fraction.

7) Heat shock changes the distribution of 70kDa and 18kDa mRNAs within the polyribosomal RNP particles. Heat shock mRNAs maximally accumulate on the free-cytoplasmic and membrane-associated polyribosomes suggesting that translation of heat shock messages in heat-shocked cells is largely independent of the putative cytoskeletal containing fraction. In contrast, control messages remain predominantly associated with this fraction during heat shock.

8) Prolonged heat shock and recovery from heat shock are characterized by a temperature-independent dissociation of heat shock messages from the ribosomes of polyribosomes. Dissociation in not accompanied by a re-association of these messages with the non-polyribosomal RNP particles suggesting that the messages released from the polyribosomes are degraded.

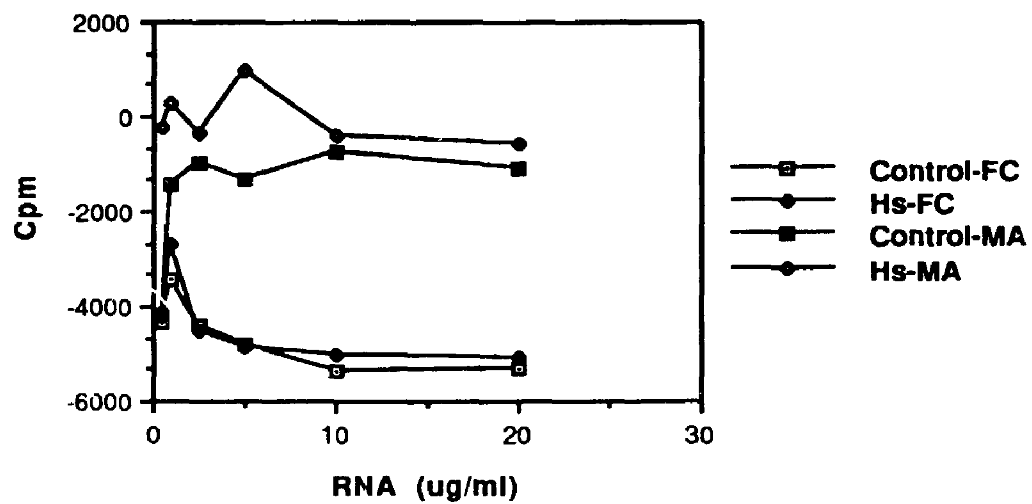
9) The stability and kinetics of association of low molecular weight mRNAs with the polyribosomes during prolonged heat shock and recovery suggest that the proteins encoded by these mRNAs are integral to the mechanism(s) regulating these responses.

10) The kinetics of message accumulation in the non-polyribosomal and polyribosomal RNP particles during prolonged heat shock, together with the accumulation of

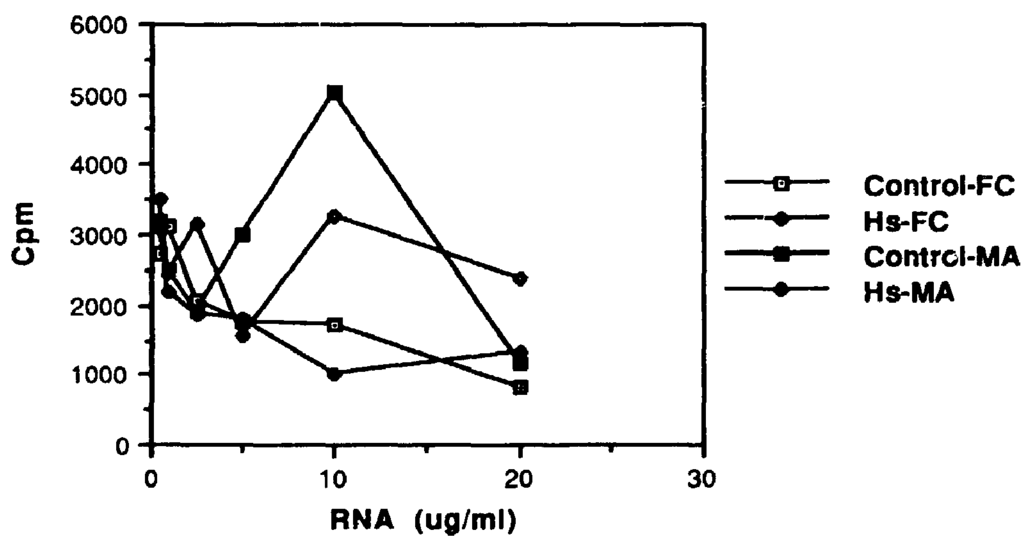
messages in free-cytoplasmic non-polyribosomal putative 48S initiation complexes, suggest that heat shock messages associate with the non-polyribosomal RNP prior to integration into polyribosomes.

Appendix 1: Incorporation of label into proteins synthesized by non-polyribosomal RNP or non-polyribosomal RNP RNA. A comparison of the relative incorporation of ^{35}S-methionine (cpm) into acid-precipitable, in vitro translation products as a function of the amount of exogenously added free-cytoplasmic (FC) or membrane-associated (MA) non-polyribosomal RNP (A) or non-polyribosomal RNP RNA (B) extracted from control or heat-shocked maize plumules. Values represent mean cpm from three independent determinations.

A.

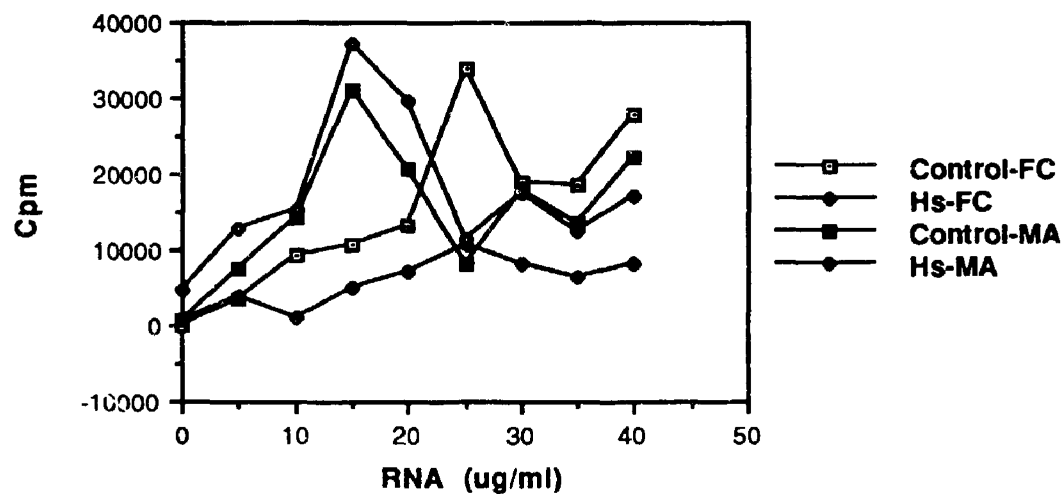


B.

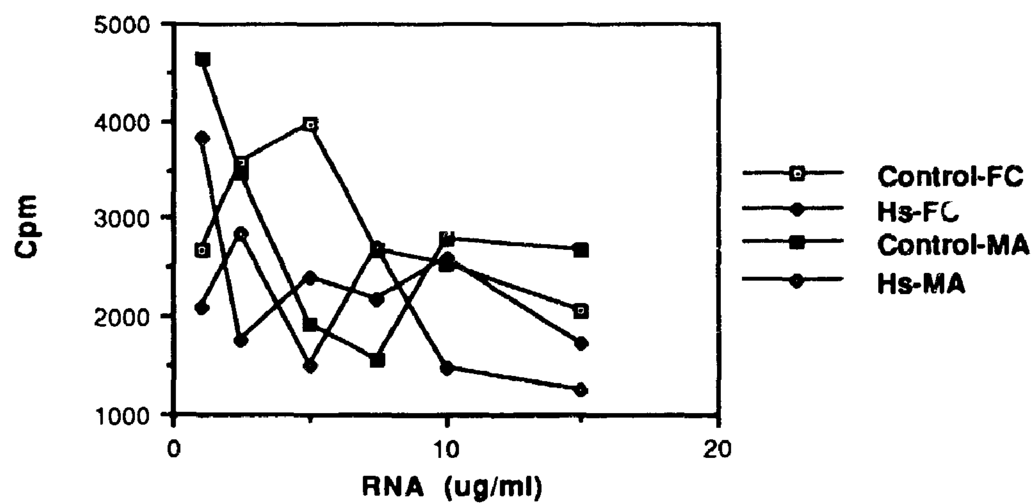


Appendix 2: Incorporation of label into proteins synthesized by polyribosomal RNP or polyribosomal RNP RNA. A comparison of the relative incorporation of ^{35}S-methionine (cpm) into acid-precipitable products synthesized in vitro by increasing concentrations of free-cytoplasmic (FC) or membrane-associated MA) polyribosomal RNP (A) or polyribosomal RNP RNA (B) extracted from control or heat-shocked maize plumules. Values represent mean cpm from three independent determinations.

A.

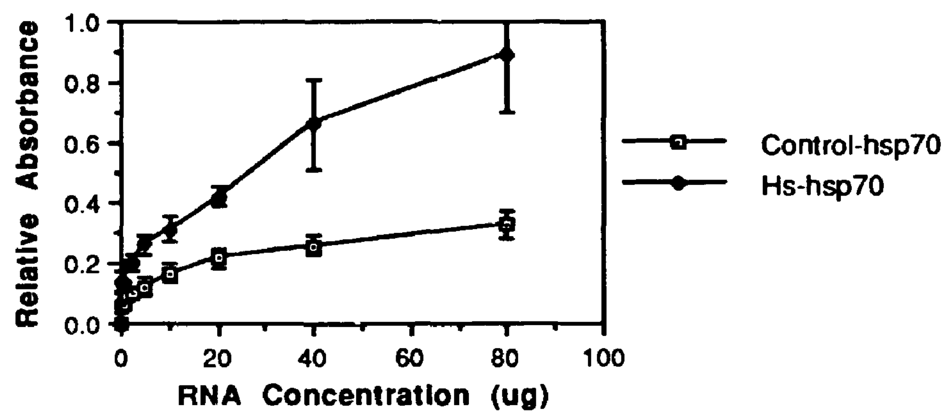


B.

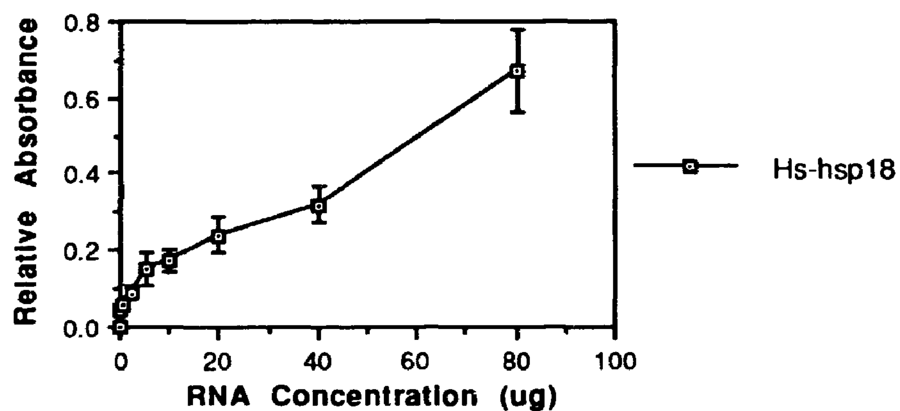


Appendix 3: Relationship between relative absorbance and increasing concentrations of total cellular RNA. Values represent mean relative absorbances for hsp70 (A), hsp18 (B), and actin (C) under control and heat shock conditions as calculated from three independent determinations. The graphs in (B) and (C) closely approximate those obtained when relative absorbance was plotted as a function of increasing polyribosomal RNP RNA (not shown)

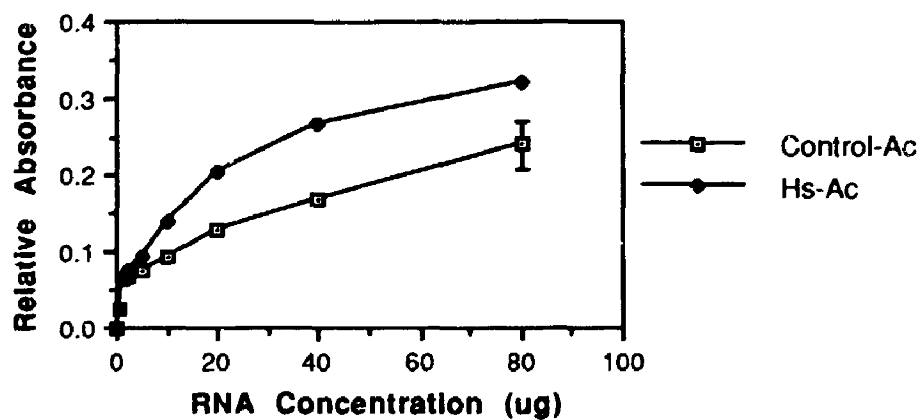
A.



B.

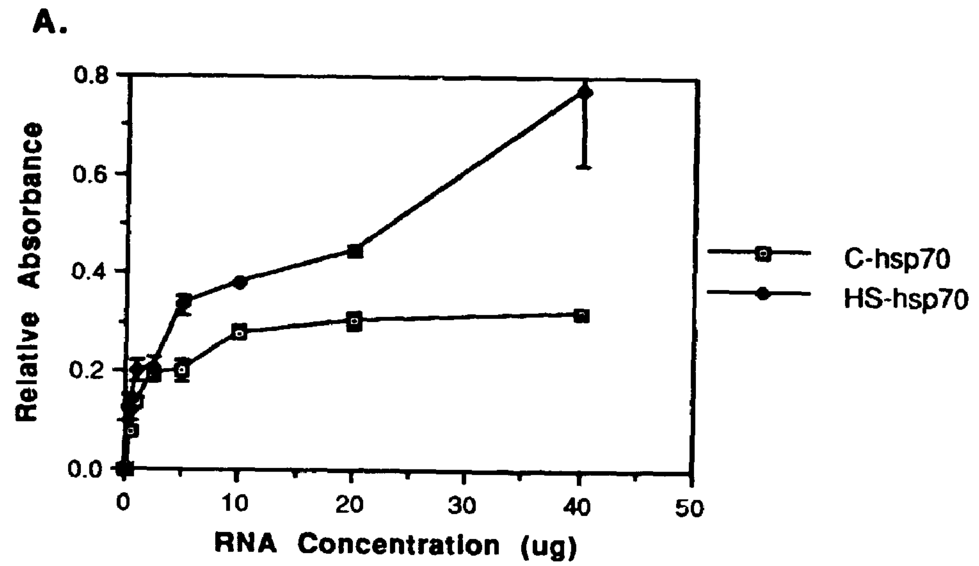


C.

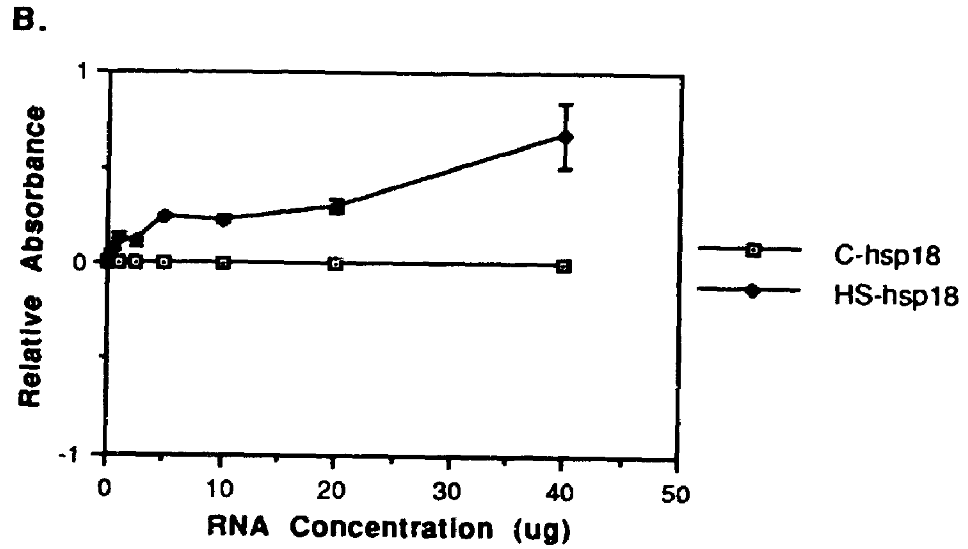


Appendix 4: Relationship between relative absorbance and increasing concentrations of total cellular poly(A)+ RNA. Values represent mean relative absorbances for hsp70 (A) and hsp18 (B) under control and heat shock conditions as calculated from three independent determinations.

A.

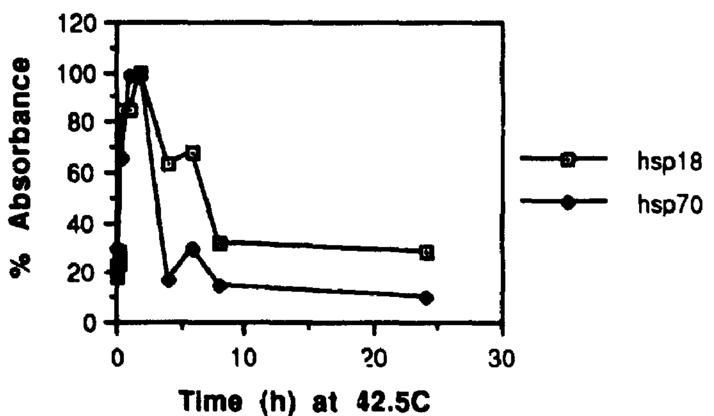


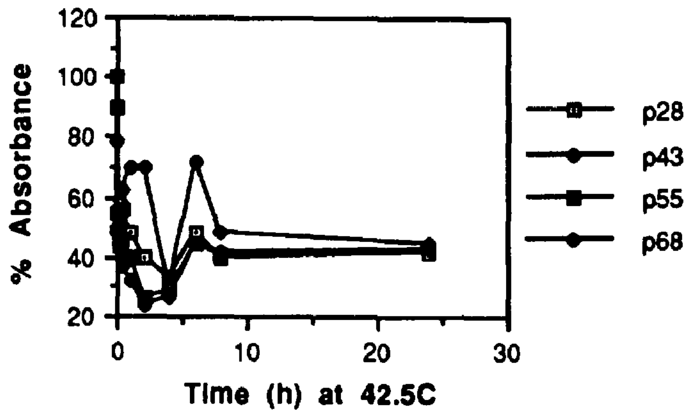
B.



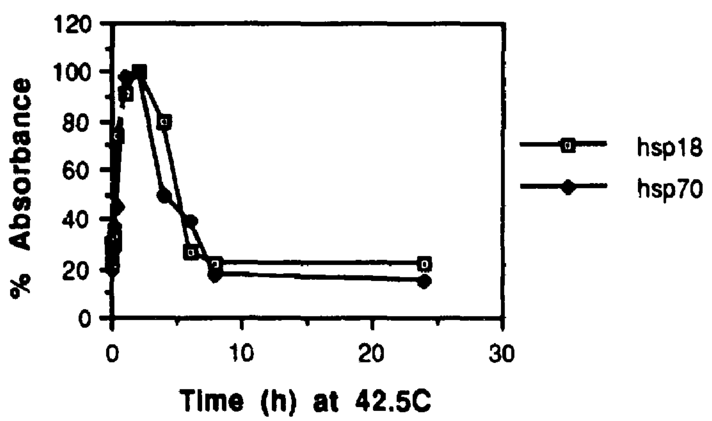
Appendix 5: In vitro synthetic levels of selected control proteins and hsp's during prolonged heat shock. Fluorograms in section 4.3.2, Figure 22, were quantified by densitometric scans to show relative trends. The relative quantity of protein synthesized at a given time is plotted as a percentage of the maximum incorporation rate for that protein. (A) Profiles for hsp70, hsp18, and four randomly selected 25°C proteins with M_r's of approximately 28000, 43000, 55000, and 68000 synthesized in vitro by free-cytoplasmic polyribosomal poly $(A)^+$ RNA. (B) Profiles for proteins, with similar M_r's to those in (A), synthesized in vitro by membrane-associated polyribosomal poly (A) RNA.

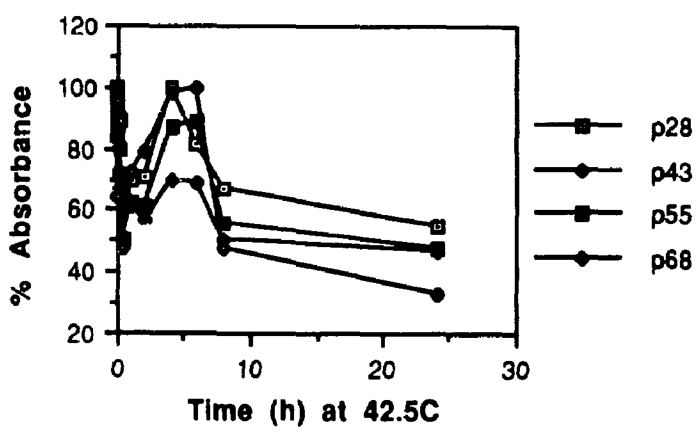
A. Free-cytoplasmic





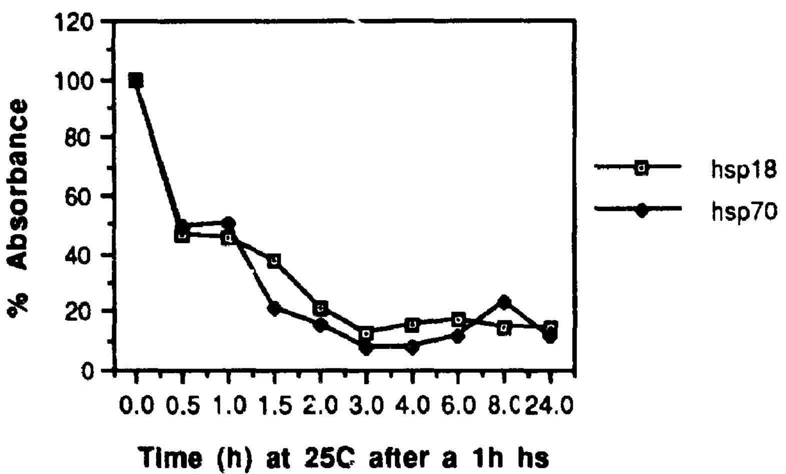
B. Membrane-associated

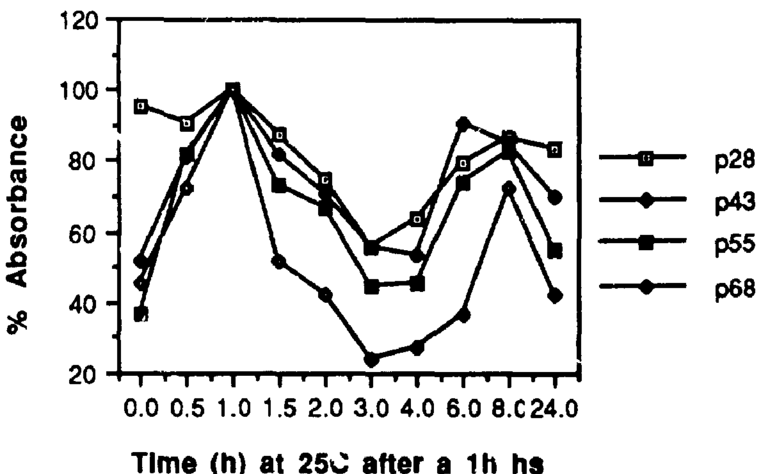




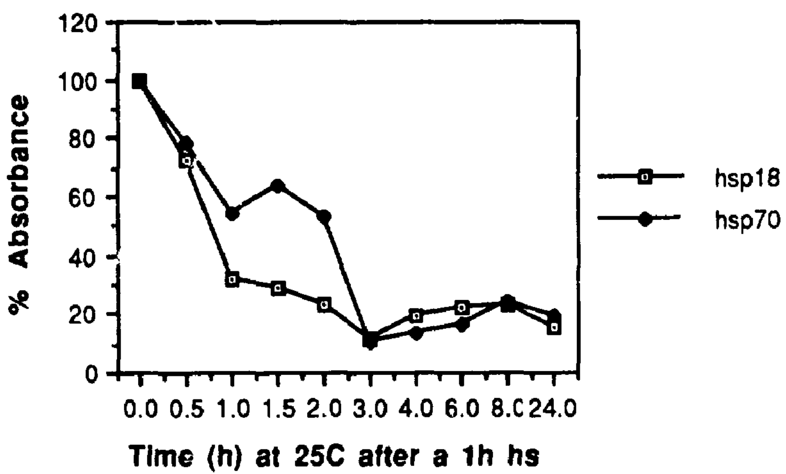
Appendix 6: In vitro synthetic levels of selected control proteins and hsp's during recovery at 25°C from a 1h heat shock at 42.5°C. Fluorograms in section 4.3.3, Figure 26, were quantified by densitometric scans to show relative trends. The relative quantity of protein synthesized in vitro at any given time is plotted as a percentage of the maximum incorporation rate for that protein. (A) Profiles for hsp70, hsp18, and four randomly chosen 25°C proteins synthesized in vitro by free-cytoplasmic polyribosomal poly $(A)^+$ RNA during recovery. (B) Profiles for proteins synthesized in vitro by membrane-associated polyribosomal poly $(A)^+$ RNA with similar M_r's as those scanned in (A).

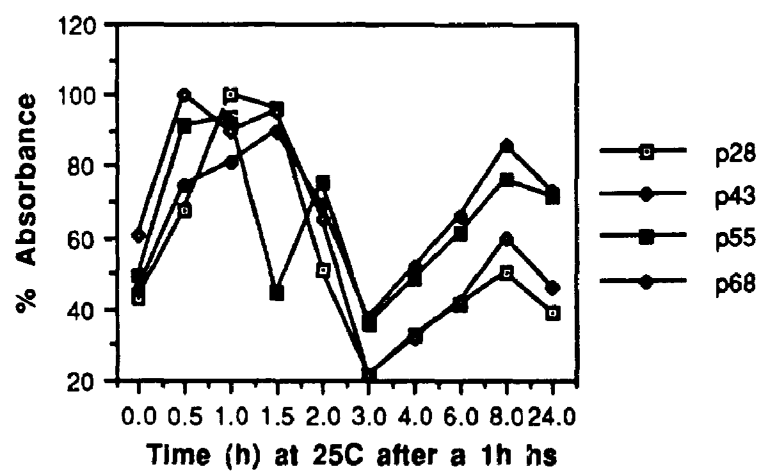
A. Free-cytoplasmic





B. Membrane-associated





Appendix 7: Determination of sedimentation values. Sedimentation values were estimated using the procedure of McEwen (1967).

1. Estimate z_o for the rotor and gradient using the following formula:

$$z_o = \frac{z_1 r_2 - z_2 r_1}{r_2 - r_1}$$

where: z_1=minimum % w/w of sucrose gradient (ie. 10%)

z_2=maximum % w/w of sucrose gradient (ie. 25%)

r_1=minimum radial distance from the centrifugal axis in centimeters (cm)(ie. the gradient meniscus for the SW28 is 7.53 cm)

r_2=maximal radial distance from the centrifugal axis in cm (ie. the bottom of an SW28 tube is 16.1 cm)

z_o=solute concentration corresponding to extrapolation of a linear distribution to zero radius (ie. for the SW28, z_o=-3.2 which is rounded off to -5)

2. Determine the starting and ending sucrose concentrations (determine by interpolation from the density plot).
3. Determine the time integral (I) values for sucrose at the meniscus and at the separated zone for the particle from the appropriate tables for gradient centrifugation (see McEwen, 1967).

   I (10%)=1.9427

   I (25%)=2.2361

   I (25%)-I (10%)=0.2934=$s_{20,w}w^2t$

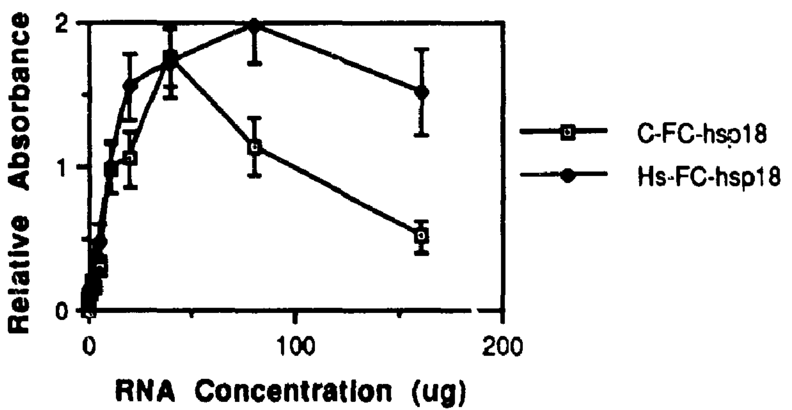
4. Determine w_2t (can read from the ultracentrifuge or compute). In this case, $w_2t=2.02\times10^{11}$.
5. Determine $s_{20,w}$:

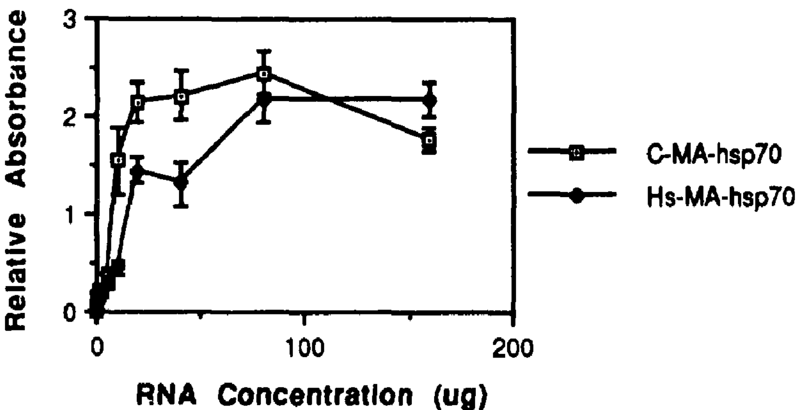
$$s_{20,w} = \frac{I}{w^2t} = 14.5\times10^{-13} = 14.5S$$

This value closely approximates the actual value of 16S.

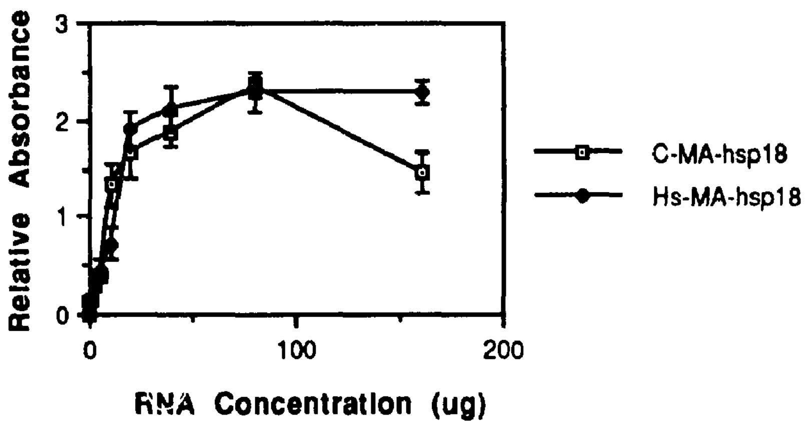
Appendix 8: Relationship between relative absorbance and increasing concentrations of non-polyribosomal RNP RNA. Values represent mean relative absorbances for hsp70 and hsp18 under control and heat shock conditions as calculated from three independent determinations.

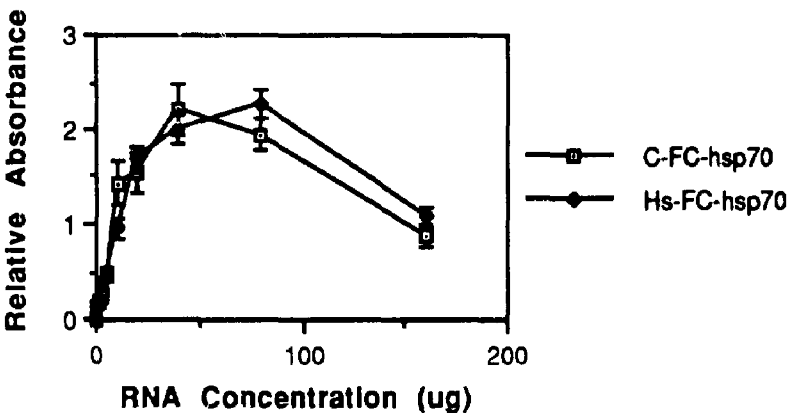
A. Free-Cytoplasmic





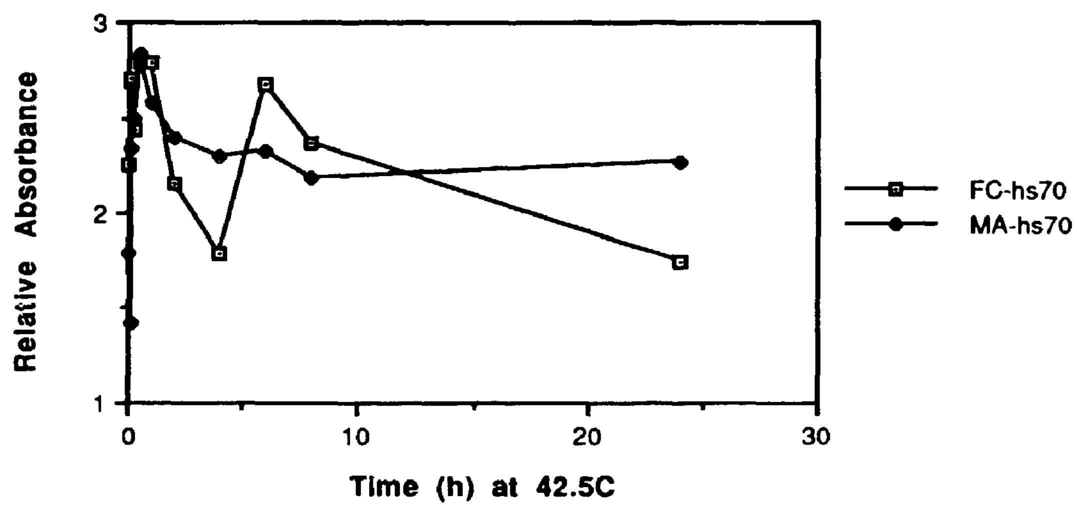
B. Membrane-Associated



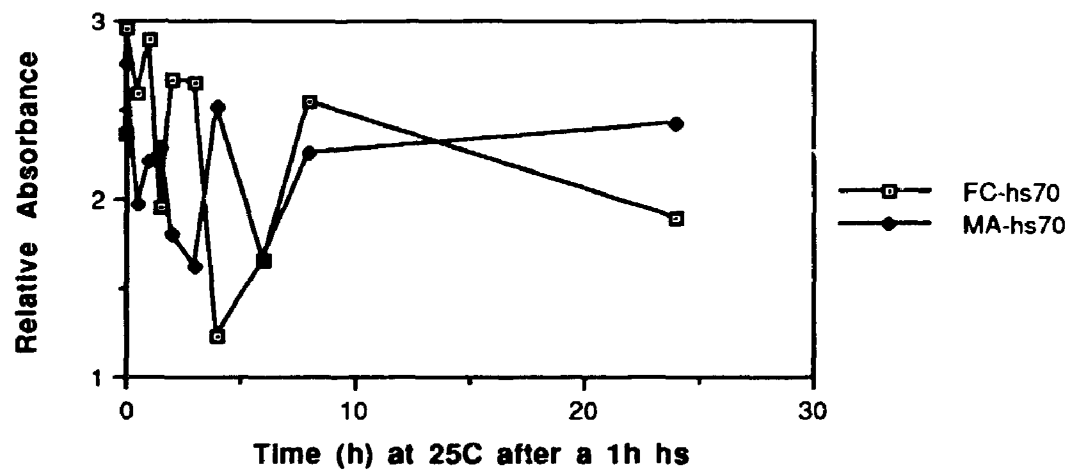


Appendix 9: A comparison of the relative levels of 70kDa mRNA associated with the free-cytoplasmic and membrane-derived non-polyribosomal RNPs during prolonged heat shock (A) or recovery from a brief heat shock (B). Autoradiograms of RNA dot blots probed with a hsp70 gene fragment (section 5.3.3, Figure 33) were scanned with a laser densitometer and the relative RNA levels (expressed as relative absorbance) plotted as a function of time (h) at a given temperature. Values represent mean relative absorbances from two determinations.

A.

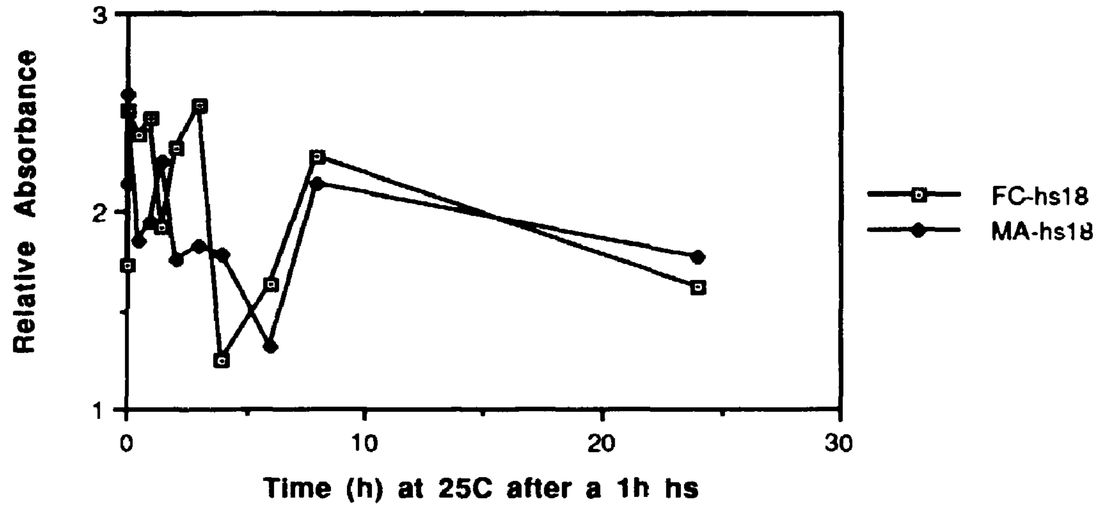


B.

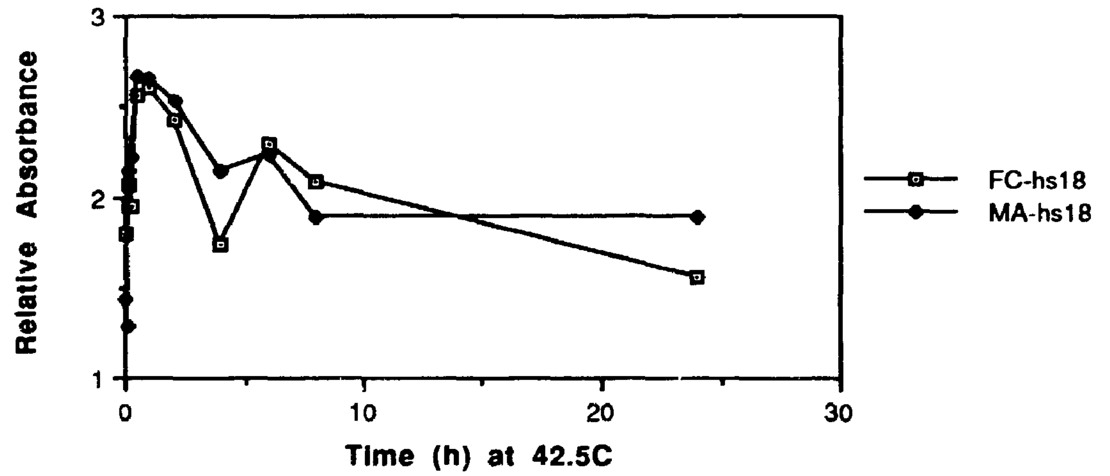


Appendix 10: A comparison of the relative levels of 18kDa mRNA associated with the free-cytoplasmic and membrane-derived non-polyribosomal RNPs during prolonged heat shock (A) or recovery from a 1h heat shock at 42.5°C (B). Autoradiograms similar to those in section 5.3.3, Figure 35, were scanned using a laser densitometer and the relative mRNA levels plotted as a function of time at a given temperature. Values represent mean relative absorbances from two determinations.

B.



A.



REFERENCES

Adams, S. A., Choi, Y. D., Dreyfuss, G. (1986). The interaction of mRNA with proteins in VSV infected cells. J. Virol. 57: 614-622.

Akhayat, O., Infante, A. A., Infante, D., Martins de Sa, C., Grossi de Sa, M. -F., and Scherrer, K. (1987a). A new type of prosome-like particle, composed of small cytoplasmic RNA and multimers of 21-kDa, inhibits protein synthesis in vitro. Eur. J. Biochem. 170: 23-33.

Akhayat, O., Grossi de Sa, F., and Infante, A. A. (1987b). Sea urchin prosome: Characterization and changes during development. Proc. Natl. Acad. Sci. 84: 1595-1599.

Altschuler, M., and Mascarenhas, J. P. (1985). Transcription and translation of heat shock and normal protiens in seedlings and developing seeds of soybean exposed to a gradual temperature increase. Plant Mol. Biol. 5: 291-297.

Altschuler, M., and Mascarenhas, J. P. (1982). Heat shock proteins and effects of heat shock in plants. Plant Mol. Biol. 1: 103-115.

Alwine, J. C., Kemp, D. J., Parker, B. A., Reiser, J., Renart, J., Stark, G. R., and Wahl, G. M. (1979). Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxy-methyl paper. In: Methods in Enzymology, Vol. 68, Recombinant DNA, ed., Wu, R., Academic Press, Inc., NY.

Amin, J., Ananthan, J., and Voellmy, R. (1988). Key features of heat shock regulatory elements. Mol. Cell. Biol. 8: 3761-3769.

Ananthan, J., Goldberg, A. L., and Voellmy, R. (1986). Abnormal proteins serve as eucaryotic stress signal and trigger the activation of heat shock genes. Science 232: 522-524.

Anderson, J. M., and Key, J. L. (1971). The effects of diethyl pyrocarbonate on the stability and activity of plant polyribosomes. Plant Physiol. 48: 801-805.

Anderson, M. L. M., and Young, B. D. (1986). Quantitative Filter Hydridization. In: Nucleic Acid Hybridization-a practical approach, eds., Hames, B. D., and Higgins, S. J., IRL Press Limited, Oxford, England.

Arrigo, A. -P., Suhan, J. P., and Wesch, Wl Jl (1988a). Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. Mol. Cell. Biol. 8: 5059-5071.

Arrigo, A. -P., Tanaka, K., Goldberg, A. L., and Welch, W., J. (1988b). Identity of the 19S "prosome" particle with the large multifunctional protease complex of mammalian cells (the pro'easome). Nature (London) 331: 192-193.

Arrigo, A. -P., and Pauli, D. (1988). Characterization of hsp27 and three immunologically related polypeptides during Drosophila development. Exptl. Cell Res. 175: 169-183.

Arrigo, A. -P., and Welch, W. J. (1987). Characterization and purification of the small 28,000-dalton mammalian heat shock protein. J. Biol. Chem. 262: 15359-15369.

Arrigo, A. -P. (1987). Cellular localization of hsp23 during Drosophila development and following subsequent heat shock. Dev. Biol. 122: 39-48.

Arrigo, A. -P., Darlix, J. -L:., Khandjian, E. W., Simon, M., and Spahr, P. -F. (1985). Characterization of the prosome from Drosophila and its similarity to the cytoplasmic structures formed by the low molecular weight heat-shock proteins. EMBO J. 4: 399-406.

Arrigo, A. -P., and Ahmad-Zadeh, C. (1981). Immunofluorescence localization of a small heat shock protein (hsp23) in salivary gland cells of Drosophila melanogaster. Mol. Gen. Genet. 184: 73-79.

Arrigo, A. -P., Fakan, S., and Tissieres, A. (1980). Localization of the heat shock induced proteins in Drosophila melanogaster tissue culture cells. Dev. Biol. 78: 86-103.

Ashburner, M., and Bonner, J. J. (1979). The induction of gene activity in Drosophila by heat shock. Cell 17: 241-254.

Ashburner, M. (1982). The effects of heat shock and other stresses on gene activity: an introduction. In: Heat Shock from Bacteria to Man, eds., Schlesinger, M. J., Ashburner, M., and Tissieres, A., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Atkinson, B. G., Ling, L., Goping, I. S., and Walden, D. B. (1989). Expression of the genes encoding hsp73, hsp18, and ubiquitin in radicles of heat-shocked maize seedlings. Genome (in press).

Atkinson, B. G., and Walden, D. B. (1985). Changes in eukaryotic gene expression in response to environmental stress. Academic Press, Inc., New York.

Atkinson, B. G. (1981). Synthesis of heat shock proteins by cells undergoing myogenesis. J. Cell. Biol. 89: 666-673.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987). Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, John Wiley and Sons, NY.

Ayme, A., Southgate, R., and Tissieres, A. (1985). Nucleotide sequences responsible for the thermal inducibility of the Drosophila small heat-shock proteins in monkey COS cells. J. Mol. Biol. 182: 469-475.

Bag, J., and Pramanik, S. (1986). Attachment of mRNA to the cytoskeletal framework and translational control of gene expression in rat L6 muscle cells. Biochem. Cell Biol. 65: 565-575.

Bag, J. (1984). Cytoplasmic mRNA-protein complexes of chicken muscle cells and their role in protein synthesis. Eur. J. Biochem. 141: 247-254.

Bag, J., and Sarkar, S. (1975). Studies on a non-polyribosomal messenger ribonucleoprotein containing actin messenger RNA in chicken embryonic muscles. Biochemistry 14: 3800-3807.

Ballinger, D. G., and Pardue, M. L. (1985). Mechanism of translational control in heat-shocked Drosophila cells. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson, B. G., and Walden, D. B., Academic Press Inc., New York.

Ballinger, D. G., and Pardue, M. L. (1983). The control of protein synthesis during heat shock in Drosophila cells involves altered polypeptide elongation rates. Cell 33: 103-114.

Ballinger, D. G., and Pardue, M. L. (1982). The subcellular localization of messenger RNAs in heat shocked Drosophila cells. In: Heat Shock from Bacteria to Man, eds., Schlesinger, M. J., Ashburner, M., and Tissieres, A., Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Banerji, S. S., Berg, L., and Morimoto, R. I. (1986). Transcription and post-transcription regulation of avian hsp70 gene expression. J. Biol. Chem. 261: 15740-15745.

Banerji, S., Theodorakis, N. C., and Morimoto, R. I. (1984). Heat shock-induced translational control of hsp70 and globin synthesis in chicken reticulocytes. Mol. Cell. Biol 4: 2437-2448.

Bardwell, J. C. A., and Craig, E. A. (1987). Eukaryotic Mr 83,000 heat shock protein has a homologue in Escherichia coli. Proc. Natl. Acad. Sci. USA 84: 5177-5181.

Barwell, J. C. A., and Craig, E. A. (1984). Major heat shock gene of Drosophila and the Escherichia coli heat inducible dnaK gene are homologous. Proc. Natl. Acad. Sci. USA 81: 848-852.

Barnett, T., Altschuler, M., McDaniel, C. N., and Mascarenhas, J. P. (1980). Heat shock induced proteins in plant cells. Dev. Gen. 1: 331-340.

Baszczynski, C. L. (1989) Gene expression in Brassica tissues and species following heat shock. (in press).

Baszczynski, C. L. (1986) Immunochemical analysis of heat-shock protein synthesis in maize (Zea mays L.). Can. J. Gen. Cytol. 28: 1076-1087.

Baszczynski, C. L., Walden, D. B., and Atkinson, B. G. (1985). Maize genome response to thermal shifts. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson, B. G., and Walden, D. B., Academic Press Inc., New York.

Baszczynski, C. L. (1984). Thermal shift induced gene expression and regulation in maize (Zea mays L.). Ph. D. Thesis, University of Western Ontario, London, Ontario.

Baszczynski, C. L., Walden, D. B., and Atkinson, B. G. (1983) Regulation of gene expression in corn (Zea mays L.) by heat shock. II. In vitro analysis of RNAs from heat-shocked seedlings. Can. J. Biochem. 61: 393-403.

Baszczynski, C. L., Walden, D. B., and Atkinson, B. G. (1982). Regulation of gene expression in corn (Zea mays L.) by heat shock. Can. J. Biochem. 60: 569-579.

Baulieu, E., -E., and Catelli, M., -G. (1989). Steroid hormone receptors and heat shock protein M_r 90,000 (HSP90): A functional interaction? In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Baulieu, E., -E. (1987). Steroid hormone antagonists at the receptor level. A role for the heat-shock protien MW 90.000 (hsp90). J. Cell. Biochem. 35: 161-174.

Belanger, F. C., Brodl, M. R., and Ho, T. -H. D. (1986). Heat shock causes destabilization of specific mRNAs and destruction of endoplasmic reticulum in barley aleurone cells. Proc. Natl. Acad. Sci. 83: 1354-1358.

Bell, J., Neilson, L., and Pellegrini, M. (1988). Effect of heat shock on ribosome synthesis in Drosophila melanogaster. Mol. Cell. Biol. 8: 91-95.

Berendes, H. J. (1968). Factors involved in the expression of gene activity. Chromosoma 24: 418-437,

Berger, S. L., and Kimmel, A. R. (1987). Methods in Enzymology: Guide to Molecular Cloning Techniques. Academic Press, Inc., Toronto.

Berry, J., and Bjorkman, O. (1980). Ann. Rev. Plant Physiol. 31: 491-543.

Bienz, M., and Pelham, H. R. B. (1987). Mechanisms of heat-shock gene activation in higher eukaryotes. Adv. Genet. 24: 31-72.

Bienz, M. (1985). Transient and developmental activation of heat-shock genes. Trends Biochem. Sci. 10: 157-161.

Bienz, M., and Pelham, H. R. B. (1982). Expression of a Drosophila heat-shock protein in Xenopus oocytes: conserved and divergent regulatory signals. EMBO J. 1: 1583-1588.

Bienz, M., and Gurdon, J. B. (1982). The heat shock response in Xenopus oocytes is controlled at the transcriptional level. Cell 29: 811-819.

Biessmann, H., Falkner, F. -G., Saumweber, H., and Walter, M. F. (1982). Disruption of the vimentin cytoskeleton may play a role in heat-shock response. In: Heat Shock from Bacteria to Man, eds., Schlesinger, M. J., Ashburner, M., and Tissieres, A., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Bochner, B., Zylicz, M., and Georgopoulos, C. (1986). Escherichia coli DnaK protein possesses 5'-nucleotidase activity that is inhibited by AppppA. J. Bacteriol. 168: 931-935.

Bond, U. (1988). Heat shock but not other stress inducers leads to the disruption of a sub-set of snRNPs and inhibition of in vitro splicing in HeLa cells. EMBO J. 7: 3509-3518.

Bond, U., and Schlesinger, M. J. (1987). Heat-shock proteins and development. Adv. Genet. 24: 1-29.

Bond, U., and Schlesinger, M. J. (1986). The chicken ubiquitin gene contains a heat shock promoter and expresses an unstable mRNA in heat-shocked cells. Mol. Cell. Biol. 6: 4602-4610.

Bond, U., and Schlesinger, M. J. (1985). Ubiquitin is a heat shock protein in chicken embryo fibroblasts. Mol. Cell. Biol. 5: 949-956.

Bonner, J. J. (1985). Mechanism of transcriptional control during heat shock. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson B. G., and Walden, D. B., Academic Press, Inc., New York.

Bonner, J. J., and Pardue, M. L. (1976). The effect of heat shock on RNA synthesis in Drosophila tissues. Cell 8: 43-50.

Bonner, W. M., and Laskey, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83-88.

Brawerman, G., Mendecki, J., and Lee, S. Y. (1972). A procedure for the isolation of mammalian messenger ribonucleic acid. Biochemistry 11: 637-641.

Breen, M. D., Whitehead, E. I., and Kenefick, D. G. (1971). Requirements for extraction of polyribosomes from barley tissue. Plant Physiol. 49: 733-739.

Brugge, J. S., Yuonemoto, W., and Darrow, D. (1983). Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. Mol Cell. Biol. 4: 2697-2704.

Brugge, J. S., Erikson, E., and Erikson, R. L. (1981). The specific interaction of the Rous sarcoma virus transforming protein, $pp60^{src}$, with two cellular proteins. Cell 25: 363-372.

Catelli, M. G., Binart, N., Jung-Testas, I., Renoir, J. M., Baulieu, E. -E., Feramisco, J. R., and Welch, W. J. (1985). The common 90-kd protein component of non-transformed "8S" steroid receptors is a heat-shock protein. EMBO J. 4: 3131-3135.

Cervera, M., Dreyfuss, G., and Penman, S. (1981). Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa cells. Cell 23: 113-120.

Chappell, T. G., Welch, W. J., Schlossman, D. M., Palter, K. B., Schlesinger, M. J., and Rothman, J. E. (1986). Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell 45: 3-13.

Cheng, M. Y., Hartl, F. -U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L., and Horwich, A. L. (1989). Mitochondrial heat-shock protein hsp60 is essential for assembly of proteins imported into yeast mitochondria. Nature (London) 337: 620-625.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5298.

Chirico, W. J., Waters, M. G., and Blobel, G. (1988). 70kDa heat shock related proteins stimulate protein translocation into microsomes. Nature (London) 332: 805-810.

Civelli, O., Vincent, A., Maundrell, K., Buri, J. -F., and Scherrer, K. (1980). The translation repression of globin mRNA in free-cytoplasmic ribonucleoprotein complexes. Eur. J. Biochem. 107: 577-585.

Civelli, O., Vincent, A., Buri, J. -F., and Scherrer, K. (1976). Evidence for a translational inhibitor linked to globin mRNA in untranslated free-cytoplasmic messenger ribonucleoprotein complexes. FEBS Lett. 72: 71-76.

Clemens, M. J. (1986). Purification of eukaryotic messenger RNA. In: Transcription and translation-a practical approach, eds., Hames, B. D., and Higgins, S. J., IRL Press Limited, Oxford, England.

Clewel, D. B., and Helsınki, D. R. (1972). Nature of Col E1 plasmic replication in Escherichia coli in the presence of chloramphenicol. J. Bact. 110: 667-676.

Collier, N. C., Heuser, J., Levy, M. A., and Schlesinger, M. J. (1988). Ultrastructural and biochemical analysis of the stress granule in chicken embryo fibroblasts. J. Cell Biol. 106: 1131-1139.

Collier, N. C., and Schlesinger, M., J. (1986). The dynamic state of heat shock proteins in chicken embryo fibroblasts. J. Cell Biol. 103: 1495-1507.

Comings, D. E., and Tack, L. O. (1972). Similarities in the cytoplasmic proteins of different organs examined by SDS gel electrophoresis. Expt. Cell Res. 75: 73-78.

Cooper, P., and Ho, T. -H. D. (1987). Intracellular localization of heat shock proteins in maize. Plant Physiol. 84: 1197-1203.

Cooper, P., Ho, T. -H. D., and Hauptmann, R. M. (1984). Tissue specificity of the heat-shock response in maize. Plant Physiol. 75: 431-441.

Cooper, P., and Ho, T. -H. D. (1983). Heat shock proteins in maize. Plant Physiol 71: 215-222.

Corces, V., Holmgren, R., Freund, R., Morimoto, R., and Meselson, M. (1980). Four heat shock proteins of Drosophila melanogaster coded within a 12-kilobase region in chromosome subdivision 67B. Proc. Natl. Acad. Sci. USA 77: 5390-5393.

Craig, E. A., Kramer, J., Shilling, J., Werner-Washburne, M., Holmes, S., Kosic-Smithers, J., and Nicolet, C. M. (1989). SSC1, an essential member of the yeast hsp70 multigene family encodes a mitochondrial protein. Mol. Cell. Biol. 9: 3000-3008.

Craig, E. A., Kramer, J., and Kosic-Smithers, J. (1987). SSC1, a member of the 70kDa hsp multigene family of Saccharomyces cerevisiae is essential for growth. Proc. Natl. Acad. Sci. USA 84: 4156-4160.

Craig, E. A. (1985). The heat shock response. CRC Critical Review of Biochemistry 18: 239-280.

Craig, E. A., and Jacobsen, K. (1985). Mutations in cognate genes of Saccharomyces cerevisiae hsp70 result in reduced growth rate at low temperatures. Mol. Cell. Biol. 5: 3517-3524.

Czarnecka, E., Nagao, R. T., Key, J. L., and Gurley, W. B. (1988). Characterization of Gmhsp26-A, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol. Cell. Biol. 8: 1113-1122.

Czarnecka, E., Gurley, W. B., Nagao, R. T., Mosquera, L. A., and Key, J. L. (1985). DNA sequence and transcript mapping of a soybean gene encoding a small heat shock protein. Proc. Natl. Acad. Sci. USA 82: 3726-3730.

Czarnecka, E., Edelman, L., Schoffl, F., and Key, J. L. (1984). Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. Plant Mol. Biol. 3: 45-58.

Davies, L. G., Dibner, M. D., and Battey, J. F. (1986). Preparation and analysis of RNA from eukaryotic cells. In: Basic Methods in Molecular Biology. Elsevier, NY.

Davies, M. E., and Exworth, C. P. (1973) Transient inhibition by cycloheximide of protein synthesis in cultured plant cell suspensions: A dose response paradox. Biochem. Biophys. Res. Commun. 50: 1075-1080.

Davies, E., Larkins, B. A., and Knight, R. H. (1972). Polyribosomes from peas-An improved method for their isolation in the absence of ribonuclease inhibitors. Plant Physiol. 50: 581-584.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A., and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) 332: 800-805.

DiDomenico, B. J., Bugaisky, G. E., and Lindquist, S. (1982a). Heat shock and recovery are mediated by differential translation mechanisms. Proc. Natl. Acad. Sci. 79: 6181-6185.

DiDomenico, B. J., Bugaisky, G. E., and Lindquist, S. (1982b). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell 31: 593-603.

Dietz, T. J., Cartwright, I. L., Gilmour, D. S., Siegfried, E., Thomas, G. H., and Elgin, S. C. R. (1989). The chromatin structure of hsp26. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Dreyfuss, G. (1986). Structure and function of nuclear and cytoplasmic ribonucleoprotein particles. Ann. Rev. Cell Biol. 2: 459-498.

Dreyfuss, G., Adam, S. A., and Choi, Y. D. (1984). Physical change in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription. Mol. Cell. Biol. 4: 415-423.

Duband, J. L., Lettre, F., Arrigo, A. -P., and Tanguay, R. M. (1987). Expression and localization of hsp-23 in unstressed and heat-shocked Drosophila culture cells. Can. J.. Genet. Cytol. 28: 1088-1092.

Duck, N., McCormick, S., and Winter, J. (1989). Heat shock protein hsp70 cognate gene expression in vegetative and reproductive organs of Lycopersicon esculentum. Proc. Natl. Acad. Sci. USA 86: 3674-3678.

Duncan, R., and Hershey, J. W. B. (1984). Heat shock-induced translational alterations in HeLa cells. J. Biol. Chem. 259: 11882-11889.

Edelman, L., Czarnecka, E., and Key, J. L. (1987). Induction and accumulation of heat shock-specific poly(A)$^{+}$ RNAs and proteins in soybean seedlings during arsenite and cadmium treatments. Plant Physiol. 86: 1048-1056.

Ellgaard, E. G., and Clever, U. (1971). RNA metabolism during puff induction in Drosophila melanogaster. Chromosoma 36: 60-78.

Ellis, R. J., and MacDonald, I. R. (1970). Specificity of cycloheximide in higher plant systems. Plant Physiol. 46: 227-232.

Ellis, R. J. (1969). Chloroplast ribosomes: Stereospecificity of inhibition by chloramphenicol. Science 163: 477-478.

Ellis, R. J., and MacDonald, I. R. (1967). Activation of protein synthesis by microsomes from aging beet disks. Plant Physiol. 42: 1297-1302.

Falkenburg, P. E., Haass, C., Kloetzel, P. M., Niedel, B., Kopp, F., Kuehn, C., and Dahlmann, B. (1988). Drosophila small cytoplasmic 19S ribonucleoprotein is homologous to the rat multicatalytic proteinase. Nature (London) 331: 190-192.

Falkner, F. G., Saumweber, H., and Biessmann, H. (1981). Two Drosophila melanogaster proteins related to intermediate filament proteins of vertebrate cells. J. Cell. Biol. 91: 175-183.

Ferrer, A., Delseny, M., and Guitton, Y. (1979). Isolation and characterisation of subribosomal ribonucleoprotein particles from radish seeds and seedlings. Plant Sci. Lett. 14: 31-42.

Fey, E. G., Ornelles, D. A., and Penman, S. (1986). Association of RNA with the cytoskeleton and the nuclear matrix. J. Cell Sci. Suppl. 5: 99-119.

Finley, D., Ozkaynak, E., and Varshavsky, A. (1987). The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. Cell 48: 1035-1046.

Findly, and Pederson, T. (1981). Regulated transcription of the genes for actin and heat-shock proteins in cultured Drosophila cells. J. Cell Biol. 88: 323-328.

Frankel, J. (1970) An analysis of the recovery of Tetrahymena from effects of cycloheximide. J. Cell Physiol. 76: 55-64.

Gander, E. S., Stewart, A. G., Morel, C. M., and Scherrer, K. (1973). Isolation and characterization of ribosome-free cytoplasmic messenger-ribonucleoprotein complexes from avian erythroblasts. Eur. J. Biochem. 38: 443-452.

Gander, E. S., Luppis, B., Stewart, A., and Scherrer, K. (1972). Dissociation and reassociation of globin-synthesizing polyribosomes from immature avian red cells. Eur. J. Biochem. 29: 369-376.

Geoghegan,T., Cereghini, S., and Brawerman, G. (1979). Inactive mRNA-protein complexes from mouse sarcoma-180 ascites cells. Proc. Natl. Acad. Sci. USA 76: 5587-5591.

Glisin, V., Crkvenjakov, R., and Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13: 2633-2637.

Glover, C. B. C. (1982). Heat shock induces rapid dephosphorylation of a ribosomal protein in Drosophila. Proc. Natl. Acad. Sci. USA 79: 1781-1782.

Greenberg, J. R., and Carroll, E. III. (1985). Reconstitution of functional mRNA-protein complexes in a rabbit reticulocyte cell-free translation system. Mol. Cell. Biol. 5: 342-351.

Greenberg, J. R. (1981). The polyribosomal mRNA-protein complex is a dynamic structure. Proc. Natl. Acad. Sci. USA 78: 2923-2926.

Greenberg, J. R. (1980). Proteins crosslinked to messenger RNA by irradiating polyribosomes with UV light. Nucl. Acids Res. 8: 5685-5701.

Griffo, J. A., Tahara, S. M., Leas, J. P., Morgan, M. A., Shatkin, A. J. (1982). Characterization of eukaryotic initiation factor 4A, a protein involved in ATP-dependent binding of globin mRNA. J. Biol. Chem. 257: 5246-5253.

Grossi de Sa, M. -F., Martins de Sa, C., Harper, F., Coux, O., Akhayat, O., Pal, J. K., Florentin, Y., and Scherrer, K. (1988). Cytolocalization of prosomes as a function of differentiation. J. Cell Sci. 89: 151-165.

Gupta, R. S., and Siminovitch, L. (1978). The isolation and preliminary characterization of somatic cell mutants resistant to the protein synthesis inhibitor-emetine. Cell 9: 213-219.

Gurley, W. B., Czarnecka, E., Nagao, R. T., and Key, J. L. (1986). Upstream sequences required for efficient expression of a soybean heat shock gene. Mol. Cell. Biol. 6: 559-565.

Haas, I. G. and Wabl, M. (1983). Immunoglobulin heavy chain binding protein. Nature (London) 306: 387-389.

Haass, Ch., Falkenburg, P. E., and Kloetzel, P. -M. (1989). The molecular organization of the small heat shock proteins in Drosophila. In: Stress-Induced Proteins, M. L. Pardue, J. R. Feramisco, and S. Lindquist, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Hall, B. G. (1983) Yeast thermotolerance does not require protein synthesis. J. Bacteriol. 156: 1363-1365.

Hallberg, R. L., and Hallberg, E. M. (1989). Heat shock in Tetrahymena induces the accumulation of a small RNA homologous to eukaryotic 7SL RNA and E. coli 4.5S RNA. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Hallberg, R. L., Kraus, K. W., and Hallberg, E. M. (1985) Induction of acquired thermotolerance in tetrahymena thermophila: Effects of protein synthesis inhibitors. Mol. Cell. Biol. 5: 2061-2069.

Hallberg, R. L., Kraus, K. W., and Findly, R. C. (1984) Starved tetrahymena thermophila cells that are unable to mount an effective heat shock response selectively degrade their rRNA. Mol. Cell. Biol. 4: 2170-2179.

Hallberg, R. L., and Hallberg, E. M. (1983) Characterization of a cycloheximide-resistant tetrahymena thermophila mutant which also displays alter growth properties. Mol. Cell. Biol. 3: 503-510.

Hallberg, R. L., and Wilson, P. G. (1981) Regulation of ribosome phosphorylation and antibiotic sensitivity in tetrahymena thermophila: A correlation. Cell 26: 47-56.

Hallberg, R. L., and Bruns, P. J. (1976) Ribosome biosynthesis is tetrahymena pyriformis. J. Cell Biol. 71: 383-394.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166: 557-580.

Hanson, J. B., and Hodges, T. K. (1963). Uncoupling action of chloramphenicol as a basis for the inhibition of ion accumulation. Nature (London) 200: 1009.

Hanson, J. B., and Krueger, W. A. (1966). Impairment of oxidative phosphorylation by D-threo- and L-threo-chloramphenicol. Nature (London) 211: 1322.

Heikkila, J. J., Cosgrove, J. W., and Brown, I. R. (1981). Cell-free translation of free and membrane-bound polysomes and polyadenylated mRNA from rabbit brain following administration of d-lysergic acid diethylamide in vivo. J. Neurochem. 36: 1229-1238.

Hemmingsen, S. M., Woolford, C., van der Vies, S. M., Tilly, K., and Dennis, D. T. (1988). Homologous plant and bacterial proteins chaperone oligeromeric protein assembly. Nature (London) 333: 330-334.

Hickey, E., Brandon, S. E., Potter, R., Stein, G., Stein, J., and Weber, L. A. (1986). Sequence and organization of genes encoding the human 27kDa heat shock protein. Nucleic Acids. Res. 14: 4127-4145.

Hickey, E. D., and Weber, L. A. (1982). Preferential translation of heat-shock mRNAs in HeLa cells. In: Heat Shock from Bacteria to Man, eds., Schlesinger, M. J., Ashburner, M., and Tissieres, A., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hightower, L. E., Guidon, P. T., Whelan, S. A., and White, C. N. (1985). Stress responses in avian and mammalian cells. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson, B. G., and Walden, D. B., Academic Press Inc., New York.

Hightower, L. E. (1980). Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. J. Cell. Physiol. 102: 407-427.

Hiromi, Y., and Hotta, Y. (1985). Actin gene mutations in Drosophila; heat shock activation in the indirect flight muscles. EMBO J. 4: 1681-1687.

Holmgren, R., Corces, V., Morimoto, R., Backman, R., and Meselson, M. (1981). Sequence homologies in the 5' regions of four Drosophila heat shock genes. Proc. Natl. Acad. Sci. USA 78: 3775-3778.

Howe, J. G., and Hershey, J. W. B. (1984). Translation initiation factor and ribosome association with the cytoskeletal framework fraction from HeLa cells. Cell 37: 85-93.

Hultmark, D., Klemenz, R, and Gehring, W. J. (1986). Translational and transcriptional control elements in the untranslated leader of the heat-shock gene hsp22. Cell 44; 429-438.

Hunt, C., and Morimoto, R. I. (1985). Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. Proc. Natl. Acad. Sci. USA 82: 6455-6459.

Imaizumi-Scherrer, M. -T., Maundrell, K., Civelli, O., and Scherrer, K. (1982). Transcriptional and post-transcriptional regulation in duck erythroblasts. Dev. Biol. 93: 126-138.

Ingolia, T. D., and Craig, E. A. (1982). Four small Drosophila heat shock proteins are related to each other and to mammalian a-cytstallin. Proc. Natl. Acad. Sci. USA 79: 2360-2364.

Jachymczyk, W. J., and Cherry, J. H. (1968) Studies on messenger RNA from peanut plants: In vitro polyribosome formation and protein synthesis. Biochim. Biophys. Acta. 157: 368-377.

Jackson, A. O., and Larkins, B. A. (1976). Influence of ionic strength, pH, and chelation of divalent metals on isolation of polyribosomes from tobacco leaves. Plant Physiol. 57: 5-10.

Jain, S. K., and Sarkar, S. (1979). Poly(riboadenylate)-containing messenger ribonucleoprotein particles of chick embryonic muscles. Biochemistry 18: 745-753.

Jakobsen, B. K., and Pelham, H. R. B. (1988). Consitutive binding of heast heat shock factor to DNA in vivo. Mol. Cell. Biol. 8: 5040-5042.

Jones, K. A., Yamamoto, K. R., and Tjian, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell 42: 559-572.

Kassenbrock, C. K., Garcia, P. D., Walter, P. , and Kelley, R. B. (1988). Heavy-chain binding protein recognizes aberrant polypeptides translocated in vitro. Nature (London) 333: 90-93.

Kay, R. J., Russnak, R. H., Jones, D., Mathias, C., and Candido, E. P. M. (1987). Expression of intron-containing C. elegans heat shock genes in mouse cells demonstrates divergence of 3' splice site recognition sequences between nematodes and vertebrates, and an inhibitor effect of heat shock on the mammalian splicing apparatus. Nucleic Acids Res. 15: 3723-3741.

Kelley, P. M., and Schlesinger, M. J. (1982). Antibodies to two major chicken heat shock proteins crossreact with similar proteins in widely divergent species. Mol. Cell. Biol. 2: 267-274.

Kelley, P. M., and Schlesinger, M. J. (1978). The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. Cell 15: 1277-1286.

Key, J. L., Kimpel, J., Vierling, E., Lin, C. -Y., Nagao, R. T., Czarnecka, E., and Schoffl, F. (1985). Physiological and molecular analyses of the heat shock response in plants. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson B. G., and Walden, D. B., Academic Press Inc., New York.

Key, J. L., Czarnecka, E., Lin, C. -Y., Kimpel, J., Mothershed, C., and Schoffl, F. (1983). A comparative analysis of heat shock response in crop plants. In: Current Topics in Plant Biochemistry and Physiology Symposium, eds., Randell, D. D., Blevins, D. G., Larson, R. L., and Rapp, B. J., Univ. Missouri Press, Columbia.

Key, J. L., Lin, C. Y., and Chen, Y. M. (1981). Heat shock proteins of higher plants. Proc. Natl. Acad. Sci. 78: 3526-3530.

Kimpel, J. A., and Key, J. L. (1985). Heat shock in plants. Trends in Biochem. Sci. 10: 353-357.

Kingston, R. E., Schuetz, T. J., and L: rin, Z. (1987). Heat-inducible human factor that binds to a human hsp70 promoter. Mol. Cell. Biol. 7: 1530-1534.

Kingston, R. E., Baldwin, A. S., and Sharp, R. A. (1984). Regulation of heat shock protein 70 gene expression by c-myc. Nature (London) 312: 280-282.

Klemenz, R., Hultmark, D., and Gehring, W. J. (1985). Selective translation of heat shock mRNA in Drosophila melanogaster depends on sequence information in the leader. EMBO J. 4: 2053-2060.

Kloetzel, P. -M., and Bautz, E. K. F. (1983). Heat-shock proteins are associated with hnRNA in *Drosophila melanogaster* tissue culture cells. EMBO J. 2: 705-710.

Kloppstech, K., Meyer, G., Schuster, G., and Ohad, I. (1985) Synthesis, transport and localization of a nuclear coded 22-kd heat shock protein in the chloroplast membranes of peas and *Chlamydomonas reinhardtii*. EMBO J. 4: 1901-1909.

Kraus, K. W., Good, P. J., and Hallberg, R. L. (1987). A heat shock-induced, polymerase III-transcribed RNA selectively associates with polysomal ribosomes in *Tetrahymena thermophila*. Proc. Natl. Acad. Sci. USA 84: 383-387.

Kraus, K. W., Hallberg, E. L., and Hallberg, R. L. (1986). Characterization of a *Tetrahymena thermophila* mutant strain unable to develop normal thermotolerance. Mol. Cell. Biol. 6: 3854-3861.

Kremp, A., Schliephacke, M., Kull, U., and Schmid, H. -P. (1986). Prosomes exist in plant cells too. Exptl. Cell Res. 166: 553-557.

Kruger, C., and Benecke, B. -J. (1981). *In vitro* translation of *Drosophila* heat-shock and non-heat-shock mRNAs in heterologous and homologous cell-free systems. Cell 23: 595-603.

Kurtz, S., Rossi, J., Petko, L., and Lindquist, S. (1986). An ancient developmental induction in *Saccharomyces* sporulation and *Drosophila* oogenesis. Science 231: 1154-1157.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 6[illegible] 585.

Larkins, B. A., and Davies, E. (1975). Polyribosomes from peas- V. An attempt to characterize the total free and membrane-bound polysomal population. Plant Physiol. 55: 749-756.

Laskey, R. A., and Mills, A. D. (1975). Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56: 335-341.

Lee, P. C., Bochner, B. R., and Ames, B. N. (1983). AppppA, heat-shock stress, and cell oxidation. Proc. Natl. Acad. Sci. USA 80: 7496-7500.

Leicht, B. G., Biessmann, H., Palter, K. B., and Bonner, J. J. (1986). Small heat shock proteins of Drosophila associate with the cytoskeleton. Proc. Natl. Acad. Aci. USA 83: 90-94.

Lengyel, J. A., and Pardue, M. L. (1975). Analysis of hnRNA made during heat shock in Drosophila melanogaster cultured cells. J. Cell. Biol. 67: 240a.

Lenk, R., and Penman, S. (1979). The cytoskeletal framework and poliovirus metabolism. Cell 16: 289-301.

Lerner, M. P., Wettstein, F. O., Herschman, H. R., Stevens, J. G., and Fridlender, B. R. (1971). Distribution of polysomes in mouse brain tissue. J. Neurochem. 18: 1495-1507.

Lin, C. -Y., Roberts, J. K., and Key, J. L. (1984). Acquisition of thermotolerance in soybean seedlings: Synthesis and accumulation of heat shock proteins and their cellular localization. Plant Physiol. 74: 152-160.

Lindquist, S., and Craig, E. A. (1988). The heat-shock proteins. Annu. Rev. Genet. 22: 631-677.

Lindquist, S. (1986). The heat-shock response. Ann. Rev. Biochem. 55: 1151-1191.

Lindquist, S., and DiDomenico, B. (1985). Coordinate and noncoordinate gene expression during heat shock: A model for regulation. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson B. G., and Walden, D. B., Academic Press Inc., New York.

Lindquist, S., DiDomenico, B. J., Bugaisky, G., Kurtz, S., Petko., L., and Sonoda, S. (1982). Regulation of the heat-shock response in Drosophila and yeast. In: Heat Shock from Bacteria to Man, eds., Schlesinger, M. J., Ashburner, M., and Tissieres, A., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Lindquist, S. (1981). Regulation of protein synthesis during heat shock. Nature (London) 294: 311-314.

Lindquist, S. (1980a). Translational efficiency of heat-induced messages in Drosophila melanogaster cells. J. Mol. Biol. 137: 151-158.

Lindquist, S. (1980b). Varying patterns of protein synthesis in Drosophila during heat shock: Implications for regulation. Dev. Biol. 77: 463-479.

Lis, J. T., Xiao, H., and Perisic, O. (1989). A structural unit of heat shock regulatory regions. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

MacDonald, R. J., Swift, G. H., Przybyla, A. E., and Chirgwin, J. M. (1987). Isolation of RNA using guanidinium salts. In: Methods in Enzymology 152: 219-227.

MacDonald, I. R., and Ellis, R. J. (1969) Does cycloheximide inhibit protein synthesis specifically in plant tissues? Nature (London) 222: 791-792.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular cloning-a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mans, R. J., and Novelli, D. G. (1960). A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. Biochem. Biophys. Res. Commun. 3: 540-548.

Mansfield, M. A., and Key, J. L. (1987) Synthesis of the low molecular weight heat shock proteins in plants. Plant Physiol. 84: 1007-1017.

Marcus, A., and Feeley, J. (1966). Ribosome activation and polysome formation in vitro: requirement for ATP. Proc. Natl. Acad. Sci. USA 56: 1770-1777.

Martins de Sa, C., Rollet, E., Grossi de Sa, M. -F., Tanguay, R. M., Best-Belpomme, M., and Scherrer, K. (1989). Prosomes and heat shock complexes in Drosophila melanogaster cells. Mol. Cell. Biol. 9: 2672-2681.

Martins de Sa, C., Grossi de Da, M. -F, Akhayat, O., Broders, F., Scherrer, K., Horsch, A., and Schmid, H. -P. (1986). Prosomes: Ubiquity and inter-species structural variation. J. Mol. Biol. 187: 479-493.

Mascarenhas, J. P., and Altschuler, M. (1985). Responses to environmental heat stress in the plant embryo. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson B. G., and Walden, D. B., Academic Press Inc., New York.

Mayrand, S., and Pederson, T. (1983). Heat shock alters nuclear ribonucleoprotein assembly in Drosophila cells. Mol. and Cell. Biol. 3: 161-171.

Mazzarella, R. A., and Green, M. (1987). ERp99, and abundant, conserved glycoprotein of the endoplasmic reticulum, is homologous to the 90-kDa heat shock protein (hsp90) and the 94-kDa glucose regulated protein (GRP94). J. Biol. Chem. 262: 8875-8883.

McEwen, C. R. (1967). Tables for estimating sedimentation through linear concentration gradients of sucrose solutions. Anal. Biochem. 20: 114-149.

McGarry, T. J., and Lindquist, S. (1985). The preferential translation of Drosophila hsp70 mRNa requires sequences in the untranslated leader. Cell 42: 903-911.

McKenzie, S. L., Henikoff, S., and Meselson, M. (1975). Localization of RNA from heat-induced polysomes at puff sites in Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 72: 1117-1121.

McMaster, G. K., and Carmichael, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA 74: 4835-4838.

McMullin, T. W., and Hallberg, R. L. (1988) A highly evolutionarily conserved mitochondrial protein is structurally related to the protein encoded by the Escherichia coli groEL gene. Mol. Cell. Biol. 8: 371-380.

McMullin, T. W., and Hallberg, R. L. (1987) A normal mitochondrial protein is selectively synthesized and accumulated during heat shock in Tetrahymena thermophila. Mol. Cell. Biol. 7: 4414-4423.

McMullin, T. W., and Hallberg, R. L. (1986) Effect of heat shock on ribosome structure: Appearance of a new ribosome-associated protein. Mol. Cell. Biol. 6: 2527-2535.

Milarski, K. L., and Morimoto, R. I. (1986). Expression of human HSP70 during the synthetic phase of the cell cycle. Proc. Natl. Acad. Sci. USA 83: 9517-9521.

Mirault, M. E., Goldschmidt-Clermont, M., Moran, L., Arrigo, A. P., and Tissieres, A. (1978). The effect of heat shock on gene expression in Drosophila melanogaster. Cold Spring Harbor Symp. Quant. Biol. 42: 819-827.

Moore, M., Schaack, J., Baim, S. B., Morimoto, R. I., Shenk, T. (1987). Induced heat shock mRNAs excape the nucleocytoplasmic transport block in adenovirus-infected HeLa cells. Mol. Cell. Biol. 7: 4505-4512.

Morel, C., Kayibanda, B., and Scherrer, K. (1971). Proteins associated with globin messenger RNA in avian erythroblasts isolation and comparison with the proteins bound to nuclear messenger-like RNA. FEBS Lett. 18: 84-88.

Morgan, W. K., Williams, G. T., Morimoto, R. I., Greene, J., Kingston, R. E., and Tjian, R. (1987). Two transcriptional activators, CCAAT-box-binding transcription factor and heat shock transcription factor, interact with a human hsp70 gene promoter. Mol. Cell. Biol. 7: 1129-1138.

Mosser, K. K., Theodorakis, N. G., and Morimoto, R. I. (1988). Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. Mol. Cell. Biol. 8: 4736-4744.

Munro, S., and Pelham, H. R. (1986). An hsp70-like protein in the ER: Identity with the 78kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46: 291-300.

Munro, S, and Pelham. H. R. B. (1985). What turns on heat shock genes? Nature (London) 317: 477-478.

Nagao, R. T., Kimpel, J. A., Vierling, E., and Key, J. L. (1986). The heat shock response: a comparative analysis. In: Oxford Surveys of Plant Molecular and Cell Biology, ed., B. J. Miflin, Oxford Univ. Press, Oxford.

Nagao, R. T., Czarnecka, E., Gurley, W. B., Schoffl, F., and Key, J. L. (1985). Genes for low-molecular-weight heat shock proteins of soybeans: Sequence analysis of a multigene family. Mol. Cell. Biol. 5: 3417-3428.

Nebiolo, C. M., and White, E. M. (1985) Corn mitochondrial protein synthesis in response to heat shock. Plant Physiol. 79: 1129-1132.

Neumann, D., zur Nieden, U., Manteuffel, R., Walter, G., Scharf, K. -D., and Nover, L. (1987). Intracellular localization of heat shock proteins in tomato cell cultures. Eur. J. Cell Biol. 43: 71-81.

Neumann, K., Scharf, K. -D., and Nover, L. (1984). Heat shock induced chnages of plant cell ultrastructure and autoradiographic localization of heat shock proteins. Eur. J. Cell Biol. 34: 254-264.

Nevins, J. R. (1987). Isolation and analysis of nuclear RNA. In: Methods in Enzymology 152: 234-241.

Newton, K. J. (1988). Plant mitochondrial genomes: organization, expression and variation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39: 503-532.

Nichols, J. L., and Welder, L. (1983). The proteins in free cytoplasmic poly(A) - ribonucleoprotein complexes from maize. Plant Sci. Lett. 29: 61-66.

Nieto-Sotelo, J., and Ho, T. -H. D. (1987). Absence of heat shock protein synthesis in isolated mitochondria and plastids from maize. J. Biol. Chem. 262: 12288-12292.

Nover, L., Scharf, K. -D., and Neumann, D. (1989). Cytoplasmic heat shock granules are formed from precursor particles and are associated with a specific set of mRNAs. Mol. Cell. Biol. 9: 1298-1308.

Nover, L. (1984). Heat shock response of eukaryotic cells. Springer-Verlag KG, Berlin.

Nover, L., and Scharf, K. -D. (1984) Synthesis, modification and structural binding of heat-shock proteins in tomato cell cultures. Eur. J. Biochem. 139: 303-313.

Nover, L., Scharf, K. -D., and Neumann, D. (1983) Formation of cytoplasmic heat shock granules in tomato cell cultures and leaves. Mol. Cell. Biol. 3: 1648-1655.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

Oishi, K., Sumnicht, T., and Tewari, K. K. (1981). Messenger ribonucleic acid transcripts of pea chloroplast deoxyribonucleic acid. Biochemistry 20: 5710-5717.

Ovchinnikov, L. P., Avanesov, A. T., Seriakova, T. A., Alzhanova, A. T., and Radzhabov, H. M. (1978). A comparison of the RNA-binding proteins with the proteins of polyribosomal messenger ribonucleoproteins in rabbit reticulocytes. Eur. J. Biochem. 90: 527-535.

Palter, K. B., Watanabe, M., Stinson, L., Mahowald, A. P., and Craig, E. A. (1986). Expression and localization of Drosophila melanogaster hsp70 cognate proteins. Mol. Cell. Biol. 6: 1187-1203.

Panniers, R., Stewart, E. B., Merrick, W. C., and Henshaw, E. C. (1985). Mechanism of inhibition of polypeptide chain initiation in heat-shocked Ehrlich cells involves reduction of eukaryotic initiation factor 4F activity. J. Biol. Chem. 260: 9648-9653

Panniers, R., and Henshaw, E. C. (1984). Mechanism of inhibition of polypeptide chain initiation in heat-shocked Ehrlich ascites tumor cells. Eur. J. Biochem. 140: 209-214.

Pardue, M. L., Feramisco, J. R., and Lindquist, S. (1989). Stress-induced proteins, UCLA symposia on molecular and cellular biology, New series, Volume 96, Alan R. Liss, Inc., New York.

Parker, C. S., and Topol, J. (1984). A Drosophila RNA polymerase II transcription factor contains a promoter region specific DNA binding activity. Cell 36: 357-369.

Pauli, D., Arrigo, A. -P., Vazquez, J., Tonka, C. -H., and Tissieres, A. (1988). Expression of small heat shock genes during Drosophila development: comparison of the accummulation of hsp23 and hsp27 mRNAs and polypeptides. Genome (in press).

Pauli, D., Spierer, A., and Tissieres, A. (1986). Several hundred base pairs upstream of Drosophila hsp23 and 26 genes are required for their heat induction in transformed flies. EMBO J. 5: 755-761.

Pearson, J. A. (1969). Isolation and characterisation of polysomes and polysomal RNA from radish leaves. Exptl. Cell Res. 57: 235-239.

Pelham, H. (1988). Heat-shock proteins: Coming in from the Cold. Nature (London) 332: 776-777.

Pelham, H. R. B. (1986). Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell 46: 959-961.

Pelham, H. R. B. (1985). Activation of heat-shock genes in eucaryotes. Trends Genet. 1: 31-35.

Pelham, H. R. B. (1984). Hsp70 accelerates recovery of nucleolar morphology after heat shock. EMBO J. 3: 3095-3100.

Pelham, H. R. B., and Bienz, M. (1982). A synthetic heat-shock promoter element confers heat-inducibility on the herpes simplex virus thymidine kinase gene. EMBO J. 1: 1473-1477.

Pelham, H. R. B. (1982). A regulatory upstream promoter element in the Drosophila hsp70 heat-shock gene. Cell 30: 517-528.

Pelletier, J., and Sonnenberg, N. (1985). Photochemical crosslinking of cap binding proteins to eukaryotic mRNAs: effect of mRNA 5' secondary structure. Mol. Cell. Biol. 11: 3222-3230.

Perlman, J., and Feldman, J. F. (1982) Cycloheximide and heat shock induce new polypeptide synthesis in Neurospora crassa. Mol. Cell. Biol. 2: 1167-1173.

Peterson, R., and Lindquist, S. (1988). The Drosophila hsp70 message is rapidly degraded at normal temperatures and stabilized by heat shock. Gene (in press).

Pratt, G., Deveraux, Q., and Rechsteiner, M. (1989). Ubiquitin metabolism in stressed mammalian cells. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Ramagopal, S., and Hsiao, T. C. (1973). Polyribosomes from maize leaves isolation at high pH and amino acid incorporation. Biochim. et Biophys. Acta. 299: 460-467.

Ramsey, J. C., Steele, W. J. (1977). Quantitative isolation and properties of nearly homogeneous populations of undegraded free and bound polysomes from rat brain. J. Neurochem. 28: 517-527.

Rees, C. A. B., Hogan, N. C., Walden, D. B., and Atkinson, B. G. (1986). Identification of mRNAs encoding low molecular mass heat-shock proteins in maize(Zea mays L.). Can. J. Genet. Cytol. 28: 1106-1114.

Ritossa, F. M. (1964). Behaviour of RNA and DNA synthesis at the puff level in salivary gland chromosomes of Drosophila. Exp. Cell Res. 36: 515-523.

Rochester, D. E., Winter, J. A., and Shah, D. M. (1986). The structure and expression of maize genes encoding the major heat shock protein, hsp70. EMBO J. 5: 451-458.

Rodriguez, R. L., and Tait, R. C. (1983). Recombinant DNA Techniques: An Introduction. Addison-Wesley Publishing Company, Don Mills, Ontario.

Rubin, G. M., and Hogness, D. S. (1975). Effect of heat shock on the synthesis of low molecular weight RNAs in Drosophila: accumulation of a novel form of 5S RNA. Cell 6: 207-213.

Russnak, R. H., and Candido, E. P. M. (1985). Locus encoding a family of small heat-shock genes in Caenorhabditis elegans: Two genes duplicated to form a 3.8-kilobase inverted repeat. Mol. Cell. Biol. 5: 1268-1278.

Sachs, M. M., and Ho, T. -H. D. (1986). Alteration of gene expression during environmental stress in plants. Ann. Rev. Plant Physiol. 37: 363-376.

Sakakibara, Y. (1988). The dnaK gene in Escherichia coli functions in initiation of chromosome replication. J. Bacterio. 170: 972-979.

Saleem, M., and Atkinson, B. G. (1976). Isoelectric points and molecular weights of salt-extractable ribosomal proteins. Can. J. Biochem. 54: 1029-1033.

Sanchez, E. H., Toft, D. O., Schlesinger, M. J., and Pratt, W. B. (1985). Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat-shock protein. J. Biol. Chem. 260: 12398-12401.

Scharf, K. -D., and Nover, L. (1987). Control of ribosome biosynthesis in plant cell cultures under heat shock condition. II. Ribosomal proteins. Biochim. Biophys. Acta 909: 44-57.

Scharf, K. -D., and Nover, L. (1982). Heat shock induced alterations of ribosomal protein phosphorylation in plant cell cultures. Cell 30: 427-437.

Schlesinger, M. J., Collier, N. C., Agell, N., and Bond, U. (1989). Molecular events in avian cells stressed by heat shock and arsenite. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96,

eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Schlesinger, M. J., Kelley, P. M., Aliperti, G., and Malfer, C. (1982). Properties of three major heat-shock proteins and their antibodies. In: Heat Shock from Bacteria to Man, eds., Schlesinger, M. J., Ashburner, M., and Tissieres, A., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schmid, H. -P., Akhayat, O., Martins de Sa, C., Puvion, F., Koehler, K., and Scherrer, K. (1984). The prosome: an ubiquitous morphologically distinct RNP particle associated with repressed mRNPs and containing specific ScRNA and a characteristic set of proteins. EMBO J. 3: 29-34.

Schmid, H. -P., Schonfelder, M., Setyono, B., and Kohler, K. (1983). 76-kDa poly(A)-protein is involved in the formation of 48S initiation complexes. FEBS 157: 105-110.

Schmid, H. -P., Kohler, K., and Setyono, B. (1982). Possible involvement of messenger RNA-associated proteins in protein synthesis. J. Cell Biol. 93: 893-898.

Schoffl, F., Raschke, E., and Nagao, R. T. (1984). The DNA sequence analysis of soybean heat-shock genes and identification of possible regulatory promoter elements. EMBO J. 3: 2491-2497.

Schoffl, F., and Key, J. L. (1983). Identification of a multigene family for small heat shock proteins in soybean and physical characterization of one individual gene coding region. Plant Mol. Biol. 2: 269-278.

Schoffl, F., and Key, J. L. (1982). An analysis of mRNAs for a group of heat shock proteins of soybean using cloned cDNAs. J. Mol. and Appl. Gen. 1: 301-314.

Schonfelder, M., Horsch, A., and Schmid, H. -P. (1985). Heat shock increases the synthesis of the poly (A)-binding protein in HeLa cells. Proc. Natl. Acad. Sci. USA 82: 6884-6888.

Schuster, G., Even, D., Kloppstech, K., and Ohad, I. (1988). Evidence for protection by heat-shock proteins against photoinhibition during heat shock. EMBO J. 7: 1-6.

Scott, M. P., and Pardue, M. L. (1981). Translational control in lysates of Drosophila melanogaster cells. Proc. Natl. Acad. Aci. USA 78: 3353-3357.

Scott, M. P. (1980). Translational control of protein synthesis in Drosophila. Ph.D. thesis, M.I.T., Cambridge, Massachusetts.

Setyono, B., and Greenberg, J. R. (1981). Proteins associated with poly(A) and other regions of mRNA and hnRNA molecules as investigated by crosslinking. Cell 24: 175-183.

Shah, D. M., Rochester, D. E., Krivi, G. G., Hironaka, C. M., Mozer, T. J., Fraley, R. T., and Tiemeier, D. C. (1985). Structure and expression of maize hsp 70 gene. In: Cellular and Molecular Biology of Plant Stress, eds., Key and J. L., and Kosuge, T., Alan R. Liss, Inc., NY.

Shah, D. M., Hightower, R. C., and Meagher, R. B. (1983). Genes encoding actin in higher plants: Intron positions are highly conserved but the coding sequences are not. J. Mol. and Appl. Gen. 2: 111-126.

Shapiro, S. Z., and Young, J. R. (1981). An immunochemical method for mRNA purification. J. Biol. Chem. 256: 1495-1498.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46: 659-667.

Sherwood, A. C., John-Alder, K., and Sanders, M. M. (1989). Anion transport is linked to heat shock induction. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Simcox, A. A., Cheney, C. M., Hoffman, E. P., and Shearn, A. (1985). A deletion of the 3' end of the Drosophila melanogaster hsp70 gene increases stability of mutant mRNA during recovery from heat shock. Mol. Cell. Biol. 5: 3397-3402.

Sinibaldi, R. M., and Turpen, T. (1985) A heat shock protein is encoded within mitochondria of higher plants. J. Biol. Chem. 260: 15382-15385.

Sinibaldi, R. M., and Morris, P. W. (1981). Putative function of Drosophila melanogaster heat shock proteins in the nucleoskeleton. J. Biol. Chem. 256: 10735-10738.

Sonnenberg, N. (1981). ATP/Mg^{++}-dependent crosslinking of cap binding proteins to the 5' end of eukaryotic mRNA. Nucleic Acids Res. 9: 1643-1650.

Sorger, P. K., and Pelham, H. R. B. (1987a). Purification and characterization of a heat-shock element binding protein from yeast. EMBO J. 6: 3035-3041.

Sorger, P. K., Lewis, M. J., and Pelham, H. R. B. (1987). Heat shock factor is regulated differently in yeast and HeLa cells. Nature (London) 329: 81-84.

Sorger, P. K., and Pelham, H. R. B. (1987b). The glucose-regulated protein grp94 is related to heat shock protein hsp90. J. Mol. Biol. 194: 341-344.

Southgate, M. -E., Mirault, A. A., and Tissieres, A. (1985). Organization, sequences, and induction of heat shock genes. In: Changes in Eukaryotic Gene Expression in Response to Environmental Stress, eds., Atkinson, B. G., and Walden,D. B., Academic Press, Inc , New York.

Spirin, A. S. (1969). Informosomes. Eur. J. Biochem. 10: 20-35.

Spohr, G., Kayibanda, B., and Scherrer, K. (1972). Polyribosome-bound and free-cytoplasmic-hemoglobin-messenger RNA in differentiating avian erythroblasts. Eur. J. Biochem. 31: 194-208.

Spohr, G., Granbouland, N., Moul, C., and Scherrer, K. (1970). Messenger RNA in HeLa cells: an investigation of free and polyribosome bound cytoplasmic messenger RNP particles by kinetic labelling and electron microscopy. Eur. J. Biochem. 17: 296-318.

Spradling, A., Pardue, M. L., and Penman, S. (1977). Messenger RNA in heat-shocked Drosophila cells. J. Mol. Biol 109: 559-587.

Stephan, D., and van Huystee, R. B. (1980). Peroxidase biosynthesis as part of protein synthesis by cultured peanut cells. Can. J. Biochem. 58: 715-719.

Storti, R. V., Scott, M. P., Rich, A., and Pardue, M. L. (1980). Translational control of protein synthesis in response to heat shock in D. melanogaster cells. Cell 22: 825-834.

Subjeck, J. R., and Shyy, T. -T. (1986). Stress protein systems of mammalian cells. Am. J. Physiol. 250: C1-C17.

Sutton, C. A., Ares, M., and Hallberg, R. L. (1978) Cycloheximide resistance can be mediated through either ribosomal subunit. Proc. Natl. Acad. Sci. USA 75: 3158-3162.

Tanguay, R. M., and Vincent, M. (1982). Intracellular translocation of cellular and heat shock induced proteins upon heat shock in Drosophila Kc cells. Can. J. Biochem. 60: 306-315.

Theodorakis, N. G., and Morimoto, R. I. (1987). Posttranscriptional regulation of hsp70 expression in human cells: Effects of heat shock, inhibition of protein synthesis, and Adenovirus infection on translation and mRNA stability. Mol. Cell. Biol. 7: 4357-4368.

Thomas, P. S. (1983). Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. In: Methods in Enzymology, ed., Wu, R. 100: 255-266.

Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. 77: 5201-5205.

Thomas, G. P., and Mathews, M. B. (1982). Control of polypeptide chain elongation in the stress response: a novel translation control. In: Heat Shock from Bacteria to Man, eds., Schlesinger, M. J., Ashburner, M., and Tissieres, A., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Tissieres, A., Mitchell, H. K., and Tracy, U. M. (1974). Protein synthesis in salivary gland chromosomes of D. melanogaster. Relation to chromosome puffs. J. Mol. Biol. 84: 389-398.

Topol, J., Ruden, D. M., and Parker, C. S. (1985). Sequences r ,uired for in vitro transcriptional activation of a Drosophila hsp70 gene. Cell 42: 527-537.

van Venrooij, W. J., Sillekens, P. T. G., van Eekelen, C. A., and Reinders, R. J. (1981). On the association of mRNA with the cytoskeleton in uninfected and adenovirus-infected human KB cells. Exptl. Cell Res. 135: 79-91.

Varshavsky, A. (1983). Deadenosine 5', 5'''-P1, P4-tetraphosphate: a pleiotropically acting hormone. Cell 34: 711-712.

Velazquez, J. M., and Lindquist, S. (1984). HSP70: Nuclear concentration during environmental stress; cytoplasmic storage during recovery. Cell 36: 655-662.

Venkatesan, N., and Steele, W. J. (1972). Free and membrane-bound polysomes of rat liver: separation in nearly quantitative yield and analysis of structure and function. Biochim. et Biophys. Acta 287: 526-537.

Vierling, E., Harris, L. M., and Chen, Q. (1989) The major low-molecular-weight heat shock protein in chloroplasts shows antigenic conservation among diverse higher plant species. Mol. Cell. Biol. 9: 461-468.

Vierling, E., Nagao, R. T., DeRocher, A. E., and Harris, L. M. (1988) A heat shock protein localized to chloroplasts is a member of a eukaryotic superfamily of heat shock proteins. EMBO J. 7: 575-581.

Vierling, E., Mishkind, M. L., Schmidt, G. W., and Key, J. L. (1986) Specific heat shock proteins are transported into chloroplasts. Proc. Natl. Acad. Sci. USA 83: 361-365.

Vincent, A., Akhayat, O., Goldenberg, S., and Scherrer, K. (1983). Differential repression of specific mRNA in erythroblast cytoplasm: a possible role for free mRNP proteins. EMBO J. 2: 1869-1983.

Vincent, A., Goldenberg, S., and Scherrer, K. (1981). Comparison of proteins associated with duck-globin mRNA and its polyadenlyated segment in polyribosomal and repressed free messenger ribonucleoprotein complexes. Eur. J. Biochem. 114: 179-193.

Vincent, A., Civelli, O., Maundrell, K., and Scherrer, K. (1980). Identification and characterization of the translationally repressed cytoplasmic globin messenger-ribonucleoprotein particles from duck erythroblasts. Eur. J. Biochem. 112: 617-633.

Walker, P. R., and Whitfield, J. F. (1985). Cytoplasmic microtubules are essential for the formation of membrane-bound polyribosomes. J. Biol. Chem. 260: 765-770.

Walter, M. F., and Biessmann, H. (1987). A non-filamentous configuration of intermediate-sized filament proteins in Drosophila Kc tissue culture cells. Dev. Biol. 23: 453-458.

Waters, M. G., Chirico, W. J., Henrigques, R., and Blobel, G. (1989). Purification of yeast stress proteins based on their ability to facilitate secretory protein translocation. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Watowich, S., and Morimoto, R. I. (1988). Complex regulation of heat shock- and glucose-responsive genes in human cells. Mol. and Cell. Biol. 8: 393-405.

Welch, W. J., Mizzen, L. A., and Arrigo, A. -P. (1989). Structure and function of mammalian stress proteins. In: Stress-Induced Proteins, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 96, eds., Pardue, M. L., Feramisco, J. R., and Lindquist, S., Alan R. Liss, Inc., New York.

Welch, W. J. and Mizzen, L. A. (1988). Characterization of the thermotolerant cell. II. Effects on the intracellular distribution of heat-shock protein 70, intermediate filaments, and small nuclear ribonucleoprotein complexes. J. Cell Biol. 106: 1117-1130.

Welch, W. J., and Suhan, J. P. (1986). Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell Biol. 103: 2035-2052.

Welch, W. J., and Suhan, J. P. (1985). Morphological study of the mammalian stress response: characterization of changes in cyoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. J. Cell Biol. 101: 1198-1211.

Welch, W. J., and Feramisco, J. R. (1985). Disruption of the three cytoskeletal networks in mammalian cells does not affect transcription, translation, or protein translocation changes induced by heat shock. Mol. and Cell. Biol. 5: 1571-1581.

Werner-Washburne, M., Stone, D. E., and Craig, E. A. (1987). Complex interactions among members of an essential subfamily of hsp70 genes in Saccharomyces cerevisiae. Mol. Cell Biol. 7: 2568-2577.

Wiederrecht, G., Shuey, D. J., Kibbe, W. A., and Parker, C. S. (1987). The Saccharomyces and Drosophila heat shock transcription factors are identical in size and DNA binding properties. Cell 48: 507-515.

Wolin, S. L , and Walter, P. (1988). Ribosome pausing and stacking during translation of a eukaryotic mRNA. EMBO J. 7: 3559-3569.

Wu, C. H., Warren, H. L., Sitaraman, K., and Tsai, C. Y. (1988). Translational alterations in maize leaves responding to pathogen infection, paraquat treatment, or heat shock. Plant Physiol. 86: 1323-1329.

Wu, B. J., Williams, G. T., and Morimoto, R. I. (1987). Detection of three protein binding sites in the serum-regulated promoter of the human gene encoding the 70-kDa heat shock protein. Proc. Natl. Acad. Sci. USA 84: 2203-2207.

Wu, B. J., Kingston, R. E., and Morimoto, R. I. (1986a). Human HSP70 promoter contains at least two distinct regulatory domains. Proc. Natl. Acad. Sci. USA 83: 629-633.

Wu, B. J., Hurst, H. C., Jones, N. C., and Morimoto, R. I. (1986b). The ElA 13S product of adenovirus 5 activates transcription of the cellular human hsp70 gene. Mol. Cell. Biol. 6: 2994-2999.

Wu, B., Hunt, C., and Morimoto, R. (1985). Structure and expression of the human gene encoding major heat shock protein HSP70. Mol. Cell. Biol. 5: 330-341.

Wu, C., Wilson, S., Walker, B., Dawid, I., Paisley, T., Zimarino, V., and Ueda, H. (1987). Purification and properties of Drosophila heat shock activator protein. Science 238: 1247-1253.

Wu, C. (1985). An exonuclease protection assay reveals heat-shock element and TATA box DNA-binding proteins in crude nuclear extracts. Nature (London) 317: 84-87.

Wu, C. (1984a). Two protein-binding sites in chromatin implicated in the activation of heat-shock genes. Nature (London) 309: 229-234.

Wu, C. (1984b). Activating protein factor binds in vitro to upstream control sequences in heat-shock genes. Nature (London) 311: 81-84.

Wu, C. (1980). The 5' ends of Drosophila heat-shock genes in chromatin are hypersensitive to DNase 1. Nature (London) 286: 854-860.

Yost, H. J., and Lindquist, S. (1988). Translation of Unspiced Transcripts after Heat Shock. Science (in press).

Yost, H. J., and Lindquist, S. (1986). RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. Cell 45: 185-193.

Zimarino, V., and Wu, C. (1987). Induction of sequence-specific binding of Drosophila heat shock activator protein without protein synthesis. Nature (London) 327: 727-730.

Zimmerman, J. L., Petri, W. L., and Meselson, M. (1983). Accumulation of specific subsets of D. melanogaster heat shock mRNAs in normal development without heat shock. Cell 32: 1161-1170.

Zylicz, M., LeBowitz, J. H., McMacken, R., and Georgopoulos, C. P. (1983). The dnaK protein of Escherichia coli possesses an ATPase and autophosphorylating activity and is essential to an in vitro DNA replication system. Proc. Natl. Acad. Sci. USA 80: 6431-6435.