THE ROLE OF CYTOSTRUCTURE IN THE CONTROL OF PROTEIN SYNTHESIS AND THE PROMPT HEAT SHOCK RESPONSE

by

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ABSTRACT

Cytochalasin D was shown to be a reversible inhibitor of protein synthesis in HeLa cells. The inhibition was detected at a concentration typically used to perturb cell structure and increased in a dose-dependent manner. The drug released messenger RNA from the cytoskeletal framework in direct proportion to the inhibition of protein synthesis. The released messenger RNA was unaltered in its translatability as measured in vitro but was no longer translated in the cytochalasin-treated cells. The residual protein synthesis occurred on polyribosomes that were reduced in amount but displayed a normal sedimentation distribution. The cytoskeletal framework was not altered in composition or amount of protein by cytochalasin D. Electron microscopy using resinless sections revealed the profound reorganization effected by the drug but did not indicate substantial disaggregation of the cytoskeletal elements.

Elevated temperatures induced the synthesis of a number of new proteins in Drosophila melanogaster cells. These included the conventional heat shock (HS) proteins and a set of proteins resembling the prompt HS proteins of mammalian cells. The synthesis of the prompt HS proteins was insensitive to actinomycin D and apparently directed by pre-existing but normally untranslated messenger RNA. The prompt HS proteins were rapidly induced by exposure of cells to 36.5°C but, unlike the conventional HS proteins, only two of fourteen could be detected at 33°C. The prompt HS proteins were found exclusively in the nuclear matrix-intermediate filament scaffold (NM-IF) and not in any other subcellular fraction. Synthesis of the prompt HS proteins was compared to the synthesis of the heat- and actinomycin D-insensitive HPS-1 viral proteins. Although both prompt HS and viral protein synthesis proceeded at high temperatures and was unaffected by actinomycin D, virus-directed protein synthesis was independent of temperature. The prompt HS proteins were not be detected in the NM-IF after exposure to alternative inducers of the conventional HS proteins.

(continued)

ABSTRACT (continued)

Approximately fifteen prompt HS proteins of HeLa cells were identified. In contrast to the prompt HS proteins of Drosophila cells, a variable subset of the HeLa prompt HS proteins were rapidly induced by alternative inducers (arsenite, cadmium, zinc) of the stress response as well as agents not known to induce HS proteins in HeLa cells (ethanol, dinitrophenol). Synthesis of the chemically-induced prompt HS proteins was unaffected by the inhibition of transcription. The synthesis of specific prompt HS proteins appeared to stop at different times during prolonged exposure (16 hours) to cadmium or arsenite.

The NM-IF of both control and heat shocked cells displayed well-defined morphologies when examined by transmission electron microscopy of resinless sections. The nuclear matrix consisted of networks of fibers of varying sized bounded by the nuclear lamina. After heat shock, the NM-IF of Drosophila cells displayed some empty regions in the inter of the nuclear matrix and areas of dense amorphous material. Some of the amorphous material may have resulted from non-nuclear matrix proteins that accumulated in the NM-IF at elevated temperature. In HeLa, the additional protein that accumulated in the NM-IF appeared to contribute to a more elaborate, interconnected fiber network visible in the RNP-containing nuclear matrix.

Thesis Supervisor: Sheldon Penman
Title: Professor of Cell Biology

Biographical Note

Born December 19, 1957 of a diverse racial and cultural heritage, I was raised and educated in Honolulu, Hawaii. I chose to study biology at a very early stage in life and so I naturally majored in electrical engineering while pursuing a minor course of study in mathematics and biology from September, 1975 through August, 1980 at the University of Hawaii. Between the Departments of Reproductive Biology, Biomedical Engineering, and Physiology, I engaged in a multidisciplinary program of research, supported by a M.A.R.C. fellowship. My close association with Drs. Ruth Kleinfeld and Fred Greenwood of the School of Medicine and Dr. Frank Koide of the School of Engineering shaped my decision to study in such an environment as is found at the Massachusetts Institute of Technology. My graduate studies were made possible by the support of my wife and long-time friend, Roanne, and through a Graduate Research Fellowship of the National Science Foundation and training grant from the National Institutes of Health to the Department of Biology.

Acknowledgements and Dedication

Foremost, I wish to acknowledge the very special contribution of my advisor, Sheldon Penman, to my training and growth as a scientist. His style as a scientist and teacher is almost beyond words--at least words suitable for polite company. His approach to science, which I hope to have acquired in part, can be described as ruthless; all effort is to be brought to bear directly on the problem with no fussing, no waffling and, especially, nothing that "doesn't make sense". In the same style, results are not treated delicately. Logical implications or inconsistencies that follow from valid results are addressed directly with no respect for the entrenched paradigms of man or beast.

On the practical side of my training, I offer my gratitude for the contributions of all the members of the Penman lab. I especially want to thank Drs. Min Min Wan and Ted Fey for their help as well as friendship and good laughs. Towards the twilight of my career at M.I.T., Dr. Jeff Nickerson has been a friend, teacher and appreciated busy-body. Especially in the wee hours of the morning, I found Dr. Bill Bendena of the Pardue lab was a welcome and helpful friend. The beautiful and more beautiful electron microscopy was possible only with the patience and skill of Gabriela Krochmalnic.

On an even more practical side, I recognize and appreciate the contributions of taxpayers and other willing supporters of the National Science Foundation and the National Institutes of Health.

Finally and most importantly, the contribution of my family(ies) has been immeasurable. From childhood, my parents have given me the freedom and means to grow. My mother continued to provided this encouragement and support for myself and my wife, Roanne, right up until my mother's death not long after my arrival at M.I.T. Through all of these times, Roanne and her family have accepted and supported all of my choices and directions. The support, encouragement, friendship, and love of my wife has made this effort possible and meaningful (even fun at times!) It is to Roanne that I owe and dedicate this thesis.

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CHAPTER 1

Overview and Introduction

Overview

The physical organization of the cell is the product of a coordinated and regulated pattern of gene expression. The remarkable expression of form and function during the development of a multicellular organism is a large-scale manifestation of this program of control. The converse of this observation, that gene expression is controlled by the physical organization of the cell, is equally true. Clearly the constituents of the nucleus and cytoplasm include the molecular machinery necessary to transcribe and translate the genetic information. However, cytostructure modulates gene expression more than just as effectors of gene expression. In a broad sense, the results presented in this thesis describe an aspect of this reciprocal nature. More specifically, protein synthesis in the HeLa cell, and many other cell types, is modulated by the requirement that mRNA be associated with the complex, filamentous structure in the cytoplasm termed the cytoskeletal framework (CSKF). Protein synthesis can be diminished by releasing messenger RNA from the CSKF. In turn, part of the "reprogramming" of protein synthesis that occurs at elevated temperature includes the rapid translational induction of a small set of novel proteins. These proteins, the prompt heat shock (HS) proteins, appear to be incorporated exclusively into the filamentous matrix of the nucleus and associated intermediate filaments.

The experimental background and literature related to these results is discussed in this chapter, the Overview and Introduction. Detailed experimental methods are presented in Chapter 2. As required, pertinent aspects of these procedures are summarized with the results in each subsequent chapter. The association of mRNA with the CSKF is analyzed in Chapter 3. In this chapter, it was shown that the fungal metabolite, cytochalasin D, can be used to dislodge active mRNA from cytostructure and elicit a dose-dependent

inhibition of protein synthesis. The implications of this association of active mRNA with the CSKF were exploited in the results presented at the end of this thesis, in Chapter 7. In this chapter, it is suggested that some of the translationally-regulated mRNA for the prompt HS proteins may occur as untranslated, structure-bound mRNA in the normal cell. A large part of the research presented here is on the prompt HS response. This response was initially observed by T. Reiter while working with Professor Penman. In HeLa cells, elevated temperatures appeared to induce the synthesis of a large number of previously unidentified proteins that specifically associated with the nuclear matrix and intermediate filaments. Unlike the major, "conventional" HS proteins, the synthesis of these proteins did not depend on continued transcription. In Chapter 4, the prompt HS response is described in cultured cells of Drosophila melanogaster. The results contained in Chapter 5 show that the Drosophila prompt HS proteins were not induced by agents that induce the conventional HS proteins while a subset of the prompt HS proteins were induced by such agents in HeLa cells. The heat-induced alterations in the morphology of the filament networks of the cytoplasm and nucleus were analyzed in Chapter 6 using transmission electron microscopy of unembedded, extracted cells. Finally, future prospects and the implications of these findings are briefly presented in the conclusion.

Cytostructure

Cytoskeletal filaments. The cytoplasm of most eukaryotic cells contains an elaborate array of filamentous structures. The most readily visualized and well studied members of this organization are the three cytoskeletal filament networks: microfilaments, microtubules, and intermediate filaments.

Microfilaments are the most chemically homogenous of the three major filament systems, composed of the acidic protein, actin (Clarke and Spudich, 1977; Korn, 1982). In its simplest form, the actin microfilaments (f-actin) appear to be a duplex helical filament, 6-9 nm in width, containing an axial rise of 2.73 nm (Huxley and Brown, 1967; Hanson, 1967; reviewed in DeRosier and Tilney, 1984). Despite an apparently simple composition, the microfilaments form some of the most heterogenous structures among the three major cytoskeletal filaments. Immunofluorescent microscopy has revealed large, parallel arrangements of microfilaments termed stress fibers in several types of cultured cell lines and cells in vivo (Lazarides and Weber, 1974; Lazarides, 1975; Wong et al, 1983). Also using immunofluorescence microscopy, closely-packed, paracrystalline arrays of actin filaments have been observed in the cytoplasm and nucleus of cells under a variety of conditions (Sanger et al, 1980; Iida et al, 1986; Pekkala et al, 1984). By binding the heavy meromyosin fragment to microfilaments in situ, the polarity of actin filaments could be determined with electron microscopy (Ishikawa et al, 1969). This approach also revealed the existence of still other forms of f-actin such as the anastomosing networks found at the cortex (Wessells et al, 1971) and periphery (Schliwa and van Blerkom, 1981; Small, 1981; Schliwa, 1982) of many cells. The polymorphic nature of f-actin in cultured cells appears to result from the extensive number of associated proteins that modulate the mechanical properties of the microfilament and regulate the assembly of actin monomers into filaments (reviewed in Schliwa, 1981; Stossel, 1984). In addition, DeRosier and associates have suggested that the angular disorder possible within a single actin filament enables the filament to engage in a wide variety of structural interactions, presumably through the same binding domains of the monomer (Egelman et al, 1982; DeRosier and Tilney, 1984).

The microtubules were named in 1963 after the introduction and widespread use of the fixative glutaraldehyde confirmed the tubular nature of these structures in plants and animals (Slautterback, 1963; Ledbetter and Porter, 1963). The microtubules arise from the assembly of dimeric subunits, composed of two distinct, acidic proteins (α- and β-tubulin). The dimeric subunits associate as parallel protofilaments, 13 of which typically form the tubular structure recognized as the microtubule (Ledbetter and Porter, 1963). Immunofluorescent microscopy was used to identify microtubules in both the mitotic spindle and in astral arrays, emanating from structures near the nucleus in the cytoplasm of cultured cells (Weber et al, 1975; Brinkley et al, 1975; Fuller et al, 1975). Microtubules, as well as microfilaments, have been implicated in a diverse collection of motile events such as the separation of chromosomes at mitosis or meiosis, cellular motility, intracellular transport, and the establishment and maintenance of cell shape (reviewed recently in Sakai et al, 1982; Dustin, 1984; McKeithan and Rosenbaum, 1984). Recent studies on the assembly of microtubules <u>in vivo</u> has led to a model of assembly characterized by three features: 1) nucleation at one end of the polymer; 2) the rapid assembly/disassembly of monomeric units at the opposite end which is modulated by hydrolysis of GTP bound to β-tubulin; and 3) the stabilization of the growing end of the microtubule or entire microtubule by mechanisms not yet understood (reviewed in Eutener, 1986; Kirschner and Mitchison, 1986). Unlike actin filaments, microtubules have not been shown to form interconnected webs. Nonetheless, the models of microtubule dynamics implies at least the transient association with a number of macromolecules to establish nucleation in some cases, capping, and stabilization. Numerous microtubule-associated proteins have been identified by <u>in vitro</u> and <u>in situ</u> means (reviewed in Dustin, 1984; Drubin et al, 1985).

The third major filament system of the cytoskeleton was initially identified by electron microscopy as filaments with a diameter between that of actin microfilaments and of myosin thick filaments, giving rise to the term "intermediate" filaments (Ishikawa et al, 1968). Shortly after, immunological and morphological studies revealed the common occurrence of related, 8-10 nm filaments in established cell lines (c.f. Hynes and Destree, 1978). Combined biochemical, immunological, and morphological studies have demonstrated that the intermediate filaments are composed of chemically heterogenous subunits. Although the filaments of wide-spread origin exhibit morphological similarity, the immunological and chemical properties of the subunits distinguish five major groups: (1) desmin filaments, found primarily in muscle cells; (2) glial filaments, found in cells of glial origin; (3) neurofilaments, found in differentiated neurons; (4) keratin filaments, in cells of epithelial origin; and (5) vimentin filaments, found in cells grown in culture and un(pre)differentiated cells. The extensive literature on the biochemistry, molecular biology, and developmental regulation of these proteins has received thorough review (Lazarides, 1982; Weber and Geisler, 1985; Geisler et al, 1985; Traub, 1985; Shay, 1986). By comparison, the functions of the diverse intermediate filament proteins in different cells types is largely unknown. Intermediate filament-associated proteins in non-muscle cells (Lazarides, 1982) have not been characterized as well as those associated with microfilaments or microtubules. However, extensive morphological (Mueller and Franke, 1983; Fey et al, 1984; Bologna et al, 1986) and biochemical (Franke et al, 1981; Geiger et al, 1983) criteria demonstrate an association between cytokeratin and desmin filaments with the desmosomal proteins. Also, by exploiting the repeated assembly-disassembly of intermediate filaments and shared immunological features, other related proteins have been identified (Pachter and Liem, 1985; Aynardi et al, 1984). Intermediate-sized filaments that share immunological

properties with vimentin in Drosophila Kc have been reported (Falkner, et al, 1981; Palter et al, 1986; Walter and Biessman, 1984).

The intermediate filaments and most of the constituent proteins are solubilized only by moderately chaotropic conditions (8 M urea and reducing agents). In part, this property contributes to the co-isolation of the intermediate filaments with the nucleus and nuclear lamina after extraction with high salt (Lehto et al, 1978; Woodcock, 1980; Capco et al, 1982; Granger and Lazarides, 1982; Staufenbiel and Deppert, 1982). However, the major proteins of the nuclear lamina have been shown to share a region of extensive amino acid homology (McKeon et al, 1986; Fisher et al, 1986) with a conserved structural motif found in nearly all intermediate filament proteins (Marchuk et al, 1984; Weber and Geisler, 1985; Steinert et al, 1985). In addition, the nuclear lamina isolated from Xenopus oocytes appeared as a meshwork of 10 nm filaments (Aebi et al, 1986). These investigators found that filaments, formed in vitro from the purified lamin proteins of other organisms, resembled those formed by the in vitro assembly of neurofilaments. In reviewing these results, Franke (1987) suggested that the nuclear lamins and intermediate filaments are related by more than just similar biochemical properties and may constitute members of a multigene family.

The cytoskeletal framework and the microtrabecular lattice. The interactions (and possible relationship) between intermediate filaments and the nuclear lamina is one of many interactions in which the major cytoskeletal filaments participate. The extent of interactions between the well-defined filament systems and between the filament systems and cell membrane has been extensively studied (recently reviewed in Porter, 1984; Shay, 1986). One approach to studying the functional significance of this complex cytostructure is afforded by the gentle extraction of the cell with non-ionic detergent. The application of this

methodology to the study of cultured cells was largely pioneered by Spudich and associates (Brown et al, 1976). The extracted cell or cytoskeletal framework (CSKF) could be visualized directly by electron microscopy after dehydration and critical point drying (Fulton et al, 1980; Fulton et al, 1981). Lacking two-thirds of the protein and nearly all of the phospholipids, the cytoplasmic space of the extracted cell still contained an elaborate network of filaments that terminated on the nuclear lamina and made intimate contact with the cytoplasmic membrane (Lenk et al, 1977; Lenk and Penman, 1979). By morphological criteria as well as biochemical means, the CSKF was shown to include a large portion of integral proteins of the plasma membrane which formed a near-continuous lamina (Ben-Ze'ev et al, 1979; Fulton et al, 1981).

Such extracted structures were soon used for the coordinate morphological and biochemical analysis of the major filament systems (Trotter et al, 1978). In addition, the application of detergent extraction with whole-mount electron microscopy furthered an understanding of the poorly defined cytoplasmic ground substance of the cell. Using the extreme penetrating power and depth of field of the million-volt electron microscope, Porter and associates proposed that the cytoplasm was organized into an elaborate, anastomosing network of fibers with very little unbound substance termed the microtrabecular lattice (Wolosewick and Porter, 1975, 1976). The controversy surrounding the validity of these observations (Wolosewick and Porter, 1979; Porter, 1986) was in part addressed by the application of detergent extraction by Schliwa and associates (Schliwa and van Blerkom, 1981; Schliwa, 1982). The interior of the cytoplasm was shown to be organized in a hierarchy that could be dissected by biochemical means. The major cytoskeletal filament systems were shown to engage in interactions with each other, directly in some cases and most often through a network of 2 to 3 nm diameter filaments (Schliwa and van Blerkom, 1981).

The images obtained by Schliwa and van Blerkom suggested that the path of microtubules may be physically imposed by interactions with the structural elements present in the cytoplasm in addition those found at organizing centers. The formation of the marginal band of microtubules in erythrocytes appears to represent a striking confirmation of this prediction (Miller and Solomon, 1984). In as much as the major cytoskeletal filaments were a subset of the detergent extracted cell, the CSKF could be considered a subset of the loosely-knit microtrabecular lattice.

The reality of the microtrabecular lattice continues to be debated although the techniques of freeze-substitution to avoid the use of fixatives (Heuser and Kirschner, 1980) and refinements in the fixation and drying of unembedded samples (Ris, 1985) have generated only modifications to the shape and form of the elements of the microtrabecular model (Porter, 1986). A growing body of evidence suggests that many aspects of cell metabolism are spatially and temporally organized (Welch and Clegg, 1985); many of the enzyme complexes may be obliged to form weak or transient associations with an underlying substructure in the cytoplasm.

The nuclear matrix. Further dissection of cell structure by detergent and salt extraction has enabled additional study into the cytoplasmic and nuclear structural networks. Berezney and Coffey (1974, 1975, 1977) reported the isolation of an internal, fibrillar protein network from the nuclei isolated from rat liver. The nuclei, which were isolated by low-ionic strength buffers, were subjected to a sequence of nuclease digestion, extraction with high-salt (2 M NaCl), and extraction by non-ionic detergent. During the course of these studies, these investigators monitored the ultrastructure and biochemical composition as they developed the isolation protocol. The final structure showed a remarkable degree of similarity to the nucleus visualized "<u>in situ</u>", by examining the non-chromatin structures of the

nucleus with the regressive stain methodology of Bernhard (1969). The importance of complementary analysis by electron microscopy (or by appropriate structural criteria) has been stressed in recent reviews addressing the large amount of conflicting results in this area (Kaufmann and Shaper, 1984; Berezney, 1984; Nelson et al, 1986). For example, the procedure developed for the isolation of the rat liver nuclear matrix appears to take advantage of the high level of endogenous nucleases present in the isolated tissue. Moreover, the procedure has been found unsuitable for the isolation of the nuclear matrix of tissue culture cells such as HeLa cells (Berezney, 1984). Not surprisingly, reports on the composition and function of the nuclear matrix appear highly variable although a number of functions have been attributed to the nuclear matrix. DNA replication (Berezney and Coffey, 1975; Pardoll et al, 1980), RNA synthesis and processing (Herman et al, 1978; Zeitlin et al, 1987), hormone receptor binding (Barrack and Coffey, 1980; Siemmen et al, 1986), some tumor antigen attachment (Staufenbiel and Deppert, 1983) appear to be part of the nuclear matrix. Nuclear matrix structure functions in viral metabolism as well (Ben-Ze'ev and Aloni, 1983; Zhai et al, 1987; Pinard et al, 1987).

The application of sequential fractionation in conjunction with electron microscopic analysis suggested that the nuclear matrix and associated nuclear lamina was intimately connected with many components of the cytoplasm (Capco et al, 1982; Fey, et al, 1984), particularly the intermediate filaments (Lehto et al, 1978; Woodcock, 1980; Capco et al, 1982; Granger and Lazarides, 1982; Staufenbiel and Deppert, 1982). Morphological criteria suggested that the chromatin-depleted elements of nuclear structure (nuclear matrix proper) were best studied <u>in situ</u> while retaining associations with the salt- and detergent-resistant elements of the CSKF. This structure has been designated the nuclear matrix-intermediate scaffold (NM-IF).

Protein composition of the nuclear matrix. Gel electrophoretic analysis of the NM-IF scaffold prepared from HeLa (Capco et al, 1982; Verheijen et al, 1986) and other mammalian cells in culture (Fey et al, 1984; Staufenbiel and Deppert, 1984) reveal a large number of diverse proteins. The identity, significance, and intracellular location of most of the proteins identified by electrophoretic analysis remains unknown. However, several components of this cellular compartment are well studied and may serve as models for the development of suitable methods of study. The intermediate filaments are perhaps the best studied "component" of the NM-IF. Although the biochemical properties of these highly insoluble structures would suggest the coincidental isolation of the intermediate filaments with the NM-IF, the homologies to the nuclear lamin proteins (discussed previously in association with the intermediate filaments) and the intimate physical contacts observed between the intermediate filaments and the nucleus suggests a more meaningful relationship. In this regard, it may be significant to note that the intermediate filament proteins (cytokeratins in particular) are differentially expressed in various tissues and at different stages of differentiation and transformation (Lazarides, 1982; Moll et al, 1982; Steinert et al, 1984). The NM-IF derived from Madin-Darby canine kidney cells is rapidly and profoundly affected by the action of tumor promoters and ultimate carcinogens (Fey and Penman, 1984) suggesting that the physical organization of the NM-IF is also very sensitive to transformation. However, chemically transformed cells in culture display dramatic changes in the nature of synthesis for intermediate filament proteins as well as other proteins of the NM-IF. These results suggest that the synthesis of NM-IF proteins in general may be sensitive to transformation and differentiation (Fey and Penman, 1986). The composition of the nuclear matrix prepared by other means has also been found to be a sensitive indicator of changes in

differentiation (Brasch and Peters, 1985; Setterfield et al, 1985).

The components of the nuclear lamina and associated nuclear pores constitute a significant portion of the NM-IF. The high-salt method of isolating the nuclear matrix employed by Berezney and Coffey (1974) was related to the method developed by Blobel and associates to isolate morphologically pure nuclear envelopes (Aaronson and Blobel, 1975). The proteins, shown by immunocytochemical means to be part of the pore complex, as well as part of the detergent- and salt-resistant "nuclear matrix-pore complex-lamina" (Fisher et al, 1982) include: a 180-190 kD glycoprotein which is immunologically conserved between a wide variety of organisms (Gerace et al, 1982; Filson et al, 1985); a group of seven diverse proteins (an additional related protein is released by detergent extraction) bearing O-linked N-acetylglucosamine residues (Snow et al, 1987); a related glycoprotein which is essential for nuclear transport <u>in vitro</u> (Davis and Blobel, 1986; Finlay et al, 1987); a myosin-like ATPase (Berrios and Fisher, 1986); and perhaps actin (Schindler and Jiang, 1986).

The nuclear lamins represent between one to five proteins that form the meshwork about the nuclear envelope in a variety of organisms (reviewed in Gerace et al, 1984). The properties and relationship of these proteins to the intermediate filament proteins was discussed previously with the intermediate filament proteins. However, citing unpublished data, Berezney (1984) noted that a large group of proteins of similar molecular weights to the nuclear lamin proteins could be distinguished immunologically from the lamins. It was not clear whether this represented antigenic differences in the lamins due to posttranslational modifications, as the phosphorylated forms of lamins A and C are not incorporated into the fibrous meshwork of the lamina (Ottaviano and Gerace, 1985). Nonetheless, Berezney has provided suggestive evidence that a large amount of the nuclear matrix protein of similar size to the nuclear lamins

is in fact distinct from the lamins (Berezney, 1980). Berezney suggests that the lamin-sized proteins serve to form part of the internal fibrous network of the nuclear matrix. Admittedly, the coincidental equivalence of molecular weights for the putative internal fiber protein and the three lamins is unusual. However, a much more subtle form of organization and molecular evolution may be represented here. The work of Fisher and Berrios on the nuclear membrane-associated ATPase initially suggested that the ATPase was identical to the major 180 kD, conserved glycoprotein. However, these investigators subsequently reported that the glycoprotein and ATPase, while sharing identical mobility during two-dimensional gel electrophoresis, were in fact distinct polypeptides, with different affinities to nucleotides and proteolytic peptide fragments (Berrios et al, 1983).

The intermediate filament proteins, the nuclear lamins, and the proteins of the nuclear pore complex are a large portion of the NM-IF by mass. However, these proteins represent a small fraction of the distinct polypeptides that are isolated in preparations similar to the NM-IF (Nelson et al, 1986). While the interior structure and composition of the nuclear matrix remains controversial (see especially Berezney, 1984) it may be likely that much of the additional protein seen by gel electrophoresis is part of the interior organization. Immunocytochemical results are providing increasing amounts of evidence for an internal filament network and superstructure. Monoclonal antibodies were prepared against a group of non-histone proteins that were tightly bound to HeLa DNA (Bhorjee et al, 1983). Some of the antibodies prepared by these investigators recognized fibrous domains within nucleus by indirect immunofluorescence microscopy. Keller and Riley previously suggested that the internal protein networks reorganize at mitosis and form part of the metaphase chromosome scaffold (Keller and Riley, 1976). At least one of the antibodies prepared by Bhorjee and associates defined an antigen with such properties.

Nonetheless, few of the proteins that form the internal matrix have been identified. One of the first proteins of prior note to be identified in the interior of a DNA-depleted nuclear matrix was topoisomerase II, which is a major protein of the Drosophila embryo nuclear matrix (Berrios et al, 1985). Indirect antibody labeling has identified proteins that occur at the lamina as well as the interior of the nucleus (Fisher et al, 1982; Werner et al, 1981). Using immunofluorescence microscopy of whole nuclear preparations, additional images have been obtained suggesting the presence of internal structure in the nuclear matrices from a variety of mammalian, insect and plant cells (Chaly et al, 1985; Chaly et al, 1986; Verheijen et al, 1986).

Protein Synthesis and the Cytoskeletal Framework

Morphological observations. The application of high voltage electron microscopy to intact, well-spread cells revealed that ribosomes were incorporated into the substance of microtrabecular lattice (Wolosewick and Porter, 1975; Wolosewick and Porter, 1976). Moreover, stereoscopic analysis of these images by Porter and associates suggested that the polyribosomes were clustered about membranes and at the nexi of the filaments (Wolosewick and Porter, 1976). By fluorescence microscopy, the polyribosomes of intact fetal lung fibroblasts appeared to colocalize with actin stress fibers (Toh et al, 1980). Fulton et al (1980) used acridine fluorescence to localize RNA-containing structures (viz. ribosomes) in intact and extracted 3T3 cells. This approach provided suggestive evidence that the polyribosomes in intact as well as extracted cells were distributed in a non-random manner and associated with an underlying organizing matrix.

Analysis by detergent extraction. The release of "soluble" constituents by extraction with non-ionic detergents afforded further analysis for the role of the CSKF in protein synthesis. As observed by Porter and associates

in the intact cell, the polyribosomes in the CSKF also occurred at the junctions of interconnecting fibers (Lenk et al, 1977; van Venrooij et al, 1981; Schliwa et al, 1981) and occurred in a similar distribution as seen in intact cells (Fulton et al, 1980). The monoribosomes, prominent in the intact cells, were not seen in extracted cells (Lenk et al, 1977; Lenk and Penman, 1979). Sucrose density gradient analysis confirmed that nearly all of the polyribosomes were retained with the CSKF while ribosomes, as 80S monomers or as dissociated subunits, were released by the extraction (Lenk et al, 1977; Cervera et al, 1981; Ben-Ze'ev et al, 1981; van Venrooij et al, 1981; Howe and Hershey, 1984). Similar results were obtained by investigators using modified extraction procedures. In cultured lens cells, Bloemendal and associates reported the retention of approximately 60% of the polyribosomes after extraction with Triton X-100 in a buffer containing 25 mM KCl at pH 7.4. Using a buffer containing 10 mM KCl and 0.1% Triton X-100, Bagchi et al (1987) found essentially all of the polyribosomes associated with the CSKF of rat myoblasts.

Significance of messenger RNA association. Many results suggest that ribosomes associate with the CSKF only while engaged in translation or associated with messenger RNA. Inhibiting protein synthesis with agents that disrupt polyribosome integrity such as heat shock, high salt, sodium fluoride, pactamycin, and verucarin resulted in the release of ribosomes from the CSKF (Lenk et al, 1977; Cervera et al, 1981; van Venrooij et al, 1981). These results also suggest that messenger RNA mediates the association of ribosomes with the CSKF. However, some findings suggest that free ribosomes may interact weakly with the CSKF. Under some conditions, Lenk et al (1979) observed that not all of the ribosomes released by sodium fluoride were released by the initial extraction. In addition, Howe and Hershey (1984) reported that the ribosomes released from mRNA by EDTA did not leave the CSKF.

Unlike the near-complete retention of polyribosomes, approximately 60-85 per cent of the cytoplasmic poly(A)+ RNA remained associated with CSKF of mammalian cells (Lenk et al, 1977; Cervera et al, 1981; van Venrooij et al, 1981; Howe and Hershey, 1984; Bonneau et al, 1985). As discussed previously, the association of the mRNA was not dependent on the presence of polyribosomes. Using viral and cellular mRNA as specific models, Bonneau et al (1985) concluded that the association was not dependent on the 5' terminal cap structure or the 3' poly(A) tail. The poly(A)- RNA of SV40 was also translated while bound to the CSKF (Ben-Ze'ev et al, 1981). Lawrence and Singer reported that 60-75 per cent of actin mRNA but 85-100 per cent of tubulin and vimentin mRNA remains associated with the CSKF (unpublished results cited in Lawrence and Singer, 1986). Using a minimally-disruptive method of <u>in situ</u> hybridization (Lawrence and Singer, 1985), these investigators established distinct patterns of intracellular localization for these mRNA in intact chick embryonic muscle cells. The concentration of vimentin and actin mRNA corresponded with striking similarity to the intracellular location of their respective proteins. The work of Fulton suggests that the assembly of many structural proteins occurs at the site of synthesis and that this spatial localization is not due to association of the nascent protein (Fulton et al, 1980; Fulton and Wan, 1983; Fulton, 1984). Variability in the extent of mRNA binding to the CSKF reported by various research groups could be attributed to procedural differences; however, such examples of different mRNA species with different binding properties in the same cell suggests the existence of some form of discrimination based on mRNA structure or sequence.

The non-uniform distribution of mRNA in the oocytes and embryos of several organisms (Capco and Jackle, 1982; Capco, 1984; Jeffery, 1984; op. cit.) is an example of similar discrimination based on sequence or content. In the oocyte of the ascidian, <u>Styelea plicata</u>, (Jeffery, 1984) and

fertilized oocyte of Xenopus (Moon et al, 1983) the segregation appears to be maintained by an association with the underlying CSKF. However, this segregation does not occur until fertilization. Shortly after fertilization, maternal mRNA are translated only after binding to the developing CSKF (Moon et al, 1983).

Evidence for the obligatory association of mRNA with the CSKF was also suggested by studying viral protein synthesis. Newly synthesized VSV mRNA was translated only while in association with the CSKF (Cervera et al, 1981). Pulse-chase labeling of the mRNA revealed that older mRNA was released into the soluble fraction where it was not translated. The preferential association of newly synthesized mRNA in the presence of unbound older mRNA suggested that the binding was not an inherent (artifactual) property of the mRNA. Similar results were observed by Ben-Ze'ev et al (1981) studying the metabolism of SV40 RNA. These investigators found that both poly(A)+ and poly(A)- viral RNA progressed from the CSKF into the soluble phase. The associated viral RNA could be released by treating the cells with cytochalasin B. The mRNA for two viruses exhibiting a strong cytopathic effect, poliovirus and adenovirus, were nonetheless translated while in association with the CSKF (Lenk and Penman, 1979; van Venrooij et al, 1981). During the course of infection, host mRNA was released into the soluble compartment as host protein synthesis was inhibited. However, it is not likely that this is the primary means of inhibiting host protein synthesis (Schneider and Shenk, 1987). One significant observation arising from these studies that is frequently overlooked is that the functional association between actively translated (viral) mRNA and the CSKF was retained despite the severe disruption of gross cytoskeletal morphology (Weed et al, 1986; Zhai et al, 1987).

Other components involved in protein synthesis associate with cytostructure. The initiation factors eIF-2, eIF-3, eIF-4A, and eIF-4B were enriched in the HeLa CSKF (Howe and

Hershey, 1984). A high-molecular-weight aminoacyl-tRNA synthetase complex was similarly enriched in the triton-insoluble fraction (Mirande et al, 1985). These investigators suggested that the association they observed may serve to provide a high local a concentration of the enzymes needed for translation. A monoclonal antibody, reactive with a related protein to the 5' cap-binding protein (Trachsel et al, 1980; Sonenberg and Trachsel, 1982), decorated a filament system resembling the intermediate filament network in hamster cells (Zumbe et al, 1982). However, in other mammalian cells, polyribosomes were not observed in association with the intermediate filaments, suggesting that mRNA did not bind to this filament system (Lenk et al, 1977; Woloswewick and Porter, 1979; van Venrooij et al, 1981). The functional significance of these additional associations and observations was not understood.

Structures involved in the binding mRNA to the cytoskeletal framework. The specific elements of the CSKF that binds mRNA, as mRNP, is unknown. In established mammalian cell lines, it appears that the integrity of the microtubule system is not required for the association, as most extraction procedures in the previously cited studies failed to preserve microtubule integrity. However, Physarum protein synthesis could be rapidly inhibited by drugs that disrupt microtubule integrity and assembly (Vernstam et al, 1980). In these studies, it was suggested that the inhibition may have resulted from a disruption in the transduction of cell surface signals mediated by the cytoskeleton. The association of mRNA with membranes in rat liver was disrupted by prior exposure (in vivo) to microtubule-disruptive drugs (Walker and Whitfield, 1985). In these same studies, microfilament-disruptive drugs did not effect any change. However, the "membrane-bound" polyribosomes analyzed in these studies were prepared by standard techniques involving centrifugation in discontinuous sucrose gradients (Ramsey and Steele, 1979). The hydrostatic

pressures (110,000 x g) applied during separation of free and bound polyribosomes has been shown to disrupt a filamentous cytomatrix (Palmer et al, 1978; Adams et al, 1983). By avoiding high-speed centrifugation while isolating "free" and membrane-bound polyribosomes from rat liver, Adams et al (1983) found that both classes of polyribosomes were associated with structures in a manner that depended on the integrity of actin filaments. The polyribosomes isolated by the method of Adams et al. were capable of sustained protein synthesis in vitro, in contrast to those isolated by differential centrifugation. In these findings, membrane bound polyribosomes were associated with a residual cytomatrix. This result was not unexpected as the integral membrane protein of VSV is also synthesized on membrane bound polyribosomes and the mRNA for this protein was associated with the CSKF in HeLa cells (Cervera et al, 1980).

Most investigators that achieved a disruption of the association between mRNA and the CSKF found that the state of actin filament organization was important. Suggestive evidence was provided by the finding that cytochalasins B and D would release mRNA, polyribosomes and initiation factors from the CSKF (Howe and Hershey, 1984; Lenk et al, 1977; Raemekers et al, 1980, 1983). DNase I, which depolymerizes f-actin (Sheetz, 1979), was found to release polyribosomes from extracted cells (Raemakers et al, 1980) and tissue (Adams et al, 1983). In the latter case, the DNase I-induced dissociation was prevented by stabilizing microfilaments with phalloidin. However, none of the evidence establishes a direct connection between mRNA and actin microfilaments. In view of the complex nature of the CSKF, no conclusions are yet possible from these results. In particular, the effects of the cytopathic viruses (Lenk and Penman, 1979; van Venrooij et al, 1981) and conditions that clearly disturb the organization of the three cytoskeletal filament systems suggest that such gross disruptions can be sustained without

affecting protein synthesis or the association of mRNA with the CSKF (Cervera et al, 1981; Welch and Feramisco, 1985).

Further understanding of the significance and details of the association of mRNA and polyribosomes with the CSKF will most likely be advanced by studies on specific mRNA (see especially Lawrence and Singer, 1986) or on primary or well-differentiated cells. For example, the synthesis of a major protein of the mammalian lens, termed MP26, was observed in primary explants of bovine lens epithelial cells. Ramaekers et al (1980) found that DNase I released a fraction of polyribosomes that were enriched in the mRNA for MP26. Hamster lens epithelial cells that were transformed with SV40 and maintained in suspension culture also synthesized MP26 on polyribosomes associated with the CSKF (Ramaekers et al, 1983). However, the preferential release of polyribosomes synthesizing MP26 could no longer be achieved by exposure to cytochalasin.

The Heat Shock Response and Heat Shock Protein Synthesis

Overview of the heat shock response. In response to elevated temperatures, the cells of all organisms display a rapid, systematic reprogramming of metabolic activities. Ritossa first observed this as an new set of puffs, corresponding to regions of high transcriptional activity, in the salivary gland polytene chromosomes of Drosophila (Ritossa, 1962). The induction of a small set of proteins, termed heat-shock proteins (HS proteins), was found to coincide with the change in chromosome activity (Tissieres et al, 1974). Shortly after, the new puff sites were identified as the site of vigorous synthesis for the mRNA encoding the HS proteins (McKenzie (Lindquist) et al, 1975; Spradling et al, 1977) and the HS response rapidly became an intensively studied model of gene expression. It was recognized since the earliest studies of the HS response that heat was not the only inducer of the response (Ritossa, 1962). Because of

similar induction by a collection of agents or conditions that are generally toxic or disruptive to cellular metabolism, (see especially Nover, 1984; Ashburner and Bonner, 1979), the response has also been referred to as a stress response.

Apparently as a result of this metabolic reprogramming and (in some cases) the synthesis of the HS proteins, cells, tissues, or organisms demonstrate a degree of resistance to further stress. In embryos, this resistance appears as a resistance to heat-induced phenocopy induction in Drosophila by additional heat (Mitchell et al, 1979) or teratogenesis in mammals (Walsh et al, 1985). Many tissues and cultured cells display a resistance to subsequent lethal heat treatments or exposure to toxic agents (Hahn, 1982; Hahn and Li, 1982). The extent of disruption in RNA synthesis (Scharf et al, 1985) and protein synthesis (McCormick and Penman, 1969; Petersen and Mitchell, 1981) is reduced by prior heat treatment and expression of the HS proteins.

The conventional heat shock proteins. The HS response generally entails the specific transcriptional induction of the small number of HS genes (reviewed in Craig, 1985; Lindquist, 1985; Ashburner and Bonner, 1979). In most organisms, these genes encode proteins that can be grouped into three classes: the high-molecular weight group; the 70,000 dalton, highly conserved proteins; and the low-molecular weight group. The high molecular weight proteins include hsp83 of Drosophila and the homologous protein in mammalian cells, hsp80. Mammalian and other vertebrates express HS proteins of approximately 90, 100 and 110 kD. The transcript for hsp83 is unique among all of the Drosophila HS transcripts in that it contains a single intron (Hackett and Lis, 1983). The absence of introns in the remaining HS genes has been suggested to allow these transcripts to bypass the block in RNA processing at high temperature. In support of this hypothesis, Mayrand and Pederson (1983) have demonstrated mRNA derived from transcripts lacking introns

(histone and interferon) continue to be processed at elevated temperature while export of hsp83 mRNA fails at high temperature.

The 70,000 kD proteins are the most highly conserved and abundant HS proteins. Homologies ranging between 60 and 80 per cent have been noted for the proteins from molds, plants, worms, insects, and mammals (see especially Craig, 1985). The dnaK protein of E. coli, a heat-inducible protein required at least for bacteriophage lambda replication, shares 40 to 50% homology with the hsp70 proteins of eukaryotes (Bardwell and Craig, 1984). In Drosophila, at least 5 hsp70 genes exist, all of which share similar 5' non-coding regions and regulatory elements. The related protein(s), hsp68, has not been as well characterized although it too shares extensive homology with hsp70. In human and monkey cells, the 70 kD family of HS proteins (hsp72, hsp73) includes proteins that are constitutively expressed as well as proteins that appear induced only during stress. Pelham has recently suggested the nomenclature of "hsx" for the constitutive form and "hsp" for the strictly inducible form (Pelham, 1986). However, it appears that the constitutive and inducible nature of a particular group can change upon expression or integration of the ras or adenovirus transforming genes (Franza et al, 1986). In several cell types, the existence of heat shock "cognate" proteins have contributed to some confusion in the literature. These proteins share extensive homology with the HS proteins but are not significantly induced by heat (Craig et al, 1983; Lowe and Moran, 1984; Palter et al, 1986; Sorger and Pelham, 1987).

The low molecular weight group of HS proteins are perhaps the least conserved between species although each organism appears to express between one (humans, Hickey et al, 1986) to as many as thirteen (tomato plants, Nover and Scharf, 1984) of these. In Drosophila, the four small HS proteins are encoded by 4 closely-linked genes (Ingolia and

Craig, 1981). Analysis of the predicted amino acid sequence has revealed that these proteins share domains of structural similarities to the bovine α-crystallin lens protein (Ingolia and Craig, 1982; Hickey et al, 1986); this similarity may account for the tendency of these proteins to form aggregates and isolate with nuclear preparations (Nover et al, 1983). The human hsp27 protein was not identified in studies using [^{35}S]methionine to label proteins as the mature protein contains no methionine residues (Hickey et al, 1986).

Minor and translationally-controlled heat shock proteins. Although not "minor", histone H2b in Drosophila has been identified as a transcriptionally-induced conventional HS protein (Sanders, 1981; Tanguay et al, 1983). In yeast and chicken embryo fibroblasts, ubiquitin has been identified as a HS protein by conventional criteria (Bond and Schlesinger, 1985; Finley and Varshavsky, 1985). The possible existence of minor, less prominent heat-induced proteins has received little consideration. As an inducible model system for the study of gene expression, the conventional HS proteins may be unsurpassed; naturally, studies have focused on these prominent proteins. However, during some of the earliest applications of polyacrylamide gel electrophoresis to the HS response, McKenzie (Lindquist) noted a number of "minor" proteins that labeled with [^{3}H]leucine or [^{35}S]methionine (McKenzie, 1976). These proteins were of low molecular weight, smaller than 50,000 daltons. In a comprehensive study of two-dimensional electrophoretic pattern of Drosophila HS proteins, Buzin and Petersen (1982) noted the existence of a number of minor heat-induced proteins, with molecular weights between 44,000 and 66,000. These authors observed that all of the HS proteins of a given molecular weight and many of the isoelectric variants were synthesized during the in vitro translation of mRNA isolated from heat-shocked cells. Interestingly, the minor 44 to 66 kD HS proteins were also synthesized from mRNA isolated from unstressed cells although

these proteins were not detected among the proteins labeled in vivo. This lead these investigators to suggest that some of the minor HS proteins were in fact translationally regulated. This observation has not received much attention in the subsequent literature.

Several reports have described the occurrence of translationally controlled HS proteins in other organisms. Reiter and Penman (1983) initially described the translational induction and nuclear matrix localization of the prompt HS proteins in HeLa cells. The heat-induced changes in the composition of a similar preparation, the nucleolar protein matrix from Chinese hamster ovary cells, was examined by Amalric and associates. These investigators noted that several pre-existing proteins appeared in the nucleolar matrix after heat shock. Using one-dimensional gel electrophoresis, they also found several new proteins of 30 to 65 kD that did not occur in the unstressed cell. They suggested that these could represent translationally-induced HS proteins (Caizergues-Ferrer et al, 1984). A similar set of translationally-induced proteins was described in salt-resistant, cytoplasmic and nuclear granules that formed upon heat shock in tomato cells (Nover and Scharf, 1984). In Tetrahymena pyriformis, the rapid induction (2.5 minutes) of approximately 10 polypeptides was observed upon heat shock. The synthesis of these proteins was not inhibited by the presence of actinomycin D (Galego and Rodrigues-Pousada, 1985). The mRNA for these Tertrahymena proteins could be identified by in vitro translation of RNA from unstressed cells. Although not a "minor" HS protein, the 70 kD HS protein of Xenopus oocytes appeared to be synthesized by the recruitment and translation of preformed maternal mRNA (Bienz and Gurdon, 1982). However, this finding has been challenged by King and Davis (1987) who suggest that the observation may have resulted from contaminating follicle cells. If the assertion by King and Davis is correct, it would appear that all translationally-regulated HS proteins are of a minor

nature and many are isolated in close association with the nucleus or structural components of the cell.

Using very-high-resolution, two-dimensional gel electrophoresis, Young and associates identified 11 HS protein in rat thymocytes that were synthesized under translational control (Maytin et al, 1985). The intracellular location of these proteins was not determined. This approach also suggested the existence of approximately 50 distinct, but rare, transcriptionally-regulated HS proteins. Also using a high-resolution system of two-dimensional gel electrophoresis, Anderson et al found a number of minor heat-induced proteins that had not been described (Anderson et al, 1982). Included among these proteins were a small number of minor proteins that were rapidly induced upon heat shock. The synthesis of these proteins also rapidly declined after the heat shock, in contrast to the behavior of the conventional HS proteins identified in these studies. Although inhibitors of RNA synthesis were not applied in these studies, the rapid and transient induction of the minor HS proteins was suggestive of the translational inductions described previously.

Essentially nothing is known about the synthesis and function of the minor HS proteins described. However, a number of investigators have suggested that an understanding of the establishment and maintenance of thermotolerance, for example, may require further understanding of minor heat-induced proteins (Hanh, 1982; Nover, 1984; Craig, 1985).

Localization of the conventional HS proteins and insight into the function of the major heat shock protein. In an effort to understand the function of the conventional HS proteins, many investigators have applied immunofluorescence microscopy and cell fractionation techniques to localize the HS proteins. Tanguay (1985) has most recently reviewed the data acquired in Drosophila. In this organism, hsp83 is consistently found in the cytoplasm by microscopic localization and is generally released from cells by

extraction with non-ionic detergents. The low molecular weight HS proteins are almost exclusively associated with salt-resistant substructures of the nucleus. Microscopic evidence has suggested that these structures are localized within the nucleus or about the nuclear periphery during HS and return to the cytoplasm during recovery (Arrigo and Ahmad-Zadeh, 1981). Subsequently, this group reported that the small HS proteins may occur associated with RNA in a cytoplasmic particle similar to the prosome. The intracellular localization of Drosophila hsp70 has provided suggestive clues as to its function. Using immuno-fluorescence and autoradiography with light and electron microscopy, a large proportion of hsp70 has been found within the nucleus and concentrated within the nucleolus during heat shock (Arrigo et al, 1980; Velazquez et al, 1980; Velazquez and Lindquist; 1984). After the heat shock, hsp70 was primarily cytoplasmic. However, when induced to a comparable amount by arsenite rather than heat, hsp70 remained cytoplasmic (Vincent and Tanguay, 1982; Tanguay, 1985). The arsenite-induced hsp70 would move to the nucleus if the cells were exposed to elevated temperature.

Pelham and associates have explored the significance of hsp70 localization in the nucleus and nucleolus. This effort has provided the most extensive analysis of a putative function for any of the conventional HS proteins. In mammalian cells, the induced 70 kD HS protein (hsp73) was found to associate with nucleoli during and shortly after heat shock (Welch and Feramisco, 1984). Pelham (1984) constitutively expressed the Drosophila hsp70 gene in monkey (COS) cells from a transfected expression vector. The Drosophila protein was found to migrate to the nucleoli upon heat shock as was observed for mammalian hsp70. More significantly, the presence hsp70 in the nucleoli was correlated with a rapid recovery of the heat-induced disruption in nucleoli morphology. The portion of the molecule responsible for nucleolar localization was later

identified (Munro and Pelham, 1985) and was found to be distinct from the previously reported ATP-binding domain (Welch and Feramisco, 1985a). Because the hsp70 that was bound to the nucleoli in the isolated nucleus could be released by very low concentrations of ATP but not non-hydrolyzable analogs (Lewis and Pelham, 1985), Pelham suggests that hsp70 binds to denatured or disrupted structures and through an energy-driven process, solubilizes the heat-induced aggregates. Support for such a model occurs in the independent findings that the ATP-dependent, clathrin uncoating enzyme is also a member of the hsp70 family (Chappell et al, 1986; Ungewickell, 1985).

Nonetheless, the possible functions for hsp70 appear diverse. Lindquist and associates (1982) have demonstrated that the level of synthesis for both the mRNA for hsp70 and the protein itself quantitatively correlated with amount of functional hsp70 present in the cell. Using inhibitors of very different modes of action and amino acid analogues, these investigators varied the amount of hsp70 mRNA transcribed as well as the amount of functional hsp70 produced. The return to normal patterns of translation and transcription after a heat shock at any given temperature required precisely the same amount of functional hsp70. The absolute amount varied with the severity of the stress. The "feedback" inhibition on the transcription of hsp70 mRNA could be accounted for by the nuclear presence of hsp70. However, the feedback inhibition of hsp70 translation cannot be as easily explained by the action of hsp70 in the nucleolus. Perhaps it is significant that the mammalian hsp70 exhibits no such self-limiting regulation, rather the regulation of hsp70 synthesis appears to occur primarily at the level of transcription and that turnover and transcription is not dependent on new protein synthesis (Widelitz et al, 1987). Furthermore, hsp70 has been reported to form associations with poly(A) RNA (Schonfelder et al, 1985), hnRNP (Kloetzel and Bautz, 1983), cytoplasmic

ribosomes (Welch and Suhan, 1986), the transformation-associated tumor antigen p53 (Pinhasi-Kimhi et al, 1986), membrane glycoproteins (Hughes and August, 1982), as well as microfilaments (La Thangue, 1984), intermediate filaments (Wang et al, 1980) and "cycled" microtubules (Lim et al, 1984). At present, the work of Pelham is the most convincing demonstration of a function for the Drosophila hsp70 albeit in mammalian nucleoli.

Studies on the localization of the remaining mammalian and Drosophila HS proteins have not yielded comparable success in the search for functions for the HS proteins (reviewed in Burdon, 1986; Subjeck and Shyy, 1986; Pelham, 1986; Schlesinger, 1986). In brief, the association of hsp80 and hsp90 with several membrane receptors and proteins processed through the endoplasmic reticulum has suggested to some investigators that these proteins mediate in the delivery of integral membrane proteins to the plasma lamina. The association of hsp100 with the Golgi complex after heat shock has suggested that this protein is engaged in carbohydrate processing or exocytocysis. The postulated functions must accommodate the fact that all but the inducible form of hsp70 are prominent in unstressed cells (Mathews et al, 1981; Welch and Feramisco, 1985a; Welch and Feramisco, 1984).

Heat shock and the cytoskeletal framework. In light of the requirement for mRNA association with the CSKF, a common speculation was that HS inhibited protein synthesis by disrupting this association (Welch and Feramisco, 1985b). From the work of Cervera et al (1981) and Howe and Hershey (1984), this simple explanation is not correct. Both normal and HS mRNA remain bound to the CSKF during a heat shock. Each of the three major cytoskeletal filament systems suffer some disruption in certain circumstances. Thus any alterations between the association of protein synthesis and the CSKF would be manifest as a more subtle change in function and it seems likely that the gross disruptions to

the cytoskeletal filaments that have been observed are not directly involved (Welch and Feramisco, 1985b).

The principle disruption observed after heat shock occurs in the intermediate filament system. In mammalian cells (Thomas et al, 1982; Welch and Feramisco, 1985b; Welch and Suhan, 1985), chicken embryo fibroblasts (Collier and Schlesinger, 1986) and Drosophila cells (Biessman et al, 1982), a nuclear cap of vimentin filaments was observed (or filaments analogous to vimentin in the case of Drosophila). Similar rearrangements have been noted after cells were exposed to microtubule depolymerizing agents (Forry-Schaudies et al, 1986; op. cit.) however a parallel disruption of the microtubule system was not noted in the examples cited above.

The heat-induced disruption of the vimentin filaments would be reversed in time although normal morphology would be prevented if actinomycin D was added during the heat shock (Collier and Schlesinger, 1986). These authors suggested that the HS proteins contributed to the recovery. A similar finding was reported by Schamhart et al (1984) who found that the adherent Reuber H35 rat hepatoma cells would round upon heat shock. During recovery, the cells would resume a flat appearance and could resist further heat-induced alterations in morphology if protein synthesis was permitted during the recovery. This group later reported alterations in different filament systems of different cells. In hepatoma cells, the actin microfilaments were disrupted. In contrast, the microfilaments of neuroblastoma cells were unaffected by heat shock but the microtubules were disorganized which presumably lead to the perinuclear cap of vimentin filaments (van Bergen en Henegowen et al, 1985). A fairly severe heat shock (45.5^{o}C), applied briefly to Chinese hamster ovary cells during mitosis, disrupted the spindle and contractile ring and prevented reformation of the spindle upon return to normal temperatures. Although such a heat shock is not lethal to 85% of the cells in an unsynchronized population,

every cell heat-shocked during mitosis failed to survive as assessed by clonogenic potential (Coss et al, 1982). The authors suggested that the mitotic spindle or contractile ring was particularly labile to the effects of heat although other targets were not excluded.

Preferential translation of Drosophila heat shock mRNA. The synthesis of the conventional HS proteins occurs under a form of translational control. During and shortly after a moderate heat stress in Drosophila the HS mRNA are preferentially translated while the translation of pre-existing mRNA is repressed (McKenzie et al, 1975; Lindquist, 1980a, 1981). This effect is observed in mammalian cells, although primarily during moderately severe stresses (McCormick and Penman, 1969; Mathews et al, 1981; Reiter and Penman, 1983). In Drosophila, Gehring and associates conclusively demonstrated that structural features of the Drosophila hsp70 mRNA, rather than its synthesis during heat shock, conferred translatability at the elevated temperatures. The gene for alcohol dehydrogenase (Adh) was placed under the control of the heat-inducible promoter and could be induced in vivo by heat shock. However, the Adh mRNA synthesized at high temperature was translated only at normal temperatures. A separate Adh gene was created that contained 95 nucleotides of the 5' leader from an hsp70 mRNA. This modified Adh mRNA was not transcribed at elevated temperatures; however, the mRNA made at normal temperature was efficiently translated during heat shock (Klemenz et al, 1985).

The 5' untranslated leaders of Drosophila HS mRNA contain a high percentage of adenine residues and are unusually long (Holmgren et al, 1981). However, the addition of 39 bases to the (naturally long) 5' end of an hsp70 mRNA abolished the translational advantage seen at elevated temperatures (McGarry and Lindquist, 1985). In mRNA not containing this insertion, either one of two conserved octameric sequences in the leader was required for

translation at elevated temperatures. Using P-element mediated transformation, Gehring and associates were able to show that as much as 86% of the hsp22 leader was not required for the preferential translation of the altered mRNA (Hultmark et al, 1986). These investigators showed that while the length of the untranslated leader, per se, was not critical for enhanced translation, a 26 base region at the 5' end of the mRNA was sufficient to confer preferential translation during heat shock. Together, these results suggested that there is a positive control signal present in the vicinity of the ribosome recognition site in Drosophila HS mRNA that enables the near-exclusive translation of HS mRNA under stress.

In mammalian cells, examples of structural features in mRNA that permit translation during other periods of reduced protein synthesis have been described. During the inhibition of protein synthesis that occurs during poliovirus infection, only mRNA lacking the 5' cap structure are translated (Detjen, et al, 1981; Nomoto et al, 1976). Late in a lytic adenovirus infection, host and early adenovirus mRNA are no longer translated; late adenovirus mRNA, however, escape this inhibition. The late viral messages contain a tripartite, untranslated 5' leader sequence that has been shown to confer translatability during this stage of the infection (Thummel et al, 1983; Logan and Shenk, 1984).

Translational advantage of vertebrate mRNA during heat shock. However, structural features similar to those in the Drosophila HS mRNA have not been described in vertebrate HS mRNA. Moreover, there are several examples that support the contention that only mRNA synthesized during a stress are translated or that an RNA factor synthesized during stress is required for utilization of the HS mRNA. In chicken reticulocytes, the mRNA for hsp70 is a major species, approximately 2 per cent of the amount of globin mRNA (Morimoto and Fodor, 1984). Upon heat shock, the synthesis of hsp70 is strongly enhanced while globin synthesis ceases.

However, the transcription rate of the hsp70 gene did not change, implying that the enhanced synthesis of hsp70 was a translational response to heat shock. In contrast to prompt HS protein synthesis (Reiter and Penman, 1983), synthesis of the chicken hsp70 could be inhibited by inhibitors of transcription (actinomycin D or 5,6-dichloro-1-β-ribofuranosylbenzimidazole--DRB) (Banerji et al, 1984). While unknown side effects of the inhibitors could affect the results, the most direct interpretation is that the continued synthesis of an RNA product was required for the translation of hsp70 mRNA during heat shock or that only newly synthesized mRNA was translated.

The early work of McCormick and Penman (1969) suggested the existence of a short-lived RNA species that enhances the reformation of polysomes shortly after the return to normal temperatures. The polysomes were later shown to be reforming on newly-made HS mRNA as well as normal mRNA (Hickey and Weber, 1982), thus the presumptive short-lived RNA may have served to enhance translation of HS and normal mRNA. The small, VAI RNA of adenovirus has been shown to be required for translation late in adenovirus infection (Thimmappaya et al, 1982; Kitajewski et al, 1986). The relationship between VA RNA and the tripartite leader is not apparent however, as VA RNA was shown to enhance the translation of viral as well as cellular mRNA. Interestingly, this RNA polymerase III product was found to prevent the phosphorylation of the alpha subunit of eIF-2 by the dsRNA-induced kinase (Kitajewski et al, 1986). The phosphorylation of the alpha subunit of eIF-2 is a lesion that is also observed in heat shocked cells (Ernst et al, 1982). The presence of a this modification appears to non-competitively inhibit the exchange of GDP for GTP in the formation of the ternary complex (Siekierka et al, 1984).

In addition to the conventional HS mRNA, other mRNA have been described that are able to escape the heat-induced inhibition of translation. These include mRNA for the

structural proteins of the HPS-1 RNA virus of Drosophila (Scott, et al, 1982), N and NS proteins of vesicular stomatitus virus, (Scott and Pardue, 1981), histone H2B (Sanders, 1982; Tanguay et al, 1983), and ubiquitin (Bond and Schlesinger, 1985). The translation of the Drosophila HS mRNA during reduced levels of protein synthesis has been attributed to the ability of the conventional HS mRNA to better compete for limiting factors during the stress (Jackson, 1986). A similar suggestion has been made with regards to the regulation of HS protein synthesis in chicken myotube cultures (Bag, 1983). However, evidence for this competitive ability has not been demonstrated in cell-free translation systems (Storti et al, 1980; Lindquist, 1981; Kruger and Bencke, 1981).

Translational discrimination against normal mRNA during heat shock. The cells of multicellular organisms show a translational discrimination against the mRNA molecules synthesized before heat shock under suitable conditions. McCormick and Penman (1969) showed that temperatures above 42^{o}C would disaggregate polyribosomes in HeLa cells. These investigators suggested that heat introduced a lesion at the initiation step of protein synthesis. They were able to show that low levels of cycloheximide, an inhibitor of elongation, could restore polyribosomes in the absence of new RNA synthesis. These results also suggested that the mRNA molecules made before the heat shock were intact and functional. Subsequent work of Hickey and Weber confirmed these observations and also provided evidence suggesting that the preferential translation of the HS mRNA in HeLa cells could be explained by the ability of the HS mRNA to escape the initiation defect (Hickey and Weber, 1982). The translational discrimination against mRNA formed before the heat shock or stress has been observed in the cells of organisms as diverse as mammals (Hickey and Weber, 1982), sea urchins (Roccheri et al, 1981), insects (Mirault et al, 1978; Kruger and Benecke, 1981; Storti et al, 1980), and plants

(Nover and Scharf, 1983). In these examples, the preexisting mRNA remain intact and available for translation after the heat shock or stress is removed. However, the inhibition of protein synthesis during a stress by this form of discrimination is not universal. In both E. coli and yeast, organisms that display a transcriptionally-induced heat shock response, normal protein synthesis is inhibited by allowing the preexisting mRNA to degrade during the stress (Lemaux, et al, 1978; McAllister and Finkelstein, 1980; Lindquist, 1981). Somewhat surprisingly, the degradation of the existing mRNA in yeast was shown to require the synthesis and nuclear export of RNA (McAllister and Finkelstein, 1980).

The molecular details of the heat-induced defect in translation in higher organisms are not well understood. However in the case of Drosophila, it has been possible to reproduce the discrimination against normal mRNA in a cell-free translation system derived from heat shocked cells (Scott and Pardue, 1981; Storti et al, 1981; Kruger and Benecke, 1982; Sanders et al, 1985). With these systems, only HS mRNA were translated when added to heat shock-derived lysates. Mixing experiments suggested that a diffusible inhibitor was not contributing to the discrimination (Kruger and Benecke, 1982). However, the ability to recognize and translate normal mRNA could be restored by crudely fractionated factors from normal cells. One group reported that partial activity could be restored by adding a crude preparation of high-salt-washed ribosomes (Storti et al, 1981). In contrast, another group found that nearly complete translational activity could be restored by adding a soluble component to the heat shocked-derived system (Sanders et al, 1985). In this system, ribosomes prepared with isotonic buffers did not provide any restoration. Other searches for related heat-induced changes in the translational apparatus have revealed several covalent modifications that could affect translation during heat shock. The ribosomal protein of the small subunit, S6, was rapidly dephosphorylated after

heat shock (Glover, 1982). During the cell cycle, reduced levels of S6-phosphorylation have coincided with reduced levels of protein synthesis (Thomas, 1980). However, because normal activity of the second heat shock-derived lysate discussed above could be restored with a soluble component but not with a ribosomal component, and because it was possible to separate the inhibition of protein synthesis from the dephosphorylation of S6 during recovery (Olsen et al, 1983), it seems likely that the inhibition is not soley the result of the dephosphorylation of S6. In accord with this possibility, other changes in the initiation factors of mammalian cells have been noted. As mentioned previously, activation of the hemin-regulated protein kinase was observed after heat shock (Ernst et al, 1982). This enzyme phosphorylates and inactivates the alpha-subunit of eIF-2 and inhibits initiation. In addition, several other forms of covalent modifications in the initiation components of mammalian cells were detected using two-dimensional gel electrophoresis and in vitro reconstruction (Duncan and Hershey, 1984).

Elongational inhibition during heat shock. Besides the defect in initiation, an additional, poorly understood alteration in protein synthesis has been postulated to occur in mammalian and Drosophila cells during hyperthermia or stress. Although Lindquist found the rates of elongation on Drosophila HS mRNA were among the fastest measured for eukaryotes (Lindquist, 1980b), two groups of investigators have argued that elongation on normal mRNA is inhibited in parallel with the reduction in initiation (Ballinger and Pardue, 1983; Thomas and Mathews, 1983; Thomas and Mathews, 1984). The evidence for this suggestion lies primarily in the observation that although the HS proteins can be nearly the exclusive product synthesized in vivo, normal mRNA species are present in the polysomes, apparently bound to ribosomes even though their translation is not observed in the cell. The association of elongationally-inhibited

ribosomes with mRNA was suggested by several lines of evidence: (1) puromycin would shift the sedimentation of the messages in sucrose density gradients, presumably by prematurely terminating elongation and releasing ribosomes from the mRNA (Thomas and Mathews, 1982); (2) the mRNA remained associated polyribosomes in sufficiently high salt to dissociate free ribosomes and some mRNP particles (Ballinger and Pardue, 1983); (3) the apparent density of the HS polyribosomes in metrizamide was identical to control polyribosomes (Ballinger and Pardue, 1983); and (4) the isolated polyribosomes could direct the synthesis of the normal proteins in the reticulocyte-derived lysate, presumably by run-off elongation, even when initiation was prevented by the presence of edeine (Ballinger and Pardue, 1983). However, in the inhibition observed in Drosophila, something of a paradox remains in that the optical density of the polyribosomes isolated from heat shocked cells appeared to be reduced by an amount greater than was suggested by direct measurement of representative mRNA species in the polysome region or by indirect measurement through in vitro translation of the phenol-purified mRNA in the polysomes (Ballinger and Pardue, 1983).

With regards to this novel mechanism of translational control, Nover (Nover, 1984) has suggested that the elongational arrest observed during heat shock may be related to the elongational arrest seen in the class of mRNA encoding proteins destined to enter the ER via signal sequence. Translation of these mRNA molecules stops after approximately 70 amino acids have been synthesized, corresponding to a mRNA loaded with two ribosomes. The site specific arrest, brought about by binding of the signal recognition particle to the nascent polypeptide, is released by association with the signal receptor protein in the rough microsomes (Gilmore and Blobel, 1982, 1983; Meyer et al, 1982). The mRNA and ribosome pair would be attached to an 11S RNP particle. Although this model would not account for the presence of

mRNA migrating with a sedimentation coefficient much greater than 100S, it is conceivable that an as yet undetermined RNP-complex is formed with the translation unit upon heat shock. This extended initiation complex may be poorly defined or poorly preserved under the conditions used to analyze polyribosomes and consequently display heterogenous sedimentation behavior. It is not impossible that some component of the cytoskeletal framework serves to bind the mRNA-ribosome complex for proteins not destined for secretion or posttranslational processing.

CHAPTER 2

Materials and Methods

Materials and Special Reagents

Radiochemicals. Radioactive amino acids and nucleosides were purchased from New England Nuclear, Boston, MA. L-[^{35}S]methionine was obtained as 8.3 mCi/ml, 1064 Ci/mmol and used between 50 - 300 μCi/ml for labeling cells, at 830 μCi/ml for <u>in vitro</u> translation. L-[4,5-^{3}H]leucine and L-[4,5-^{3}Hlysine were obtained at 55 and 100 Ci/mmol respectively and used at 10 - 100 μCi/ml. [5,6-^{3}H]uridine was obtained at 37 Ci/mmol and used to label ribosomal RNA overnight at 0.1 - 0.5 μCi/ml, mRNA for 2 - 3 hours at 100 μCi/ml, hnRNA for 10 minutes at 100 μCi/ml. ^{3}H-Polyuridine was obtained at 2 - 10 Ci/mmol UMP. ^{14}C-labeled molecular weight standards were obtained from Amersham, IL, and included lysozyme (14,300 kD), carbonic anhydrase (30,000 kD), ovalbumin (46,000 kD), bovine serum albumin (69,000 kD), phophorylase b (92,500 kD) and myosin (200,00 kD).

Enzymes and general reagents. Reagents for electrophoresis were electrophoresis-grade, obtained from Bio-Rad, Richmond, CA and are detailed under the section on electrophoresis. Ampholytes (Ampholines brand) were purchased from LKB, Sweden. Isoelectric standards with pI values between 4.7 to 10.6 were obtained from BDH Chemicals Ltd., Poole, England. Inorganic reagents for <u>Drosophila</u> cell culture medium were Cell-Culture Grade, obtained from Sigma, St. Louis, MO. Serum for cell culture, certified as mycoplasm-free, was obtained from Irvine Industries, Santa Anna, CA. Other reagents used in cell culture were obtained from Gibco, Grand Island, NY. Distilled phenol was obtained from International Biotechnologies, New Haven, CT. DNase-free pancreatic RNase A was prepared immersing a 10 mg/ml solution of RNase in low salt buffer in boiling water for 4 minutes. Proteinase K was prepared at 20 mg/ml in 100 mM NaCl, 0.5% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5. Contaminating enzymes were inactivated by autodigestion at 37^{o}C for 30 minutes before dispensing and freezing as

aliquots. All other enzymes and chemicals were typically obtained from Sigma, St. Louis, MO or Calbiochem, La Jolla, CA.

Antibiotic inhibitors and drugs. Actinomycin D was used from a stock solution (stored at -70°C) of 2 mg/ml in 70% ethanol kept in light-tight containers. Actinomycin D concentration was adjusted spectrophotometrically (Saicki and Godman, 1974). In mammalian cells, actinomycin D was used at 5 μg/ml to inhibit total RNA synthesis, 0.04 μg/ml to preferentially inhibit ribosomal RNA synthesis (Perry and Kelly, 1968). 5-fluorouridine was used at 10 μg/ml to preferentially inhibit ribosomal RNA synthesis. Cycloheximide was used to inhibit protein synthesis between 1 and 300 μg/ml from a stock solution (stored at -20°C) of 10 mg/ml in dimethyl sulfoxide. Emetine hydrochloride was used to inhibit protein synthesis at 10 - 250 μg/ml from a 1 mg/ml stock solution (stored at -20°C) in water. Cytochalasin D was used from a stock solution of 5 mg/ml in dimethyl sulfoxide. Carrier DMSO was used in all controls involving cytochalasin D treatment and the amount of DMSO added to each sample adjusted to 1.5% of the total cell culture volume. Sodium arsenite, sodium fluoride, cadmium sulfate, zinc sulfate were added from freshly prepared concentrated stock solutions in water. 2,4-dinitrophenol was added from a stock solution in acetone that was stored at -20°C in the dark. Iodoacetamide was prepared directly in the culture medium immediately before use.

RNase-free DNase I. "RNase-free" bovine pancreatic DNase was obtained from Worthington Biochemicals, Freehold, NJ then purified from trace contaminants of RNase A by adsorption against 5'-(4-aminophenylphosphoryl)-uridine-2'(3')phosphate coupled to agarose (pUp agarose, from Miles Scientific, Naperville, IL). Crystalline DNase was dissolved in 5 mM sodium acetate, 5 mM $MgCl_2$, pH 5.0 and mixed with pUp agarose equilibrated with the same buffer. The slurry was agitated on ice for four 1 hour intervals. At the end of

each interval, the agarose was pelleted, purified DNase removed with the supernatant solution, and additional buffer added to release more DNase from the agarose matrix. The supernatant fractions were pooled and concentrated by ethanol precipitation (Wilchek and Goreki, 1969). Protein content was determined by the method of Bradford (1976) using a commercially available kit from Bio-Rad.

Cell Culture

HeLa cell culture. HeLa S3 cells were maintained at $4x10^5$ cells/ml in suspension culture in 2.5 liter spinner bottles at 37°C in Eagle's minimum essential medium (MEM) supplemented with 7% horse serum . MEM was purchase as a preformulated, powdered mixture from GIBCO Laboratories. A portion of the medium containing the HeLa S3 cells in culture was replaced daily to maintain the cells in exponential growth with a generation time of 24 hours. Adherent HeLa cells, American Type Culture Collection CCL2.2, were maintained in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's MEM supplemented with 10% horse serum. Monolayer cells were passaged twice weekly at 1:5 to 1:40 dilutions and typically used 32 to 48 hours after plating.

Rabbit reticulocytes (used in Chapter 3) were prepared from anemic rabbits by standard procedures and shipped on wet ice for use within 16 hours of their isolation (prepared by and purchased from Green Hectares, Madison, WI).

HeLa cell protein labeling. Suspension grown HeLa cells were pelleted at 650 x g, washed in phosphate buffered saline (PBS) and typically concentrated three-fold in the appropriate medium as indicated in the text and figure legends. The cells were resuspended in 50 ml glass tubes fitted with a suspended magnetic stirrer mounted in a rubber stopper which was maintained at the proper temperature (+/- 0.5°C in a circulating water bath. The air space in the

culture tube was replaced with air containing 5% CO_2. Protein was labeled at the indicated temperature with 25-200 μCi/ml L-[^{35}S]methionine (8.3 mCi/ml, 1064 Ci/mmol) for the times indicated. Protein prepared for gel electrophoresis was typically labeled for 30 minutes with the higher concentration of radiolabel in methionine-free media. Protein synthetic rates were determined with a 3 minute pulse, terminated by the rapid addition of 1000-fold excess of unlabeled methionine. Time-critical experiments utilizing monolayer cells were conducted in a 37^{o}C warm room. The media in such experiments was further buffered by the addition of 10 mM 4-[2-hydroxethyl]-1-piperazine-ethanesulfonic acid (Hepes).

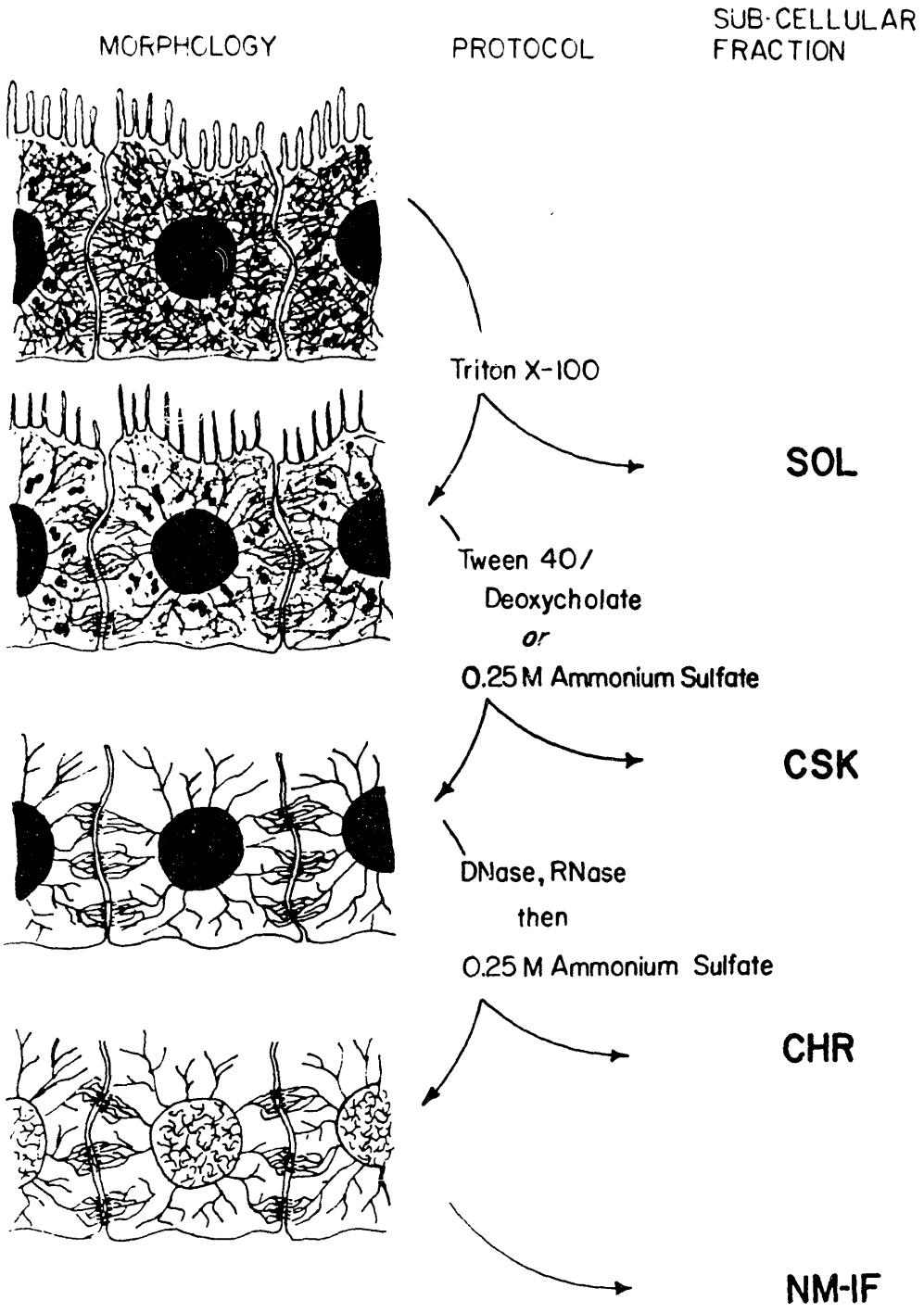
Drosophila cell culture. Schneider 2-L Drosophila melanogaster cells, kindly provided by Professor Mary Lou Pardue, M.I.T., were maintained in 75 cm^2 flasks as a loosely adherent monolayer or were maintained at 2 - 4 x 10^6 cells/ml in spinner culture at 25^{o}C. The generation time was approximately 32 hours. The Drosophila cells were cultured in Shields and Sang (Shields and Sang, 1977) medium containing penicillin and streptomycin supplemented with 12.5% heat inactivated fetal calf serum, 2 mg/ml TC Yeastolate (Difco) and 2.5 mg/ml Bactopeptone (Difco). Medium was replaced daily to adjust the concentration of cells in spinner culture to no more than 4 x 10^6 cells/ml. Cells in monolayer cultured were passaged twice weekly at 1:4 through 1:20 dilutions.

Drosophila cell protein labeling. Drosophila cells were resuspended at 1 - 2 x 10^6 cells per ml in polypropylene conical tubes (which cause minimal cell sticking) or in siliconized 50 ml glass tubes as for HeLa cell protein labeling (above) in methionine-free Shields and Sang medium containing 12.5% dialyzed fetal calf serum. If indicated, actinomycin D (Sigma) was added to 2.0 μg/ml at this point and the cells were allowed to adapt at 25^{o}C for 10 min before shifting to the appropriate temperature. Where indicated,

camptothecin was used in place of actinomycin D and was added from a stock solution of 2 mg/ml in methanol to a final concentration of 20 μg/ml. [^{35}S]methionine was added at the indicated times to a concentration of 50-300 μCi/ml. Incorporation of the label was stopped by transferring the cultures to an ice bath followed by the addition of an excess of ice cold PBS.

Storage and retrieval of frozen cell lines. Adherent cells in exponential growth were removed by 0.01% trypsin. The protease was neutralized by the addition of fresh serum and the cells removed from the mixture. Suspension-grown or non-adherent cells were washed with fresh media. The cells were gently resuspended at 10 x normal density in normal growth medium containing 20% serum and 10% dimethyl sulfoxide. 1.0 ml aliquots were dispensed in freezing vials and the cells frozen in a controlled-freezing insert above liquid nitrogen before permanent storage in liquid nitrogen. Frozen cells were rapidly thawed at 37^{o}C with gentle swirling. Once thawed, the vial was immersed in 70% ethanol to sterilize the container and the cells gently transferred to a 10 ml sterile culture tube. The original volume of media (typically 10 ml) at room temperature was added over a 5 minute period. Initially, medium was added dropwise with gentle agitation. Sensitive cell-lines were gentle pelleted and resuspended in fresh media before transferring to normal culture conditions.

Figure 1


MORPHOLOGY
PROTOCOL
SUB-CELLULAR FRACTION
Triton X-100
SOL
Tween 40/
Deoxycholate
or
0.25 M Ammonium Sulfate
CSK
DNase, RNase
then
0.25 M Ammonium Sulfate
CHR
NM-IF

Cell Fractionation

Standard solutions for cell fractionation and cell culture. All solutions were prepared from pyrogen-free, glass distilled water and sterilized by autoclaving or filtration through Millipore (Bedford, MA) 0.22 μm filters.

Phosphate-buffered saline (PBS): 137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 2 mM KH_2PO_4 and 1 mM $MgCl_2$. Solutions were prepared from 10 x concentrated solutions, filter-sterilized and stored at 4°C. PBS lacking magnesium was used only when necessary.

Cytoskeleton buffer (CSK Buffer): 100 mM NaCl, 300 mM sucrose, 10 mM 1,4-piperazinediethane sulfonic acid (Pipes), pH 6.8, 3 mM $MgCl_2$, and 1 mM ethylene glycol-bis(β-aminoethyl ether-N,N,N',N'-tetraacetic acid (EGTA). CSK buffer was filter-sterilized and stored as aliquots at -20°C. Typically, triton X-100 was added to 0.5% from a 10% solution (stored at -20°C) and the serine protease inhibitor, phenylmethylsulfonylfluoride (PMSF) was added to 1.5 mM from a 100 mM stock solution in 100% ethanol immediately before use.

Reticulocyte Standard Buffer (RSB): 10 mM NaCl, 3 mM $MgCl_2$, Tris-HCl, pH 7.4. RSB was stored at -20°C in aliquots. Typically 1.0% deoxycholate and 0.5% Tween-40 was added from a 10X concentrate mixture as well as 15 mM PMSF to strip salt-labile elements from the extracted cell and purify the nucleus.

Extraction Buffer: 250 mM $(NH_4)_2SO_4$, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES, pH 6.8. Stored as aliquots at -20°C. Used after the addition of 0.5% Triton X-100 and protease inhibitors to extract salt-labile proteins from the cytoskeletal framework and extract non-core histones from chromatin *in situ*.

Digestion Buffer: 50 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH 6.8, 3 mM $MgCl_2$, and 1 mM EGTA. Stored in small aliquots at -20°C. Supplemented with 0.5% Triton X-100, protease

inhibitors and nuclease (RNase, DNase, or both) for digesting nucleic acids in situ.

HeLa cell fractionation. The biochemical and morphological correlates of the sequential fractionation procedure used for mammalian cells has most recently been described in (Fey et al, 1984; Fey et al, 1986) and then some). The fractionation protocol is illustrated in schematic form with an epithelial cell line in Figure 1. HeLa cells grown in suspension were washed with PBS (4°C) and extracted for 3 minutes on ice with CSK buffer containing 0.5% Triton X-100, and 1.5 mM PMSF to release the SOL fraction. Monolayer cells were extracted in culture plates floating on an ice bath. The volume of CSK buffer was greater than 10 x the volume of the packed cell pellet. Previous work in this laboratory has shown that greater than 97% of the phospholipid is released by this step (Lenk et al, 1977). The nuclei-containing cytoskeletal frameworks (CSKFs) of suspended cells were isolated by centrifugation at 650 x g for 5 minutes (4°C). The supernatant fraction was termed the SOL fraction. The SOL fraction from monolayer cells was removed directly from the culture plate. The cytoskeletal frameworks were transferred to RSB with 1.0% deoxycholate and 0.5% Tween-40 either by resuspending the pellet directly or removing the CSKFs from the culture plate with a rubber policeman in RSB containing the detergents. The nuclei and associated intermediate filaments were isolated by homogenizing the structure in RSB-double detergent with 4 strokes of a teflon-coated pestle in a glass Potter homogenizer and depositing the nuclei at 1000 x g for 5 minutes. Alternatively, the extracted cells were not removed from the substrate and the salt-labile proteins of the CSKF and non-core histones were extracted by Extraction Buffer for 5 - 15 minutes at 4°C. The supernatant fraction, containing most of the extractable actin, all of the polyribosomes and approximately 25% of the protein was termed the CSK fraction.

The non-adherent nuclear structures were resuspended in Digestion Buffer with 0.5% Triton X-100 and 1.5 mM PMSF. The nuclear matrix-intermediate filament scaffold NM-IF was obtained by digesting both DNA and RNA in situ with 200 μg/ml DNase I and 200 μg/ml RNase A for 25 min at 23°C. Extraction with 250 mM $(NH_4)_2SO_4$ for 5 minutes at 23°C released the nucleic acids, chromatin-associated proteins and hnRNP-associated proteins comprising the CHR fraction. The NM-IF of non-adherent cells was isolated by centrifugation at 2000 x g. For analysis by gel electrophoresis, the NM-IF pellet was resuspended directly in the urea-containing IEF Sample Buffer and solubilized by brief sonication while on ice. The RNP-containing nuclear matrix (RNP-NM) was obtained by removing only DNA with 200 μg/ml RNase-free DNase I in place of the DNase/RNase mixture and extracting chromatin-associated proteins as above.

Drosophila cell fractionation. The variation applied to the Drosophila cells was found optimal for preserving nuclear architecture as well as for enabling biochemical analysis of the nuclear matrix. Drosophila cells were washed twice with ice cold PBS then resuspended in CSK buffer containing a mixture of freshly prepared protease inhibitors (15 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 25 μM pepstatin from Boehringer Mannheim) with 0.5% Triton X-100. The Schneider L-2 Drosophila cells appeared to require a broader range of protease inhibitors. Extraction of the soluble fraction (SOL) was complete after three min at 4°C. The resulting nuclei-containing cytoskeletal frameworks were gently pelleted (500 x g) and resuspended in digestion buffer with 100 μg/ml RNAse-free DNAse I and the preparation lightly digested for 5 min at 23°C. 1 M $(NH_4)_2SO_4$ was then added to a final concentration of 0.25 M and the cytoskeletal proteins extracted for 5 min at 23°C. The partially digested nuclei were pelleted (800 x g) then resuspended in digestion buffer with 100 μg/ml each DNAse I and RNAse. After a 15 min digestion at 23°C the chromatin was removed by the addition

of 1 M $(NH_4)_2SO_4$ to a final concentration of 0.25 M followed by extraction at 23°C for an additional 5 min. The resulting subcellular structures, also referred to as the nuclear matrix-intermediate filament scaffold (NM-IF), were pelleted in a microfuge and the two ammonium sulfate-nuclease supernatant fractions were pooled before analysis as the cytoskeletal-chromatin (CSK-CHR) fraction.

Electron Microscopy

Materials. All solutions were sterilized and filtered through 0.22 μM Millipore filters. Anhydrous organic solvents were stored in the presence of suitable drying agents. Reagents and supplies were obtained from Ted Pella, Inc. or Polysciences, Warrington, PA.

Scanning electron microscopy. HeLa cells (CCL 2.2) were grown on sterilized, 5 x 5 mm cover glass and used while subconfluent. The cells were treated as described in the text and figure legends then fixed in 2% glutaraldehyde in the appropriate buffer or PBS for 30 minutes at 4°C. The structures were washed three times in 0.1 M sodium cacodylate, pH 7.2 (5 min/wash, 4°C), briefly postfixed with 1% OsO4 in 0.1 M sodium cacodylate for 3 min at 4°C and subsequently washed in 0.1 M sodium cacodylate. The fixed cells were dehydrated through a series of increasing ethanol concentrations ending with three changes of 100% ethanol. The sample was dried through the CO_2 critical point using a SamDri Model LSI 120 fitted with dehydrating filters on the CO_2 inlet. The cover glass fixed onto a mounting stub with conductive paint, thinly sputter coated with gold/palladium and viewed in the lower stage of an ISI model DS 130 scanning electron microscope.

Whole-mount transmission electron microscopy. HeLa cells (CCL 2.2) were grown on formvar and carbon coated gold grids and used while subconfluent. The cells were treated and fractionated as described in the text and figure

legends. The resulting structures were fixed in 2% glutaraldehyde in CSK buffer for 30 min at 4°C, washed in sodium cacodylate, post-fixed and dried through the CO_2 as described under Scanning Electron Microscopy. The extracted whole mounts were lightly carbon coated and viewed directly after critical point drying as above. Typically, 24 to 100 individual cells were examined on 3 to 6 grids.

Resinless section electron microscopy. Suspension-grown HeLa or loosely adherent monolayers of Drosophila cells were harvested and fractionated as described except the NM-IF structures were harvested by gentle centrifugation (500 x g) for 15 minutes. The NM-IF structures were fixed in 2% glutaraldehyde in CSK buffer for 30 min at 4°C. The structures were washed three times in 0.1 M sodium cacodylate, pH 7.2 (5 min/wash, 4°C), briefly postfixed with 1% OsO_4 in 0.1 M sodium cacodylate for 3 min at 4°C and subsequently washed in 0.1 M sodium cacodylate. The fixed structures were allowed to sediment under normal gravity between each step. The specimens were dehydrated through a series of increasing ethanol concentrations ending with three changes of 100% ethanol and processed for embedding in diethylene glycol distearate (DGD, Polysciences Inc.) as described (Capco et al, 1985) by immersing in n-butyl alcohol (n-BA)/ethanol (2:1) then in nBA/ethanol (1:2) followed by four changes in 100% n-BA for 15 min each. The structures were transferred to DGD through a series of n-BA/DGD mixtures of 2:1 then 1:2 for 10 min each at 60°C followed by three changes of 100% DGD, 1 hr each. Embedded structures were allowed to solidify and DGD blocks were cut using glass knives at an angle of 10° on an MT-2B Porter-Blum ultramicrotome. Sections were placed on parlodion and carbon coated grids. DGD was removed by immersing grids in 100% n-BA at 23°C (3 washes, 1 hr/wash). The grids were returned to 100% ethanol through a graded series of ethanol/n-BA mixtures and dried through the CO_2 critical point. Sections were examined in a JEOL 100 B electron microscope at 80 kv.

Typically, 50 to 300 cells were examined on 3 to 6 separte sections.

Epon-araldite embedment. Samples were fractionated as described, fixed with gluteraldehyde, postfixed with OsO_4, and dehydrated as above. Samples were transferred through a graded series of propylene oxide/resin mixtures (1:3, 1:1, 3:1) to epon-araldite. After infiltration with three changes of the resin, the samples were transferred to fresh resin and allowed to polymerize at 60°C for 48 hours. Thin sections (gold interference) were cut, poststained with 10% uranyl acetate and lead citrate before viewing.

Polyacrylamide Gel Electrophoresis

Standard solutions for polyacrylamide gel electrophoresis. All chemicals used for electrophoresis were obtained from Bio-Rad and were Electrophoresis-Purity reagent grade. Abbreviations used: Bis, N,N'-methylene-bis-acrylamide; 2-ME, 2-mercaptoethanol; SDS, sodium dodecylsulfate; TEMED, N,N,N',N'-tetramethylethylenediamine; Tris, Tris [hydroxymethyl] aminomethane.

Isoelectric Focusing (IEF) Sample Buffer: 9.5 M Urea, 2% (w/v) NP-40, 5% 2-ME, 2% Ampholines (0.4% pH range 3.5-10.0, 1.6% pH range 5-7 or adjusted to match the Ampholines used in the focusing gel). 0.5 ml aliquots were stored frozen at -20°C and thawed immediately before use.

IEF Sample Overlay Solution: 9.0 M Urea, 1% Ampholines as for IEF Sample Buffer, above. 0.5 ml aliquots were stored frozen at -20°C and used for no more than 5 freeze/thaw cycles.

IEF Acrylamide Mixture: 28.38% (w/v) acrylamide, 1.62% (w/v) Bis in deionized, glass-distilled water was stored at 4°C in amber bottles.

SDS Gel Equilibration Buffer: 10.0% (v/v) glycerol, 5.0% 2-ME, 2.3% SDS, 62.5 mM Tris-HCl, pH 6.8. Small volumes (250

ml) were stored at 4°C in amber bottles and used within 3 months of preparation.

SDS Protein Sample Buffer: 10% glycerol, 2% SDS, 5% 2-ME, 50 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue. Stored at room temperature without 2-ME which was added at the time of use.

SDS-Urea Protein Sample Buffer: 9.0 M urea, 2% SDS, 5% 2-ME, 50 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue. Used for analysis of intermediate filament-containing or nuclear matrix-containing fractions. Stored at room temperature without 2-ME which was added at the time of use.

SDS Glycine Electrode Buffer: 200 mM glycine, 0.1% SDS, 25 mM Tris-HCl, pH 8.3. Prepared as a 5X stock, stored at room temperature.

SDS Acrylamide Mixture: 30.0% (w/v) acrylamide, 0.8% (w/v) Bis prepared in deionized, glass distilled water. Stored at 4°C in amber bottles.

Electrophoresis in SDS-containing, polyacrylamide gels. Unless otherwise stated, the electrophoretic separation was conducted with gels containing 10% acrylamide. The stacking gel contained 5% acrylamide. With the exceptions of these modifications, the method was essentially identical to that described by Laemmli (1970) but performed in slab gels. The separating gel solution, containing 10% acrylamide (added from the SDS Acrylamide Mixture), 1% SDS, 0.5 M Tris-HCl, pH 8.8 was degassed under vacuum for 1 minute before adding ammonium persulfate to 0.05% (from a freshly prepared 10% solution) and 0.05% tetramethylene TEMED. Slab gels (12 cm x 12 cm x 1.5 mm) were formed in a Hoeffer multiple gel apparatus. After polymerization under an overlay of 0.1% SDS, a 3.5 cm separating gel (with slot-formers when appropriate) was poured. The stacking gel, containing 5% acrylamide (from the SDS Acrylamide Mixture), 1% SDS, 0.125 M Tris-HCl, pH 6.8 was polymerized after the addition of 0.1% ammonium persulfate and 0.05% TEMED.

Protein samples in SDS Protein Sample Buffer or SDS-Urea Sample Buffer (nuclear matrix-, intermediate filament-

containing samples) were denatured in boiling water for 90 seconds before applying by microliter syringe or micropipette. Without cooling the gel, the samples were electrophoresed through the stacking gel at 100V (initial current of approximately 20 mA per gel) and then separated (after the dye front entered the separating gel) at 150V for approximately 4 hours. Using the Hoeffer multiple gel apparatus, the gels were uniformly immersed in SDS Glycine Electrode Buffer and cooled. Under these conditions, more consistent results were obtained by running the gels under constant current, typically 20 mA per gel for stacking and 30 mA per gel for separating.

IEF polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis. Isoelectric focusing was performed essentially as described by O'Farell (1975). Size-separation (second dimension) was by the method of Laemmli (1970). IEF gels were prepared in siliconized 13.5 cm glass tubes with an inner diameter of 2 mm. The polymerized gel contained 9.5 M urea, 3.77% acrylamide, 0.215% Bis, 2% NP-40, 2% Ampholines (for pH 3.5 - 10 separation: 0.4% pH range 3.5-10.0, 1.6% pH range 5-7; for pH 5.0 - 7.0 separation: 0.2% pH range 5-7, 1.8% pH range 3.5-10.0). The gels were prepared by mixing 5.5 g solid urea, 1.33 ml IEF Acrylamide Mixture, 2 ml 10% NP-40, 0.5 ml 40% Ampholine mixture, and 1.95 ml water at 37^{o}C until the urea was dissolved. 0.15% ammonium persulfate was added (from a 10% solution) and the mixture degassed under vacuum for 1 minute with stirring. Polymerization was completed by adding TEMED to 0.07% (7 μl per 10 ml solution). The glass tubes, previously sealed at one end with Parafilm, were filled from the bottom to 11.0 cm with a syringe fitted with a suitable hypodermic needle. An overlay of 8.0 M urea was floated on top of the acrylamide solution before polymerization. After polymerization, the 8M urea overlay solution was replaced with IEF Sample Buffer while preparation continued.

The pH gradient was established by prerunning the gels with fresh IEF Sample Buffer for 200V for 15 minutes, 300V for 30 minutes, and 400V for 30 minutes. 0.01M phosphoric acid was used as the anode solution, extensively degassed 0.02M NaOH was used as the cathode solution. After removing the buffers, up to 200 μg protein in up to 40 μl IEF Sample Buffer was loaded. If necessary, protein samples were concentrated by precipitation at -20°C with 5 volumes of ice-cold acetone. Protein samples were treated with 10 - 200 μg/ml RNase A (DNase I was included if nuclear samples were analyzed) at a step prior to concentration and electrophoresis. 10 μl IEF Sample Overlay Solution was added and the sample was electrophoresed at 400 V for typically 6800 volt-hours (5000 - 10,000 limits). Isoelectric focusing standards were electrophoresed in parallel where indicated. The gel containing the standards was extruded and stained with 0.1% Coomassie Brilliant Blue-R in 50% methanol, 7% acetic acid and then destained in the same buffer until the background was clear.

The IEF tube gels were extruded under air pressure applied with a syringe and tubing attachment into 10 volumes of SDS Gel Equilibration Buffer followed by agitation for 30 minutes at room temperature. The gel was affixed to the 5% stacking gel of the SDS polyacrylamide gel system with a solution of 10% agarose containing 2% 2-ME and 0.01% bromphenol blue. ^{14}C-labeled molecular weight standards were prepared in advance by mixing an appropriate amount of standard in the acrylamide solution used to prepare the IEF tube gels. The acrylamide was polymerized in glass tubes, extruded, cut into 0.5 cm sections and then stored at -70°C until use. The gel slices were removed and equilibrated briefly with SDS Gel Equilibration Buffer before being attached to the SDS polyacrylamide gel system with the IEF tube gel. This method of obtaining molecular weight standards yielded the most reproducible results for two-dimensional gel electrophoresis.

Immunoblot analysis. Two-dimensional gel electrophoresis was performed using approximately 200 μg protein. The proteins from each gel were transferred overnight onto two sheets of nitrocellulose paper as described by Towbin (1979). The nitrocellulose sheet in contact with the gel was incubated for 12 h in a 2% solution of hemoglobin in PBS (containing 0.01% sodium azide) at 4°C, rinsed three time with PBS, and incubated for 2 h at room temperature with the appropriate antibody diluted in PBS. Excess antibody was removed with four 15 minute washes in PBS at room temperature. The sheet was then incubated for one hour at room temperature with a 1:400 dilution of goat anti-mouse (or goat anti-rabbit as required) IgG conjugated to horseradish peroxidase (Cappel Laboratories, Cochranville, PA), washed with four 15 min changes of PBS at room temperature, and then developed in 0.4 mg/ml 4-chloro-1-napthol with 0.01% (vol/vol) H_2O_2. Proteins were visualized on the second nitrocellulose sheet by staining the sheet with amido black (Fisher) in 50% methanol, 7% acetic acid for 1 h then destaining with 50% methanol, 7% acetic acid until background staining was eliminated.

Fluorography. After separation by gel electrophoresis, proteins labeled with ^{35}S, ^{14}C or ^{3}H were visualized by the fluorographic method of Bonner and Laskey (1974). With constant agitation, 1.5 mm thick gels were fixed in 50% methanol, 7% acetic acid for 45 minutes, dehydrated in two changes of dimethyl sulfoxide (technical grade) for 30 minutes each and infiltrated by soaking in 4 volumes of a 20% solution of 2,5-diphenyloxazole (PPO) in DMSO for 1.5 to 3.0 hours. The scintillant was precipitated by immersing the gel in running water for 30 - 60 minutes insuring complete removal of the dimethyl sulfoxide before the gel was dried onto a Whatman filter paper support. The dried gel was exposed to Kodak X-Omat AR that was prefogged sufficiently to yield a linear response to further exposure (Laskey and Mills, 1975). The film was exposed at -70°C. Exposure times

were standardized for ^{35}S-labeled proteins such that two-dimensional electropherograms of a complex mixtures such as the SOL fraction or whole-cell lysates were exposed for 7.2 x 10^6 cpm-hours (24 hours for a 300,000 cpm sample). Unless otherwise stated, exposures of fluorograms in this thesis were prepared by analyzing equal amounts of radioactivity and exposing for a comparable amount of time.

Acetate overlay method for qualitative comparison of two-dimensional gel electropherograms. This procedure was used when the comparison of several electropherograms was required, particularly those prepared under different conditions. The positions of radioactive proteins contained in a single gel was recorded from a series of exposures (typically 3.6, 7.2 and 28.8 x 10^6 cpm-hours) onto a clear acetate overlay. Colors were used to mark prominent proteins that occurred in several cell fractions to serve as reference points. The acetate overlays were then compared between samples when fluorographic spreading or spot density precluded direct comparison of the fluorograms. Typical reference proteins (which are not identified in the figures) included actins, tubulins, constitutive heat shock proteins, non-muscle tropomyosin, α-actininin as well as other unidentified proteins. Molecular weight and isoelectric pH was determined from the distances measured from a well defined origin without mathematical transformations using computer-aided interpolation (Bevington, 1969) with the standard values prepared with each gel.

RNA Isolation and <u>In Vitro</u> Translation

RNA purification. All procedures were conducted with sterile technique. All glassware was sterilized then autoclaved in the presence of 0.1% diethyl pyrocarbonate (DEP). Deionized, glass-distilled water was treated with 0.1% DEP for 12 hours before autoclaving and using to preparing solutions. Yeast carrier tRNA was prepared by

phenol-extracting commercial-grade tRNA (Baker) and removing residual poly(A)+ RNA by two cycles of oligo(dT) chromatography. The purified poly(A)- RNA was further phenol purified as described below. A final solution of 1 mg/ml in 10 mM sodium acetate was stored at -20°C in 1 ml portions. RNA was isolated from cells that were fractionated in the presence of 20 mM vandyl adenosine or 100 units/ml RNasin (Promega Biotec, Madison, WI) as inhibitors of RNase. The cell fractions were made up to 150 mM NaCl, 5-10 mM EDTA, 1% SDS, 100 mM Tris-HCl, pH 7.6, and proteinase K added to 200 μg/ml. If the buffer precluded the use of SDS (potassium present) or prevented phenol extraction (high magnesium concentration), the samples were precipitated in the presence of 200 mM sodium ion and tRNA carrier at 50 μg/ml (if needed) before proteinase K digestion. Proteinase K digestion was conducted at 30°C for 30 min (up to 1 hour at 37°C if necessary). After proteinase K digestion, the samples were phenol extracted essentially as described by Penman (1966) with repeated cycles of extraction with water-saturated phenol containing 0.2% 8-hydroxyquinoline followed by extraction with 30:1 chloroform:isoamyl alcohol.

Polysome analysis. Where indicated, ribosomal RNA was labeled overnight with [5,6-^{3}H]uridine (37 Ci/mmol) at a concentration of 0.1-0.5 μCi/ml. After extraction with buffers containing 20 mM vanadyl adenosine, portions of each sample representing equivalent numbers of cells (up to 2 x 10^7) were applied to a 15-40% linear sucrose gradient in HSB (0.5 M NaCl, 50 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). The high salt concentration in the gradient increased the separation of nontranslated mRNA and mRNA associated with ribosomes. Ribosomes not actively engaged in translation were dissociated into subunits and mRNP was released (Zylber and Penman, 1970). The gradients were centrifuged for 90 minutes at 40,000 rpm in a Beckman SW41 rotor or for 200 minutes at 40,000 rpm in a Beckman SW50.1 rotor at 4°C. Fractions were collected from the bottom of the gradient through a

peristaltic pump while the absorbance at 260 nm was monitored. Radioactive samples were diluted to no more than 10% sucrose for scintillation counting in Hydrofluor (National Diagnostics, Sommerville, NJ).

RNA isolation from sucrose gradients. Before isolation, the fraction collecting apparatus was sequentially cleaned over several hours with solutions of 0.5% SDS, 0.1 N NaOH and DEP-treated water. Fractions were collected of the appropriate size, purified yeast tRNA was added as carrier to approximately 50 μg/ml, and the samples ethanol precipitated overnight at -20°C. The precipitate was pelleted, resuspended in 10 μM sodium acetate, made up to 150 mM NaCl, 5 mM EDTA, 100 mM Tris-HCl, pH 7.6, 1% SDS and digested with 200 μg/ml proteinase K at 30°C before further purification as described. Typically, material from 2 x 10^7 HeLa cells was fractionated in a Beckman 15 ml, SW41 centrifuge tube and yielded 8 μg polysomal poly(A)+ RNA, 2 μg poly(A)+ RNA in the postpolysomal region (<80S), and 3 μg poly(A)+ RNA in the SOL fraction (> 95% sedimenting slower than 80S).

Poly(A)+ RNA selection. Poly(A)+ RNA was isolated essentially by the method of Aviv and Leder (1972). Columns containing 0.1 g oligo(dT)-cellulose, Type III, from Collaborative Research (Woburn, MA) were prepared in Pasteur pipettes. Up to 150 μg poly(A)+ RNA was bound by passing 1-2 ml of the RNA mixture in 400 mM NaCl, 10 mM Tris-HCl (pH 7.2), 0.5% SDS four times over the column. The poly(A)+ RNA was eluted with four 0.5 ml fractions of 10 mM Tris-HCl, 0.05% SDS. The eluant was pooled and heated to 65°C for 5' then cooled rapidly in an ice bath. The solution was made to binding conditions and the cycle repeated. The purified RNA was precipitated (in the presence of 50 μg/ml tRNA carrier if needed) from 200 mM NaCl then 300 mM potassium acetate. The purified RNA was stored at -70°C in 10 μM potassium acetate for <u>in vitro</u> translation or 10 μM sodium acetate for other purposes.

Poly(U) hybridization assay for poly(A) content. Poly(A) was measured indirectly by its capacity to protect ^{3}H-poly(U) from RNase A degradation. The procedure used was identical to that described Milcarek et al (1974). Replicate RNA samples were mixed with molar excess ^{3}H-poly(U) in 2X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.4). Secondary structure was disrupted by heating for 10 minutes at 45^{o}C. Hybridization was allowed to continue on ice for 10 minutes then single stranded RNA was digested with pancreatic RNase A and the TCA-precipitable radioactivity determined and compared with a standard series using purified poly(A). The measurement was expressed as equivalent mRNA by assuming that cellular mRNA contains 4% poly(A) by mass. Values in the linear range of the standard curve were averaged. Excess poly(U) was verified by observing a constant amount of protection when additional ^{3}H-poly(U) was added.

In vitro translation. mRNA was translated in vitro using the rabbit reticulocyte lysate system from Bethesda Research Labs (Gaithersburg, MD). The method was based on that developed by Pelham and Jackson (1976). All reactions were performed in 1.5 ml disposable microtubes in a final reaction volume of 10 - 30 μl. RNA samples to be translated were precipitated from potassium acetate and lyophilized from a minimal volume of 10 μM potassium acetate before resuspending in sterile water. [^{35}S]methionine was used to label proteins at approximately 1 μCi/μl (1000 Ci/mmol). Both polysomal and nonpolysomal mRNA was optimally translated in the presence of 1.1 mM Mg^{2+}, 135 mM K^+. Incorporation of [^{35}S]methionine into protein was linear with added purified poly(A)+ RNA up to 100 ng RNA per 10 μl reaction mixture. When total RNA was used, incorporation was linear up to 150 ng equivalent (assuming mRNA contains 4% poly(A) by mass) poly(A)+ RNA per 10 μl reaction.

Translation was performed for 60 min at 30^{o}C during which time the incorporation of [^{35}S]methionine remained linear. Protein synthesis was measured by transferring a 1-2

μl aliquot of the reaction mixture to a 5 ml glass tube then degrading charged tRNA molecules and bleaching the aliquot with 0.5 ml of 0.25 M H_2O_2, 0.5 N NaOH. Bovine serum albumin was added as a carrier to 2 mg/ml and the sample precipitated on ice with 5 volumes of 10% trichloroacetic acid containing 2% hydrolyzed casein amino acids. The precipitate was collected on fiber filters, rinsed with ice cold 10% trichloroacetic acid, 100% ethanol, and air dried before scintillation counting. Reaction mixtures that were not primed with mRNA (blank) received approximately 0.5 μg purified carrier tRNA per 10 μl reaction volume in place of the mRNA.

CHAPTER 3

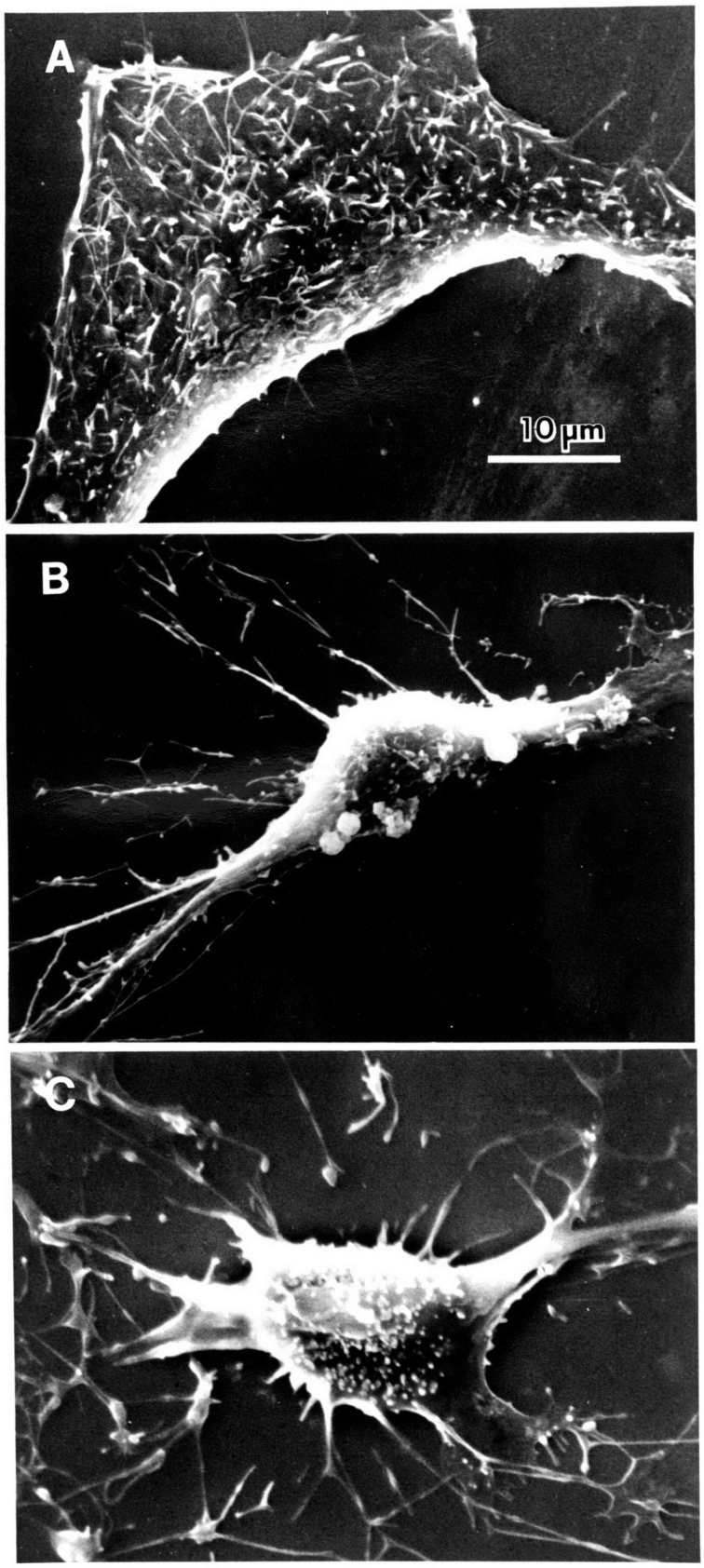
Cytochalasin D Elicits the Release of mRNA from the Cytoskeletal Framework and Inhibits Protein Synthesis

INTRODUCTION

Cellular architecture appears to play a role in eukaryotic protein synthesis. Considerable evidence indicates that eukaryotic polyribosomes are bound to the structural networks of the cell. Biochemical evidence for this association is largely based on detergent extraction which removes the soluble phase from cell structure. In these studies, polyribosomes remain bound to the detergent isolated cell structures (Ben-Ze'ev et al, 1981; Cervera et al, 1981; Fulton et al, 1980; Howe and Hershey, 1984; Lenk and Penman, 1979; Lenk et al, 1977; van Venrooij et al, 1981). There are mRNA molecules free in the cytoplasm but these are not translated (Cervera et al, 1981).

Morphological studies show that the spatial distributions of polyribosomes and mRNA are not random. Polyribosomes in 3T3 cells are preferentially localized in perinuclear regions (Fulton et al, 1980). A discrete distribution of actin, histone and vimentin mRNA in fusing myoblasts has been described (Singer and Ward, 1982; Lawrence and Singer, 1986) as well as a differential localization for histone and actin mRNA in the xenopus oocyte (Jeffery, 1984). Such topographical concentrations are difficult to envision without an underlying, organizing structure to which the polyribosomes and mRNA can bind. Those studies using both detergent extraction and morphological criteria found that the amount and the spatial distribution of polyribosomes were similar before and after the extraction (Fulton et al, 1980). It is, therefore, unlikely that the observed polyribosome binding to skeletal structures is due to extraction artifacts. Other components of the protein synthetic apparatus, including initiation factors (Howe and Hershey, 1984), and an aminoacyl-tRNA synthetase complex (Mirande et al, 1985) also appear structure bound. Furthermore, immunofluorescence experiments have indicated the co-localization of a protein homologous to the cap

Figure 2. Scanning electron micrographs of monolayer HeLa cells treated with CD. Cells were grown on glass coverslips and exposed to DMSO (1.5%) (A), 2 μg/ml CD (B) or 32 μg/ml CD (C) for 30 minutes prior to gluteraldehyde fixation and processing as described in the Materials and Methods. Bar in Figure 2A indicates 10 μm.

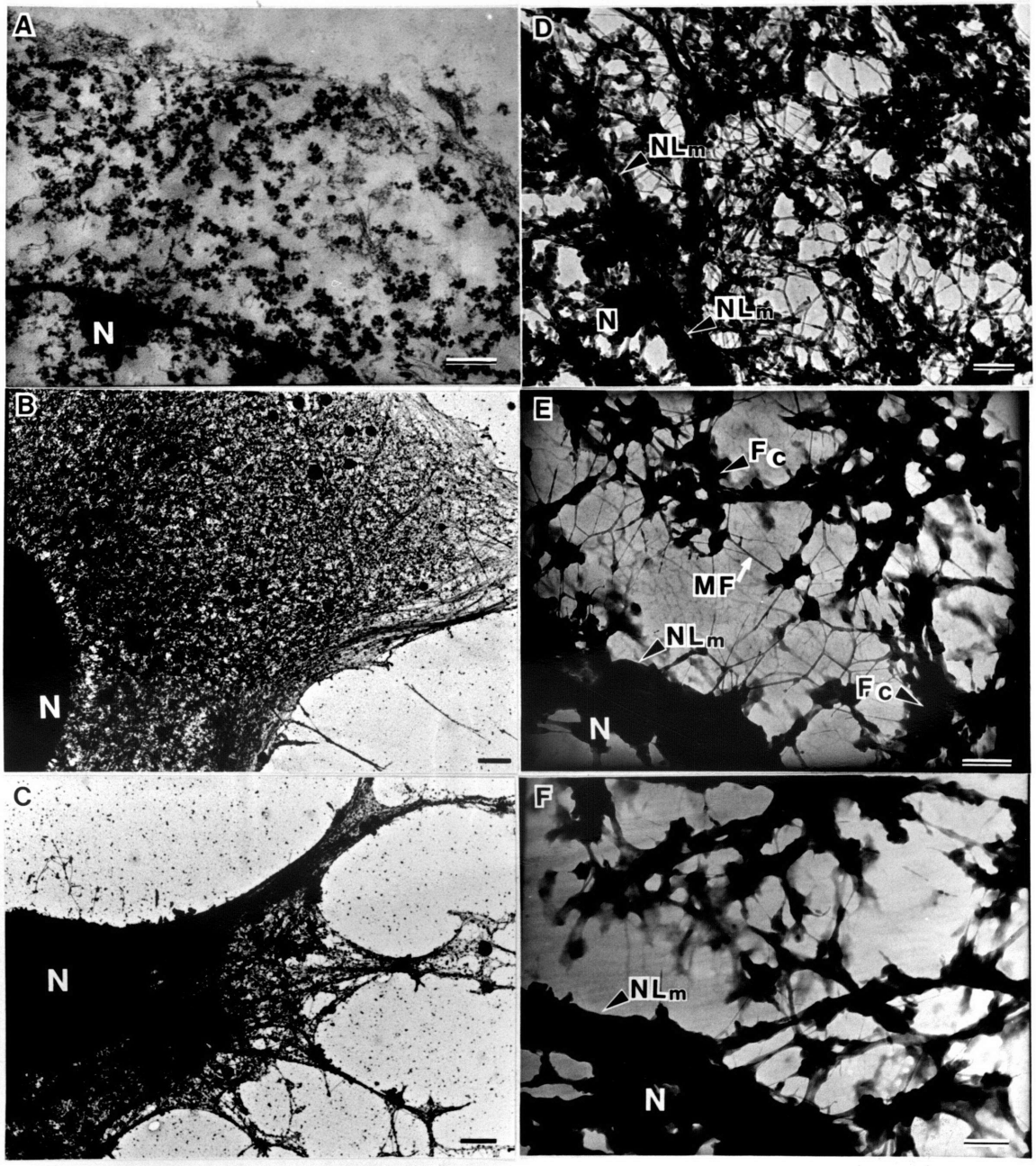

A
10 µm
B
C

binding protein with elements of the cytoskeleton (Zumbe et al, 1982; reviewed in Nielsen et al, 1983).

Some experiments have suggested that the association of polyribosomes with cell structure may be essential for translation in most eukaryotic cells. For example, newly formed VSV mRNA functions while bound to the cytoskeletal framework (CSKF) and ceases translation when released to the soluble phase (Cervera et al, 1981). Maternally inherited mRNAs in the oocyte begin to function only after binding to the newly forming CSKF (Moon et al, 1983).

This chapter examines the association of polyribosomes with the CSKF by using cytochalasin D (CD) to disaggregate polyribosomes and release mRNA. These data show that when mRNA is released from the CSKF it ceases translation. Most important, the mRNA remaining on the CSKF functions at a normal rate. In contrast, the reticulocyte, with its presumably soluble protein synthetic system, shows no sensitivity to the drug.

Figure 3. Transmission electron micrographs of HeLa cells and CSKFs from normal and CD-treated cells. (N) nucleus, (NLm) nuclear lamina, (MF) presumptive microfilaments, (Fc) CD-induced foci. A) Untreated suspension grown HeLa cells were extracted with Triton X-100 in CSK buffer, fixed and processed for epon embedding as described in the Materials and Methods. Bar = 0.2 μm. B) Whole mount view of control monolayer HeLa CSKF. Bar = 5 μm. C) Whole mount view of monolayer HeLa CSKF from cells treated with 2 μg/ml CD for 30 min. Bar = 5 μm. D) Resinless section of suspension grown control HeLa CSKF. Bar = 0.2 μm. E) Resinless section of the CSKF from HeLa cells exposed to 2 μg/ml CD for 30 min. Bar = 0.2 μm. F) Resinless section of the CSKF from cells exposed to 32 μg/ml CD for 30 min. Bar = 0.2 μm.

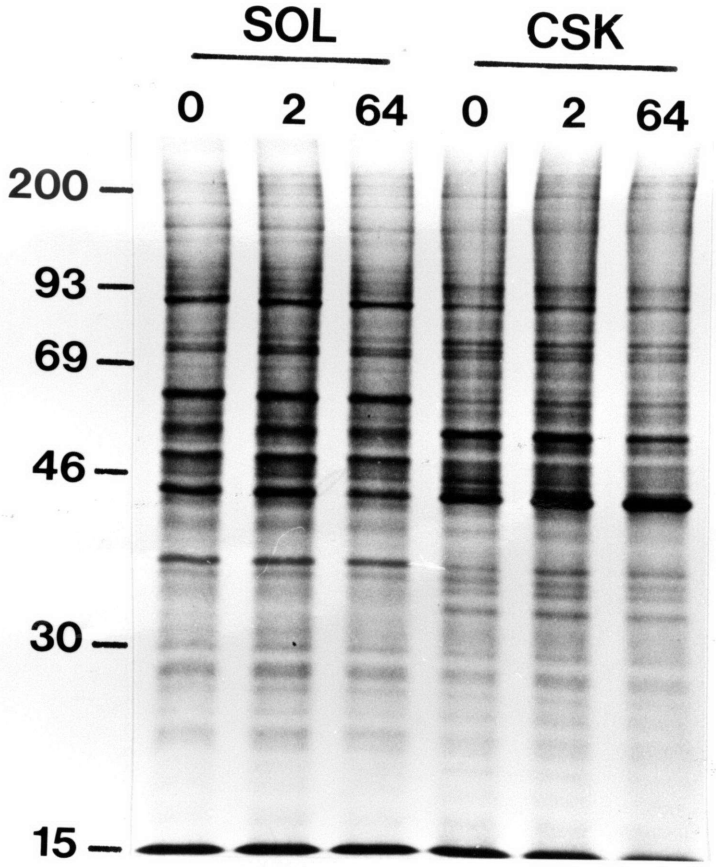

A
N
D
NLm
N
NLm
B
N
E
Fc
MF
NLm
Fc
N
C
N
F
NLm
N

RESULTS

Effect of CD on cell morphology. The fungal metabolite, cytochalasin D, is well known for its profound effects on cell morphology. The perturbations of structure are manifest at concentrations as low as 0.5 - 2 μg/ml (Miranda et al, 1974a; Miranda et al, 1974b; Schliwa, 1982). Higher concentrations of CD have little additional effect on external gross morphology but, as shown here, did further alter the organization of the internal CSKF. Figure 2 shows scanning electron micrographs of HeLa cells exposed to low (2 μg/ml) and high (32 μg/ml) concentrations of CD for 30 min. In both treatments, the cell bodies retracted leaving fine, arborized processes attached to the substrate. The alterations in morphology were almost complete at a drug concentration of 2 μg/ml; higher concentration resulted in only minor additional effects.

Effect of CD on filaments of the CSKF. The altered external morphology of CD-treated cells reflected the reorganization of the internal filamentous CSKF (Miranda et al, 1974b; Schliwa, 1982). Some changes in the fibers of the cytoskeleton, such as the dispersal of actin stress cables, are visible at the light microscope level (Weber et al, 1976). More insight into the effects of the drug required electron microscopy. However, much of the filamentous structure of the CSKF was masked in conventional embedded section electron micrographs (Figure 3A). Embedment-free electron microscopy is more suited to imaging three dimensional fiber networks (Capco et al, 1984; Capco et al, 1982). Fiber networks could be visualized in great detail in unembedded whole mounts of the CSKF provided the cells were relatively flat (Figure 3B). The CD-treated cells were no longer well spread and their CSKF was concentrated near the nucleus. The thickness of these cells precluded effective whole mount electron microscopy (Figure 3C). However, the techniques of resinless sections allowed the organization of

Figure 4. Fluorogram of prelabeled SOL and CSK proteins of HeLa cells exposed to CD. Cells were prelabeled with [^{35}S]methionine, divided into three cultures and exposed to CD at concentrations of 0, 2 and 64 μg/ml for 30 min. The SOL and CSK fractions were prepared as described in the Materials and Methods and equivalent amounts of radioactivity loaded onto the one-dimensional polyacrylamide gel. The concentration of CD used is indicated directly above each lane. The approximate position of radioactive molecular weight markers are indicated (M_r x 1000) at left. The actual radioactivity recovered for all fractions is presented in Table 1.


SOL
CSK
0
2
64
0
2
64
200
93
69
46
30
15

the CSKF from CD treated cells to be examined (Capco et al, 1984).

The CSKF of suspension grown HeLa cells was prepared and processed for resinless section electron microscopy. Control cells were compared to cells treated with either 2 or 32 μg/ml CD for 30 min. The cells were extracted with Triton X-100 in CSK buffer as described in the Materials and Methods. The CSKF was fixed, dehydrated in alcohol and infiltrated with the removable embedding compound, diethylene glycol distearate (DGD). Sections were cut and the DGD removed as described in the Materials and Methods. The self-supporting sections were dried through the CO_2 critical point and viewed directly in the electron microscope.

Figure 3D shows a resinless section image of the control HeLa cell skeletal framework. Although actin is a prominent cytoskeletal protein, the 6 nm microfilaments, marked as 'MF', constituted only a modest portion of the structure. As noted in other reports, there are many structural proteins composing the Triton extracted CSKF (Bravo et al, 1982; Fulton and Wan, 1983; Schliwa and van Blerkom, 1981). This complexity of composition is consistent with the many different structural components visualized here. CD renders the filament networks topographically heterogeneous so that regions rich in filaments alternated with empty areas. Dense foci, with many short filaments, were prominent. These foci were often adjacent to filament-depleted regions. Many fibers coalesced into loose bundles that typically followed the curvature of the nuclear lamina.

The differences observed between the effects of low (2 μg/ml) and high concentrations (32 μg/ml) of CD was one of degree of filament reorganization. In some micrographs, the larger number of short filaments suggested an increased number of free filament ends at the high concentration of CD. Such an increase in apparent ends would be in accord with the postulated action of CD in fracturing actin filaments (Hartwig and Stossel, 1979; Maruyama et al, 1980;

Selden et al, 1980) or capping filament ends (Lin et al, 1980; MacLean-Fletcher and Pollard, 1980). The high drug concentration resulted in more foci and a diminution of filamentous structures, possibly through increased lateral associations of the filaments of the CSKF or perhaps as the result of fiber scissions. The micrographs did not suggest a massive loss of cytoskeletal material. This was corroborated by direct measurements of the protein content in the structural fractions described below.

Protein content and composition of the CSKF from CD treated cells. Treatment of cells with CD had almost no effect on the measured protein content and composition of the CSKF. Suspension grown HeLa cells were prelabeled with [^{35}S]methionine, treated with CD and fractionated as described in the Materials and Methods. The soluble components (SOL) were released with non-ionic detergent extraction. The resulting nuclei-containing CSKFs were further extracted with the mixed detergent, Tween-40 and deoxycholate, in low ionic strength buffer (RSB). This second extraction removed most of the CSKF proteins from the nucleus. The fraction solubilized by the mixed detergent was termed the cytoskeleton (CSK) and it included most of the proteins of the CSKF, including the filamentous actin and essentially all of the cell polyribosomes (Lenk et al, 1977). The separated nuclear (NUC) fraction included the chromatin-associated proteins, hnRNP, and the nuclear matrix (Capco et al, 1982; Herman et al, 1978). As note by others, the intermediate filaments were retained with the isolated nucleus (Bravo et al, 1982; Capco et al, 1982; Fey et al, 1984; Staufenbiel and Deppert, 1982; Woodcock, 1980).

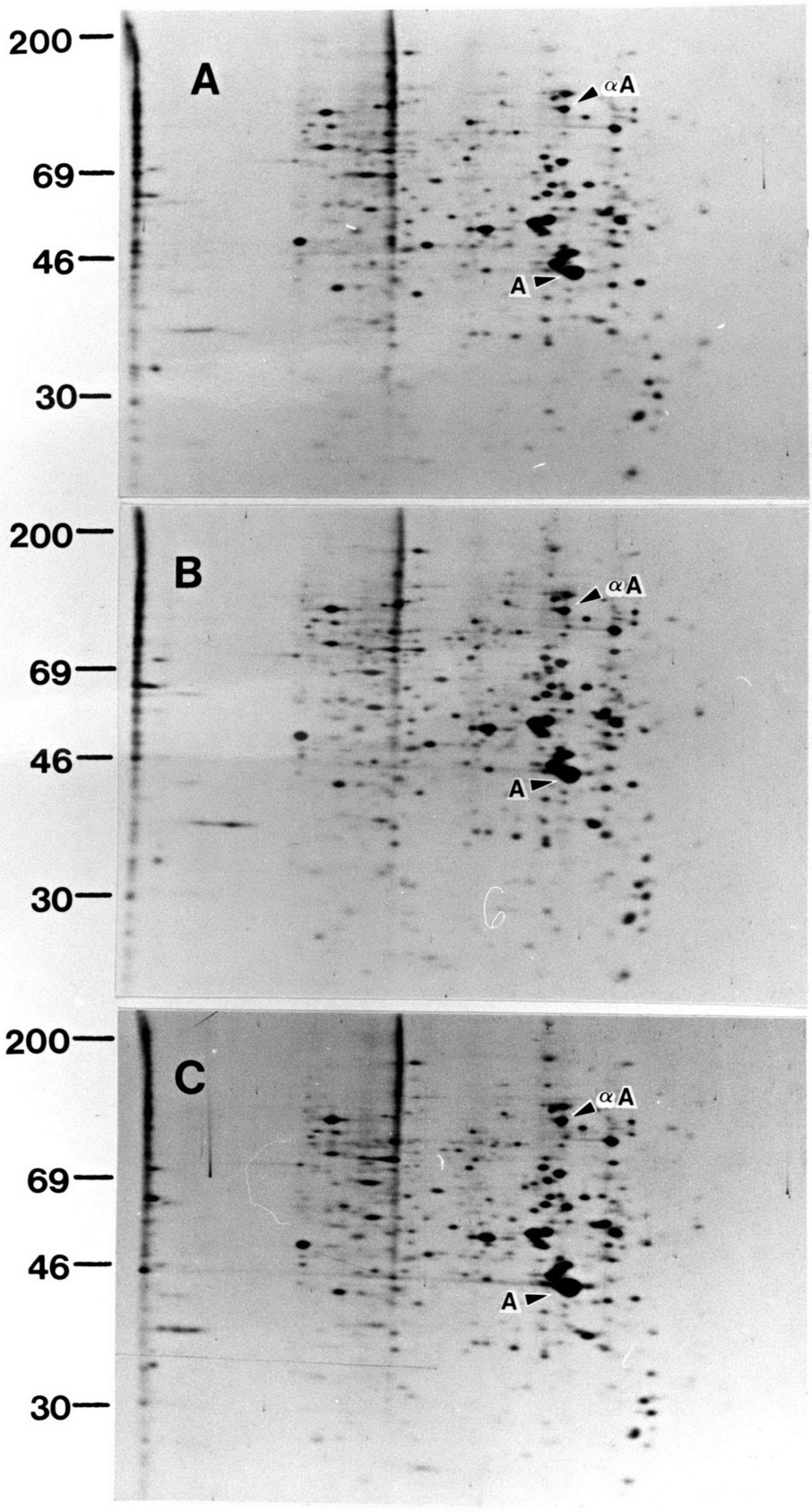
Despite the pathological appearance of CD-treated cells, the data in Table 1 show that there was no loss of total [^{35}S]methionine labeled protein. Therefore, processes leading to significant reduction in cell mass, such as cytoplasmic extrusion and enucleation, were not significant during the course of these experiments. The data in Table 1

Table 1. Distribution of ^{35}S-Methionine labeled proteins following a 30 min exposure to CD[a]

Subcellular Fraction	Conc CD (μg/ml) 0	2	64
SOL	1325	1343	1302
CSK	314	320	296
NUC	146	149	152
Total	1785	1822	1750

[a]Acid precipitable radioactivity (CPM) per 1000 cells.

Figure 5. Two-dimensional gel electropherograms of CSK proteins of HeLa cells exposed to CD. Equivalent amounts of the CSK proteins from the samples prepared for Figure 4 were analyzed by the two-dimensional gel electropherograms shown here. Control cell proteins are seen in panel A, proteins from cells exposed to 2 μg/ml CD in panel B, 64 μg/ml CD in panel C. Actin (A) and alpha-actinin (αA) are indicated based on apparent molecular weight and isoelectric pH (5). The position of radioactive molecular weight markers are indicated (M_r x 1000). Each gel was loaded with approximately 240,000 cpm and the fluorogram slightly overexposed to reveal minor proteins and any subtle changes in the pattern of proteins isolated in the CSK fraction.


200
69
46
30
A
αA
A
200
69
46
30
B
αA
A
200
69
46
30
C
αA
A

further showed that exposure to CD had no gross effect on the composition of the CSK fraction. Electropherograms of the [^{35}S]methionine labeled proteins in the SOL and CSK fractions are shown in Figure 4. The pattern of labeled CSK proteins obtained from cells exposed to 2 μg/ml CD (lane 5) was indistinguishable from the control (lane 4). The CSK proteins obtained from cells treated with 64 μg/ml CD (lane 6) were also similar to the control though two differences were apparent at the high drug concentration. There was an apparent increase in the amount of CSK proteins migrating in the region of actin at 42-44 kD and a small reduction in proteins migrating in the lower molecular weight range, between 18-27 kD. Proteins of this lower molecular weight range include the ribosomal proteins (Lastick and McConkey, 1976). Their decrease may reflect the loss of polyribosomes from the CSKF at the higher concentration of CD (described below).

The CSK proteins were further analyzed by two-dimensional gel electrophoresis. Figure 5 shows the complex pattern of CSK proteins from control and drug treated cells. Actin ('A') and alpha-actinin ('αA') are tentatively identified by their apparent molecular weight and isoelectric pH. Tubulins were largely absent since the microtubules were depolymerized by the cold extraction. The intermediate filament proteins were not present in the CSK fraction since they remained with the nucleus (Bravo et al, 1982; Capco et al, 1982; Fey et al, 1984; Staufenbiel and Deppert, 1982; Woodcock, 1980). The pattern of CSK proteins isolated from cells exposed to CD was the same as the control pattern at both low (Figure 5B) and high (Figure 5C) levels of CD. The diminution of putative ribosomal proteins at high drug levels, noted in the one-dimensional gel, is not seen here because the basic ribosomal proteins do not focus in these equilibrium gels (Lastick and McConkey, 1976).

Release of Polyribosomes from the CSKF by CD. Although CD does not change the protein composition of the CSK

fraction, high levels of the drug resulted in the release of mRNA molecules from the CSKF with the ribosomes apparently still attached (Lenk et al, 1977). This is suggested by the results shown in Figures 6 and 7. The inhibitor of protein synthesis, emetine, was applied to the HeLa cells to prevent the release of ribosomes from mRNA (Grollman, 1968). As reported by others (Cervera et al, 1981; Howe and Hershey, 1984; Lenk et al, 1977; Vazquez, 1979) and illustrated by Figure 6, the inhibition of protein synthesis is not sufficient to release mRNA from the CSKF. However, when the emetine-treated cells were exposed to CD, a large portion of intact polyribosomes were released in the initial extraction (Figure 7). It was noted that not all of the polyribosomes were released from the CSKF. Indeed, the fraction of intact polyribosomes (which remained on the CSKF) appeared approximately equal to the fractional amount of protein synthesis remaining in these cells.

The quantitative relationship between CSKF-bound polyribosomes and the inhibition of protein synthesis was further explored by first labeling total RNA with [^{3}H]uridine for 12 h. The cells were then exposed to a concentration of CD (8 μg/ml) that would inhibit protein synthesis by 50 per cent. The resulting polyribosome profiles and their partition between the SOL and CSK fractions from control and CD-treated cells is shown in Figure 8. Figure 8A shows that greater than 97% of the polyribosomes were bound to the cytoskeleton framework. This value is in agreement with previous results (Cervera et al, 1981; Lenk et al, 1977). Polyribosomes from cells exposed to 8 μg/ml CD are shown in Figure 8B. This concentration of CD inhibited protein synthesis by approximately 50%. The amount of ribosomes in polyribosomes was also reduced by 50% at this concentration. Although reduced in amount, the size (or sedimentation distribution) of the remnant polyribosomes was unchanged by CD. Few, if any, other inhibitors of protein synthesis produce this type of polyribosome pattern (Vazquez, 1979).

Figure 6. Polyribosomes remain associated with the CSKF in emetine-treated cells. Emetine was applied to monolayer HeLa cells to a concentration of 50 μg/ml and the cells maintained for 30 min at 37°C in the presence of the inhibitor. The SOL and CSK fractions were obtained and material from equivalent numbers of cells (2×10^6) was analyzed by sucrose velocity gradient centrifugation. The optical density at 260 nm was monitored with a continuous-flow ultraviolet monitor as the gradients were emptied from the the bottom. The direction of sedimentation in the gradients is indicated by the arrow along the ordinate.

Figure 6

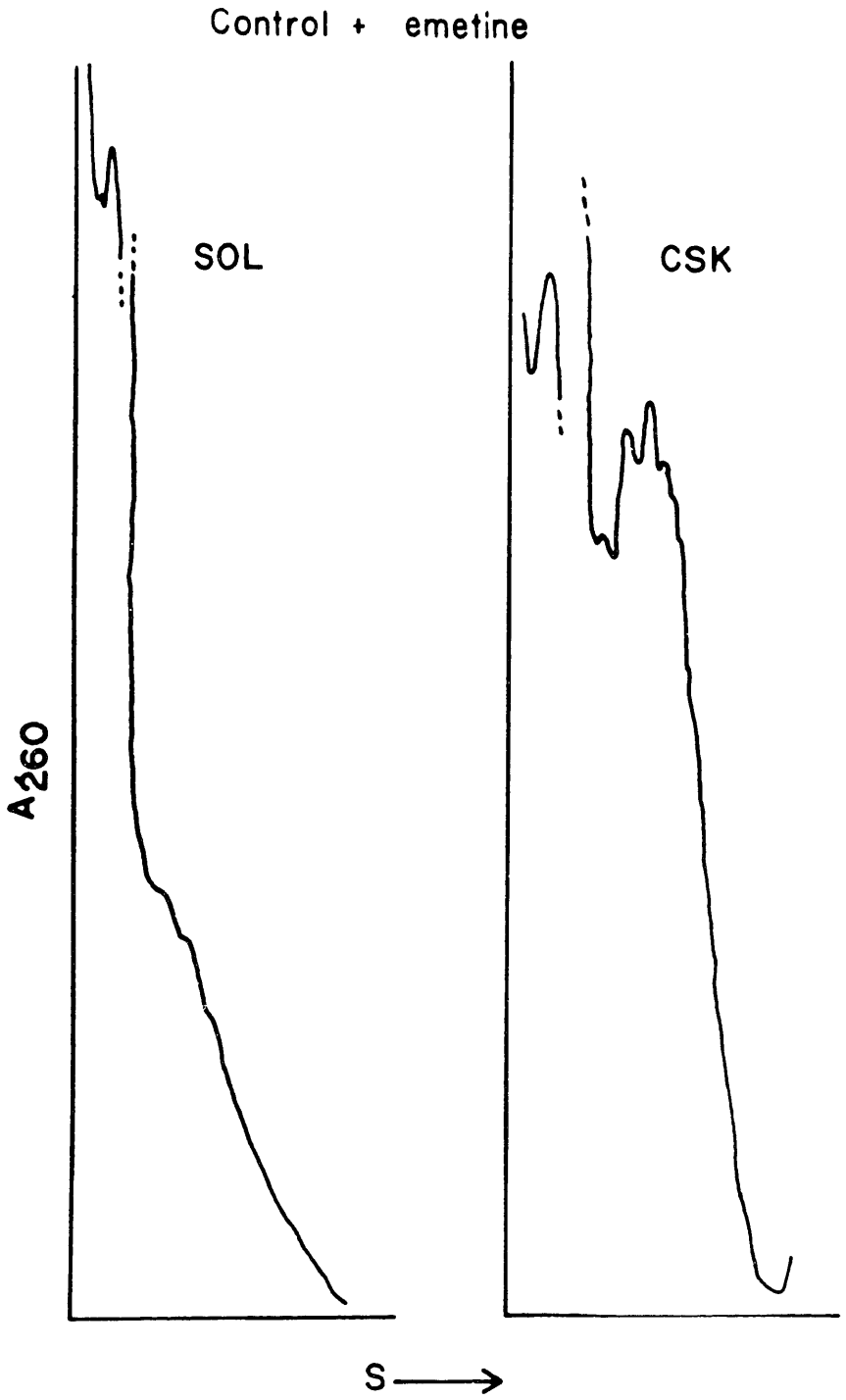
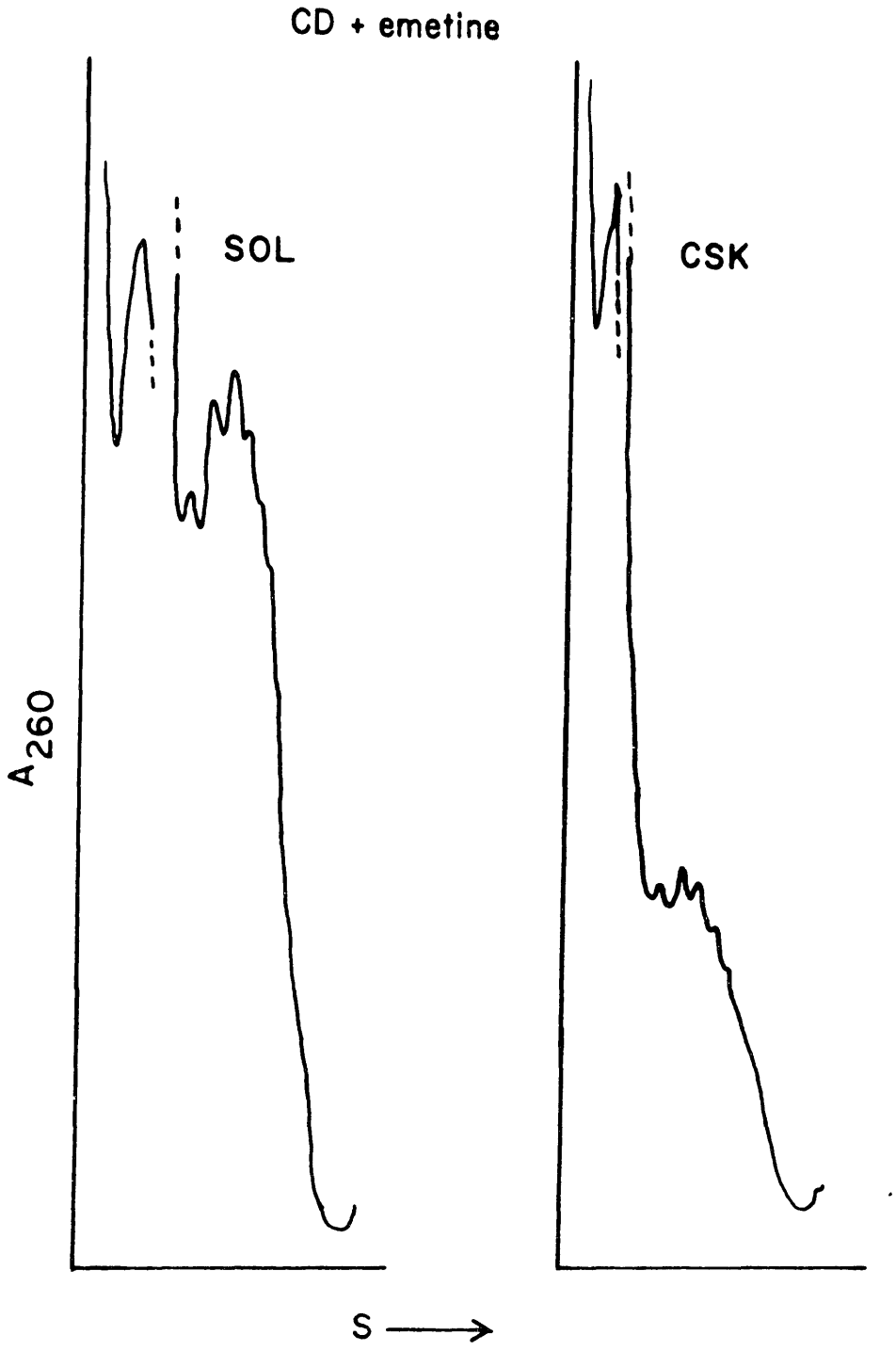


Figure 7. CD effects the release of intact polyribosomes from the CSKF in emetine-treated cells. Emetine was applied to monlayer HeLa cells to a concentration of 50 μg/ml and the cells maintained for 10 min at 37°C before CD was added to 25 μg/ml. The cells were incubated at 37°C in the presence of both drugs for an additional 20 min. The SOL and CSκ fractions were isolated and analyzed by sucrose velocity gradient centrifugation. The optical density at 260 nm was continuously monitored as the gradients were emptied from the bottom. The profiles were obtained from material derived from equal numbers of cells (2×10^6). The direction of sedimentation is indicated by the arrow along the ordinate.

Figure 7

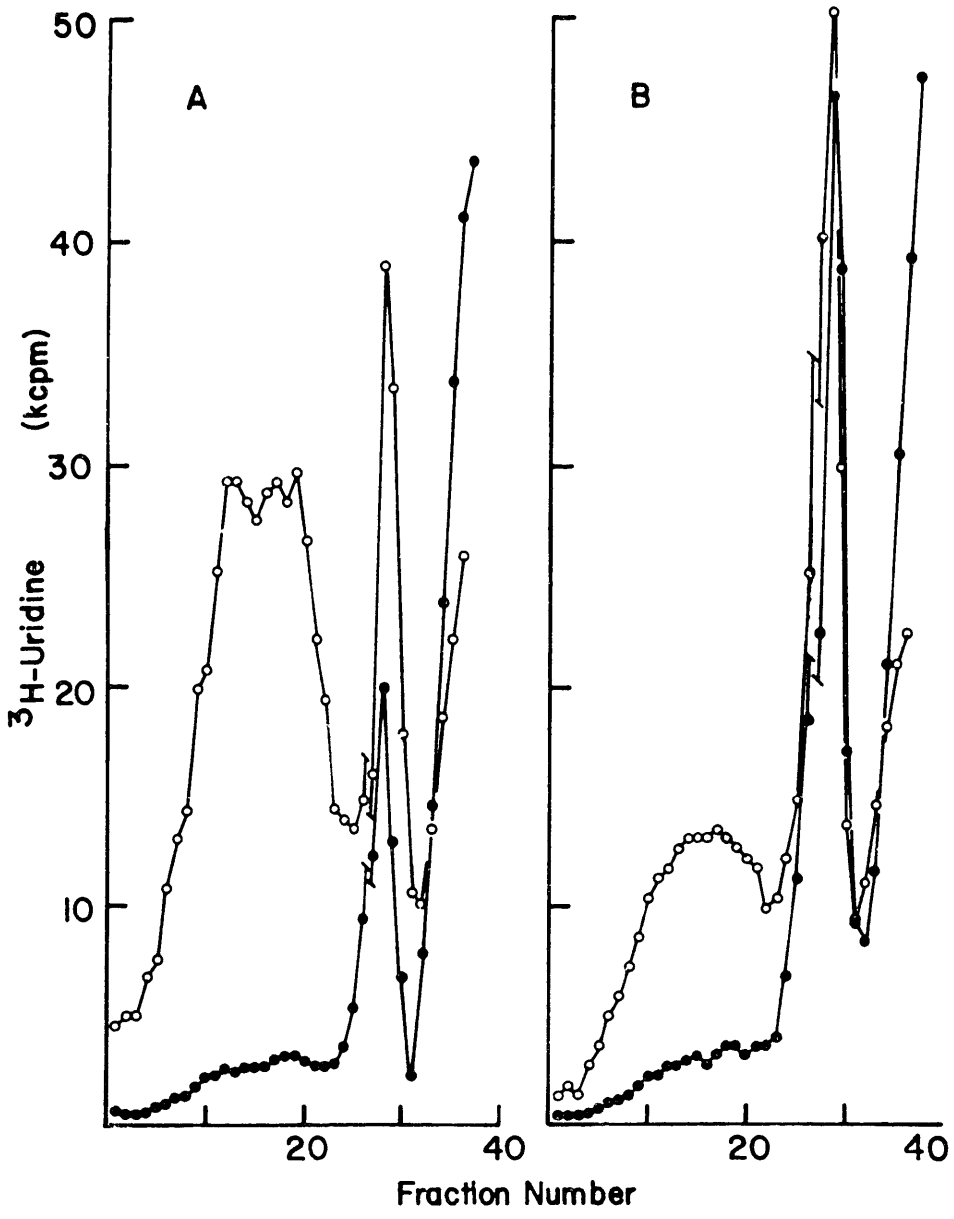


The effect of CD on both protein synthesis and polyribosomes is rapidly and completely reversible. Figure 9 compares the polyribosome profiles of cells exposed to 64 μg/ml of CD to those obtained 20 min after the removal of the drug. The amount of polyribosomes in the recovering cells returned nearly to that in the control cells and were associated with the CSKF. The recovery of protein synthesis and the reappearance of polyribosomes occurred before the cells regained normal gross morphology (data not shown).

Inhibition of protein synthesis by CD. Figure 10 shows a typical time course of inhibition of protein synthesis by CD. Cells were exposed to 8 μg/ml CD and pulse-labeled with [^{35}S]methionine at the indicated times. The inhibition of protein synthesis was complete by 15 min and remained constant over the time examined (60 min). The dose-dependent inhibition of protein synthesis by CD in subconfluent monolayer cultures is shown in Figure 11. The cells were washed and maintained in media containing CD at the concentrations indicated in Figure 11. After 20 min of exposure to the drug, the cells were pulse-labeled with [^{35}S]methionine for 3 min and the pulse terminated by the addition of excess unlabeled methionine. The data in Figure 11 shows that inhibition of protein synthesis became detectable at about 2 μg/ml of CD with a 50% level of inhibition occurring at 10 μg/ml. The inhibition curve is biphasic and can be modeled as the sum of two independent exponential functions. The apparent inhibition constants derived from a least-squares fit of the data are 1.4×10^{-5} M and 10^{-4} M. The inhibition of protein synthesis occurs at concentrations of CD almost an order of magnitude above those required to alter the morphology of the HeLa cell. These levels are in turn, an order of magnitude greater than the concentration of CD known to affect glucose transport in these cells (Miranda et al, 1974a).

Figure 8. Distribution of polyribosomes between the SOL and CSK fractions of control and CD-treated HeLa cells. Monolayer cultures of HeLa cells were labeled with [^{3}H]uridine overnight, exposed to DMSO (A) or 8 μg/ml CD for 20 minutes (B) and fractionated as described in the Materials and Methods. Material from equivalent numbers of cells were analyzed by sucrose velocity gradient centrifugation. SOL fraction: ● , CSK fraction: ○ . Total gradient radioactivity recovered from control cells was 1.29×10^6 cpm, from CD treated cells, 1.23×10^6. Values plotted for fractions above number 26 are scaled down by a factor of 0.6.

Figure 8



Release of mRNA from the CSKF by CD parallels the degree of protein synthesis inhibition. Previous results suggested that polyribosomes are linked to the CSKF by their mRNA (Cervera et al, 1981; Lenk et al, 1977). Other experiments showed that mRNA molecules not bound to the CSKF are also not translated. Taken together, these observations suggested that the binding of mRNA to the CSKF may be obligatory for its translation (Cervera et al, 1981). The experiments presented here show that CD disaggregates a portion of the polyribosomes and removes a fraction of the mRNA from translation. The remaining polyribosomes evidently function normally. This phenomena could be explained if CD released a portion of mRNA from the CSKF and these ceased functioning while the mRNA remaining bound continued to be translated. This hypothesis was supported by the findings described below in which the release of mRNA from the CSKF was shown to closely parallel the inhibition of protein synthesis.

The release of poly(A) containing RNA from the CSK to the SOL fraction by CD was measured. RNA was labeled with [^{3}H]uridine in the presence of either 5-fluorouridine or 0.04 μg/ml actinomycin D to suppress the labeling of ribosomal RNA. Cells were exposed to several concentrations of CD for 30 min. RNA was extracted from the cell fractions, purified and the poly(A) RNA selected with oligo(dT) cellulose as described in the Materials and Methods. Alternatively, unlabeled RNA was isolated from cells exposed to CD and the distribution of poly(A) among the cell fractions assayed by poly(U) hybridization. The poly(U) technique is less sensitive than radiolabeling but eliminates possible complications arising from the inhibitors of rRNA synthesis.

The results of both methods of measuring the location of poly(A) RNA agree and are presented in Table 2. Approximately 75% of the mRNA was associated with the CSKF in control cells. The remaining 25% of poly(A) RNA was in the

Table 2. Distribution of poly(A)+ RNA between the SOL and CSK fractions following a 30 min exposure to CD[a]

Assay conditions	Concentration CD (μg/ml) 0	4	16	64
[^{3}H]U, Actinomycin[b]	78.8	68.6	40.1	17.4
[^{3}H]U, 5-Fluoro-U[c]	85.2	66.3	51.8	12.9
poly(U) hybridization[d]	74.9	61.2	50.0	12.5

[a]Expressed as per cent of the total cytoplasmic poly(A)+ RNA that remains CSK associated.

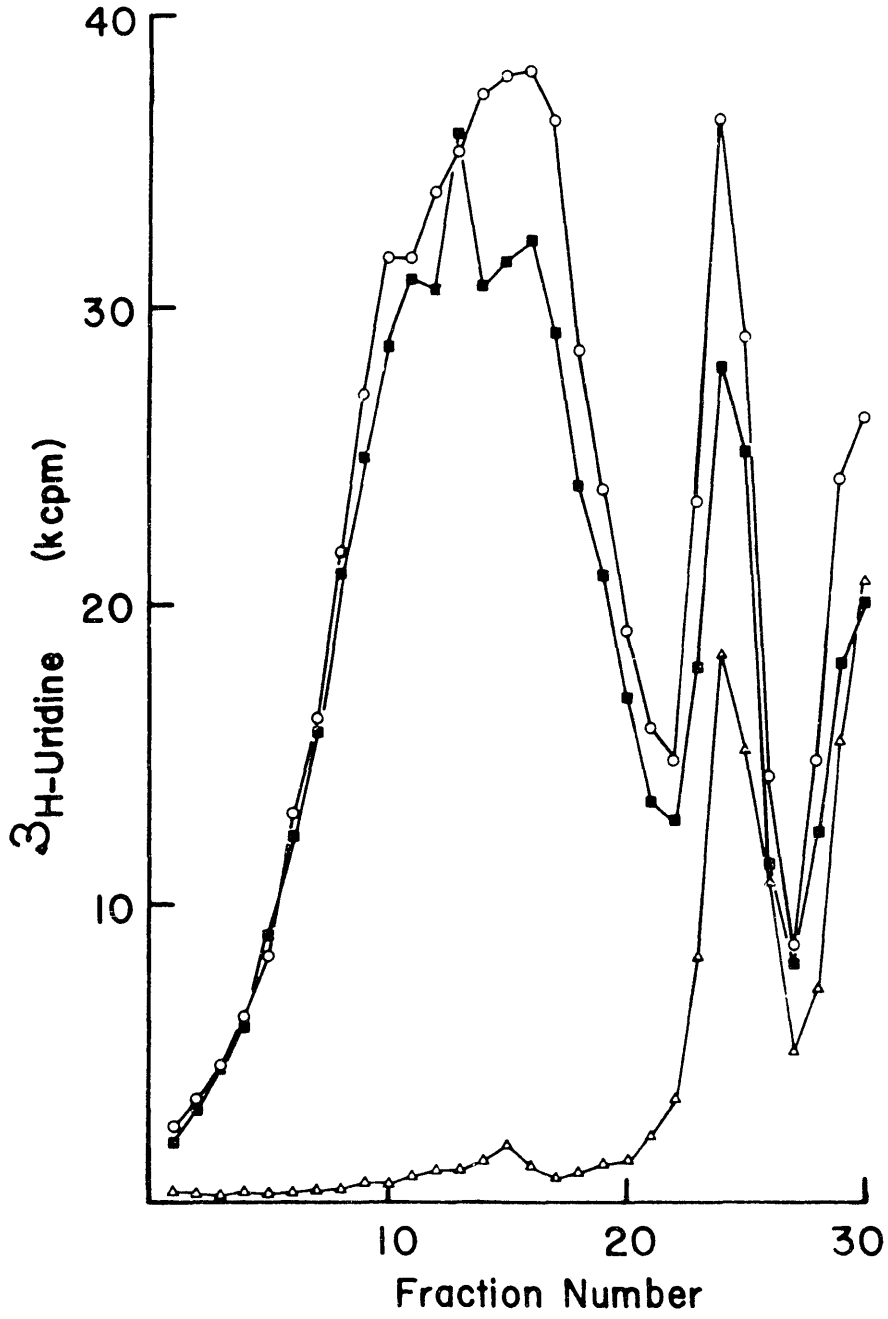
[b][^{3}H]uridine incorporated in the presence of 0.04 μg/ml Actinomycin D.

[c][^{3}H]uridine labeling in the presence of 5-fluorouridine.

[d]No inhibitors, assay by poly(U) hybridization technique.

Figure 9. Recovery of polyribosomes after disruption by high levels of CD. Suspension grown HeLa cells were labeled overnight with [^{3}H]uridine and exposed to to 64 μg/ml CD for 20 minutes. Polyribosomes derived from the CSK fraction of untreated cells (○), cells exposed to the drug (△) and cells 20 minutes after removal of the drug (■) were analyzed by sucrose velocity sedimentation. (As in Figure 8, no polyribosomes were found in the SOL fraction and this fraction is not shown.)

Figure 9

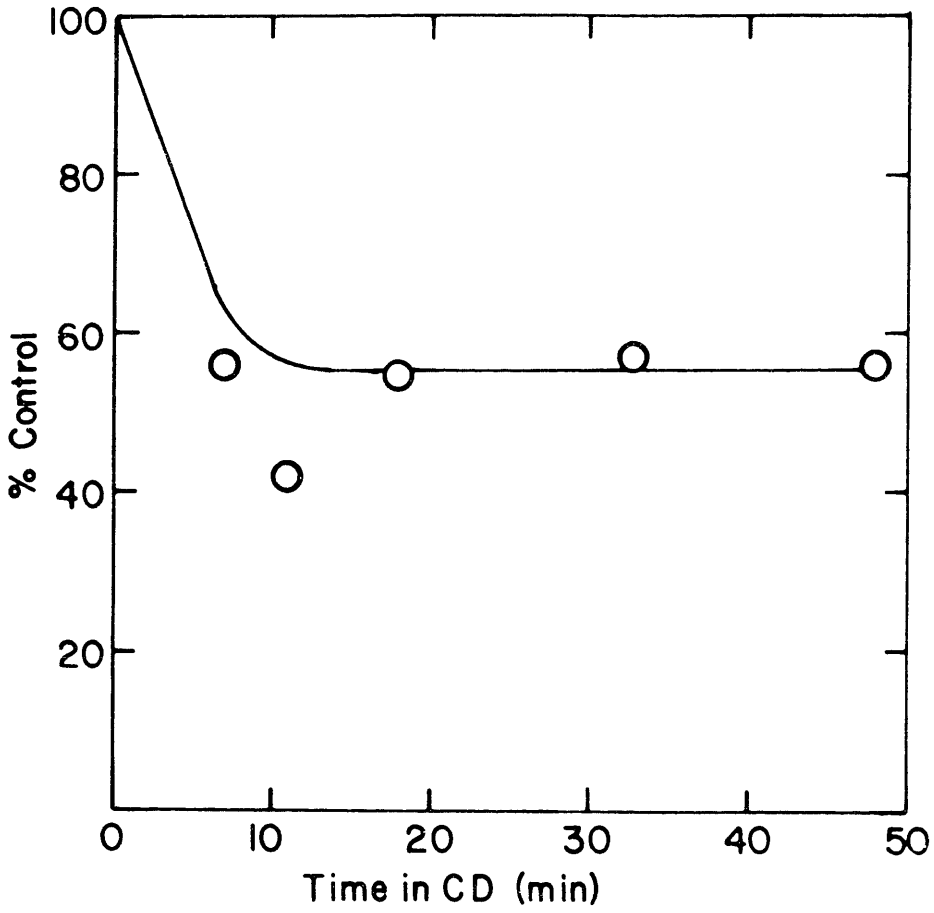


SOL fraction and was not associated with polyribosomes. The data of Table 2 indicate that mRNA was released from the CSKF in the presence of CD in a dose-dependent manner. The amount of mRNA released closely paralleled the inhibition of protein synthesis. The data from several experiments are summarized in the bar graph in Figure 12. At each dose, the proportion of poly(A) retained on the CSKF corresponds to the level of protein synthesis remaining. These data, together with the normal sedimentation profile of the remaining polyribosomes, implies that the CD-induced inhibition of protein synthesis may be the result of the release of mRNA from the CSKF.

A previous report noted that cytochalasin B preferentially dislodges polyribosomes translating a specific lens protein mRNA in lens explants (Ramaekers et al, 1980). No obvious selectivity of mRNA release was found in CD treated HeLa cells in these experiments. The pattern of newly synthesized proteins seen in the fluorogram of Figure 13 was largely unchanged by the presence of CD. Cells were exposed to 4 or 32 μg/ml CD, pulse-labeled in the presence of the drug and fractionated to yield the SOL, CSK and NUC fractions as described in the Materials and Methods. Equivalent amounts of radioactivity were loaded onto each lane of the one-dimensional SDS gel. No marked selectivity in withdrawal of certain mRNA from translation in the presence of CD is apparent. The synthesis of three proteins in the SOL fraction having apparent M_r of 37,000, 49,000 and 82,000 appeared slightly more resistant to the CD-induced inhibition. This was not the case for the majority of proteins and, to the limit of resolution afforded by the one-dimensional gels, mRNA appears to be withdrawn from translation in a uniform manner. The difference from previous findings may reflect the undifferentiated state of HeLa cells as the same investigators found that stably transformed lens fibroblasts no longer exhibited a preferential release of polyribosomes (Ramaekers et al, 1983).

Figure 10. Time course of the CD induced inhibition of protein synthesis. Replicate monolayer HeLa cultures were washed, treated with 8 μg/ml CD and pulse-labeled as described in the Materials and Methods. The time plotted indicates the end-point of a 3 min pulse. The total acid precipitable radioactive protein isolated at the end of the pulses indicated is expressed as a per cent of the untreated value.

Figure 10



Translation of mRNA release by CD. The cessation of translation of mRNA upon release from the CSK could reflect an alteration in the RNA molecules themselves. There is at least one example of changes in the translatability of mRNA accompanying major changes in the rate of protein synthesis. Anchorage-dependent fibroblasts, cultured in suspension, convert their mRNA to a form untranslatable in vitro (Farmer et al, 1978). The mRNA released from the CSKF by CD was tested for possible alterations in translatability. RNA was prepared from the SOL and CSK fraction of cells exposed to 50 μg/ml CD for 30 min. Poly(A) RNA was purified by oligo(dT) selection and the amount of poly(A) present in each fraction assayed by the poly(U) hybridization technique. Equivalent, non-saturating amounts of poly(A) mRNA were added to a reticulocyte-derived, cell free translation system. Table 3 shows the stimulation of [^{35}S]methionine incorporation into acid precipitable material by the added poly(A)-containing RNA. The results are slightly complicated by the prior existence of mRNA of low relative translatability in the soluble phase (SOL fraction) which mixes with the mRNA released from the CSKF. When suitable corrections are made, the mRNA released into the soluble phase by cytochalasin showed an unchanged translatability.

The observation of mRNA of poor translatability in the SOL fraction in control cells confirms a previous observation (Cervera et al, 1981) that there are in all cells, mRNA molecules in the SOL fraction that do not translate efficiently. These poorly translated molecules closely resemble the active mRNA on the CSKF in gross properties and are revealed only when the separation into CSK and SOL mRNA is made. The CSK mRNA was translated greater than four-fold more efficiently than an equivalent amount of SOL mRNA. In this experiment, the translational efficiency of the CSK bound, poly(A)+ RNA was comparable to that of globin mRNA. Treatment with CD releases fully translatable CSK bound mRNA into the pool of poorly translatable SOL mRNA forming a

Table 3. *In vitro* translation of poly(A)+ RNA isolated from CD treated cells[a]

Source of poly(A) RNA	Subcellular Fraction		
	SOL	CSK	"Globin"
Control	714	3303	--
50 μg/ml CD	1845	1058	--
Globin[b]	--	--	3659
Globin + CD[c]	--	--	3602

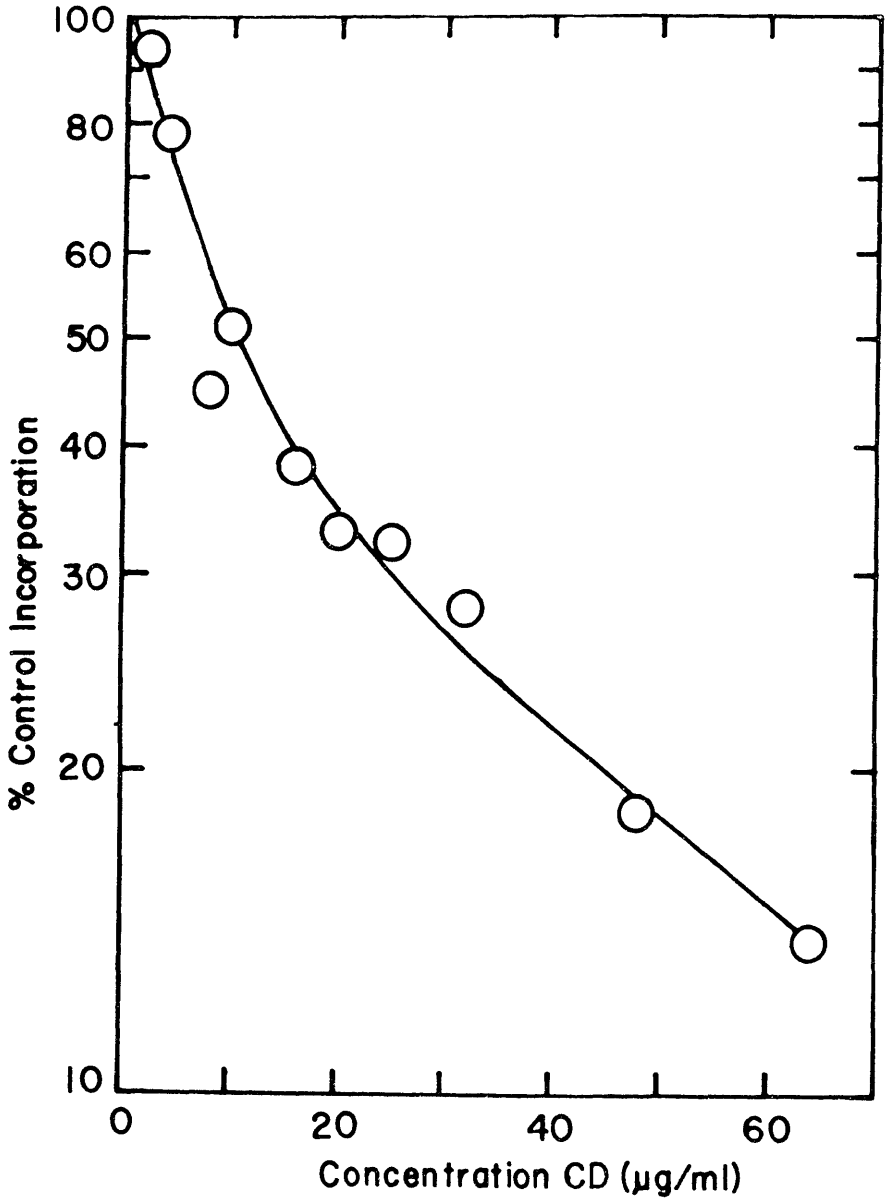
[a]Acid precipitable [^{35}S]methionine incorporated above background per ng added poly(A)+ RNA.

[b]Incorporation directed by an equivalent amount of purified globin mRNA.

[c]Incorporation directed by an equivalent amount of globin mRNA in the presence of 50 μg/ml CD in the reticulocyte translation mixture.

Figure 11. Dose dependent inhibition of protein synthesis by CD. Monolayer cultures of HeLa cells were exposed to concentrations of CD as indicated on the ordinate for 20 min and the level of protein synthesis determined by a pulse-label with [^{35}S]methionine. The total acid-precipitable radioactivity is plotted as a per cent of the untreated sample. The inhibition curve drawn through the data represents the best fit obtained by the method of least-squares fit to a biphasic function comprised of the sum of two independent exponential components. The exponential constants obtained by this manner (apparent kD of inhibition) are 7 and 54 μg/ml or 1.4×10^{-5} and 10^{-4} M.

Figure 11


100
80
60
50
40
30
20
10
% Control Incorporation
0
20
40
60
Concentration CD (µg/ml)

mixture that is less active than pure CSK message but much more active (3-fold) than SOL mRNA alone.

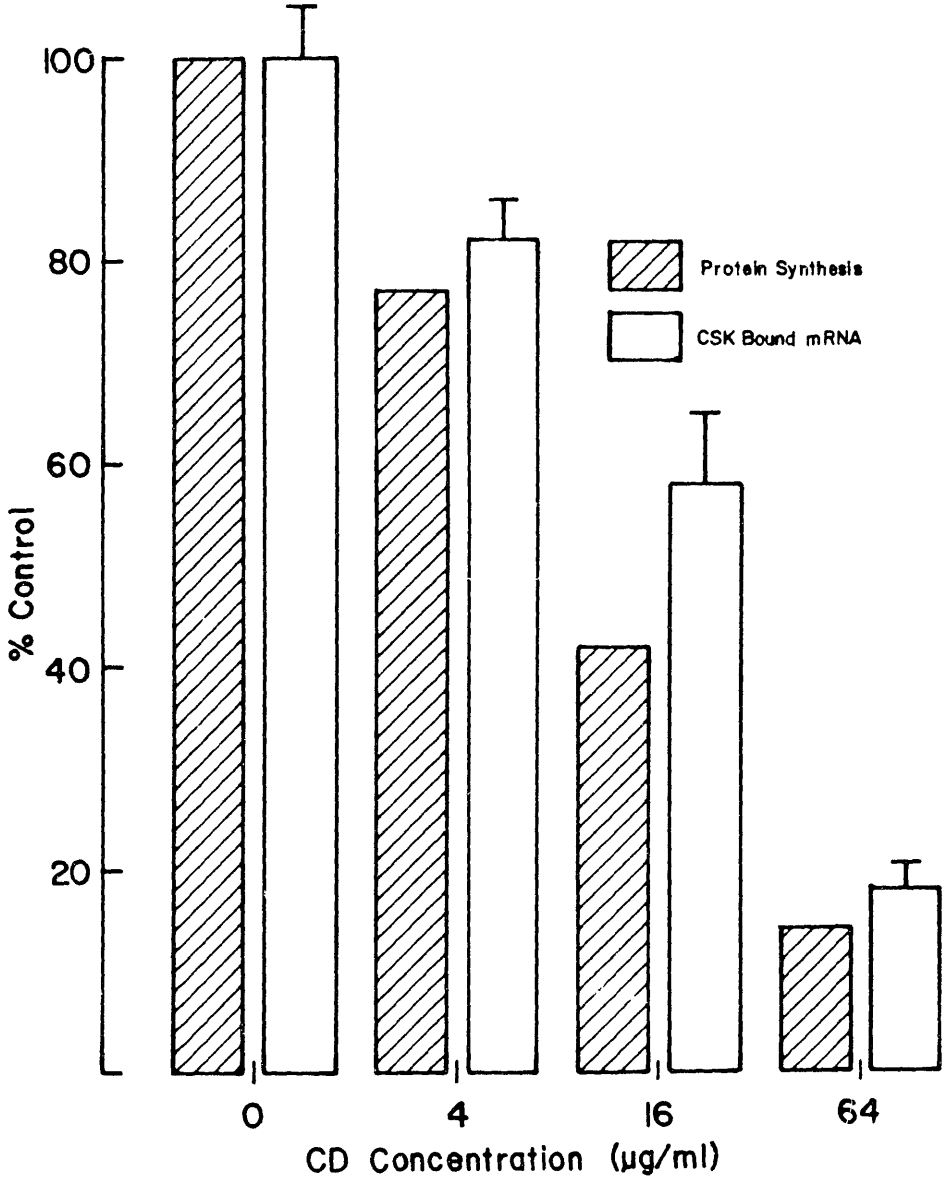
The data in Table 3 also shows that CD has no direct effect on cell-free protein synthesis. The addition of CD directly to the reticulocyte-derived, in vitro translation system shows that CD is not a direct inhibitor of translation. The total acid precipitable material obtained after translation of globin mRNA is unaffected by the presence of 50 μg/ml CD in the translation mixture. Similar results were obtained when HeLa mRNA was used (data not shown). This result suggests that CD does not act specifically at the level of the ribosome-mRNA interaction.

Insensitivity of the reticulocyte to inhibition by CD. CD clear inhibits protein synthesis in HeLa cells which have mRNA associated with the CSKF. It was of interest to examine what effect the drug would have on protein synthesis in a cell which has no apparent CSKF (Rifkind et al, 1969). The effect of CD on protein synthesis in intact rabbit reticulocytes was determined. Reticulocytes were prepared from anemic rabbits and used within 16 h of isolation at which time more than 99% of the cells excluded trypan-blue. The reticulocytes were washed in PBS and diluted in serum-free MEM with 1/10 the normal amount of methionine. CD was added in DMSO carrier as indicated in Figure 14. The cells were pulse-labeled with [^{35}S]methionine after a 30 min exposure to the drug. Suspension cultured HeLa cells were treated in a similar manner. Figure 14 shows the acid precipitable radioactivity expressed as a per cent of the untreated culture in the two cell types. HeLa cells show the expected inhibition of protein synthesis. In contrast, the rabbit reticulocytes show no detectable inhibition of protein synthesis over the range of concentrations of CD tested.

The data of Figure 15 indicate that the incorporation of [^{35}S]methionine into acid-precipitable material did in fact reflect protein synthesis. In Figure 15, the number of

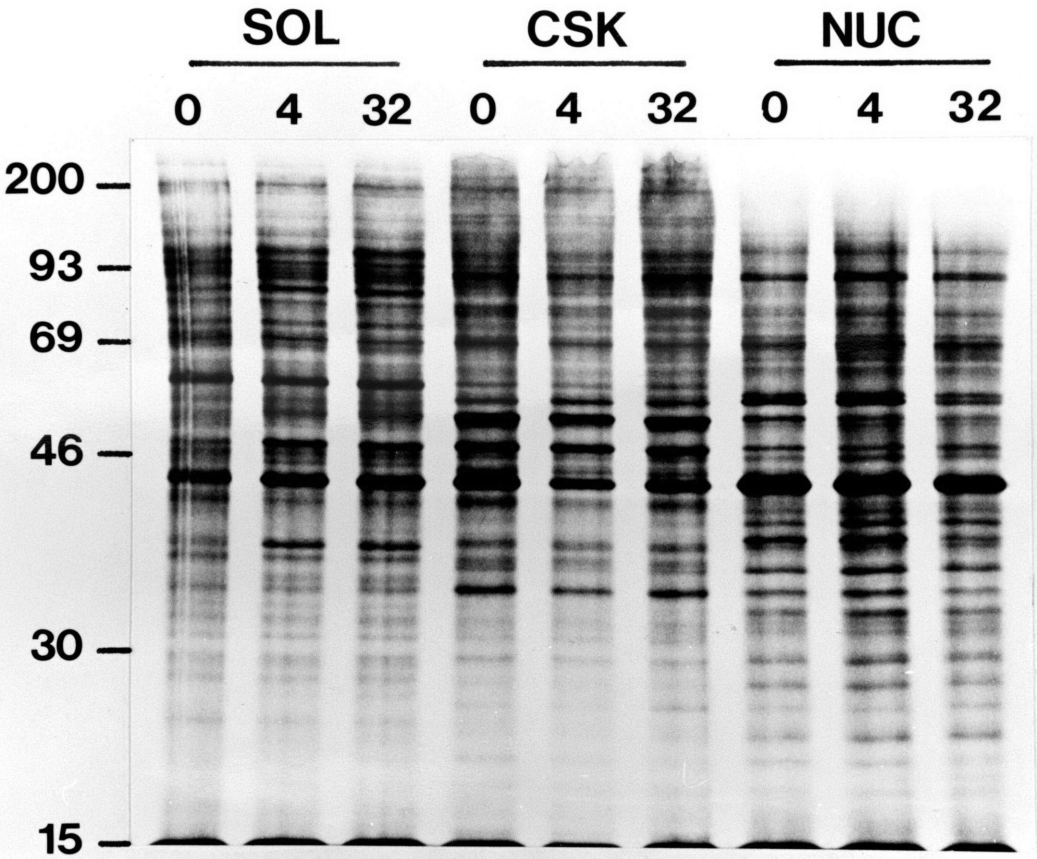
Figure 12. Concomitant inhibition of protein synthesis and release of mRNA from the CSKF. The inhibition of protein synthesis represented at each concentration indicated is obtained from the best fit curve of Figure 11. The per cent poly(A)+ RNA retained is expressed as a per cent of the cytoplasmic poly(A)+ retained on the CSKF in untreated cells. The data are taken from a number of experiments and include the data of Table 2. The SEM for each group is indicated. (0 μg/ml: N=7, 4 μg/ml: N=4, 16 g/ml: N=3, 64 μg/ml: N=5)

Figure 12


100
80
60
40
20
% Control
Protein Synthesis
CSK Bound mRNA
0
4
16
64
CD Concentration (µg/ml)

untreated cells applied to the lane was adjusted so that equal amounts of radioactivity were loaded for both control samples. The number of each cell type was not varied as the CD concentration was increased, unlike Figure 13. Consequently, the fluorogram reflects the inhibition of protein synthesis in the HeLa cells over increasing concentrations of CD and shows the absence of an effect on protein synthesis in the reticulocyte. The major protein synthesized in the reticulocyte migrated with an apparent mass of 70,000 daltons. This is clearly larger than the 46,000 dalton artifact associated with the enzymatic transfer of [^{35}S]methionine breakdown products to an endogenous protein of the rabbit reticulocyte. The identity of this protein was not determined although the 70 kD heat shock protein has been shown to be a major product of the chicken reticulocyte (Morimoto and Fodor, 1984).

Figure 13. Fluorogram of HeLa proteins synthesized in the presence of CD. Cells were exposed to 0, 4 or 32 μg/ml CD for 15 min, [^{35}S]methionine added and the cells labeled for an additional 15 min. The cells were harvested and fractionated as described in the Materials and Methods to yield the three fractions indicated. Equivalent amounts of radioactivity from each fraction was analyzed in each lane of the one-dimensional polyacrylamide gel. The concentration of CD used is indicated directly above each lane. The position of radioactive molecular weight markers are indicated (M_r x 1000) at left. Material from cells treated with 2 μg/ml was derived from approximately 1.23x the number of cells as the control; 2.6x in the 32 μg/ml treated cells.


SOL
CSK
NUC
0
4
32
0
4
32
0
4
32
200
93
69
46
30
15

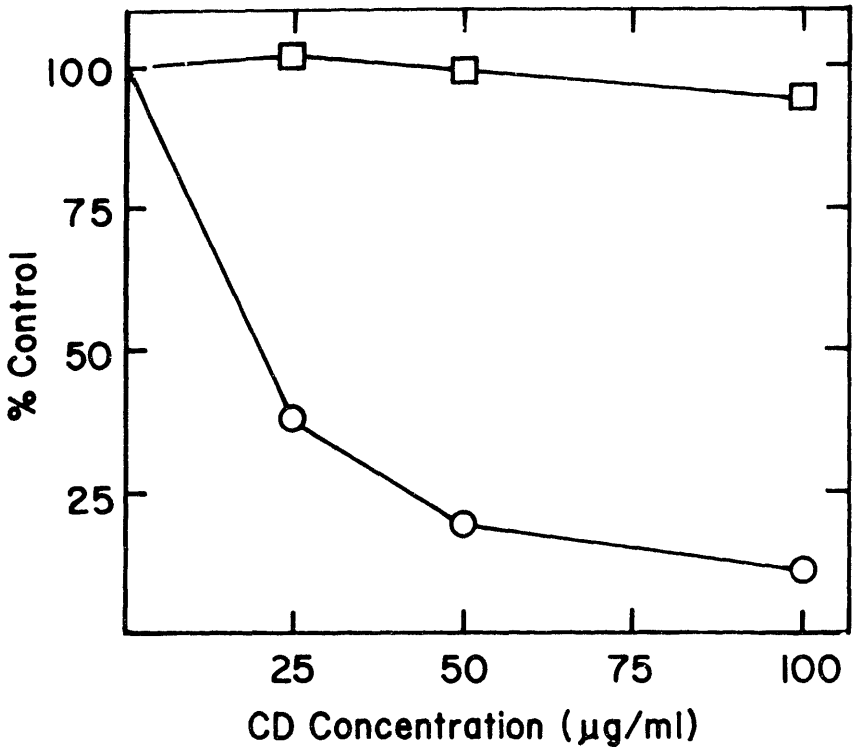
DISCUSSION

These experiments explore the functional significance of the association of polyribosomes with the CSKF. The release of mRNA from the CSKF by CD is used to probe this interaction. At sufficiently high concentrations, CD inhibits protein synthesis in an unusual manner. CD reduces the amount of polyribosomes in proportion to the degree of inhibition. The remaining polyribosomes, which retain an association with the CSKF, are unaltered in their sedimentation distribution. This pattern of inhibition could not result from simple lesions in initiation or elongation which lead to altered polyribosome distributions (Vazquez, 1979). The results are consistent with a model where, in the presence of CD, a portion of the mRNA molecules is withdrawn from active translation. In parallel with this withdrawal from translation, the mRNA molecules are released from the CSKF to the soluble phase. The mRNAs released from the CSKF cease translation while the mRNA remaining bound to the CSKF translate normally. Since a number of investigators have shown that removing the ribosomes from the mRNA by a variety of means does not release mRNA from the CSKF (Bonneau et al, 1985; Cervera et al, 1981; Howe and Hershey, 1984; van Venrooij et al, 1981) and because in the presence of emetine, intact polyribosomes are released, the mRNA is probably released prior to and not a consequence of its cessation of translation. A likely interpretation of the results is that freeing mRNA from the CSKF removes it from translation.

It was necessary to first clarify the action of high concentrations of CD on cytoskeletal organization. The drug exerts morphological effects at lower concentrations than those required to inhibit protein synthesis. The scanning electron micrographs in Figure 2 show that 2 μg/ml of CD profoundly alters cell morphology. This drug concentration has only a minor effect on protein synthesis (Figure 11). Higher concentrations of CD have only modest additional

Figure 14. CD does not affect protein synthesis in intact reticulocytes. Cells were washed with PBS and resuspended at $5x10^7$ reticulocytes or 10^6 HeLa cells per ml in media containing 1/10 normal methionine. CD in DMSO carrier was added to the concentrations indicated, adjusting the amount of DMSO to 2% of culture volume in all cases. The cultures were incubated for 30 min with the addition of 150 μCi/ml [^{35}S]methionine during the final 10 minutes of the treatment. The cells were extensively washed in PBS then lysed in buffer containing SDS. Aliquots were decolorized by H_2O_2 and NaOH and the acid precipitable radiolabeled protein determined. The results are expressed as a per cent of the untreated sample where the control level of incorporation represented 2.08 cpm per HeLa cell, 0.23 cpm per reticulocyte. HeLa protein synthesis: ◯ , reticulocyte protein synthesis: □ .

Figure 14

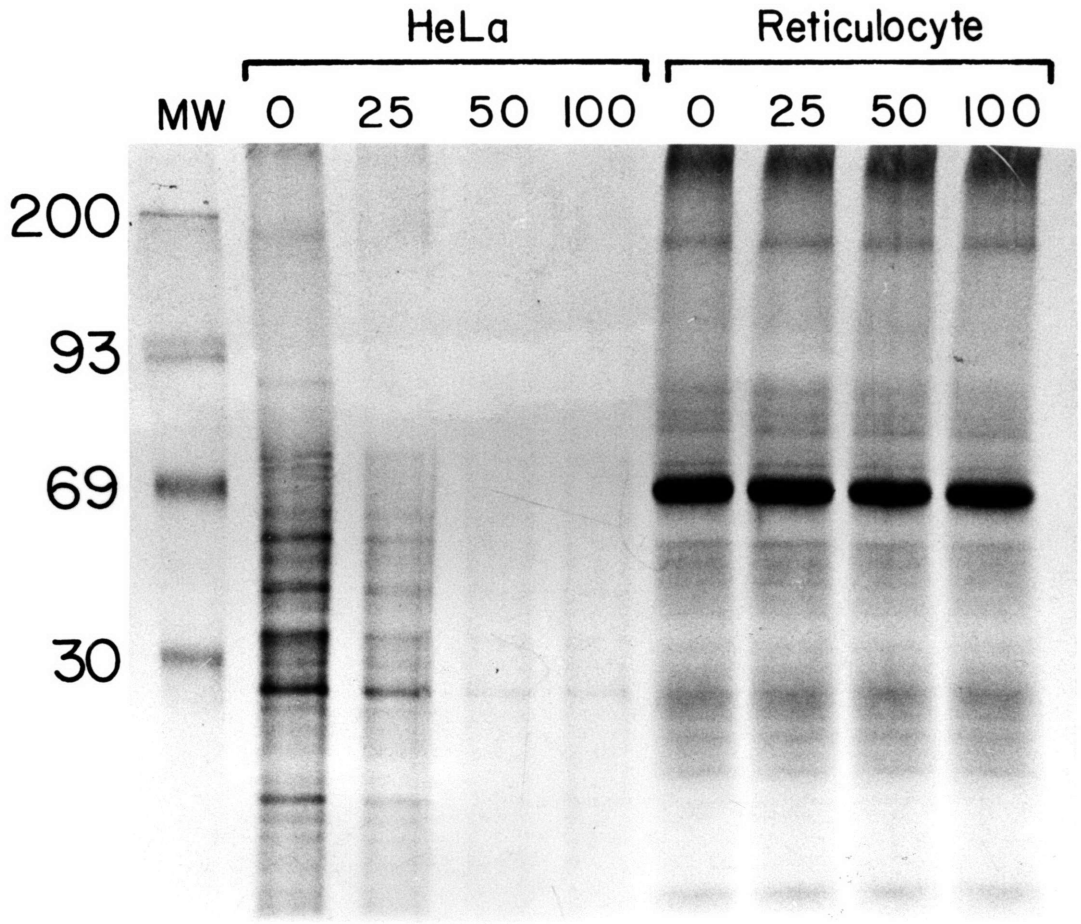


effects on external gross morphology but further alter the arrangement of the interior filament networks.

The effect of CD on cell structure is best seen by direct observation of the interior fibers. Figure 3A shows that the conventional embedded section electron micrographs give only a partial picture of the CSKF fiber networks. An effective method of imaging the CSKF is to use the techniques of embedment-free electron microscopy. The resinless sections (Figures 3D-E) afford more detailed views of the CSKF from CD treated cells. Changes in the extent and nature of interfilament associations are apparent. Fibers become clustered into dense patches or loosely connected bundles alternating with empty regions. The micrographs of Figure 3E and 3F show some of the differences between low and high drug concentrations. The frequency of dense patches is greater at the high level of CD. Presumptive microfilaments appear shortened and clustered, especially at the high drug concentration. These observations are consistent with other reports of the effects of lower doses of CD (Miranda et al, 1974b; Schliwa, 1982; Weber et al, 1976). Even high levels of CD do not diminish or disaggregate the cytoskeletal networks but do effect profound rearrangements of the cytoplasmic architecture.

The failure of CD to disassemble the CSKF is more clearly shown by direct measurements of its protein content and composition. These experiments used [^{35}S]methionine labeled cells and the same detergent extraction procedure employed for electron microscopy. The data in Table 1 and Figures 4 and 5 compare the protein content and composition of the normal and CD treated CSKF's. Neither total protein content nor the amounts of individual proteins are altered by drug concentrations much higher than those normally used to perturb cell organization. Even the amount of non-extractable actin, the presumptive target of the drug in the intact cell, does not decrease in the presence of CD.

Figure 15. [^{35}S]methionine-labeled proteins synthesized in the presence of CD from HeLa cells and reticulocytes. HeLa cells and rabbit reticulocytes were pulse labeled with [^{35}S]methionine in the presence of CD at the concentrations indicated above each lane (μg/ml). (The same cells used in association with Figure 14 are represented here.) Total cell protein was isolated and separated by electrophoresis. Protein from 1.73 x 10^4 HeLa cells was analyzed for <u>each</u> HeLa sample, protein from 1.56 x 10^6 reticulocytes analyzed for <u>each</u> reticulocyte sample. These values corresponded to 36,000 cpm [^{35}S]methionine for the untreated case. Radioactive molecular weight markers (M_r x 1000) are indicated at the left of the figure (under MW). The dye front and low-molecular weight products (globin) were inadvertently run off the gel.


HeLa
Reticulocyte
MW
0
25
50
100
0
25
50
100
200
93
69
30

CD is an effective but unusual inhibitor of protein synthesis. The inhibition becomes noticeable at concentrations just above those commonly used to alter cell morphology. (Indeed, the dose-dependent inhibition was observed over concentrations of CD that did not further alter the gross morphology of the cells.) The polyribosome profiles in Figure 8 show the unusual mode of protein synthesis inhibition. The polyribosomes in CD treated cells are reduced in amount but exhibit a normal sedimentation distribution. The reduction of polyribosomes mass closely parallels the reduction in protein synthesis (Figure 11). This implies that the remnant polyribosomes, which remain associated with the CSKF, are functioning at near normal rates. Few, if any, inhibitors have this effect on the protein synthetic machinery. Inhibitors of initiation decrease the average polyribosome size while inhibitors of elongation reduce the rate of protein synthesis without a concomitant reduction in polyribosome mass (Vazquez, 1979). In both cases the number of active mRNA molecules does not change. Furthermore, the gel patterns of proteins synthesized in presence of CD (Figure 13) appear free of the stuttering or smearing that characterize premature peptide termination. In the presence of CD, a portion of mRNA molecules are completely removed from translation while the remainder continue to function normally. The cessation of mRNA function accompanies, and may be the result of, release from the CSKF.

The mRNA molecules released from the CSKF into the SOL compartment by the action of CD are fully active in a cell-free translation system (Table 3). However, the partition of mRNA into cytoskeleton bound (CSK) and soluble (SOL) reveals a previously unseen heterogeneity in translatability. The mRNA normally found free in the soluble phase is much less translatable <u>in vitro</u>, on a molar basis, than the cytoskeleton bound RNA. Studies of these soluble mRNAs show them to be full sized with sequences that cross hybridize

extensively with those of active message (unpublished observations; S. Farmer, personal communication). Nevertheless, these mRNA molecules from the SOL fraction have an as yet undetermined lesion and do not stimulate protein synthesis in either the reticulocyte or wheat germ derived cell-free translation systems. The presence of these poorly-translated poly(A)+ molecules is masked in total cell extracts but becomes readily apparent when they are separated from the active mRNA bound to the CSKF. The presence of the inactive mRNA affects the results shown in Table 3. The mRNA released from the CSKF in the presence of cytochalasin mixes with the inactive poly(A) RNA of the SOL fraction and the composite translates with an intermediate level of efficiency. The mRNA from the CSK fraction, derived largely from bound polyribosomes, translates more effectively than mRNA from the SOL fraction. This difference is not due to extraction artifacts since, once shifted to the SOL fraction by CD, such active mRNA remains fully translatable.

Seemingly intact but inactive mRNA has been reported previously. Anchorage-dependent fibroblasts, when cultured in suspension, remove their mRNA from translation and store it in a quasi-stable form (Farmer et al, 1978). These molecules are almost untranslatable when tested in vitro. The inactive mRNA from the suspended cells is converted to an active form when the cells are allowed to reattach. Whether the lesion in mRNA from the suspended cells is the same as that in the mRNA-like molecules from the SOL fraction is not known and warrants further investigation.

The release of mRNA from the CSKF is not characteristic of agents or conditions that inhibit protein synthesis. Inhibitors of elongation, such as emetine and cycloheximide, as well as disaggregators of polyribosome structure, such as sodium fluoride, pactamycin, verucarin and heat shock, fail to change the association of cytoplasmic poly(A) RNA with the CSKF (Figure 6; Cervera et al, 1981; Howe and Hershey, 1984; Lenk et al, 1977; Vazquez, 1979). The effect of virus

infection on mRNA binding is more complex. Infection with VSV leads to the inhibition of host protein synthesis with the host mRNA remaining on the CSKF (Bonneau et al, 1985). In contrast, two viruses that show a substantial cytopathic effect, poliovirus and adenovirus, dislodge host mRNA from the CSKF and inhibit host protein synthesis (Lenk and Penman, 1979; van Venrooij et al, 1981). It is, however, not likely that the release of mRNA from the CSKF is the primary means by which host protein synthesis is inhibited (Trachsel et al, 1980; Schneider and Shenk, 1987).

CD does not appear to directly affect the components of protein synthetic system. It has no effect when added to an initiating reticulocyte in vitro system. This suggests that the drug does not act at the level of initiation and elongation and is in agreement with the normal polyribosome sedimentation distribution obtained in the presence of the drug. The reticulocyte seems to have little cytostructure and its polyribosomes are distributed uniformly throughout the cytoplasm (Rifkind et al, 1969). It seems likely that there is no cytoskeletal role in vivo for the highly specialized protein synthesis of the reticulocyte. The data in Figures 12 and 15 are consistent with this hypothesis. Levels of CD that strongly inhibit HeLa cells have no effect on protein synthesis in the intact reticulocyte. The results are consistent with the proposal that in cells with a cytoskeletal associated protein synthetic system, mRNA binding to the CSKF is necessary, though clearly not sufficient, for translation.

The nature and purpose of the association of protein synthesis with structural elements of the cell remains unknown. A review discussing this subject has recently been published (Nielsen et al, 1983). There may be several reasons for the association of mRNA with the CSKF. The seemingly obligatory binding of mRNA for translation may reflect a need for positioning the molecule near appropriate initiation (Howe and Hershey, 1984) or other regulatory

factors, including the cap-binding protein (Zumbe et al, 1982) and high molecular weight aminoacyl-tRNA synthetase complexes (Mirande et al, 1985). The binding of mRNA may also have a topographical significance. Such localization may be important for the placement of protein products at the cellular level (Capco and Jackle, 1982; Fulton and Wan, 1983) and in the segregation of maternal mRNA in the early embryo (Capco and Jackle, 1982; Moon et al, 1983).

The results presented here do not address the mechanism by which CD releases mRNA from the CSKF. In particular, it is not known how this release relates to the alteration of actin filaments. Other investigators have noted related effects of cytochalasin on the protein synthetic apparatus. Howe and Hershey (1984) found the eukaryotic initiation factors to quantitatively associate with the CSKF in HeLa. In this same study, CB released these factors as well as mRNA to the soluble phase. Cytochalasin has been reported to release polyribosomes from cultured lens cells and cytoskeletal remnants of these cells (Ramaekers et al, 1983; Ramaekers et al, 1980). Finally, polyribosomes of a rat liver preparation were found to associate with a Triton-insoluble matrix in a manner dependent on the integrity of filamentous actin (Adams et al, 1983).

Little is known of the nature of mRNA binding to the cytoarchitecture. mRNA, in form of RNP, does not require the presence of ribosomes for binding to the CSKF (Bonneau et al, 1985; Cervera et al, 1981; Howe and Hershey, 1984; van Venrooij et al, 1981). Specific mRNAs lacking a 5' cap or 3' poly(A) tract were found to bind the CSKF (Bonneau et al, 1985), suggesting that binding must include internal portions of the mRNA, possibly through the mRNA associated proteins. Because colchicine has little effect on protein synthesis and because the microtubules are depolymerized in the extraction protocol without releasing polyribosomes, the associations studied here most likely does not involve the microtubule system (Lenk and Penman, 1979). Morphological studies

indicate that the state of intermediate filament organization is probably irrelevant to protein synthesis (van Venrooij et al, 1981; Welch and Feramisco, 1985; Wolosewick and Porter, 1979). The experiments reported here and discussed previously do indicate that actin fibers are somehow involved in the functioning of the protein synthesis system. However, the data do not actually show that mRNA is bound directly to actin fibers. In view of the complex interactions possible with the actin filament system (Schliwa, 1981), no specific conclusions concerning its role in protein synthesis are possible. The finding that the presumptive actin filaments are shortened but not reduced in amount when CD releases mRNA might suggest that the actin fibers themselves are not the polyribosome binding site.

CHAPTER 4

Prompt Heat-Shock and Heat-Translocated Proteins Associated with the Nuclear Matrix in *Drosophila melanogaster* Cells

INTRODUCTION

The cells of a wide variety of organisms exhibit a well documented response to elevated temperature (recently reviewed in Craig, 1985; Lindquist, 1986; Neidhardt et al, 1984; Nover, 1984; Pelham, 1985; Schlesinger, 1986; Tanguay, 1983). This heat shock (HS) response, which is also induced by many varieties of environmental stress (see in particular Nover, 1984), results in the synthesis of a new set of mRNA that encode the small number of stress or HS proteins. Although the HS proteins occur in organisms throughout the phylogenetic scale, from archaebacteria (Daniels et al, 1984) to higher plants (Baszczynski et al, 1982; Key et al, 1981) and man (Burdon et al, 1982; Kioussis et al, 1981), they have been most intensively studied in Drosophila, both at the organismic and single cell level. It has been possible to show that the Drosophila HS proteins confer some measure of protection against the deleterious effects of elevated temperature (Arrigo, 1980; Lewis and Pelham, 1985; Mitchell et al, 1980; Petersen and Mitchell, 1981).

Recently, a different HS response has been found in mammalian cells (Reiter and Penman, 1983). Supranormal temperature induces the synthesis of still another set of proteins but, in contrast to the conventional HS proteins, these appear to be made from pre-existing, normally untranslated mRNAs. These proteins, which appeared in the nuclear matrix shortly after the shift to high temperature, have been designated as "prompt HS proteins". In contrast to the conventional HS proteins, the induction of prompt HS proteins is insensitive to transcriptional inhibitors such as actinomycin D. This result, together with the rapid appearance of the prompt proteins, suggested that the prompt HS proteins were synthesized from preexisting mRNA.

The prompt HS proteins have been found only in association with the nuclear matrix, or more accurately, with the complex composed of the nuclear matrix, nuclear lamina,

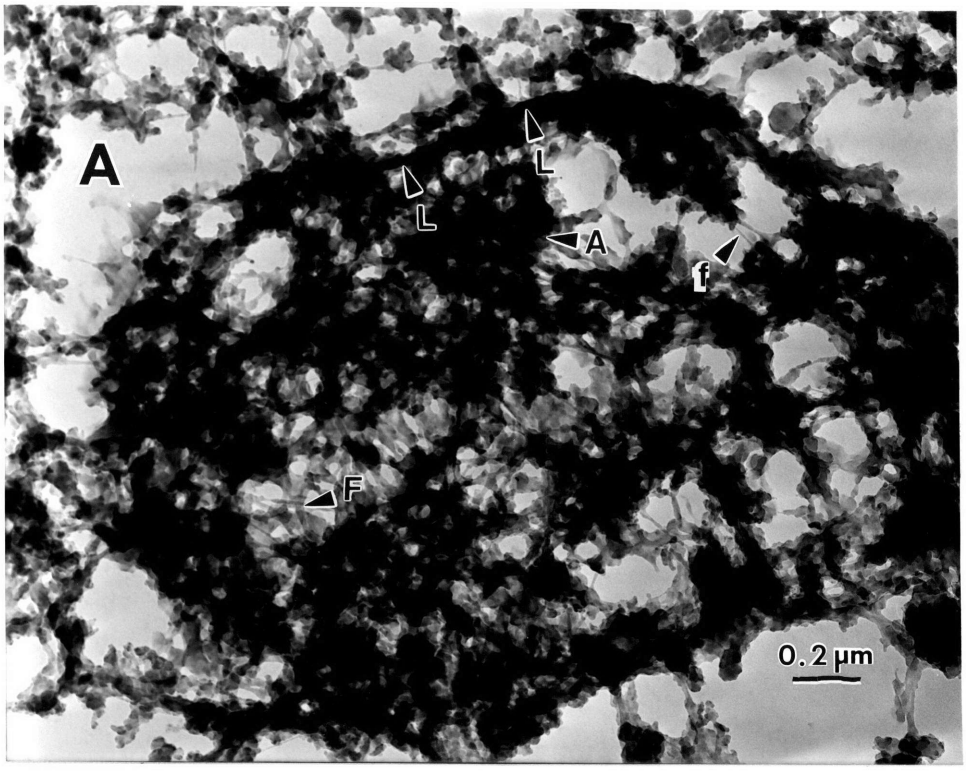
and intermediate filaments (NM-IF). Indeed, it is this association that made their detection possible. The prompt HS proteins are minor cell constituents and their labeling is difficult to detect in the presence of the much larger amount of total cell protein. However, the prompt HS proteins are readily discerned once the NM-IF proteins are separated from the remainder of the cellular proteins. The NM-IF proteins amount to less than 5% of total cell protein and their synthesis is reduced under heat shock conditions. The prompt HS proteins appear as a new set in the NM-IF against a background of reduced normal protein synthesis. The residual normal protein synthesis found in other cell fractions, if not separated by fractionation, would mask the prompt HS proteins and preclude their detection in a whole cell extract.

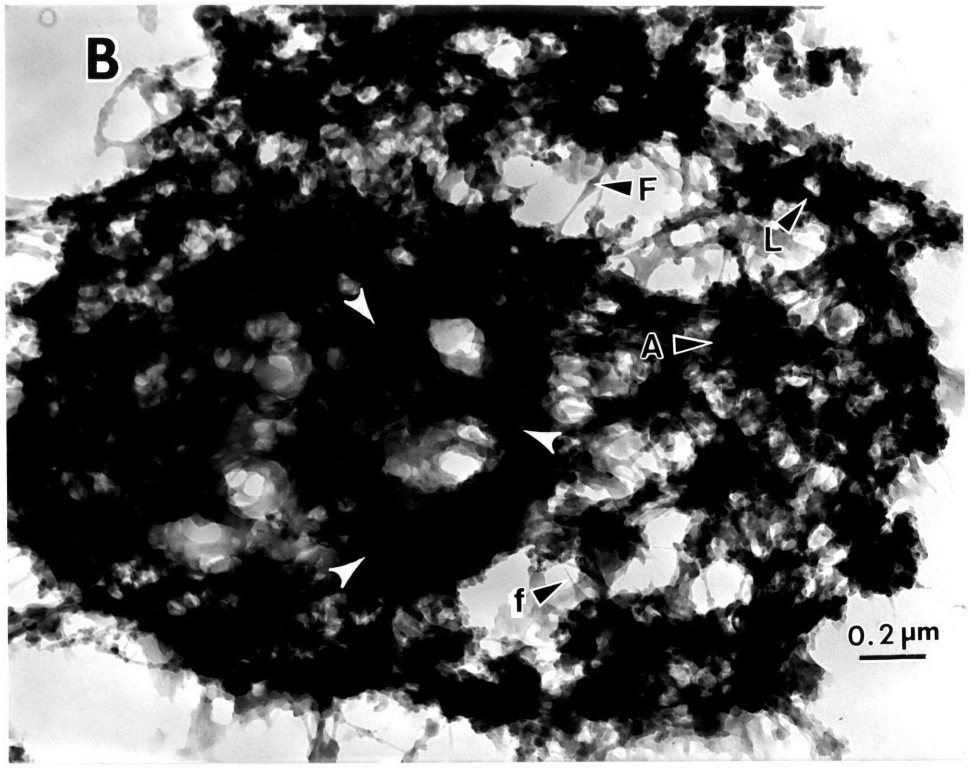
The prompt HS proteins were originally found in human and mouse cells, both derived from homeothermic mammals (Reiter and Penman, 1983). A similar transcription-independent heat shock response has been described in rat thymic lymphocytes (Maytin et al, 1985). It was natural to ask whether the phenomenon is related to homeothermy or if it occurs in cells derived from a poikilothermic organism. Drosophila was chosen because of the extensive literature characterizing its conventional heat shock response. In addition, several proteins of the Drosophila NM-IF have been described in some detail (Berrios et al, 1983a; Berrios et al, 1983b; Berrios et al, 1985; Filson et al, 1985; Fisher et al, 1982; Smith and Fisher, 1984). The present study examines the prompt HS response in the Schneider 2-L Drosophila cell line and shows it to be similar in many regards to the prompt HS response in mammals.

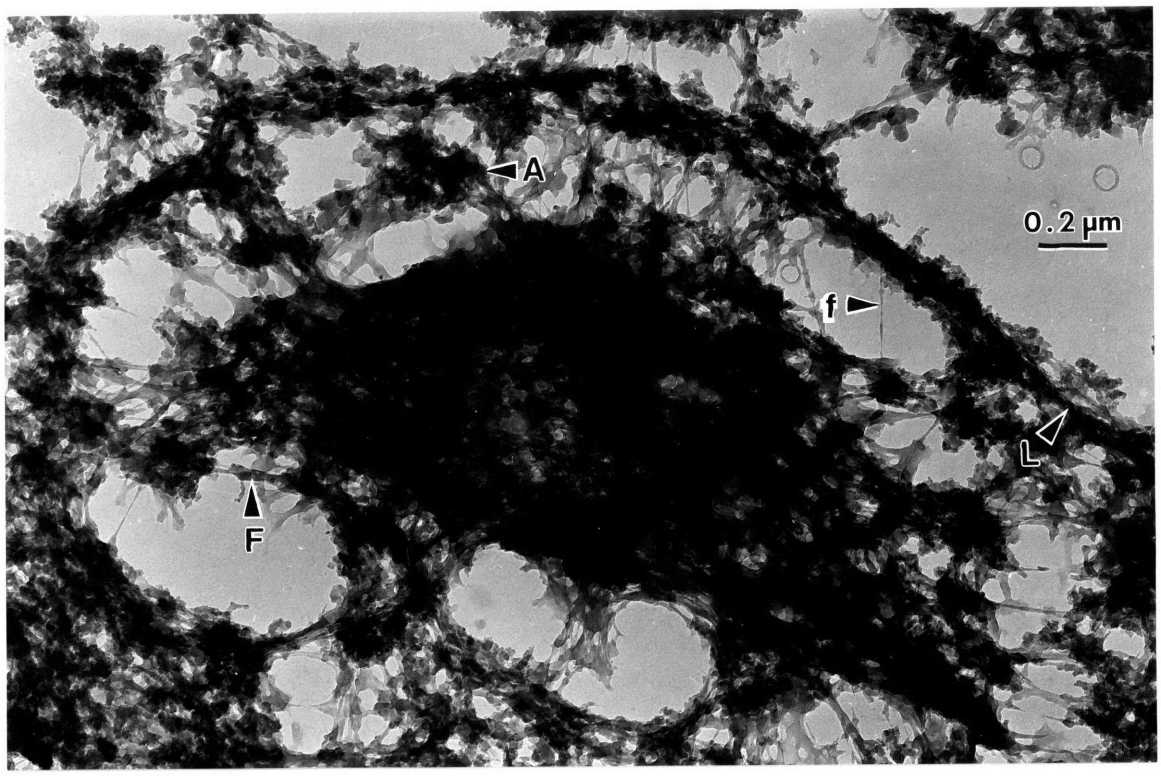
The nuclear matrix isolation procedure for Drosophila, as in other cell types, must be reevaluated for heat shocked cells. Hyperthermia elicits the reorganization of the cytoskeletal filament systems as well as an increase in the number and amount of proteins isolated in the nuclear

subfractions (Avan Bergen en Henegouwen et al, 1985; Biessmann et al, 1982; Caizergues-Ferrer et al, 1984; Murname and Li, 1985; Rabilloud et al, 1985; Roti Roti and Wilson, 1984; Sanders et al, 1982; Tanguay and Vincent, 1982; Welch and Suhan, 1985). Consequently it was necessary to distinguish the presumptive prompt HS proteins from the two other classes of proteins that appeared in the Drosophila nuclear matrix at elevated temperature. One such class consisted of the conventional HS proteins which were easily suppressed by inhibitors of transcription such as actinomycin D. The second class was more difficult to distinguish and included a group of preexisting proteins, normally only in the soluble fraction, that associated in part with the NM-IF upon heat shock. In these experiments, the prompt HS proteins were first differentiated from this background of translocated or heat-shifted (referred to here as HShift) proteins. Once the prompt HS proteins were identified, the conditions which induced them were characterized. The inducing conditions for the prompt HS proteins were compared to those for another group of actinomycin-insensitive, heat-insensitive proteins directed by an persistent virus.

Figure 16. Resinless section electron micrographs of the NM-IF from control and heat shocked Drosophila cells. The NM-IF structures from control and heat shocked cells were isolated and processed for sectioning and viewing in the absence of embedding material as described in the Materials and Methods. The NM-IF of control cells is seen in (A). The nuclear matrix is bounded by the nuclear lamina (L). Numerous thick (F) and thin (f) fibers of a non-uniform appearance can be seen within the nuclear matrix. Electron dense aggregates (A) of thick fibers are distributed about the interior of the nuclear matrix. The NM-IF imaged in (B) was isolated from cells heat shocked at 36.5°C for 45 min. The fibers within the nuclear matrix of the heat shocked cells are less uniformly distributed than in the control although thick (F) and thin (f) fibers, as well as aggregates of thick fibers (A), can be discerned. In addition to the partially disrupted fiber network, the heat shock NM-IF also contains amorphous, electron-dense regions (marked by three white arrowheads). The NM-IF shown in Figure 16C (on page following Figures 16A and 16B) illustrates a more extreme example of the amorphous aggregate seen in the nuclear interior. The sections were cut to 0.2 μm thickness. The bar in each figure represents 0.2 μm.


A
L
L
L
A
f
F
0.2 µm


B
F
L
A
f
0.2 µm


A
0.2 µm
f
L
F

RESULTS

Fractionation of Drosophila Cells to Obtain the NM-IF. Detection of the NM-IF associated prompt HS proteins in mammalian cells required the separation of NM-IF proteins from the much larger amount of remaining cellular proteins. The sequential procedure employed first separated the nucleus from soluble and cytoskeleton proteins. The NM-IF was then separated from the chromatin and its proteins analyzed. The method, as established for mammalian cells, has been described in detail elsewhere together with the molecular and morphological characterization of the subcellular fractions (Capco et al, 1982; Fey et al, 1984).

The well characterized fractionation procedure was adapted to Drosophila cells. Briefly, cells were exposed to non-ionic detergent (Triton X-100) in a near-physiological buffer. The detergent served to solubilize lipids and thus permeabilized membranes, releasing the soluble proteins. The cell structure that remained, designated the cytoskeletal framework, was then further fractionated. The cytoskeleton portion of the framework was solubilized by extraction with 0.25 M $(NH_4)_2SO_4$, leaving the intact nucleus. For these experiments the chromatin and hnRNP were then quantitatively removed by digestion with nuclease and elution with ammonium sulfate. These fractions were not analyzed separately but combined with the cytoskeletal proteins to constitute a single fraction (CSK/CHR). The remaining fraction consisted of the nuclear matrix and intermediate filaments with associated proteins and was designated the nuclear matrix-intermediate filament scaffold or NM-IF.

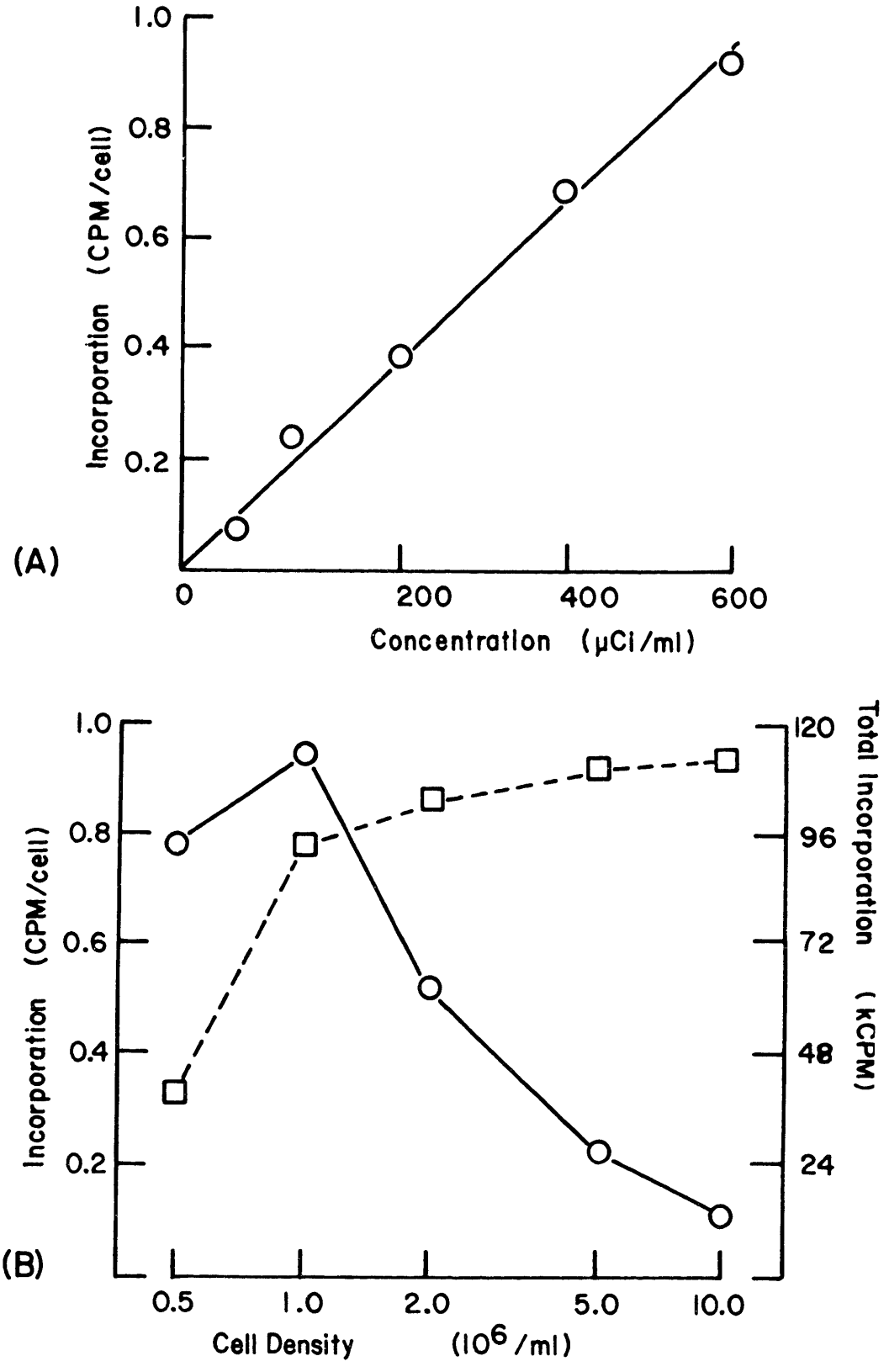
The resinless section electron micrographs in Figure 16 shows the morphology of the Drosophila NM-IF obtained from normal (Figure 16A) and heat shocked (Figure 16B) cells. An irregular lamina formed the boundary between the cytoplasmic fibers from the nuclear interior. The interior of the nucleus contained a heterogenous network of interconnecting

thick and thin fibers, many of which terminated on the nuclear lamina. In some places, thick fibers appeared aggregated. The morphology of the NM-IF isolated from heat shocked cells (Figure 16B) was similar to that of the control although several distinct changes were noted. The interior fibers were similar to those of the control nuclear matrix but had apparently pulled apart in many places creating empty regions. Whether these empty regions existed in the cell or occurred during isolation of the NM-IF was not determined. The most notable change in the heat shocked NM-IF was the appearance of regions of dense, amorphous substance. These may have resulted from the accretion of proteins not normally in the nucleus but shifted there at high temperatures. These heat-shifted proteins are examined in greater detail below.

Conditions for optimal [^{35}S]methionine labeling of Drosophila protein. Initial experiments with the Schneider Line 2 Drosophila cells yielded protein labeled at low specific activity compared to similar labeling protocols applied to mammalian cell lines (or previous Drosophila cell lines carried in the laboratory). A systematic study, undertaken to establish optimal labeling conditions for the cells used in this study revealed two unexpected results which are summarized in Figure 17. The first observation was that the incorporation of exogenously added [^{35}S]methionine in methionine-free medium was linear up to at least 600 μCi/ml (0.6 μM). Nonetheless, the amount of radioactive label in the medium was in vast excess over that incorporated into acid-precipitable material. This is in contrast to the labeling of HeLa cells at normal density which saturates at approximate 200 μCi [^{35}S]methionine/ml. The second unexpected finding was that protein in the Drosophila cells is labeled to the highest specific activity at a cell concentration that is less than the optimal concentration for growing the cells in suspension or slosh culture. In spinner culture or in a rocking tissue culture flask, cell growth is maximal when the cells are seeded at 10^6 cells/ml and

Figure 17. Incorporation of [^{35}S]methionine into protein as a function of amino acid concentration and Drosophila cell density. The incorporation of [^{35}S]methionine into protein as a function of amino acid concentration was is shown in (A). For this experiment, increasing amounts of [^{35}S]methionine in 0.1 ml methionine-free medium was added to 0.25 ml aliquots of 5 x 10^6 Drosophila cells/ml in the same medium at 25°C. The cells were incubated for 30 min before labeling was stopped by adding an excess of ice cold PBS containing a vast excess of unlabeled methionine. The cells were pelleted, resuspended in 0.5 ml of 0.5 N NaOH, and the protein precipitated on ice by trichloroacetic acid. The radioactivity in the acid-insoluble material determined and expressed as incorporated CPM per cell (◯). Incorporation as a function of cell density (B) was determined in a similar manner except the number of cells in the 0.25 ml volume was adjusted to yield the indicated final cell density. [^{35}S]methionine was added to provide a final concentration of ≈250 μCi/ml. The incorporation was expressed as CPM per cell (◯) as well as the total CPM obtained in each fraction (□).

Figure 17


(A)
Incorporation (CPM/cell)
1.0
0.8
0.6
0.4
0.2
0
200
400
600
Concentration (μCi/ml)
(B)
Incorporation (CPM/cell)
Total Incorporation (KCPM)
1.0
0.8
0.6
0.4
0.2
120
96
72
48
24
0.5
1.0
2.0
5.0
10.0
Cell Density (10^6/ml)

passaged between 4 - 5 x 10^6 cells/ml. As seen in Figure 17B, the greatest amount of labeling (on a per cell basis) occurred at a concentration of 10^6 cells/ml. In addition, the total incorporation of [^{35}S]methionine into acid-precipitable material practically remained constant as the cell density was increased above the optimal density. An inhibitory effect was seen at cell densities higher than those shown; that is, the total amount of labeled protein eventually decreased as the cell density was increased (data not shown).

Comparison of prompt and conventional HS proteins. Figure 18 shows the labeling of the conventional and the prompt HS proteins at different temperatures in each subcellular fraction. The conventional HS proteins were the principal proteins seen in each fraction when the temperature was raised. Actinomycin added prior to heat shock suppressed the conventional HS proteins and revealed the proteins changes that include the prompt HS proteins.

Cultured Drosophila cells were exposed to two elevated temperatures (33°C and 36.5°C) for 15 min and then labeled with [^{35}S]methionine for a further 30 min at the elevated temperature. The experiment was performed with and without the inhibitor of transcription, actinomycin D, present. The cells were harvested and fractionated into the soluble proteins (SOL, Figure 18A), the combined cytoskeleton-chromatin proteins (CSK/CHR, Figure 18B) and those of the NM-IF (Figure 18C). The fractionated proteins were then analyzed by one-dimensional gel electrophoresis. Approximately equal radioactivity was loaded onto each lane. The total acid-precipitable radioactivity isolated in each fraction is given in Table 4.

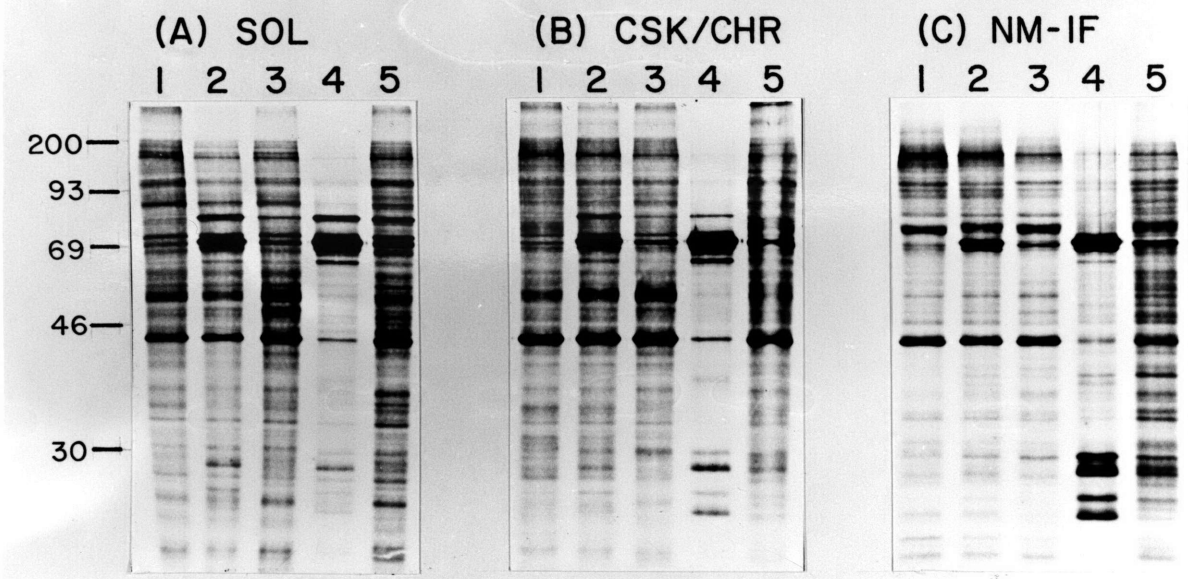
Heat shock induced the conventional HS proteins when transcription was not inhibited with actinomycin (Figure 18 lanes 2 and 4). Lane 1 in each panel shows the labeling of proteins at the control temperature, lane 2 at 33°C and lane 4 at 36.5°C. The conventional HS proteins were detected at

Table 4. Protein synthesis for each subcellular fraction of Schneider 2-L Drosophila melanogaster cells at normal and elevated temperatures[a]

	Subcellular Fraction		
	Soluble	Cytoskeletal-Chromatin	Nuclear Matrix-Intermediate Filament
Control (25°C)	453.3	53.4	2.8
33°C heat shock			
- Actinomycin D	322.6	56.8	6.5
+ Actinomycin D	187.9	41.6	6.9
36.5°C heat shock			
- Actinomycin D	123.3	66.9	17.6
+ Actinomycin D	43.3	10.3	11.4

[a]Acid-precipitable counts of incorporated [^{35}S]methionine expressed as CPM per 1000 cells.

Figure 18. <u>Drosophila</u> proteins pulse labeled with [^{35}S]methionine at normal and elevated temperatures. Cells were resuspended in methionine-free medium, incubated at 25°C (lanes 1), 33°C (lanes 2), 33°C with 2 μg/ml actinomycin D (lanes 3), 36.5°C (lanes 4), 36.5°C with 2 μg/ml actinomycin D (lanes 5). [^{35}S]methionine was added at a concentration of 200 μCi/ml 15 min after the temperature shift and the cells labeled at the appropriate temperature for an additional 30 min. The soluble proteins (SOL) are shown in (A), combined cytoskeletal-chromatin proteins (CSK/CHR) in (2) and the nuclear matrix-intermediate filament proteins (NM-IF) in (C). Approximately equivalent amounts of radioactivity were loaded on each lane; consequently the protein from increasing number of cells is represented in panels A, B and C, respectively. The actual radioactivity obtained per cell in each fraction is presented in Table 4. The position of radioactive molecular weight markers are indicated ($M_r \times 10^{-3}$) at left.

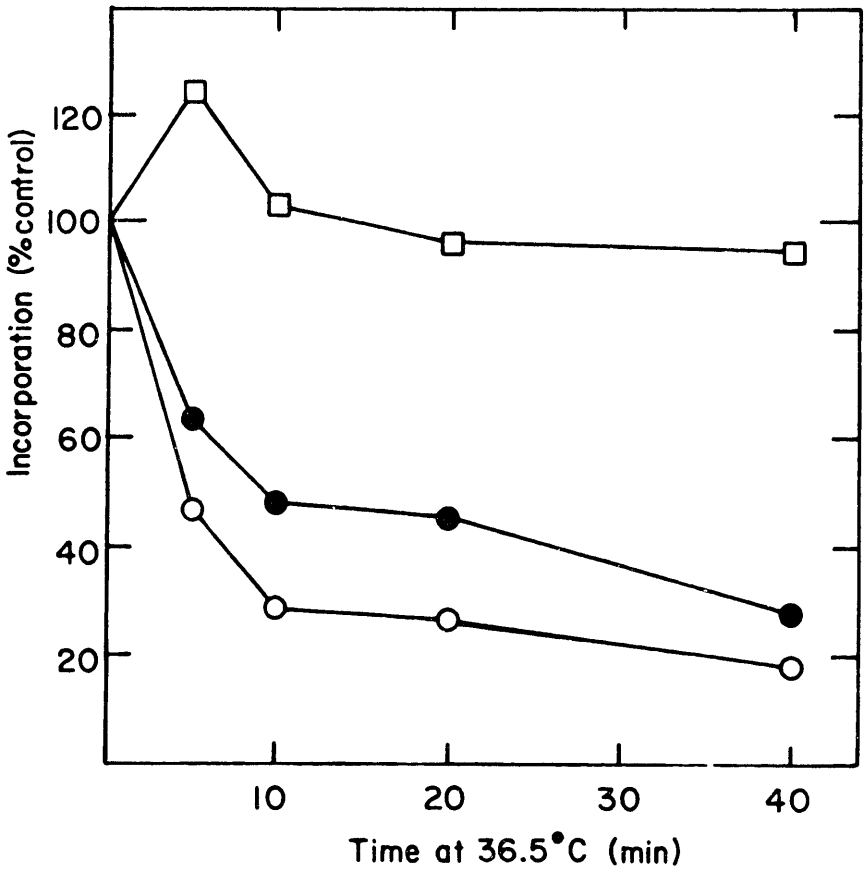

(A) SOL
1 2 3 4 5
200
93
69
46
30
(B) CSK/CHR
1 2 3 4 5
(C) NM-IF
1 2 3 4 5

33°C and were strongly labeled at 36.5°C. The most prominent HS proteins were a group of bands at 70 kD, corresponding to the well known hsp70, 80% of which appeared in the SOL fraction. (The data in Figure 18 does not show this directly since equal radioactivity was loaded into each lane. Consequently protein from increasing numbers of cells is represented in panels A, B, and C, respectively.) In contrast, the small HS proteins (hsp22, hsp23, hsp26 and hsp27), were mostly in the NM-IF (Figure 18C, lanes 2 and 4). The somewhat atypical 83 kD HS protein was only moderately labeled and appeared principally in the SOL fraction. As noted by other investigators, the hsp83 mRNA is present at a basal level in cells maintained at normal growing temperatures and is preferentially translated at elevated temperatures (Scott et al, 1980; Scott and Pardue, 1981; Vincent and Tanguay, 1982) as shown by its labeling in cells heat shocked in the presence of actinomycin D (Figure 18A, lanes 3 and 5). These data, obtained with this modified fractionation procedure for Drosophila cells, are in good agreement with previous findings for the subcellular distribution of the conventional HS proteins (Arrigo, 1980; Arrigo et al, 1980; Levinger and Varshavsky, 1981; Sinibaldi and Morris, 1981; Tanguay and Vincent, 1982; Velazquez et al, 1980; Vincent and Tanguay, 1982).

Total cellular protein synthesis declined at the elevated temperature, especially in the actinomycin treated cells. The total amount of [^{35}S]methionine incorporated by cells in the presence of actinomycin was reduced at 33°C and 36.5°C to 45% and 15% of the control levels, respectively (Table 4). Although quantitative changes could be seen in the relative labeling of proteins in the SOL fraction (Figure 18A, lanes 3 and 5) and the CSK/CHR (Figure 18B, lanes 3 and 5), the only new band is due to hsp83. However, many new proteins appeared in the NM-IF of cells labeled at the highest temperature. These included the prompt HS proteins together with proteins normally found in other subcellular

Figure 19. Kinetics of labeling of subcellular fractions of Drosophila cells exposed to a heat shock of 36.5°C in the presence of actinomycin D. The results are expressed as a percentage of the corresponding fraction prepared from cells kept at 25°C. Cells were harvested and concentrated 5-fold in medium containing one tenth normal methionine with dialyzed fetal calf serum then split into five portions of equal cell number. Actinomycin D was added to 2 μg/ml to each aliquot 45 min prior to labeling. At the appropriate times, cells were shifted to 36.5°C and 50 μCi/ml [^{35}S]methionine add at the time indicated. The pulse was stopped after 5 min by the addition of 1000-fold excess unlabeled methionine. The cells were washed with ice cold PBS, collected on ice and fractionated as described. (○ SOL, ● CSK/CHR, □ NM-IF.)

Figure 19


Incorporation (%control)
120
100
80
60
40
20
10
20
30
40
Time at 36.5°C (min)

fractions but moved to the NM-IF by heating. These new proteins could be discerned most clearly when actinomycin was used to abolish the conventional HS response.

The time course of labeling of total protein for each subcellular fraction during a heat shock at 36.5°C in actinomycin is shown in Figure 19. The labeling kinetics of each fraction was significantly different. Synthesis of the SOL proteins declined markedly, eventually reaching less than 20% of the control. The labeling of the CSK/CHR fraction also declined but less severely. In sharp contrast, incorporation into the NM-IF did not decline but, after a transient rise, remained constant. The results presented here suggest that the high level of labeling of the NM-IF at 36.5°C resulted from normal protein synthesis shutting off, the acquisition of some proteins not normally associated with the NM-IF, and the synthesis and incorporation of the prompt HS proteins.

Comparison of normal proteins of the NM-IF, normal proteins translocated to the NM-IF at high temperature and prompt HS proteins. A more complete study of the effects of high temperature on the proteins of the NM-IF required analysis using two-dimensional gel electrophoresis. The two-dimensional gel pattern of normal NM-IF proteins is shown in Figure 20A and is represented in schematic form in Figure 20B. A few of these NM-IF proteins may correspond to known NM-IF proteins in higher organisms and are indicated. The doublet labeled "A" appears to correspond to actin II and III on the basis of isoelectric pH, molecular weight, and prevalence. The apparent molecular weight of 43,000 is in good agreement with the reported value for actin (Mogami et al, 1982; Storti et al, 1978). The proteins labeled "L" may be identical to the 74 and 76 kD proteins characterized as major nuclear envelope protein in Drosophila (Smith and Fisher, 1984) which appear related to the nuclear lamins A and C of higher organisms (Gerace et al, 1978; Gerace et al,

Figure 20. Normal, HShift and prompt HS proteins of the Drosophila NM-IF analyzed by 2D gel electropherograms. Cells were labeled for 30 min with 200 μCi/ml. Actinomycin D was added to 2 μg/ml 10 min prior to the temperature shift. The position of radioactive molecular weight markers (M_r x 10^{-3}) is indicated for the schematic figures. The position of isoelectric point standards is indicated at the top of the figure. **(A and B)** Normal proteins of the Drosophila NM-IF. Arrowheads point to characteristic proteins of the NM-IF including actin (A), the presumptive nuclear lamin proteins (L), a major glycoprotein (GP), proteins cross-reactive with anti-hamster vimentin antibodies (V), and possibly the heat shock cognate protein (hsc70). The protein was derived from 6.0 x 10^6 cells; exposure was for 48 hr. The major control proteins of the NM-IF are indicated by stippled areas in all schematic figures. The derived molecular weight and isoelectric point of the control proteins are presented in Table 5. **(C and D)** NM-IF protein of cells prelabeled at 25°C then shifted to 36.5°C for 45 min. Circles mark proteins that were not present in the control NM-IF or whose appearance was markedly increase by the 45 min HS (HShift). Protein from 1.8 x 10^6 cells; the fluorogram was exposed for 48 hr. The HShift proteins are numbered and indicated by open circles in the adjacent schematic. **(E and F)** NM-IF proteins labeled in the presence of actinomycin D between 15 and 45 min at 36.5°C. Squares identify the newly synthesized proteins that are unique to the heat shock-labeled NM-IF (prompt HS). The molecular weight and apparent isoelectric point of these prompt HS proteins are enumerated in Table 6 (pHS:1 - pHS:14). Circles mark the HShift proteins that were labeled at the high temperature and were isolated in the NM-IF. The gel was loaded with material from 2.5 x 10^6 cells; the fluorogram was exposed for 48 hr. The prompt HS proteins are identified in the adjacent schematic representation by numbered, solid squares.

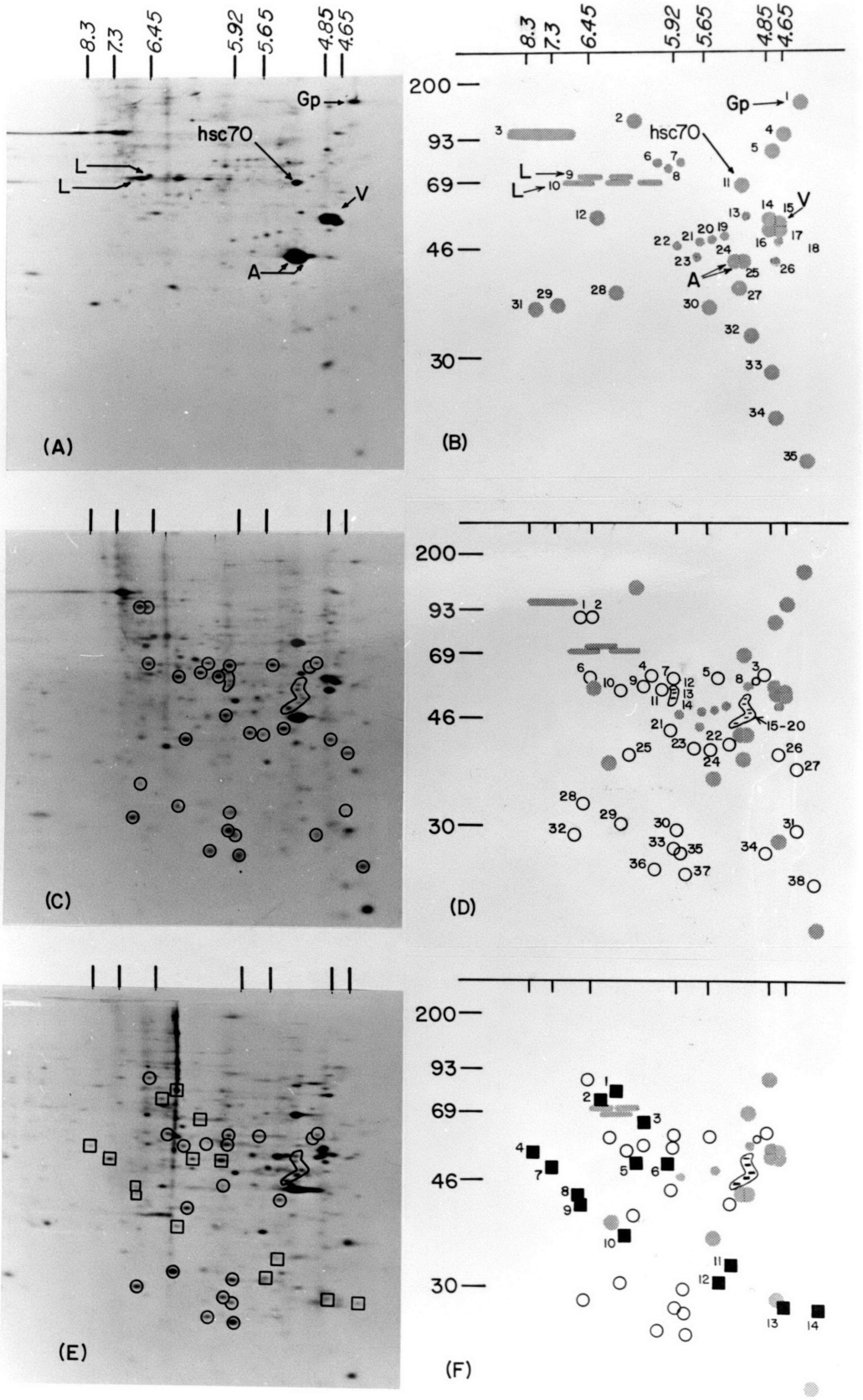
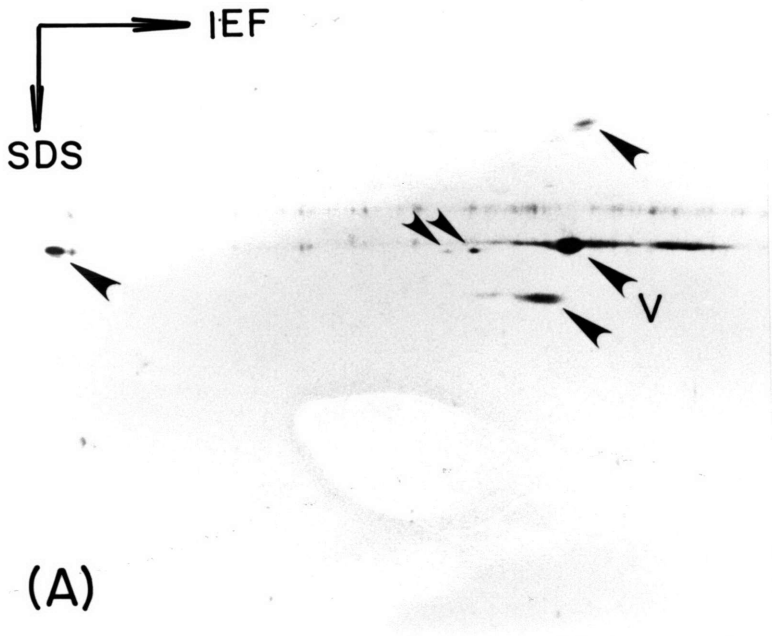
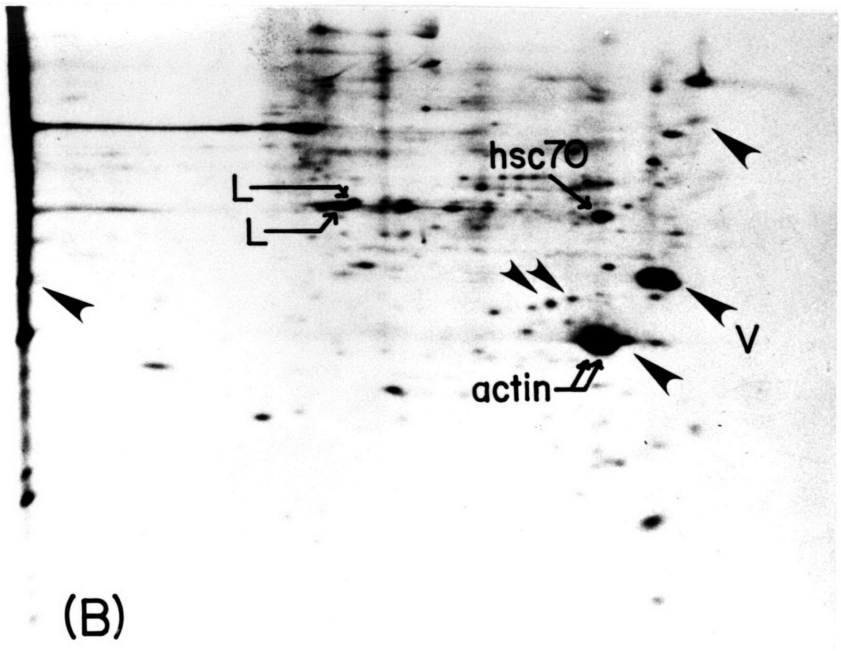

8.3
7.3
6.45
5.92
5.65
4.85
4.65
Gp
hsc70
L
L
V
A
(A)
200
93
69
46
30
(B)
(C)
(D)
15-20
(E)
(F)

Figure 21. Immunoblot analysis of the Drosophila NM-IF using antiserum against mammalian vimentin. Approximately 200 μg of Drosophila NM-IF protein was separated by two-dimensional gel electrophoresis and transferred to nitrocellulose as described in the Materials and Methods. Rabbit antisera raised against hamster vimentin was reacted with the attached proteins and the primary antibody visualized by reaction with a second antibody coupled to horseradish peroxidase (A). The strongest reactivity with the anti-vimentin antibody occurred with the protein labeled V. Heavy arrowheads mark other proteins recognized by the polyclonal antibody. (B) is a fluorogram of the the normal proteins of the Drosophila NM-IF shown for reference. Corresponding proteins recognized by the anti-vimentin antibody in (A) are marked with heavy arrowheads. Characteristic proteins of the Drosophila NM-IF are labeled as in Figure 20. From other determinations the molecular weights of these proteins are L, 73,000 and 72,000; hsc70, 70,000; V, 52,000; actin, 43,500.



(A)



(B)

1982). As shown in Figure 21, the cluster of 4 proteins (V) of 52,000 to 54,000 molecular weight reacted with antiserum raised against hamster vimentin (Hynes and Destree, 1978). In addition, limited reactivity to the same antibody was seen with the 47 kD proteins numbered 20 and 21. These proteins differ from the 46 kD cytoplasmic protein identified in Drosophila Kc cells that has been shown to be homologous to vertebrate vimentin (Falkner et al, 1981; Walter and Biessmann, 1984). The reason for this disparate result is not known, although some reactivity was seen with a protein of approximately 50 kD that failed to enter the isoelectric focusing gel (Figure 21). It was noted that the Drosophila Kc vimentin homolog may be too positively charged to enter the equilibrium focusing gel (Palter et al, 1986). "GP" denotes a protein recognized by a monoclonal antibody prepared by Filson et al. against the 190 kD glycoprotein of the Drosophila nuclear matrix-pore complex lamina (Filson et al, 1985). The 190 kD Drosophila glycoprotein was found to be homologous to a glycoprotein of the mammalian nuclear pore complex (Gerace et al, 1982). Finally, on the basis of molecular weight and isoelectric pH, the protein identified as hsc70 in Figure 20B may correspond to the product of the heat shock cognate gene, Hsc4 (Craig et al, 1983). This protein has been shown to colocalize with the intermediate filament network of embryonic Drosophila cells (Palter et al, 1986). These and other major proteins of the control Drosophila NM-IF are identified in the adjacent and subsequent schematic representations by stippled areas. These proteins are numbered in Figure 20B and the molecular weight and isoelectric pH for each presented in Table 5.

The two-dimensional gel pattern of the NM-IF was markedly altered when labeling was at sufficiently elevated temperature as shown in Figure 20E. This striking change in labeling of NM-IF proteins resembled the prompt HS response previously described in mammalian cells (Reiter and Penman, 1983). Prompt HS proteins were characterized in those

experiments as proteins synthesized only at elevated temperature, apparently from pre-existing but normally untranslated mRNAs. The prompt HS proteins evidently associate exclusively with the NM-IF. However, in Drosophila, as well as other cell types, hyperthermia also elicits an increase in the number and amount of proteins derived from other cell fractions which appear in the nuclear subfractions (Falkner and Biessmann, 1980; Rabilloud et al, 1985; Tanguay and Vincent, 1982). Under these conditions, prompt HS proteins appear in the presence of non-NM-IF proteins that are still synthesized at the elevated temperature and isolate with the NM-IF.

One means of distinguishing the prompt HS proteins from normal proteins shifted to the NM-IF is by labeling cells prior to heat shock. Those proteins which then "shift" to the NM-IF during the heat stress are identified as HShift proteins. This analysis is presented in Figure 20. The two-dimensional gel electrophoresis pattern of the normal NM-IF proteins of Drosophila (Figure 20A) was compared to the NM-IF proteins from cells that had been labeled with [^{35}S]methionine at normal temperature, chased, and then heat shocked (Figure 20C). The new proteins which appear in the NM-IF in this case have been shifted into the NM-IF by the elevated temperature. These HShift proteins, in turn, were compared to the pattern of NM-IF proteins from cells labeled during heat shock at 36.5^{o}C in the presence of actinomycin D (Figure 20E). Those proteins that appeared only when cells were labeled during heat shock are the presumptive prompt HS proteins.

Figure 20C shows the shifting of prelabeled protein from the SOL and CSK/CHR fractions into the NM-IF induced by a 45 min heat shock. Cells were labeled with [^{35}S]methionine in the presence of actinomycin for 30 min at 25^{o}C. They were then transferred to 36.5^{o}C in the presence of actinomycin and a large excess of unlabeled methionine. Circles indicate the major new proteins shifted into the NM-IF by the high

Table 5. Apparent molecular weight and isoelectric pH of the major control (Con) proteins of the *Drosophila* NM-IF.

Protein	MW	pI	Note
Con:1	165	4.45	Gp
Con:2	134	6.2	
Con:3	116	8.4, 7.7, 7.1, 7.0	
Con:4	113	4.61	
Con:5	95	4.74	
Con:6	85	5.99	
Con:7	85	5.85	
Con:8	79	5.96	
Con:9	73	6.5, 6.4, 6.3	L L L
Con:10	71	6.6, 6.3, 6.18	L L L
Con:11	70	5.27	hsc70
Con:12	55	6.5	
Con:13	55	5.20	
Con:14	54	4.75	V
Con:15	54	4.67	V
Con:16	52	4.74	V
Con:17	52	4.65	V
Con:18	49	4.72	
Con:19	49	5.51	
Con:20	48	5.58	
Con:21	47	5.69	(V)
Con:22	47	5.90	(V)
Con:23	44	5.70	
Con:24	43	5.35	actin II
Con:25	43	5.20	actin III
Con:26	43	4.71	
Con:27	37.2	5.32	
Con:28	36.5	6.4	
Con:29	34.0	7.1	
Con:30	33.7	7.9	
Con:31	33.7	5.59	
Con:32	29.8	5.04	
Con:33	25.8	4.74	
Con:34	21.3	4.70	
Con:35	16.7	4.40	

Key: MW, molecular weight (x 10^{-3}); pI, isoelectric pH; Gp, reacts with antibody specific for a major glycoprotein of the nuclear matrix-pore complex-lamina; L, possibly homologous to lamins A and C; (V), limited reactivity with anti-hamster vimentin antisera; V, strong reactivity with anti-vimentin antisera.

Table 6. Apparent molecular weight and isoelectric pH of the prompt HS (pHS) proteins of the Drosophila NM-IF.

Protein	M.W.	pI
pHS:1	86	6.3
pHS:2	78	6.5
pHS:3	65	6.17
pHS:4	53	8.2
pHS:5	50	6.2
pHS:6	50	6.01
pHS:7	49	7.5
pHS:8	42.1	6.7
pHS:9	39.5	6.7
pHS:10	34.6	6.3
pHS:11	30.4	5.51
pHS:12	27.7	5.58
pHS:13	25.5	4.70
pHS:14	24.8	4.38

Key: MW, molecular weight (x 10^{-3}); pI, isoelectric pH.

temperature. These HShift proteins are indicated by open circles in the schematic representations of Figure 20 and are referenced by an adjacent number in the schematic of Figure 20D. The pattern of HShift proteins in Figure 20C is to be compared to the prompt HS pattern in Figure 20E, the proteins of the NM-IF labeled during heat shock in the presence of actinomycin. Most of the control NM-IF proteins continue to be labeled at the high temperature. In addition, most of the HShift proteins present in Figure 20C also appear in the heat shock labeled NM-IF and are marked by circles. These are, presumably, normal temperature proteins that continue to be synthesized at high temperature and partially segregate with the NM-IF. The remaining proteins labeled at the high temperature are the presumptive prompt HS proteins.

The presumptive prompt HS proteins are those NM-IF proteins that were synthesized at the high temperature and were not accounted for by either the control NM-IF nor the HShift class of proteins. These new proteins are indicated by a square in the fluorogram (Figure 20E) and by a numbered square in the schematic diagram (Figure 20F). These proteins, designated pHS:1 through pHS:14, are candidates for the translationally controlled prompt HS proteins.

Distribution of the prompt HS and HShift proteins among the subcellular fractions. The results presented in Figure 20 established the prompt HS proteins as those proteins unique to the NM-IF that were labeled only during heat shock. To ascertain whether the prompt HS proteins associated exclusively with the NM-IF, as they do in mammalian cells, their presence was sought in the other cellular fractions. These results are presented in Figure 22 and discussed below. Cells were labeled with [^{35}S]methionine in the presence of actinomycin D at normal temperatures (Figures 22A, 22C, and 22E) or at 36.5^{o}C (Figures 22B, 22D, and 22F). Proteins from the SOL fraction are shown in Figures 22A and 22B and proteins from the combined CSK/CHR fractions in Figures 22C and 22D. These proteins were obtained from the control and

heat shocked cells of the experiment presented in Figure 20. The NM-IF fractions of the control and heat shocked cells are shown again for reference in Figures 22E and 22F, respectively. Approximately equivalent amounts of acid-precipitable radioactive protein was analyzed for the CSK/CHR and NM-IF fractions. Because of the greater number of proteins in the SOL fraction, twice the radioactivity was applied to the gel to achieve comparable labeling of individual proteins.

The prompt HS proteins in the NM-IF are indicated in Figure 22F by the numbered squares. The expected locations of the prompt HS proteins in the SOL fractions (Figures 22A and 22B) are indicated by broken squares. None of the prompt HS proteins identified in Figure 22F were detected in either SOL fraction even after an eight-fold greater exposure of the fluorograms. Although not indicated in Figures 22C and 22D, the prompt HS proteins were not visible in the CSK/CHR fractions after similar overexposure of the fluorograms. However, the presence of small amounts these prompt HS proteins in the SOL fraction cannot be completely precluded. The basic prompt HS proteins, pHS:4 (53 kD, pI 8.2) and pHS:7 (49 kD, pI 7.5), were sometimes poorly resolved in the equilibrium gels used in these studies. In addition, the predicted location of several prompt HS proteins (pHS:5 [50 kD, pI 6.2], pHS:6 [50 kD, pI 6.01], and pHS:13 [25.5 kD, pI 4.7]) might be obscured by overexposure of the adjacent proteins in the SOL fraction. Nevertheless, to the limits of resolution afforded by the gel system employed in these experiments, the prompt HS proteins appeared to exclusively associate with the NM-IF.

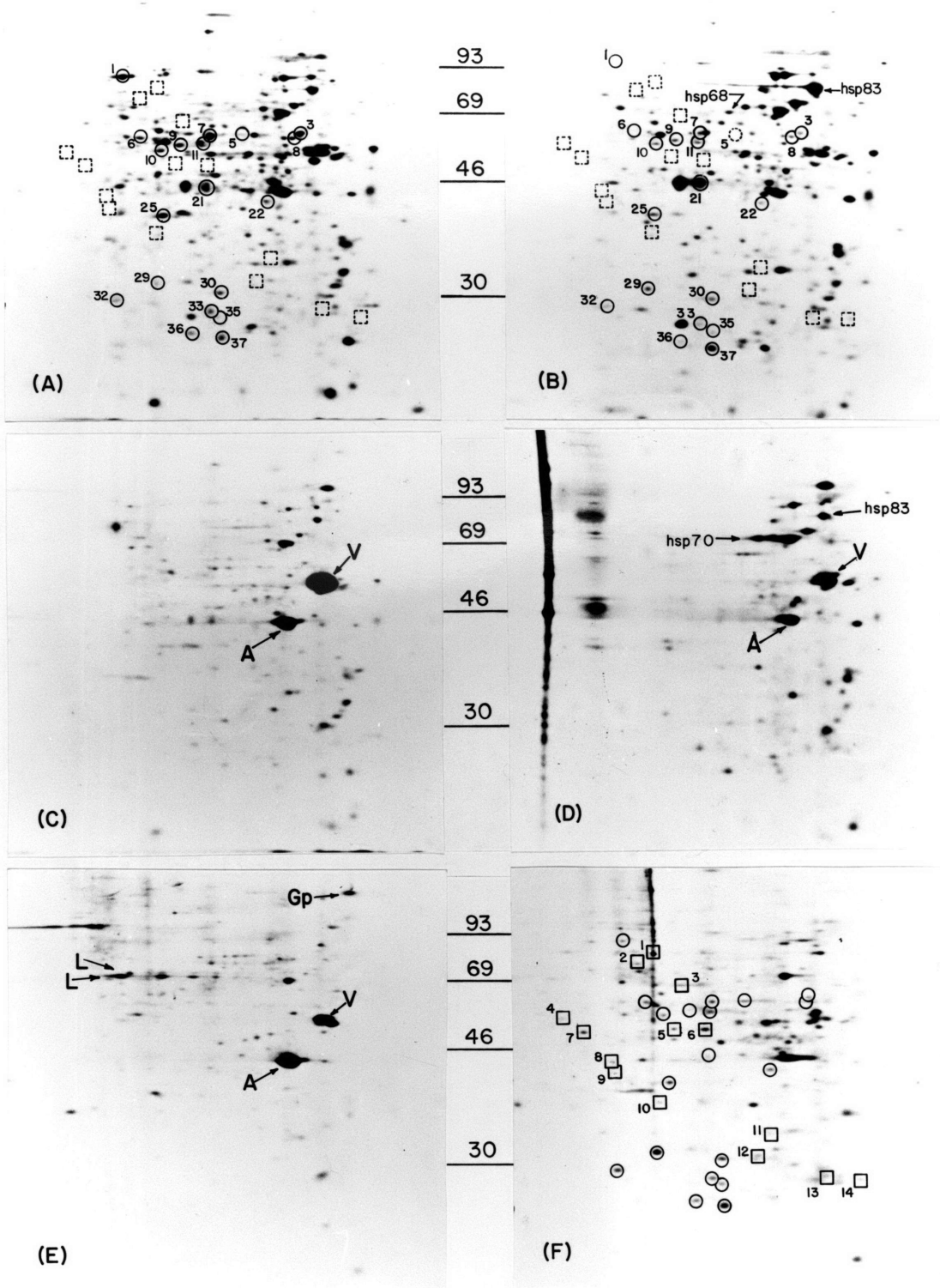
In contrast to the prompt HS proteins, each of the 38 HShift proteins identified was also found in the control SOL fraction (Figure 22A). 25 of the 38 HShift proteins were among those that also appeared in the NM-IF when cells were labeled during heat shock. Each of these HShift proteins, synthesized at the high temperature, is indicated by a closed

circle in Figure 22F and is identified by a numbered circle in Figures 22A and 22B. (The cluster located above actin, HShift:15-20, is not marked because of the complexity of the fluorogram in that region.) With the exception of HShift:5 (58 kD, pI 5.6), all HShift proteins seen in the NM-IF of cells labeled during heat shock were found in the SOL fraction of these heat shocked cells (Figure 22B). Furthermore, less than ≈30% of each HShift protein moved to the nucleus. That is, at least ≈70% of the HShift protein synthesized during the heat shock remained in the SOL fraction of the cell. The exception to this observation, HShift:5, may be a basic isoform of a prominent soluble protein. The apparent absence of this protein in the heat shock SOL fraction could reflect a different affinity for the NM-IF of different forms of the same protein.

Search for prompt HS-like proteins in the SOL and CSK/CHR subcellular fractions. The SOL and CSK/CHR fractions were further examined for the appearance of prompt HS-like proteins which did not move to the NM-IF. Several such proteins, made only at high temperature and without new transcription, were indeed found. However, these proteins appeared to result from a low level of expression of several of the conventional HS proteins. They were identical in isoelectric pH and gel mobility to known conventional HS proteins and their synthesis was markedly increased when transcription during the heat shock was permitted (see Figure 23).

Specifically, hsp68 was prominent in the heat shock SOL fraction (Figure 22B) although not detected in the corresponding control fraction at the exposure of the fluorogram presented in the figure. Hsp83 was evident in the SOL fraction isolated from unstressed cells and the synthesis of this protein was enhanced by the heat shock (Figure 22B) in agreement with previous reports suggesting that a basal level of the hsp83 mRNA exists in unstressed cells (Scott et al, 1980; Scott and Pardue, 1981; Vincent and Tanguay, 1982).

Figure 22. *Drosophila* proteins labeled with [^{35}S]methionine at normal and heat-shock (36.5^{o}C) temperatures from each subcellular fraction. Cells were shifted to either 25^{o}C (A, C, E) or 36.5^{o}C (B, D, F) in methionine-free medium containing 2 μg/ml actinomycin D. After 15 min at the indicated temperature, radioactive methionine was added and the cells labeled for an additional 30 min. The cells were fractionated and the proteins from each of the cell fractions analyzed. Equivalent amounts of acid-precipitable radioactive material was analyzed for the CSK/CHR and NM-IF fractions (B-E). Because of the complexity of the SOL fraction (A and B), twice the amount of radioactivity analyzed in the CSK/CHR or NM-IF fraction was used. All fluorograms were exposed for 48 hours. The control SOL, CSK/CHR and NM-IF proteins are seen in (A), (C), and (E) respectively. The HS labeled proteins from the SOL, CSK/CHR and NM-IF fractions are presented in (B), (D), and (E) respectively. HShift proteins are indicated by circles and are numbered in the SOL fractions (A and B). pHS proteins are indicated by closed squares and are numbered in (F). The expected locations of the pHS proteins in the SOL fraction are indicated by the broken squares.


93
69
46
30
(A)
(B)
hsp68
hsp83
(C)
(D)
hsp70
V
A
Gp
L
(E)
(F)

A small amount of hsp83 was also seen in the CSK/CHR fraction of the heat shock labeled cells (Figure 22D). A substantial increase in the relative labeling of a presumptive form of hsp70 was noted during the heat shock in the presence of actinomycin D. Most of this variant of hsp70 (indicated in Figure 22D) was isolated in the CSK/CHR fraction; none of this protein was detected in the NM-IF. This presumptive species of hsp70, of 70,000 molecular weight and pI 5.05, 5.35 and 5.65, was synthesized in the absence of new transcription. The identity of this particular protein in the CSK/CHR fraction remains to be determined.

The complexity of the SOL protein two-dimensional gel pattern may have prevented the detection of minor new proteins. In addition, for reasons that were not apparent, proteins of the CHR fraction were more difficult to analyze by two-dimensional gel electrophoresis following heat shock. The CHR proteins sometimes focused poorly and some radioactive material failed to enter the isoelectric focusing gel (Figure 22D) despite extensive nuclease treatment of the sample. However, subject to the appropriate caution, elevated temperature produced principally changes in the rates of synthesis or stability of proteins usually synthesized at normal temperatures. There was little appearance of new proteins in the subcellular fractions other than the NM-IF.

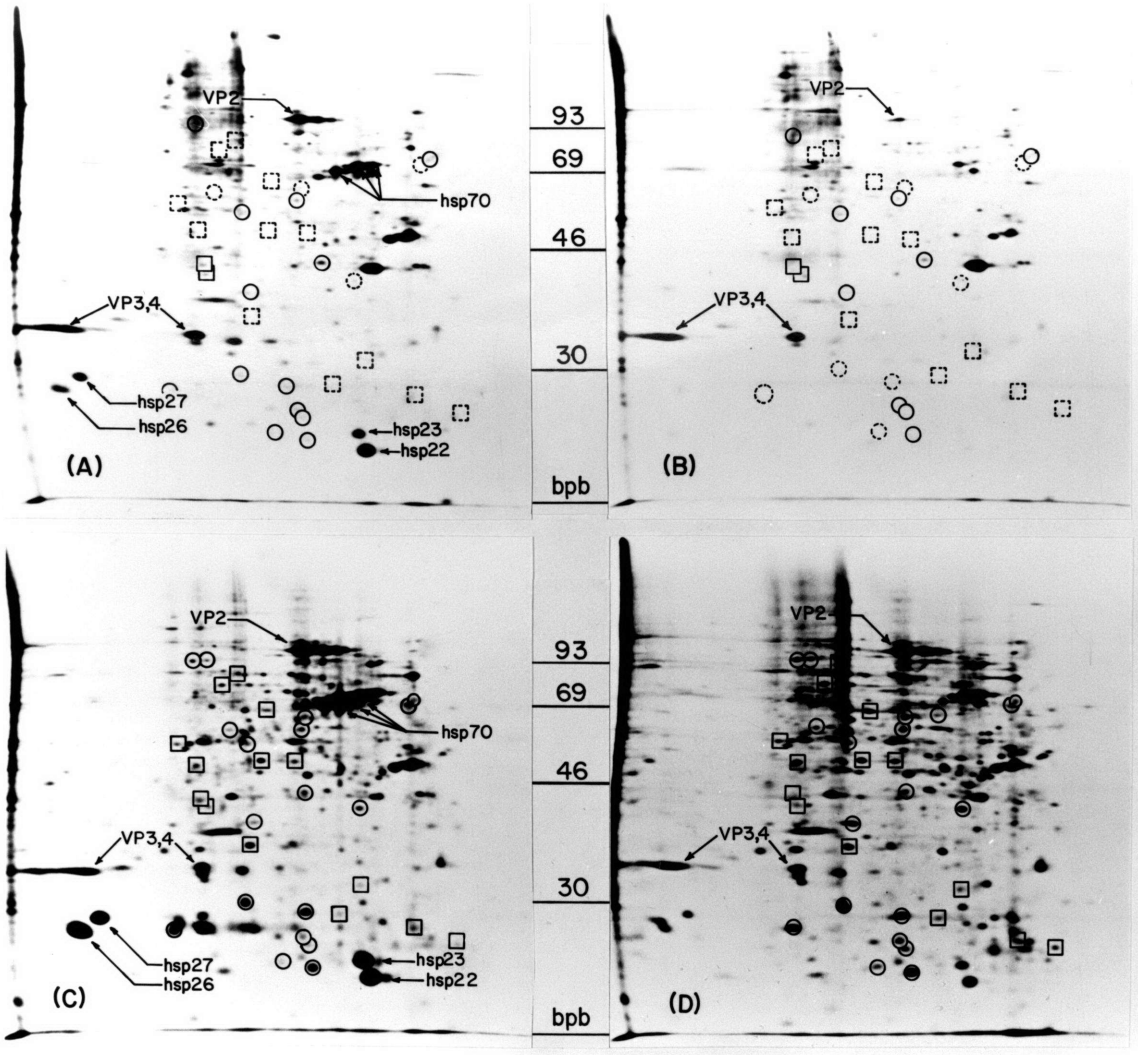
The lack of effect of actinomycin on the synthesis of the prompt HS proteins. The prompt HS response at 36.5^{o}C appeared to be regulated at the level of translation as suggested by its insensitivity to actinomycin D. The same amount of the drug abolished almost all of the transcriptionally controlled conventional HS response (Figures 20 and 22). Having identified the prompt HS proteins in the presence of actinomycin D, the possibility of partial inhibition of prompt HS protein synthesis by the drug was investigated. The expression of the prompt HS proteins was measured in the presence and absence of actinomycin and

compared to the unique set of proteins expressed by an endogenous virus. The synthesis of these viral proteins is insensitive to actinomycin D and also escapes the heat-induced inhibition of translation (Scott et al, 1980). These results are presented in Figure 23.

Drosophila cells expressing the small RNA virus described by Scott et al. were incubated at either 33°C or 36.5°C in the presence or absence of actinomycin D as indicated in the legend to Figure 23. The cells were labeled with [^{35}S]methionine between 15 and 45 min of the heat shock and the NM-IF fraction isolated and analyzed. For this experiment, material from equal numbers of cells were analyzed and the fluorograms exposed for the same length of time. The prompt HS proteins are indicated by closed squares and their expected positions marked by broken squares. The HShift proteins are indicated with closed circles and their expected positions marked by broken circles. The major conventional HS proteins are indicated by labeled arrows as are the viral proteins (VP2, VP3,4).

The electropherograms in Figures 23C and 23D reveal that the synthesis of the prompt HS proteins was largely unaffected by actinomycin D. All prompt HS proteins synthesized at 36.5°C in the presence of actinomycin D (Figure 23D) were labeled when the inhibitor was omitted (Figure 23C). The labeling of the prompt HS proteins is independent of the presence of actinomycin D. The data may even suggest that the relative labeling of some prompt HS proteins was somewhat enhanced by actinomycin D although this quantitative determination is not precise. The data are consistent with an essentially translational control for the prompt HS proteins. The fluorograms also emphasize the relatively small amount of label found in the prompt HS proteins compared to the conventional HS proteins. The results illustrate how relatively minor prompt HS proteins could easily escape detection unless selectively labeled and isolated.

Figure 23. Comparison of the conventional HS, prompt HS and HPS-1 viral protein synthesis for the NM-IF at 33°C and 36.5°C. Cells expressing the persistent virus, HPS-1, were briefly adapted to methionine-free medium and transferred to the indicated temperature in the presence or absence of 2 μg/ml actinomycin D. After 15 min at the indicated temperature, [^{35}S]methionine was added to 200 μCi/ml and proteins labeled for an additional 30 min. The NM-IF fraction was isolated and material derived from an equal number of cells analyzed. The fluorograms shown are of the NM-IF of cells labeled at 33°C in the absence (A) or presence of actinomycin D (B) and cells labeled at 36.5°C in the absence (C) or presence (D) of actinomycin D. The prompt HS proteins are indicated by closed squares, the expected position of the prompt HS proteins by broken squares. The HShift proteins are similarly indicated with closed or broken circles. The major conventional HS proteins are indicated by labeled arrows as are the viral proteins (VP2, VP3,4).


VP2
hsp70
VP3,4
hsp27
hsp26
hsp23
hsp22
(A)
93
69
46
30
bpb
VP2
VP3,4
(B)
VP2
hsp70
VP3,4
hsp27
hsp26
hsp23
hsp22
(C)
93
69
46
30
bpb
VP2
VP3,4
(D)

Prompt HS protein synthesis requires a higher temperature than the conventional HS response . The synthesis of the conventional and prompt HS proteins differed markedly in their response to temperature. The conventional HS proteins were synthesized at both 33°C (Figure 23A) and 36.5°C (Figure 23C). The low molecular weight HS proteins, hsp22, hsp23, hsp26, and hsp27, were each represented primarily by a single species. At the higher temperature of 36.5°C, both hsp27 and hsp26 were represented by several acidic variants of the major form. Several hsp70 species were synthesized at both temperatures with molecular weights of 70,000, 72,000 and 73,000 and isoelectric points between 5.05 and 5.75.

Although the conventional HS proteins were induced by the moderate HS of 33°C, only two of the prompt HS proteins (pHS:8 [42 kD, pI 6.7], and pHS:9 [59.5 kD, pI 6.67]) could be detected at this lower temperature. The broken squares in Figure 23A and 23B indicate the expected positions of the prompt HS proteins. No proteins could be detected at these positions even after an eight-fold greater exposure of the fluorograms. Unlike the conventional HS proteins in this cell line, which are fully induced by temperatures at or above 32.5°C, the unique pattern of actinomycin-insensitive prompt HS protein synthesis was detected in the NM-IF only at a temperature in excess of 36°C. A very low level of this high temperature pattern, masked in a background of normal translation at 33°C, cannot be ruled out.

HShift proteins appear in the NM-IF at lower temperature than do the prompt HS proteins. The prompt HS proteins were also differentiated from the HShift proteins by their appearance in the NM-IF as a function of the temperature. As previously discussed, the HShift proteins were those proteins normally isolated in the SOL fraction that continued to be synthesized during the heat shock and partially shift to the NM-IF at elevated temperature. The HShift proteins labeled at 36.5°C and isolated in the NM-IF are indicated by closed

circles in Figure 23C and 23D. (In the interest of clarity, the cluster directly above actin, HShift:15-20, is not marked.) These proteins were labeled to comparable levels in cells that were incubated at 36.5°C in the presence (Figure 23D) or absence (Figure 23C) of actinomycin D. Of the 25 HShift proteins labeled at 36.5°C and identified in Figure 23C, 21 of these proteins were found in the NM-IF of cells labeled at 33°C (Figure 23A). Many of the HShift proteins labeled at this lower temperature were not as heavily represented in the NM-IF as at the higher temperature but these proteins were readily identified after increased exposure of the fluorogram. Unlike the prompt HS proteins, the HShift proteins were synthesized and appeared in the NM-IF while the cells were maintained at 33°C. The data of Figure 23 also suggest that the synthesis and subsequent appearance in the NM-IF of some of the HShift proteins at 33°C was affected by actinomycin D. Of the 21 HShift proteins identified in Figure 23A, 7 were not seen when actinomycin D was present during the heat shock. The reason for this difference is not known.

Comparison of prompt HS protein synthesis to HPS-1 viral protein synthesis. The protein synthesis directed by a persistent RNA virus associated with this cell line (HPS-1) in some ways resembles that of the prompt HS. Synthesis of the viral proteins was found to be insensitive to both actinomycin D and to elevated temperatures (Scott et al, 1980) and one of the them, VP2, associates almost exclusively with the NM-IF. However, the following experiments show that the virus directed and prompt HS protein synthesis differ substantially.

A culture known to be infected was maintained and utilized for these experiments. The presence of the active virus was indicated by the reduced rate of cell growth, the constitutive presence of the conventional HS proteins, and by the appearance of large amounts of the major viral proteins. A major viral protein was found to have a molecular weight of

about 110,000 and an isoelectric point of 6.00. This is in reasonable agreement with the structural protein labeled VP2 by Scott et al. with a molecular weight of 120,000 daltons. Minor acidic variants of apparently the same protein were noted with pI values of 5.80 and 5.60. This protein associates exclusively with the NM-IF (data not shown). The doublet at 37 kD and 35 kD identified as VP3,4 by Scott et al. appeared in these experiments as two related groups of protein with similar mobility (37 and 35 kD). One form consistently appeared as a streak in the isoelectric focusing dimension extending from the basic end of the equilibrium gel to a pH of 8.5. Other components of the VP3,4 group of the same molecular weight focused at a pH of 6.8 and included related proteins of similar charge with slightly lower molecular weight (34 kD). At the higher heat shock temperature, an additional, apparently related species of 33 kD was observed. These lower molecular weight virus proteins were found in all cell fractions.

HPS-1 viral protein synthesis at 36.5°C, like that of the prompt HS proteins, was not inhibited by actinomycin D (compare Figures 23C and 23D). Also, unlike the normal temperature NM-IF proteins, synthesis of the viral proteins was not inhibited by elevated temperature. Indeed, the mRNA for the VP2 appeared to be preferentially translated at the higher temperatures (compare Figures 23C and 23D with 23A and 23B). However, at lower temperatures the synthesis of the viral proteins differed from synthesis of the prompt HS proteins. In particular, the viral mRNA was translated at the lower heat shock temperature of 33°C (Figures 23A and 23B) as well as at normal growing temperatures (data not shown). In contrast, the prompt HS mRNAs were not translated at 25°C and, with the exception of the mRNAs encoding pHS:8 (42 kD, pI 6.7) and pHS:9 (59.5 kD, pI 6.67), were apparently not translated at the moderate heat shock of 33°C. These differences in protein synthesis, seen at normal and mild heat shock temperatures suggest that the novel form of

translation directed by the HPS-1 virus and by the prompt HS mRNA are unrelated.

Fate of the prompt HS and HShift proteins upon return to normal growing temperatures. The prompt HS proteins and those proteins shifted into the NM-IF following a heat stress were further differentiated by their kinetic behavior when the cells were returned to normal temperatures. These results are presented in Figure 24. RNA synthesis was inhibited to suppress the conventional heat shock response. However, to maintain cell viability over the length of these experiments, an inhibitor of RNA synthesis was needed which could be reversed upon return to normal temperature. Camptothecin was employed, which unlike actinomycin, is fully and quickly reversible (Ableson and Penman, 1972; Wu et al, 1971).

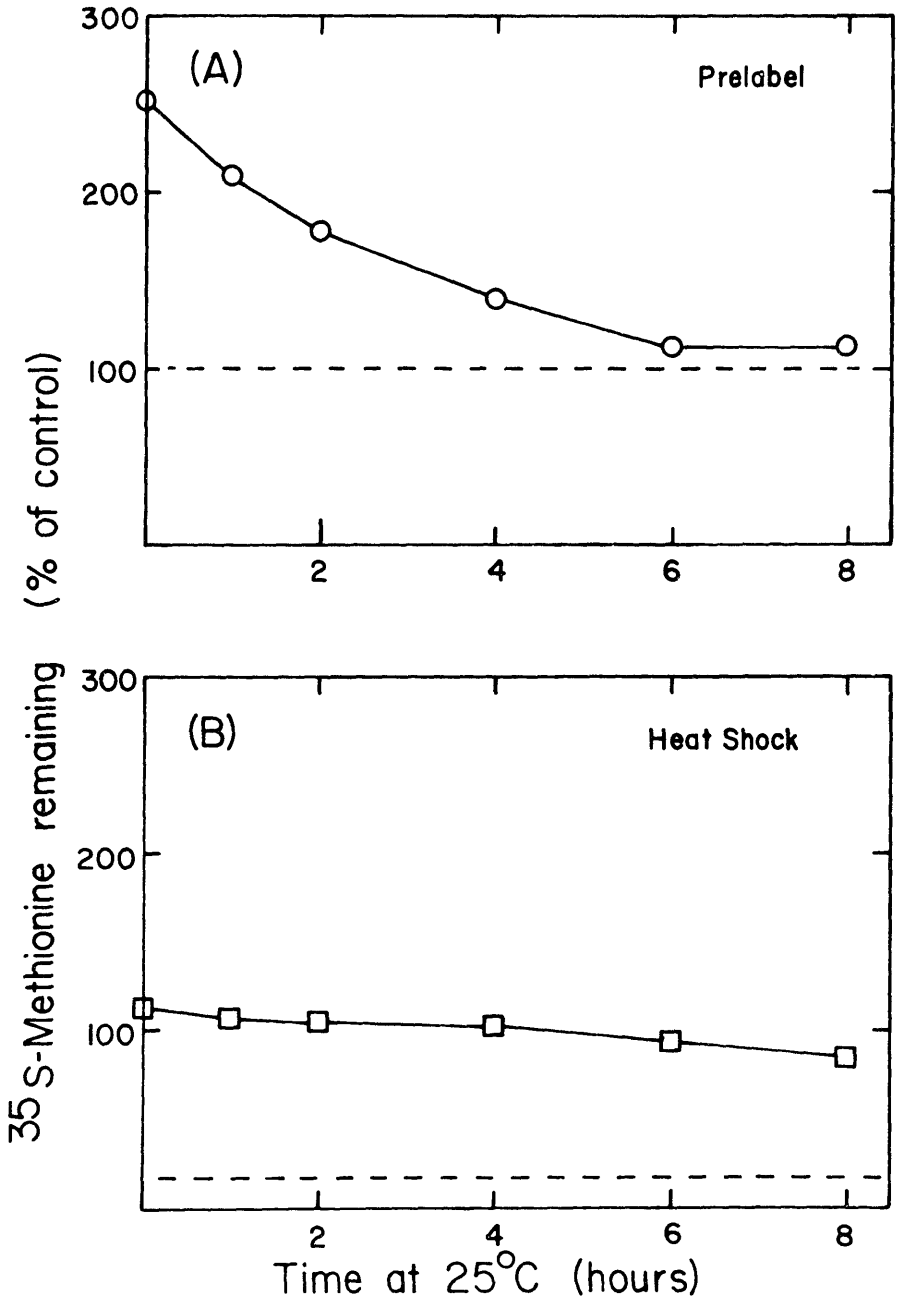
To follow the HShift proteins alone, cells were first labeled with [^{35}S]methionine for 15 min at 25^{o}C in the presence of camptothecin. The radioactive label and drug were removed and the cells chased with complete medium. These prelabeled cells were then heat shocked at 36.5^{o}C for 30 min and returned to normal growing temperatures. Following the return to normal temperature, the radioactivity of the NM-IF was determined at the indicated times. These data, plotted relative to the control at 25^{o}C, are shown in Figure 24A. These results were then compared to the fate of NM-IF proteins labeled during the heat shock. Cells were incubated at 36^{o}C in the presence of camptothecin and then labeled with [^{35}S]methionine. Thus, the NM-IF contained the prompt HS proteins and a subset of the HShift proteins as described above. The cells were returned to normal temperature and the radioactivity in the NM-IF determined at the indicated times. The fate of these heat shock-labeled NM-IF protein is shown in Figure 24B.

The radioactivity in NM-IF with only prelabeled, HShift proteins decreased steadily over the course of 6 hours at normal temperature, approaching the value of control (Figure

24A). The disappearance of radioactivity from the NM-IF with only HShift proteins may reflect the gradual return of these proteins to other subcellular compartments. Alternatively, the decline in radioactivity over time may have resulted from the specific degradation of the HShift proteins in the NM-IF. In contrast to the prelabeled proteins, the proteins pulse-labeled at the elevated temperature remained as a stable component of the NM-IF for at least 8 hours (Figure 24B). This latter result was somewhat surprising since both the prompt HS proteins and some of the HShift proteins were present. The HShift proteins might be expected to leave the NM-IF based on the data in Figure 24A but apparently did not. The experiments indicate that the proteins produced during the heat shock apparently associate differently with the NM-IF as seen in their behavior upon return to normal temperature.

Figure 24. Fate of the HShift and prompt HS proteins of the NM-IF upon return to normal temperatures. Cells were concentrated 5-fold and adapted for 15 min in serum-free medium containing one-tenth normal methionine with 20 μg/ml camptothecin. One third of the starting culture was labeled for 15 min at 25°C, chased in complete medium with camptothecin for an additional 15 min then heat-shocked at 36.5°C for 30 min. These pre-labeled cells were then washed and cultured in identical medium at 25°C. The fate of these prelabeled NM-IF proteins of cells subject to a HS is shown in (A). Another third of the cells were transferred to 36.5°C, labeled with [^{35}S]methionine for the final 15 min of a 30 min heat shock, washed with complete medium then maintained in camptothecin containing complete medium at 25°C. The chase of these prompt HS labeled proteins is shown in (B). The final third of the initial cell culture provided the control; these cells were labeled for 15 min at normal temperatures, chased and maintained with camptothecin containing complete medium at 25°C. At times indicated following the return to normal temperature, portions of each culture were fractionated and the amount of label in the NM-IF expressed as a percentage of the label in the NM-IF of the control cells. The dashed line in each figure indicates the level of total protein synthesis relative to the control cells. (Approximately 15% for the prompt heat-shock labeled cells of [B] and exactly 100% for the prelabeled cells of [A]).

Figure 24


300
200
100
(A)
Prelabel
2
4
6
8
(% of control)
35S-Methionine remaining
300
200
100
(B)
Heat Shock
2
4
6
8
Time at 25°C (hours)

DISCUSSION

This chapter describes experiments which detected and characterized the prompt HS proteins in the NM-IF of cultured Drosophila cells. Recent reports have questioned the validity of findings that localize proteins to a nuclear matrix-like subfraction through the use of cell fractionation (Evan and Hancock, 1985; Kirov et al, 1984). Indeed, the isolation of an oncogene product with a "nuclear matrix" apparently depended on conditions during isolation of nucleus and during nuclear fractionation (Evan and Hancock, 1985). The procedures employed here avoided the use of low ionic and very high ionic conditions and also did not apply elevated temperatures during cell fractionation. This approach was developed to preserve cellular architecture and facilitate biochemical analysis. In recent publications and in preliminary reports, several other investigators have described the use of mild conditions, similar to those described here, for isolation of the nuclear matrix (Belgrader and Berezney, 1986; Razin et al, 1985; Smith et al, 1986). The procedure appears to afford reproducible and consistent results.

Drosophila cells manifested a prompt response to heat shock which resembled, in many regards, that seen in HeLa cells. Cells exposed to elevated temperature immediately synthesized a set of proteins that associated exclusively with the NM-IF. This synthesis apparently proceeded from pre-existing, but normally untranslated mRNA since, unlike the conventional HS response, the prompt HS proteins were not inhibited by suppressing RNA synthesis with actinomycin. The prompt HS response is therefore not unique to homeothermic animals but is found here in an evolutionarily distant invertebrate. Similar responses to heat shock that are not dependent on de novo transcription have been described. In xenopus oocytes, the synthesis of a major HS protein in response to heat stress may result from the recruitment and

translation of pre-existing HS mRNA (Bienz and Gurdon, 1982). Using cordycepin to block the appearance of newly synthesized mRNA, Young and associates described the rapid induction of 11 transcription-independent HS proteins in thymic lymphocytes (Maytin et al, 1985). These proteins were of low abundance and their detection in the whole-cell extract was achieved by the use of high resolution, "giant" two-dimensional gel electrophoresis (Young et al, 1983). Also, the rapid induction of relatively minor, novel HS proteins has been described in hamster (CHO) cells (Caizergues-Ferrer et al, 1984) and human lymphoblastoid cells (Anderson et al, 1982). Finally, the isolation of proteins apparently analogous to both the HShift and the prompt HS proteins has been described in cultured tomato cells (Nover and Scharf, 1984).

The prompt HS proteins appear to localize exclusively to the NM-IF. They were not found in the chromatin, cytoskeleton or soluble fractions. The isolated nuclear matrix fraction is referred to here by the more accurate designation, NM-IF (for nuclear matrix-intermediate filament scaffold). Although located in the cytoplasmic space, the intermediate filaments differ from other cytoskeletal elements in that they remain quantitatively attached to the nuclear matrix under most matrix isolation procedures (Capco et al, 1982; Fey et al, 1984). Furthermore, in HeLa cells at least one half of the prompt HS proteins apparently associate with the intermediate filaments and never migrate into the nuclear space (Reiter and Penman, 1983). The actual spatial localization of the Drosophila prompt HS proteins, whether within the nuclear interior or associated externally with the intermediate filaments, is not yet known and it is most correct to term them proteins of the NM-IF.

Besides the prompt HS proteins, heat induced other changes in the Drosophila NM-IF that complicated the identification of the prompt HS proteins. Several of the conventional HS proteins associated with the NM-IF but these

were easily suppressed by inhibiting RNA synthesis. More problematic was the marked shift into the nucleus of proteins not normally associated with the nucleus. Some of these HShift proteins continued to be synthesized at the high temperature and appeared in the NM-IF as part of the heat shock response. The experiments shown in Figures 20, 22 and 24 were necessary to distinguish the prompt HS from the HShift proteins. These HShift proteins were identified first by labeling cells at 25°C and then determining those prelabeled proteins which moved to the NM-IF after heat shock. Most, but not all, of the HShift proteins also appeared in the NM-IF when the cells were labeled at elevated temperature. These were not considered as part of the prompt HS response. The HShift proteins also differed from the prompt HS proteins in that they all occurred in both the soluble fraction and the NM-IF. Also, most appeared in the NM-IF to some degree at 33°C while, with two exceptions, the prompt HS proteins did not.

These experiments cannot exclude the possibility that the prompt HS proteins were the result of aberrant translation or were proteolytic products of nascent polypeptides. However, these possibilities seem unlikely. The electropherograms showed no stuttering or smearing characteristic of premature termination or proteolysis. Perhaps most significant is that the new high temperature proteins occurred only in the NM-IF. The other fractions, SOL, CSK and CHR, amount to more than 95% of the cell protein mass and the aberrance that produced the prompt HS proteins would have to produce proteins extraordinarily specific in their localization.

The accumulation of proteins in the nucleus at high temperature in Drosophila is a well known phenomena (Biessmann et al, 1982; Rabilloud et al, 1985; Tanguay and Vincent, 1982). The possible significance of these shifted proteins has been discussed by other investigators who suggested that these proteins may modulate or disrupt

nucleolar structure and function, alter chromatin structure and composition, and potentiate thermal damage (Falkner and Biessmann, 1980; LaThangue, 1984; Lewis and Pelham, 1985; Roti Roti and Wilson, 1984; Roti Roti and Winward, 1978; Tanguay, 1983; Tanguay and Vincent, 1982; Warters et al, 1986). Alternatively, the shifting of proteins may have no physiological significance but may only reflect the heat-induced alteration of proteins that then bind to the NM-IF (Biessmann et al, 1982; Evan and Hancock, 1985; Kirov et al, 1984). These results show that much of the protein isolated in the NM-IF immediately after a heat shock is derived from the soluble protein (SOL fraction). The experiments described in Figures 20 and 24 were necessary to show that the prompt HS proteins are not simply normal temperature proteins that are labeled during heat shock and isolate with the NM-IF at the elevated temperature.

A potential source of the prompt HS proteins described in this chapter was the novel persistent virus associated with the Drosophila cell line used in these experiments (Scott et al, 1980). The protein synthesis directed by the viral genome was shown to be both heat-resistant and actinomycin-insensitive in a manner similar to prompt HS protein synthesis. However, the possibility that the prompt HS proteins described here were derived from endogenous synthesis of viral proteins or the breakdown products of the major viral products seems unlikely. The prompt HS proteins appeared equally well in cell cultures that were not expressing the virus (Figure 20) as those fully expressing the viral proteins (Figure 23). In addition, the 7.5 kb viral genome cannot contain sufficient information to encode for the both the major viral proteins as well as the 14 prompt HS proteins identified in Figure 20E. Finally, unlike the prompt HS proteins, which are synthesized only at temperatures exceeding 36^{o}C, the major viral proteins are synthesized at all temperatures.

The prompt HS response was distinct in several respects from the well studied, conventional response. The most apparent differences were the immediate appearance of the prompt HS proteins after the temperature shift and their insensitivity to actinomycin. Furthermore, the extent of synthesis of the prompt HS proteins was limited. In contrast to the large amounts of conventional HS proteins synthesized under typical conditions (DiDomenico et al, 1982; Lindquist, 1980a), the prompt HS proteins were minor cell constituents. Although the induction of some conventional HS proteins occurs as low as 29°C and all are induced by 33°C (Lindquist, 1980), expression of the prompt HS proteins required temperatures greater than 36°C. Another example of a response specific to high temperature is found in a report describing the phosphorylation and translocation to the nuclear compartment of a salt-resistant Drosophila Kc cell cytoskeletal protein (Vincent and Tanguay, 1982). This behavior was observed at 37°C but not at low temperature heat shocks of 30°C nor as the result of arsenite stress. In the following chapter, the conventional and the prompt HS proteins will be seen to differ in their response to alternative inducers of the stress response such as arsenite, cadmium, and aeration following anoxia.

The prompt HS proteins of Drosophila and HeLa have been localized entirely to the NM-IF. Many of the conventional HS proteins of diverse organisms are also preferentially localized in the nucleus and nuclear matrix (See especially Tanguay, 1985; Burdon, 1986). Other experiments in this laboratory show that the composition of the NM-IF is intimately related to patterns of transcription (Fey and Penman, 1986). Alteration in the NM-IF proteins accompanies changes in differentiation and patterns of gene expression. The changes in composition of the NM-IF during heat shock may reflect a fundamental role of the nuclear matrix in modulating gene expression.

CHAPTER 5

Alternative Inducers of the Stress Response Do Not Induce the Prompt Heat Shock Proteins in *Drosophila* but Do Induce a Subset of the Prompt Heat Shock Proteins in HeLa.

INTRODUCTION

Essentially all cells in culture, and many cells studied in vivo, respond to environmental stress, such as supranormal temperatures and metabolic poisons, by transiently reprogramming their metabolic activities. One ubiquitous aspect of this response, well-conserved between the cells of diverse organisms, is the production of a small set of stress or heat shock (HS) proteins. The HS response has attracted attention as a model for the modulation of gene expression at the molecular level and several recent reviews summarize the findings it has provided (Bonner, 1985; Craig, 1985; Neidhardt et al, 1984; Nover, 1984; Pelham, 1985; Schlesinger, 1986). The modern forerunner of these studies is widely acknowledged to be the paper of Ritossa in 1962 (Ritossa, 1962). This report described the induction of a novel pattern of puffs, corresponding to intensely active genes, in the polytene chromosomes of Drosophila hydei by elevated temperatures or exposure to dinitrophenol. Subsequently, the work of Tissieres suggested that the newly active genes encoded a limited set of novel proteins, later termed the HS proteins (Tissieres et al, 1974). Studies of Schlesinger and of Hightower linked the expression of the HS proteins with exposure to amino acid analogues and provided additional strategies by which the HS phenomenon could be studied (Kelley and Schlesinger, 1978; Hightower and Smith, 1978). The list of agents that induce the conventional HS proteins is large and growing (summarized in Burdon, 1986; Lindquist, 1986; Pelham, 1985; Schlesinger, 1985; Schlesinger, 1986). Clearly, the HS response is most accurately considered a reaction to a wide variety of metabolic stresses.

The prompt HS response constitutes another cellular response to high temperature. In this response, the very rapid production of another, distinct set of proteins designated as "prompt heat shock" has been described (Reiter

and Penman, 1983). The prompt HS response differed in many regards from previously described "conventional" HS response including, in the case of Drosophila, a significantly higher temperature of onset (Chapter 4). In this chapter, the induction of the prompt HS response by agents other than elevated temperature is examined.

The prompt HS response was originally described in HeLa and mouse 3T3 cells (Reiter and Penman, 1983) and has been identified in cultured Drosophila cells (Chapter 4). Elevated temperatures rapidly induced the synthesis of a set of proteins that were distinct from the transcriptionally-controlled, conventional HS proteins. In contrast to the conventional HS proteins, the prompt HS proteins were apparently made from preexisting, normally untranslated mRNAs. These proteins were found to associate exclusively with the nuclear matrix-intermediate filament scaffold or NM-IF. The prompt HS proteins were shown to be distinct from the preexisting, cytoplasmic proteins that become associated with the nucleus and nuclear matrix as a result of the heat stress. In further contrast to the conventional HS proteins, the synthesis of prompt HS proteins was not affected by inhibitors of transcription such as actinomycin D. The significance of the prompt HS proteins, as well as their relationship to the conventional HS response, is unknown. As a first step further characterizing the prompt HS response, alternative inducing conditions were examined.

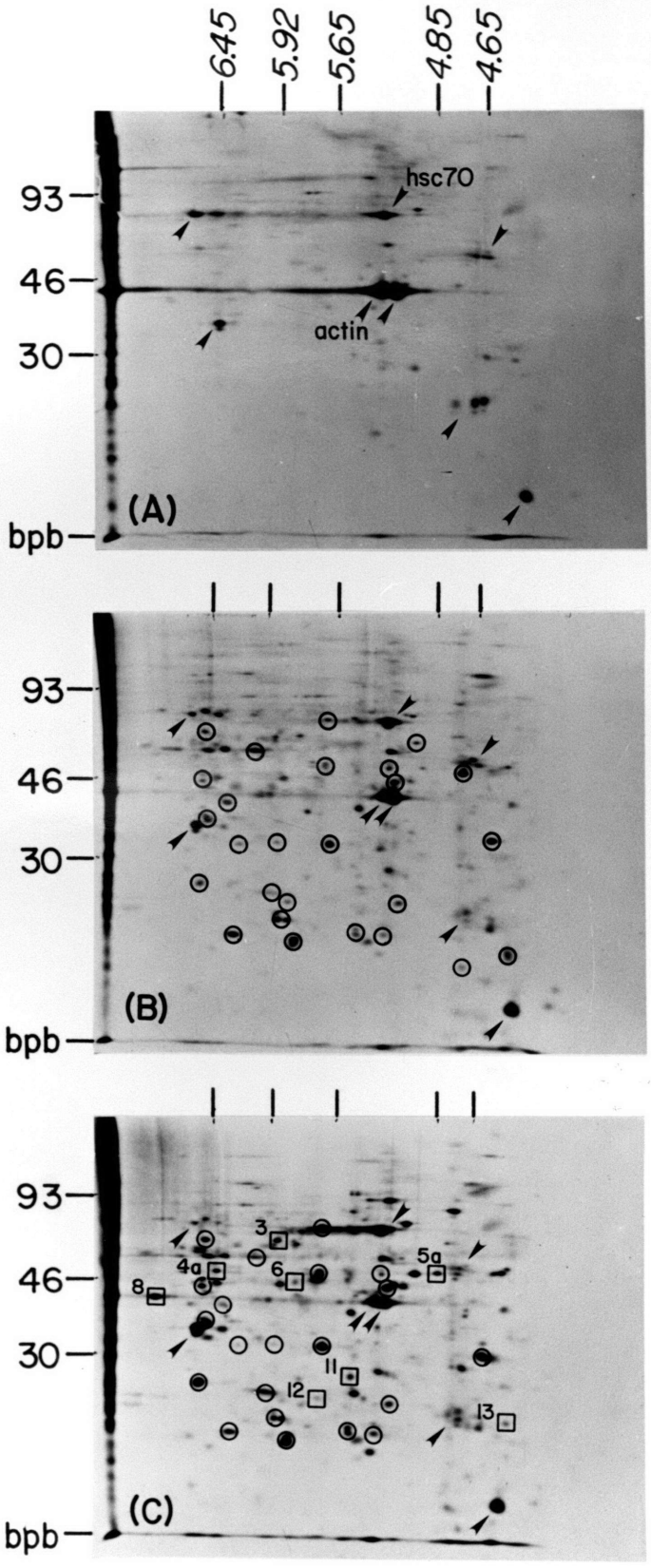
The use of inducing agents other than heat has enabled a number of investigators to further analyze the conventional HS response. Certain amino acid analogues, when incorporated into proteins, induce the synthesis of the HS proteins in Drosophila (DiDomenico et al, 1982). Lindquist and associates noted that transcription of the gene for hsp70 failed to stop under these conditions, in contrast to induction by HS. These observations lead to the discovery that hsp70 synthesis in Drosophila may be autoregulated and requires a fixed amount of functional hsp70 for the cessation

of its transcription (DiDomenico et al, 1982). In the established embryonic Drosophila cell line, Schneider's line 3, the molting hormone, β-ectdysterone, induced the synthesis of all four small HS proteins (Ireland and Berger, 1982). However, primary cultures of embryonic Drosophila cells responded to ectdysterone by synthesizing only hsp23 and 26 and not hsp22 and 29 (Buzin and Bournias-Vardiabasis, 1984). This differential induction of the small HS proteins suggests the existence of several levels of control over these genes even though all four HS genes are closely linked members of the 67B cytological locus (Craig and McCarthy, 1980). Serum starvation or the exposure to heavy metals was found to induce the human hsp70 gene (Wu and Morimoto, 1985). By examining the DNA sequences preceding the promoter in this gene, it was found that in addition to the DNA sequences that confer heat inducibility, a member of the hsp70 gene family is also under the control of a heavy metal-sensitive element (Wu et al, 1986).

In the experiments reported here, alternative inducers of the stress response were tested for their ability to induce the prompt HS proteins. The availability of alternative inducers of the prompt HS response could facilitate study of the prompt HS response in several ways. First, at the elevated temperatures best suited for identifying the prompt HS proteins, the synthesis of the prompt HS proteins is transient and occurs as cell viability diminishes. Chemically produced induction might afford greater amounts of the prompt HS proteins for biochemical studies. In addition, isolation of the heat-induced prompt HS proteins is complicated by the presence of a large background of preexisting proteins that are shifted to the NM-IF by elevated temperature. Chemical inducers have far less effect on pre-existing proteins. Finally, induction of the prompt HS proteins by agents other than heat may provide some insight into pathways common to the transcriptionally-controlled conventional and the prompt HS responses.

The results of the studies reported here indicate that the prompt HS response of Drosophila and human (HeLa) cells differ markedly with respect to the induction by agents other than heat. In Drosophila, the prompt HS proteins appeared in response to elevated temperature alone and were not induced by conditions capable of evoking the conventional HS proteins. In contrast to these results, the response of HeLa cells to alternative forms of stress was more complex. Different subsets of prompt HS proteins were induced by different agents, including some that are not known to induce the conventional HS proteins. The continued synthesis of some of the prompt HS proteins was observed during prolonged exposure to arsenite and cadmium. The induction of some of the HeLa prompt HS proteins by alternative inducers of the stress response may provide additional means by which the prompt HS proteins could be studied.

Figure 25. Normal, HShift and prompt HS proteins of Drosophila labeled with [^{35}S]methionine. The control pattern of NM-IF proteins in (A) was obtained from cells labeled for 30 min with 200 μCi/ml at 25^{o}C and then fractionated. Arrowheads point to characteristic proteins of the Drosophila NM-IF including actin II and III and a protein that may be the 70 kD HS cognate protein, hsc70. The proteins visualized in (B) were obtained from cells prelabeled at 25^{o}C, washed with complete medium and heat shocked at 36.5^{o}C for 45 min before fractionation. Circles mark those proteins that were not present in the control NM-IF or whose appearance was markedly increased by the 45 min heat shock (HShift proteins). The labeling pattern in (C) was obtained from cells shifted to 36.5^{o}C and labeled between 15 and 45 min at the elevated temperature. Circles mark the HSHift proteins that were synthesized at the high temperature and were isolated in the NM-IF. Squares identify the newly synthesized proteins that were unique to the NM-IF (prompt HS). AMD was added to 2 μg/ml 10 min prior to the temperature shift. Each fluorogram was derived from 200,000 cpm obtained from: (A), 1.04 x 10^{6} cells; (B), 0.31 x 10^{6} cells; (C) 0.74 x 10^{6} cells. The films were exposed for 36 h. The approximate position of radioactive molecular weight markers (M_r x 10^{-3}) and bromphenol blue tracking dye (bpb) is indicated at the left of the fluorogram. The approximate position of isoelectric point standards is indicated at the top of each figure.


6.45
5.92
5.65
4.85
4.65
93
46
30
bpb
hsc70
actin
(A)
(B)
(C)
3
4a
5a
6
8
11
12
13

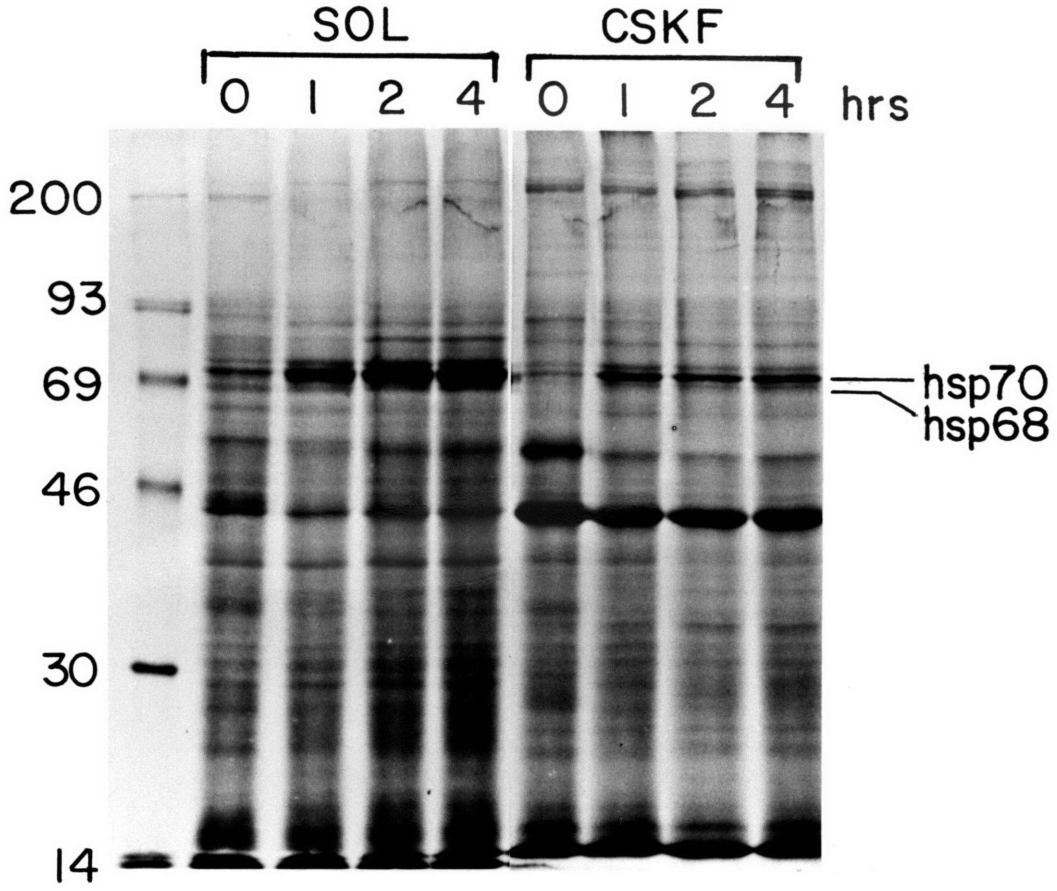
RESULTS

Isolation of the NM-IF. The prompt HS proteins of both Drosophila (Chapter 4) and HeLa (Reiter and Penman, 1983) cells were found associated almost exclusively with the NM-IF. The prompt HS proteins represent a very small proportion of the total protein synthesis that occurs during HS and are consequently difficult to detect. The association of these proteins with the purified NM-IF permitted the identification of the prompt HS proteins once they were separated from the majority (> 90 per cent) of newly synthesized cellular proteins. The NM-IF, which amounts for approximately 5 per cent of the total cell protein in the normal cell, was obtained by a sequential fractionation procedure which accounts for all cellular proteins. The procedure has been described previously in detail (Fey et al, 1984). In brief, cells were first exposed to the non-ionic detergent, Triton X-100, in a buffer of near-physiological ionic strength to solubilize lipids and release the "soluble" proteins (SOL fraction). The resulting cytoskeletal framework (CSKF) was further fractionated in situ by extracting the salt-labile proteins. The NM-IF was then obtained by removing, DNA, nuclear RNA and chromatin-associated proteins by digesting with DNase I and RNase A followed by extraction with $(NH_4)_2SO_4$. To the limit of detection afforded by the two-dimensional gel electrophoretic system, none of these protein fractions, separated from the NM-IF by this procedure including the Triton X-100 released or soluble proteins (SOL), the salt-labile cytoskeletal proteins (CSK), and the chromatin-associated and hnRNP proteins (CHR), contained the prompt HS proteins (Chapter 4, Reiter and Penman, 1983). The prompt HS proteins were detected only in the NM-IF and only the two-dimensional gel electropherograms of this fraction are presented in this chapter.

Prompt HS proteins of Drosophila cells identified in the NM-IF. A substantial change in the protein composition of the NM-IF occurred after cells were exposed to elevated temperatures. In addition to the control NM-IF proteins, three classes of proteins synthesized during HS appeared in the NM-IF. These proteins included 1) the prompt HS proteins, 2) the conventional HS proteins, and 3) proteins of the SOL fraction that became associated with the NM-IF as a result of the elevated temperature. The prompt HS proteins were easily distinguished from the conventional HS proteins by inhibiting RNA synthesis with actinomycin D (AMD) during the HS, thereby inhibiting synthesis of the conventional HS proteins. The problem of discerning the prompt HS proteins among the proteins shifted to the NM-IF from other cell fractions was addressed by the experiment presented in Figure 25. Drosophila cells were labeled with [^{35}S]methionine prior to the HS. Those prelabeled proteins which "shifted" to the NM-IF during the HS (i.e. are not found in the control NM-IF) are identified as HShift proteins. The prompt HS proteins are defined as those NM-IF proteins that are synthesized during HS that are distinct from the control and HShift proteins.

The proteins of the normal Drosophila NM-IF, labeled with [^{35}S]methionine, are seen in the two-dimensional gel electropherogram of Figure 25A. The arrowheads in Figure 25 mark major proteins that were characteristic of the Drosophila NM-IF. Some of these proteins may correspond to known NM-IF proteins of other organisms and were also indicated in the wide range gels of Chapter 4. Drosophila actin II and III was identified on the basis of the apparent molecular weight of 43,000 and isoelectric pH (pI) of 5.2 and 5.35: values which are in good agreement with those previously reported (Mogami et al, 1982; Storti et al, 1978). On the basis mobility in the two-dimensional gels, the protein identified as hsc70 in Figure 25A may be the gene product of the HS cognate gene, Hsc4 (Craig et al, 1983).

Figure 26. Drosophila protein synthesis in the presence of sodium arsenite. A culture of Drosophila cells was incubated in the presence of 100 μM sodium arsenite for 4 h. At the times indicated above each lane, a portion of the culture was removed, the cells harvested and transferred to methionine-free media containing the drug, then labeled with [^{35}S]methionine for 30 min before fractionation. The proteins released by extraction with Triton X-100 in CSK buffer (SOL) and the proteins associated with the residual cytoskeletal framework (CSKF) were analyzed by gel electrophoresis and fluorography. The approximate migration of the conventional HS proteins, hsp68 and hsp70 are indicated at the right. The position of radioactive molecular weight standards (M_r x 10^{-3}) is indicated at the left. Equivalent amounts of radioactivity was loaded onto each lane, consequently the material from approximately 3 x as many cells is represented in the CSKF fractions.


SOL
CSKF
0 1 2 4 0 1 2 4 hrs
200
93
69
46
30
14
hsp70
hsp68

This protein, which is structurally similar to the 70 kD HS protein although not induced by heat, has been shown to colocalize with the intermediate filament network of embryonic Drosophila cells (Palter et al, 1986).

Figure 25B shows the proteins shifted into the NM-IF from other cell fractions by a 45 min HS at 36.5°C. Cells were labeled with [^{35}S]methionine at normal temperature, the radioactive amino acid removed and the cells heat shocked in the presence of an inhibitor of transcription (AMD), before the NM-IF was isolated. In addition to the major control NM-IF proteins, new proteins appear. These were termed the HShift proteins, for "heat-shifted", and the more prominent are circled in Figure 25.

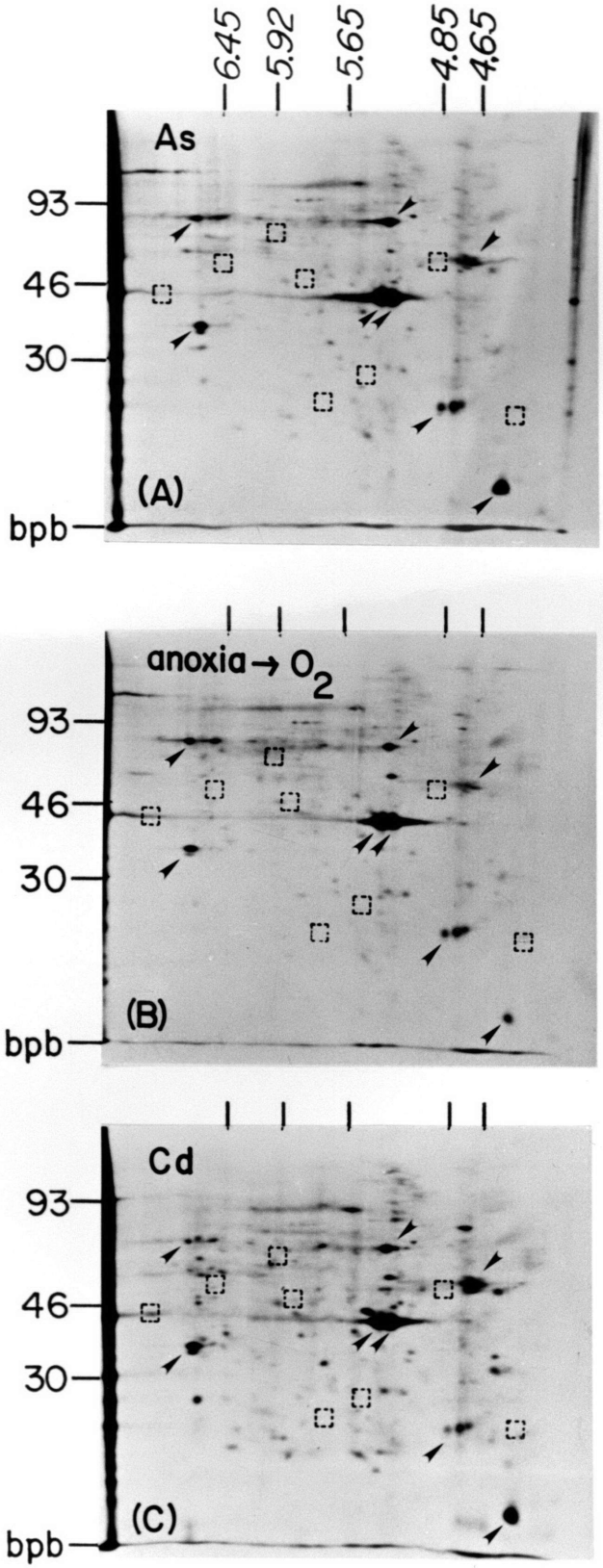
Figure 25C shows the two-dimensional gel electropherogram pattern of NM-IF proteins labeled during HS at 36.5°C in the presence of AMD. The prompt HS proteins, i.e. those synthesized at the elevated temperature in the absence of new transcription, are indicated by numbered squares. These are NM-IF proteins that were distinct from the normal proteins of the NM-IF and were distinct from the HShift proteins. Note that many of the HShift proteins continued to be synthesized at the elevated temperature and were isolated as part of the NM-IF (circled in Figure 25C). Prompt HS proteins were not detected in any of the other subcellular fractions obtained from either control or heat shocked cells (Chapter 4). The numbers designating the prompt HS proteins are those assigned in Table 6, Chapter 4. The narrow range isoelectric focusing used in these experiments revealed two additional prompt HS proteins (Drosophila pHS:4a, 52 kD, pI 6.44 and Drosophila pHS:5a, 50 kD, pI 4.83) that were not well resolved previously using wide range isoelectric focusing gels.

Alternative inducers of the stress response fail to induce the prompt HS proteins in Drosophila cells. Exposure of cultured Drosophila cells to the metabolic poison, arsenite, or the transition-series metal ion, cadmium,

induces a subset of the conventional HS proteins (Courgeon et al, 1984; Vincent and Tanguay, 1982). In addition, prolonged anoxia induces the synthesis of hsp70 in Drosophila cells shortly after the return to aerobic conditions (Velazquez and Lindquist, 1984). Figure 26 illustrates the time course of protein synthesis in the presence of 100 μm sodium arsenite when transcription was permitted. After 1 h exposure to the drug, newly synthesized hsp70 and hsp68 were readily detected among the proteins labeled with [35S]methionine. The synthesis of these two proteins increased only marginally over the course of a four hour exposure to arsenite. Similar results were obtained with 100 μm cadmium sulfate (data not shown). Arsenite failed to induce the synthesis of the low molecular weight HS proteins. Also, following treatment with either arsenite or cadmium, most of the newly synthesized hsp68/70 was released in the initial extraction; very little associated with the nucleus or nuclear subfractions, in agreement with the findings of other investigators (Tanguay, 1985; Vincent and Tanguay, 1982).

The NM-IF was isolated and from cells exposed to various stresses when transcription was inhibited by AMD. The pattern of proteins synthesized for the NM-IF after 90 min exposure to arsenite (Figure 27A) was largely unchanged from the control pattern (Figure 25A). In particular, the prompt HS proteins could not be detected in the NM-IF; the expected position of the prompt HS proteins are indicated by the broken squares in Figure 27. These could not be detected even after increased exposure of the fluorogram. In addition, the prompt HS proteins were not detected among the newly synthesized NM-IF proteins following the release from 90 min of anoxic conditions (Figure 27B). For this experiment, anoxic medium was obtained by degassing serum-free Shields and Sang media for 6 hours under humidified nitrogen. Cells were harvested and resuspended in a minimal volume of anoxic medium, transferred to the larger volume spinner culture with a teflon seal, and the air space was

Figure 27. The prompt HS proteins are not present among the Drosophila NM-IF proteins labeled after exposure to arsenite, cadmium, or after release from anoxia. Arrowheads point to the major proteins of the normal Drosophila NM-IF. Broken squares mark the expected positions of the prompt HS proteins. The pattern in (A) was obtained from cells that were exposed to 100 μM sodium arsenite for 60 min, transferred to methionine-free medium containing arsenite and labeled with 200 μCi/ml [^{35}S]methionine for 30 min. NM-IF protein from 1.13 x 10^6 arsenite-treated cells was analyzed in (A). The NM-IF protein visualized in (B) was prepared from cells labeled with [^{35}S]methionine after being maintained under anoxic conditions for 90 min. NM-IF protein from 0.66 x 10^6 cells was analyzed. The NM-IF fraction analyzed in (C) was obtained from cells exposed to 100 μM cadmium sulfate and labeled as for the arsenite treatment. Protein from 0.74 x 10^6 cells was analyzed in (C). AMD was added to 2 μg/ml 10 min before the addition of the drug (A and C) or immediately after the release from anoxic conditions (B). 200,000 cpm was analyzed in each fluorogram. Exposure was for 36 h. The position of radioactive molecular weight markers (M_r x 10^{-3}) and bromphenol blue tracking dye (bpb) is indicated at the left of each fluorogram. The approximate position of isoelectric point standards is indicated at the top of each figure.


6.45
5.92
5.65
4.85
4.65
As
93
46
30
(A)
bpb
anoxia → O_2
93
46
30
(B)
bpb
Cd
93
46
30
(C)
bpb

purged with nitrogen. AMD was added immediately after the release from anoxia.

As indicated by the broken squares in Figure 27C, none of the prompt HS proteins were detected after a 90 min exposure to 100 μM cadmium sulfate. Cadmium did, however, induce other changes in the proteins synthesized and incorporated in the Drosophila NM-IF. Numerous minor proteins were found that were not among the normal NM-IF proteins. Although not marked, some of these proteins corresponded to HShift proteins, identified in Figure 25B. Several proteins seen in the NM-IF after exposure to cadmium appeared to be distinct from the normal, HShift and prompt HS proteins. These cadmium-specific changes in the NM-IF were not further examined. Other investigators have found that longer exposures to higher concentrations of cadmium induced larger amounts of the conventional HS proteins (Courgeon et al, 1984). These more severe conditions were not examined here.

These results, obtained with a limited group of agents and conditions known to induce the conventional stress response in Drosophila cells, suggest that the prompt HS proteins of Drosophila are not elicited by alternative inducers. It is possible that a limited synthesis of these proteins would have escaped detection if the proteins were not localized to the NM-IF. To the limit of resolution afforded by the electrophoretic separation, this did not appear to be the case. More severe conditions and other inducing agents may be required to induce the prompt HS proteins of Drosophila which, so far, have only been produced in response to elevated temperature. This is in marked contrast to the prompt HS response of HeLa cells, described in the remainder of this chapter.

Normal, HShift and prompt HS proteins of the HeLa NM-IF. The prompt HS response of HeLa cells, as in Drosophila, was complicated by the shifting into the NM-IF of proteins normally found in other subcellular fractions (Roti Roti and

Wilson, 1984; Warters et al, 1986). Consequently, it was necessary to differentiate the HeLa prompt HS proteins from the normal NM-IF proteins as well as these "heat-shifted" or HShift proteins. The results of this analysis are shown in Figure 28. The pattern of NM-IF proteins that was obtained from cells labeled at normal temperatures, chased with unlabeled methionine and then heat shocked, was compared to the normal pattern of NM-IF protein. The new proteins that appeared in the NM-IF from other cell fractions were the HShift proteins. The prompt HS proteins were then identified as those unique proteins labeled during the 43.5°C HS that were neither normal NM-IF proteins nor HShift proteins.

The normal pattern of NM-IF protein (Figure 28A) was obtained from HeLa cells that were labeled with [^{35}S]methionine at 37°C. Arrowheads mark the major methionine-labeled proteins found in the NM-IF of control cells. These proteins also served as reference points when comparing electrophoretic patterns of different samples. Some of the proteins identified in Figure 28A, determined by previous immunoblot analysis, included vimentin, the lamins A and C, and a group of immunologically related cytokeratins with molecular weights between 44,000 and 54,000 (Fey et al, 1986). The HShift pattern of proteins is seen in Figure 28B. These proteins were obtained from cells that were labeled with [^{35}S]methionine at 37°C, chased at the normal temperature, and then heat shocked at 43.5°C for 45 min before isolating the NM-IF. The radioactivity in the NM-IF protein from these cells was approximately 2.4 times that obtained from unstressed cells. This additional incorporation reflected the many new (HShift) proteins seen in Figure 28B and did not result from changes in the relative abundance of the major control proteins. In Figure 28B, the major control proteins appear to be a less significant part of the NM-IF only because equivalent amounts of radioactivity were analyzed in the electropherograms of Figure 28. Because of the large number of HShift proteins that can be detected

(approximately 50), they were not individually marked in Figure 28.

Fifteen proteins were identified in the HS-labeled NM-IF that had no counterpart in either the control or HShift NM-IF proteins. These proteins, identified by numbered squares in Figure 28C, are the prompt HS proteins of HeLa cells. The apparent molecular weight and isoelectric pH of each of these proteins, referred to as HeLa pHS:1 through HeLa pHS:15, is presented in Table 7. For the experiments using HeLa cells, it was not necessary to inhibit the conventional HS response to detect the prompt HS proteins in the isolated NM-IF. However, it has been previously shown that the pattern of NM-IF proteins labeled during the first hour of treatment is unchanged by the inhibition of RNA synthesis (Reiter and Penman, 1983).

In addition to the prompt HS proteins, many of the HShift proteins, (defined as those proteins labeled at normal temperatures which associate with the NM-IF only after HS) continued to be synthesized at elevated temperature and associated with the NM-IF of cells labeled between 15 and 45 min at 43.5^{o}C (Figure 28C). The major normal proteins of the NM-IF (Figure 28A) were also synthesized to a limited extent during the HS (Figure 28C). However, at 43.5^{o}C, the translation of most messenger RNAs synthesized before the HS is reduced (Burdon et al, 1982; Hickey and Weber, 1982; McCormick and Penman, 1969). As a result of this inhibition, it was necessary to expose the electropherogram of Figure 28C for a longer period of time to visualize the normal proteins (indicated by arrowheads).

Prompt HS proteins of HeLa cells are rapidly induced by alternative inducers of the stress response. In contrast to the results obtained with Drosophila cells, metabolic poisons, transition-series metal ions, and ethanol induced the synthesis of a varied subset of the prompt HS proteins in HeLa cells. These results are seen in the two-dimensional gel electropherograms of NM-IF protein of Figure 29. For

Figure 28. Normal, HShift and Prompt HS proteins of the HeLa NM-IF labeled with [^{35}S]methionine. HeLa cells were labeled for 30 min with 150 μCi/ml at 37^{o}C and then fractionated (A) or washed with complete medium and heat shocked at 43.5^{o}C for 45 min before fractionation (B). The heat shock labeling pattern in (C) was obtained from the NM-IF protein isolated from cells shifted to 43.5^{o}C and labeled between 15 and 45 min at the elevated temperature. The approximate position of radioactive molecular weight standards ($M_r \times 10^{-3}$) is indicated at the left of each figure, the position of isoelectric point standards is indicated at the top. Approximately 300,000 cpm was analyzed in each fluorogram.

Figure 3A. NM-IF proteins of HeLa cells cultured and labeled at 37^{o}C. Arrowheads point to characteristic proteins of the HeLa NM-IF including the cytokeratins, "C"; vimentin, "V"; and the lamins A and C, "L". The gel was prepared with protein derived from 0.77 x 10^6 cells, the fluorogram was exposed for 24 h.

Figure 3B. Proteins of the NM-IF from cells prelabeled at 37^{o}C then shifted to 43.5^{o}C for 45 minutes. Arrowheads point to the characteristic proteins of the normal HeLa NM-IF. The gel was prepared with material from 0.32 x 10^6 cells, the fluorogram was exposed for 24 h.

Figure 3C. Proteins of the NM-IF from cells labeled between 15 and 45 minutes at 43.5^{o}C. Numbered squares mark the newly synthesized proteins that are unique to the NM-IF and can be identified at the exposure of the fluorogram shown (prompt HS proteins). The apparent molecular weight and isoelectric pH of each of these prompt HS proteins is enumerated in Table 1. The gel was prepared with NM-IF protein from 0.74 x 10^6 cells and the fluorogram exposed for 60 h.

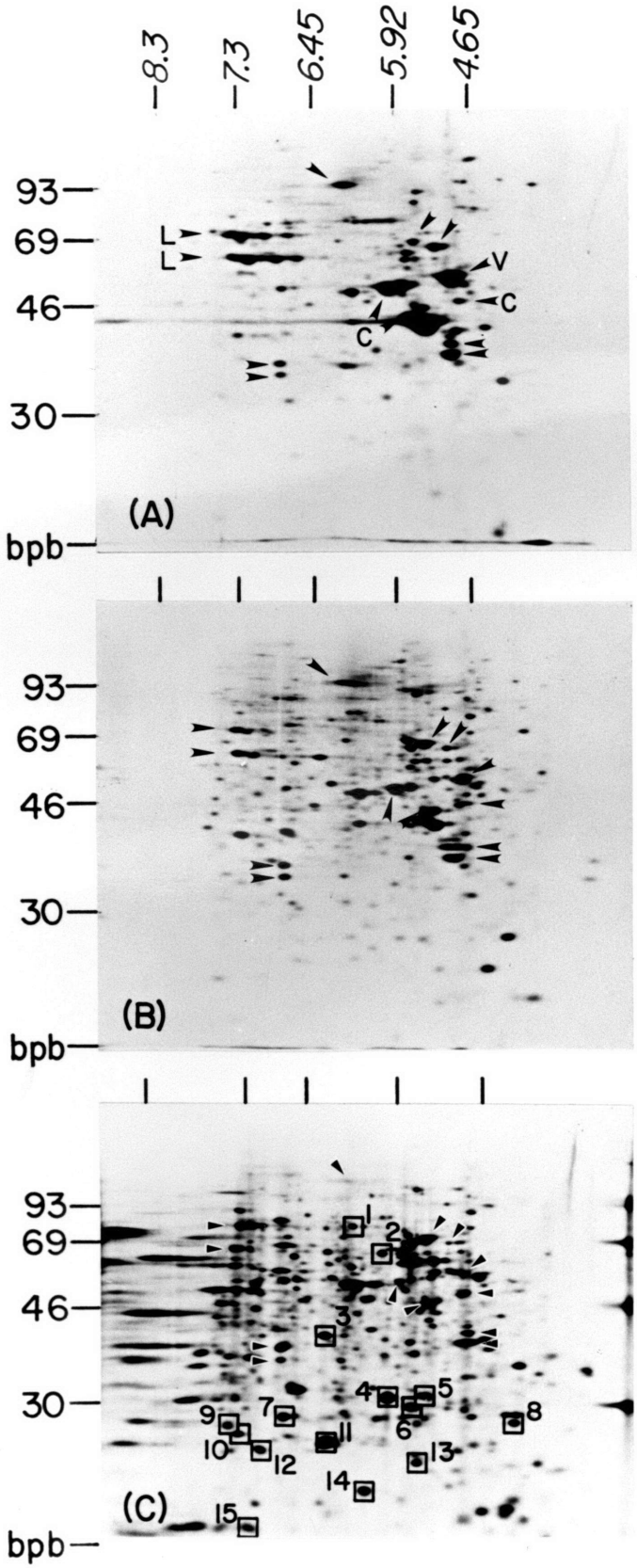

8.3
7.3
6.45
5.92
4.65
93
69
46
30
bpb
(A)
L
L
V
C
C
(B)
(C)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Table 7. Apparent molecular weight and isoelectric pH of the HeLa prompt HS proteins.

Protein Number	Molecular Weight	Isoelectric pH
pHS:1	84.0	6.2
pHS:2	65.0	6.1
pHS:3	42.0	6.35
pHS:4	33.0	5.95
pHS:5	33.0	5.7
pHS:6	31.0	5.9
pHS:7	29.0	6.6
pHS:8	28.0	4.5
pHS:9	28.0	7.6
pHS:10	27.0	7.4
pHS:11	26.0	6.4
pHS:12	24.5	6.45
pHS:13	24.3	5.7
pHS:14	19.0	6.1
pHS:15	16.0	7.3

Key: molecular weight expressed x 10^{-3}

these experiments, HeLa cells were exposed to the chemical stress for 30 min in medium lacking methionine. (Incubation in methionine-free medium alone for at least 1 hour did not induce the synthesis of any stress proteins in these cells.) [^{35}S]methionine was then added to the cultures and the cells were labeled in the presence of the inducing agent for an additional 30 min. Although ethanol and dinitrophenol are not known to induce the stress response in HeLa cells (Craig, 1985; Schlesinger, 1986), those agents do induce the stress response in other mammalian (Li and Werb, 1982) and insect cell lines (for review, see Hightower et al, 1985; Nover, 1984; Schlesinger, 1985). The numbered squares mark the HeLa prompt HS proteins detailed in Table 7. The square braces mark HeLa prompt HS proteins that were visualized with longer exposure of the electropherogram.

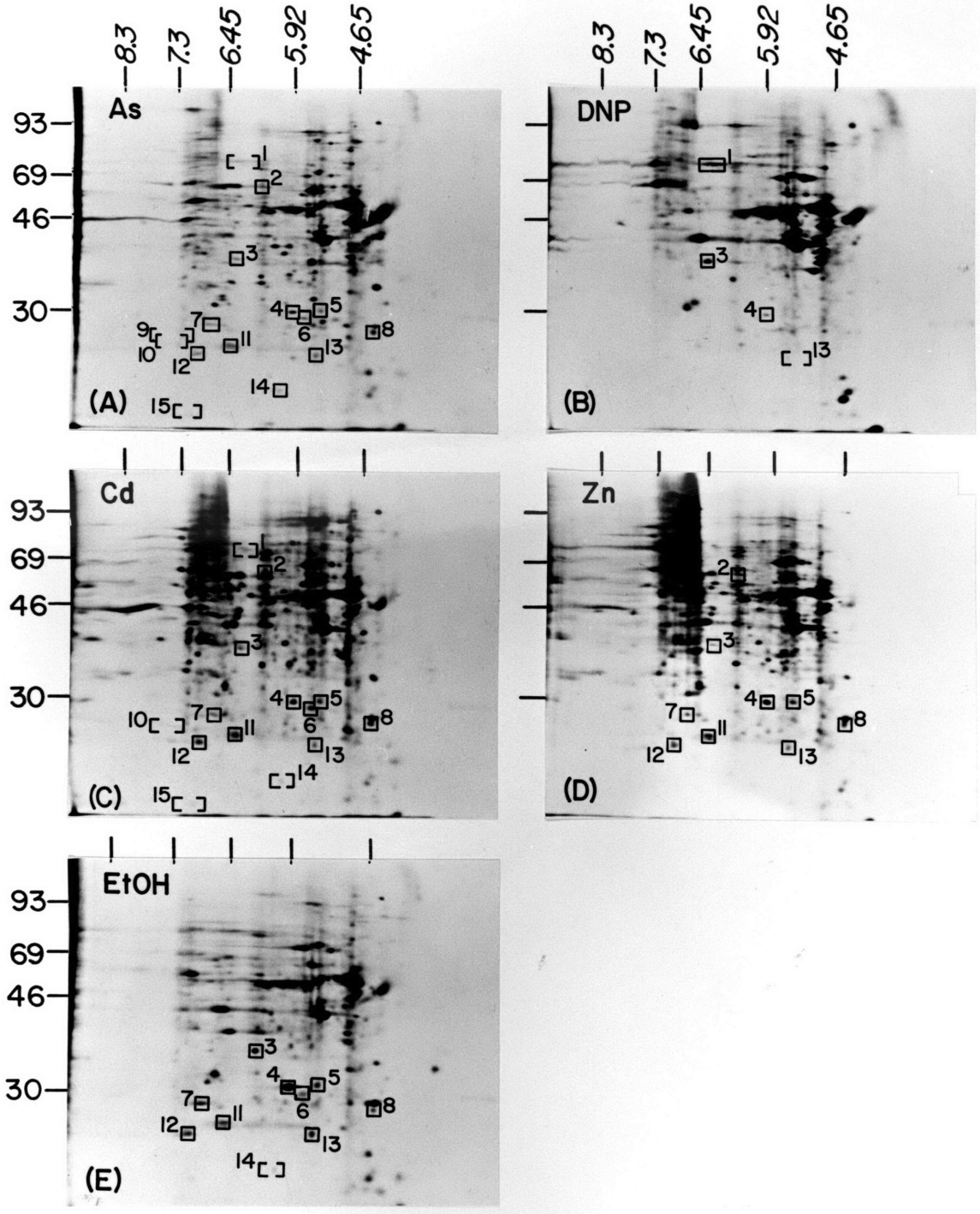
Sodium arsenite appeared to induce the complete set of HeLa prompt HS proteins. The newly synthesized protein of the NM-IF isolated from cells exposed to the 100 μM sodium arsenite is seen in Figure 29A. The total amount of [^{35}S]methionine incorporated into protein was substantially reduced relative to untreated cells during the course of the treatment (see Table 8). In addition, the relative intensities of the newly synthesized NM-IF proteins, labeled during the brief exposure to arsenite, differed from that of the control (see Figure 28A). Of the prompt HS proteins identified in Figure 29A, HeLa pHS:1, 9, 10 and 15 were more clearly visualized after increased exposure of the fluorogram. Although not indicated in Figure 29A, many of the HShift proteins were detected in the NM-IF of the arsenite treated cells.

2,4-dinitrophenol (DNP) effected a very different change in the pattern of proteins synthesized for the NM-IF (Figure 29B). DNP appeared to reduced total protein synthesis by only 20 per cent (Table 8) and only four prompt HS proteins were detected in the NM-IF. Both arsenite and DNP are well known mitochondrial poisons, undoubtedly with pleiotropic

effects. In the mitochondrion, arsenite acts primarily by binding vicinal sulfhydryl groups such as those in lipoic acid and inhibits acetyl-CoA formation (Reiss and Hellerman, 1958), while DNP uncouples the synthesis of ATP from electron transport chain by dispersing the charge gradient across the mitochondrial membrane (Kraayenhof and van Dam, 1969). Although both agents have been shown to induce the stress response in Drosophila cells, DNP is not known to induce the major HS proteins in HeLa cells (Craig, 1985; Schlesinger, 1986).

The transition series IIb metal ions, introduced as cadmium sulfate and zinc sulfate, induced different subsets of the prompt HS proteins. Nearly all (14 of 15) of the prompt HS proteins were detected among the NM-IF proteins isolated from cells exposed to 100 μM cadmium sulfate (Figure 29C). At least nine of the prompt HS proteins were detected in the NM-IF of cells treated with 100 μM zinc sulfate. The prompt HS proteins that were found in the cadmium-treated cells but were not detected in the zinc-treated cells included the basic prompt HS proteins, HeLa pHS:9 and 6, and the low molecular weight prompt HS proteins, HeLa pHS:14 and 15. Both heavy metal ions caused many of the HShift proteins to appear in the NM-IF (which are not marked in Figure 29) and additional proteins that were not further identified. In contrast to the action of the mitochondrial poisons, protein labeling (as measured by the incorporation of [^{35}S]methionine into acid-precipitable material) increased by nearly two-fold over that observed in the control (Table 8). A proportional increase in the amount of label found in the NM-IF was also observed. The appearance of the prompt HS proteins under these conditions indicates that the diminution of protein synthesis is not obligatory for the detection of the prompt HS proteins in the NM-IF. Finally, a subset of the prompt HS proteins were induced by exposing HeLa cells to 4 per cent (0.8 M) ethanol, a concentration sufficient to induce the conventional HS response in CHO cells (Li and Werb, 1982).

Figure 29. Prompt HS proteins are synthesized upon exposure to alternative inducers of the stress response in HeLa cells. HeLa cells were concentrated 3-fold into methionine-free medium and the appropriate chemical agent added from a concentrated stock. After 30 min, [^{35}S]methionine was added to 100 μCi/ml and the cells labeled for an additional 30 min in the presence of the inducer. The cells were fractionated and approximately equal amounts of radioactivity (300,000 cpm) from the NM-IF was analyzed. Numbered squares mark the prompt HS proteins. Square brackets indicate the location of prompt HS proteins that were visualized upon further exposure of the fluorogram or failed to focus to a well defined spot during electrophoresis. The agents used to induce the stress response and number of cells contributing the NM-IF protein were: (A), 100 μM sodium arsenite, 8.11 x 10^6 cells; (B), 1 mM 2,4DNP, 3.95 x 10^6 cells; (C), 100 μM cadmium sulfate, 0.29 x 10^6 cells; (D), 100 μM zinc sulfate, 0.28 x 10^6 cells; (E), 4 per cent ethanol, 6.25 x 10^6 cells (150,000 cpm was analyzed, exposure was for 48 h). The approximate position of radioactive molecular weight standards (M_r x 10^{-3}) is indicated at the left of each figure, the migration of isoelectric point standards indicated at the top. All fluorograms, except that shown in (E), were exposed for 24 h.


8.3
7.3
6.45
5.92
4.65
93
69
46
30
As
(A)
DNP
(B)
Cd
(C)
Zn
(D)
EtOH
(E)

Even though protein labeling in cells exposed to 4 per cent ethanol was reduced by 90 per cent, the proteins synthesized for the NM-IF included ten of the prompt HS proteins (Figure 29E).

Arsenite and cadmium induce the synthesis of most of the conventional HS proteins in HeLa cells. The temporal behavior of prompt HS protein synthesis could not be measured in heat-treated cells since all protein synthesis ceased after one hour at the elevated temperature (Reiter and Penman, 1983). In contrast, cells treated with arsenite or cadmium continued a high rate of protein synthesis for many hours and permitted measurement of the prompt HS response at longer times. Protein synthesis was monitored after three hours exposure to arsenite or cadmium by labeling with [^{35}S]methionine. As seen in Figure 30, the major conventional prompt HS proteins were induced under these conditions (Anderson et al, 1982; Burdon et al, 1982). The data of Figure 30 shows the newly synthesized protein in the presence (indicated by a bullet above the lane) and absence of AMD in untreated (Con), arsenite-treated (As) and cadmium-treated (Cd) cells. Before analysis, the cells were extracted to yield the SOL fraction and residual CSKF. From the one-dimensional gel electropherogram, the following salient features can be summarized: 1) in the absence of AMD, the synthesis of hsp72/73, hsp80, hsp90 and hsp100 was induced by arsenite or cadmium; 2) in the presence of AMD, arsenite or cadmium enhanced the synthesis of only one band migrating at 72 kD; 3) a 3 h exposure to AMD alone did not enhance the synthesis of any proteins, with the possible exception of hsp90; 4) the majority of the conventional HS protein induced by arsenite or cadmium was released into the SOL fraction after the initial extraction.

The kinetics of arsenite and cadmium induced prompt HS protein synthesis. The subcellular fractions of the cells analyzed in Figure 30 were analyzed by two-dimensional gel electrophoresis. The prompt HS proteins were detected only

Figure 30. Effect of AMD on protein synthesis in HeLa cells during exposure to arsenite or cadmium. Duplicate cultures of HeLa cells were exposed to 100 μM sodium arsenite (As), 100 μM cadmium (Cd) or no inducing agent (Con). AMD was added to one of each pair of cell cultures 5 min prior to the addition of the inducing agent. The cells were maintained in the presence of the drugs for 2.5 h then concentrated into methionine-free medium containing the same drugs and pulse-labeled with [^{35}S]methionine for 30 min. The cells were fractionated to yield the SOL fraction and the residual cytoskeletal framework (CSKF). Equivalent amounts of radioactivity were analyzed in each lane. The presence of AMD during the incubation and labeling is indicated by the bullet directly above the lane. The expected position of the major HS proteins is indicated at the right of the figure. Radioactive molecular weight standards and their sizes ($M_r \times 10^{-3}$) are indicated at the left of the figure.

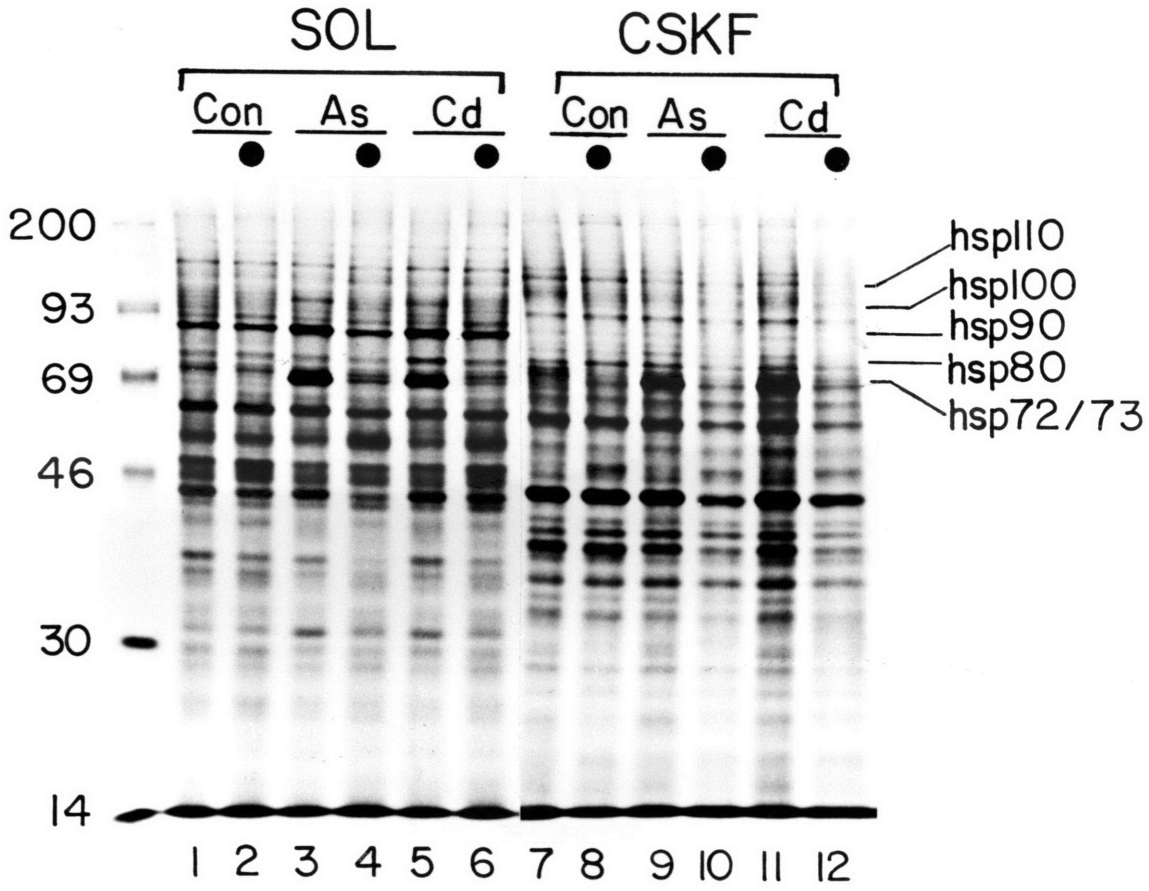
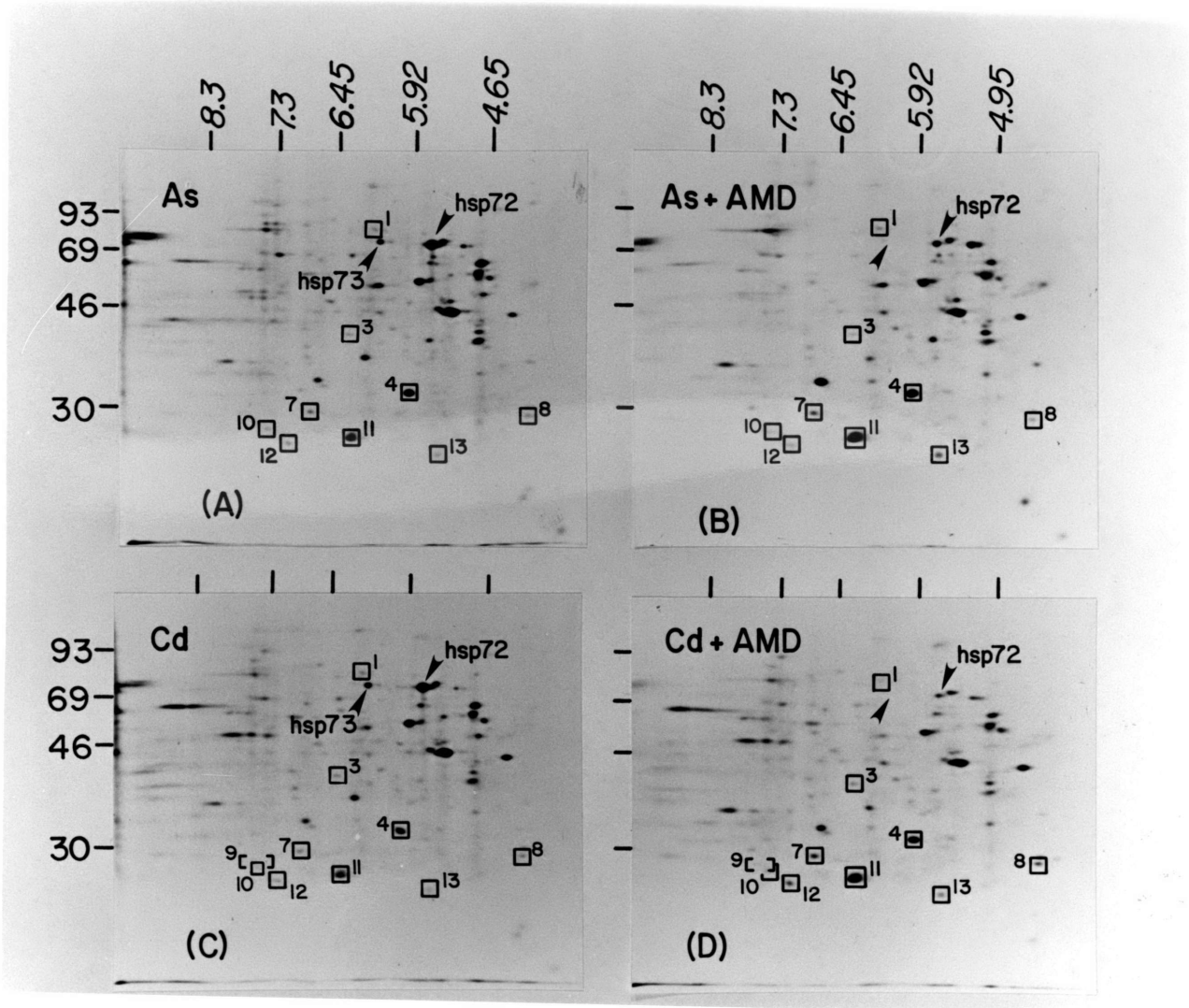

SOL
CSKF
Con
As
Cd
Con
As
Cd
200
93
69
46
30
14
hspl10
hspl00
hsp90
hsp80
hsp72/73
1 2 3 4 5 6 7 8 9 10 11 12

Figure 31. A subset of the HeLa prompt HS proteins are synthesized after 3 h exposure to arsenite or cadmium. HeLa cells were maintained in the presence of 100 μM sodium arsenite (A and B) or 100 μM cadmium sulfate (C and D) for 2.5 h, transferred to methionine-free medium containing the same drugs and labeled with [^{35}S]methionine for another 30 min. AMD was added prior to addition of the inducing agent to the cultures represented in (B) and (D). Cells were fractionated and 120,000 cpm of NM-IF protein was analyzed. An exposure of 60 h was used for the fluorograms presented. A constitutively expressed HS protein, hsp72, is indicated in each figure. Hsp73 was seen in the absence of AMD and is indicated by the labeled arrow in (A) and (C). The unlabeled arrow in (B) and (D) indicates the expected location of hsp73. NM-IF protein from the following number of cells was used for each: (A), arsenite alone, 0.95 x 10^6 cells; (B), arsenite with AMD, 6.67 x 10^6 cells; (C), cadmium alone, 0.78 x 10^6 cells; (D), cadmium with AMD, 2.86 x 10^6 cells. The approximate position of molecular weight (M_r x 10^{-3}) and isoelectric point standards is indicated at the left and top of the figures, respectively.


-8.3
-7.3
-6.45
-5.92
-4.65
-4.95
As
As + AMD
Cd
Cd + AMD
hsp72
hsp73
93
69
46
30
(A)
(B)
(C)
(D)

among the proteins of the NM-IF which are seen in the electropherograms of Figure 31. The prompt HS proteins synthesized after three hours exposure to arsenite (Figures 31A and 31B) and cadmium (Figures 31C and 31D) were very similar. The only notable difference was HeLa pHS:9, seen in the NM-IF after exposure to cadmium but not arsenite. However, the patterns at three hours differed significantly from those seen after one hour of induction. At least five of the prompt HS proteins found in the NM-IF after one hour (HeLa pHS:2, 5, 6, 14 and 15) were no longer detectable. Changes in the relative labeling intensities of several prompt HS proteins were also noted. When induced by heat, HeLa pHS:4 and HeLa pHS:11 were the most heavily labeled prompt HS proteins (Figure 28C). Following one hour exposure to arsenite or cadmium, these same two proteins were labeled only to about the same extent as the other prompt HS proteins (Figure 29). However, after a 3 hour exposure to arsenite or cadmium, the amount of label in HeLa pHS:4 and 11 had increased significantly relative to the remaining prompt HS proteins (Figure 31).

The data in Figures 31A and 31C show the prompt HS proteins synthesized after three hours when transcription was permitted (AMD omitted). These results, when compared to the results of Figure 29, indicate that the prompt HS proteins are differentially regulated over time. The data of Figures 31B and 31D suggest that this differential regulation is probably not due to the differential synthesis of new prompt HS mRNA. This appears likely because the synthesis of the prompt HS proteins, relative to each other and to other NM-IF proteins, was largely unaffected by inhibiting transcription with AMD.

Two proteins identified in the fluorograms of Figure 31 appeared to correspond to the major HS protein of mammalian cells, labeled hsp72 and hsp73. The identification of these two proteins was based on their mobility in the two-dimensional gels (Thomas et al, 1982; Welch and Feramisco,

1985a; Welch et al, 1983). The synthesis of hsp72 and hsp73 was increased over that seen in untreated cells or in cells exposed to the inducing agents in the presence of AMD (Figures 31B and 31D). The 73 kD HS protein, with isoelectric pH of 6.15, was observed in only the NM-IF of cells treated in the absence of AMD (Figures 31C and 31D). The size and charge of this protein, together with the observation that it appeared only in the induced cells suggested that it is an "inducible" HS protein, initially described in HeLa cells by Mathews et al (1982). Hsp73 was not found exclusively in the NM-IF but largely in the soluble and cytoskeletal fractions (Figure 30). Another conventional HS protein seen in Figure 31 was labeled hsp72. The synthesis of this protein was elevated after exposure to the inducing agents when transcription was permitted. However, in contrast to the inducible HS protein, hsp72 was synthesized in unstressed cells to a limited extent, as are most of the HeLa HS proteins (Burdon et al, 1982; Thomas et al, 1982; Welch et al, 1983; Pelham, 1985).

The synthesis of some of the prompt HS proteins continues after prolonged exposure to cadmium or arsenite. The mass of newly synthesized prompt HS proteins that accumulates during HS is too small to serve for biochemical analysis or as immunogens for antibody production. Consequently, the continued synthesis of prompt HS proteins after 3 h expcsure to arsenite or cadmium seen in the experiments of Figure 31 raised the possibility of isolating much larger quantities of some of the prompt HS proteins. This possibility was further explored by culturing HeLa cells in the presence of arsenite or cadmium for 16 h before pulse-labeling and isolating the newly synthesized proteins of the NM-IF. These results, shown in Figure 32, indicate that at least some prompt HS proteins continued to be synthesized during the prolonged exposure to arsenite or cadmium.

The two drugs produced different changes in the HeLa cells after the 16 h exposure. In the presence of both

Figure 32. The synthesis of four prompt HS proteins can be detected in the NM-IF after prolonged exposure to arsenite or cadmium. HeLa cells were maintained at normal density in medium containing 100 μM sodium arsenite (A) or 100 μM cadmium sulfate (B) for 16 h. Equal numbers of trypan blue-excluding cells were concentrated and labeled with 100 μCi [^{35}S]methionine per ml in methionine-free medium containing arsenite or cadmium for 30 min before fractionation. The NM-IF fraction was isolated and 100,000 cpm of NM-IF protein from 0.68 x 10^6 cells in (A) and 20 x 10^6 cells in (B) analyzed. The constitutive HS protein, hsp72 is indicated. The position of hsp73 is also indicated. The prompt HS proteins that continued to be synthesized and could be identified with the exposure shown are indicated by the closed squares. Square brackets indicate the prompt HS proteins that are visible after longer exposure of the fluorogram. The positions of molecular weight (M_r x 10^{-3}) and isoelectric point standards are indicated above and at the left of the figure, respectively.

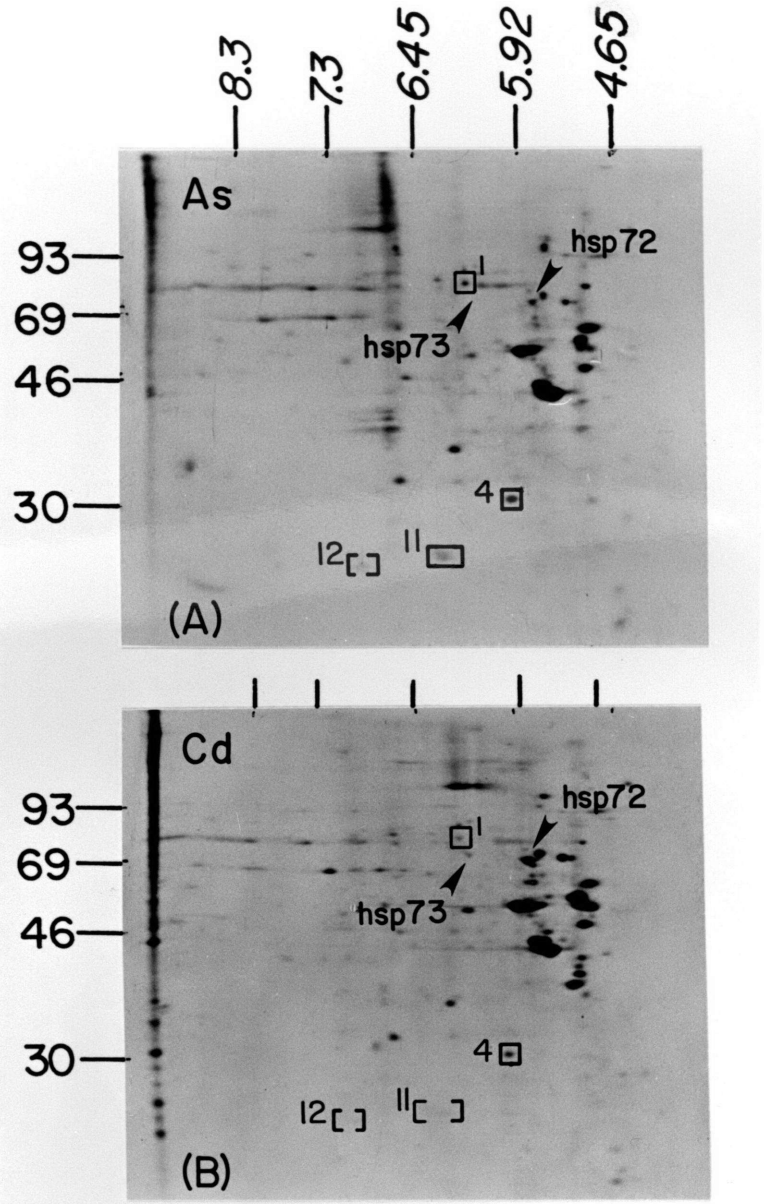

8.3
7.3
6.45
5.92
4.65
As
93
69
46
30
hsp72
hsp73
1
4
12[ ]
11
(A)
Cd
93
69
46
30
hsp72
hsp73
1
4
12[ ]
11[ ]
(B)

Table 8. Protein synthesis for the NM-IF of HeLa cells exposed to chemical stress.

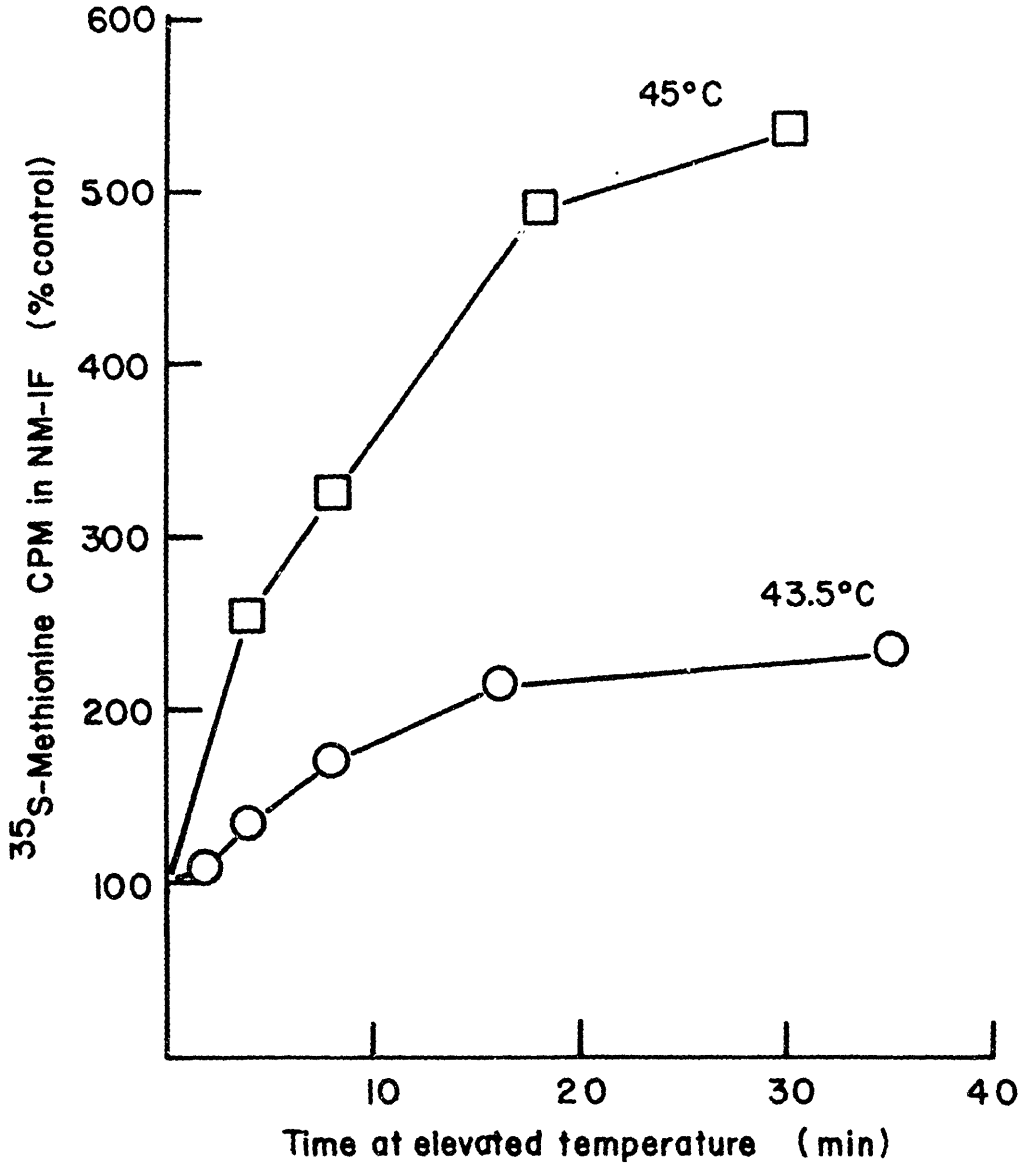
Time and Treatment	[^{35}S]methionine incorporated into acid-precipitable material	
	Total CPM per 1000 cells	Per cent label as NM-IF
1 hour:		
None	2428	4.9
Arsenite	381	9.7
DNP	2077	3.7
Cadmium	4265	24.1
Zinc	6255	17.0
EtOH	214	11.3
3 hour:		
None	2650	4.3
Arsenite	1640	7.7
Cadmium	1805	8.5
Arsenite + AMD	360	5.1
Cadmium + AMD	505	8.5
16 hour:		
None	4310	4.1
Arsenite	2560	5.8
Cadmium	120	4.8

agents, cell division ceased and the number of cells capable of excluding trypan blue diminished. The arsenite-treated cells acquired a crenelated appearance and displayed numerous cytoplasmic extrusions. Despite the pathological appearance of the cells, 91 per cent of the arsenite-treated cells continued to exclude trypan blue. By comparison, the cadmium-treated cells maintained a slightly swollen, but otherwise normal appearance when viewed with phase microscopy although only 71 per cent of these cells excluded trypan blue. Equal numbers of trypan-blue excluding cells from arsenite-, cadmium- and mock-treated cultures were removed and transferred to methionine-free medium and then labeled with [^{35}S]methionine for 30 min. The total amount of acid precipitable radioactive protein isolated from cultures exposed to arsenite was 59 per cent of the control. The amount of label incorporated into protein in the cadmium-treated cultures was only 2.9 per cent of the control. The amount of [^{35}S]methionine isolated in the NM-IF fraction in all cases remained approximately 4 per cent of the total incorporation.

Despite the large relative differences in total [^{35}S]methionine uptake and incorporation, the pattern of newly synthesized NM-IF proteins from both long-term arsenite and cadmium cultures were remarkably similar (Figure 32A and 32B, respectively). Of the prompt HS proteins, HeLa pHS:4 was prominently labeled among the newly synthesized prompt HS proteins found in the NM-IF of both drug-treated cells. In addition, HeLa pHS:1, 11, and 12 could be detected among the newly synthesized NM-IF proteins in both cases. Overall, the patterns of newly synthesized NM-IF proteins was closer to that of the control (see Figure 28A) after the prolonged exposure to the inducing agents then immediately after treatment. In particular, the newly synthesized HShift proteins were largely absent from the NM-IF and the enhanced synthesis of hsp72 and hsp73 was no longer evident. The tendency to return to a normal pattern of translation after

Figure 33. Heat shock induces a rapid increase in the amount of pre-existing, [^{35}S]methionine-labeled protein that associates with the HeLa NM-IF. HeLa cells were labeled for 30 min at 37^{o}C with [^{35}S]methionine, the radioactive amino acid was removed and the cells incubated at normal (37^{o}C) temperature for 1 h. The prelabeled cells were transferred to the indicated temperature (the temperature reached equilibrium within 90 seconds) and equal number of cells removed and fractionated at the indicated times. The amount of acid-precipitable radioactivity found in the NM-IF was plotted as a percentage of the radioactivity found in the NM-IF before the heat shock. The control value represented 4.8 per cent of the total acid-precipitable radioactivity. The total radioactivity recovered at each time point was essentially unchanged from the control value.

Figure 33


600
500
400
300
200
100
45°C
43.5°C
35S-Methionine CPM in NM-IF (% control)
10
20
30
40
Time at elevated temperature (min)

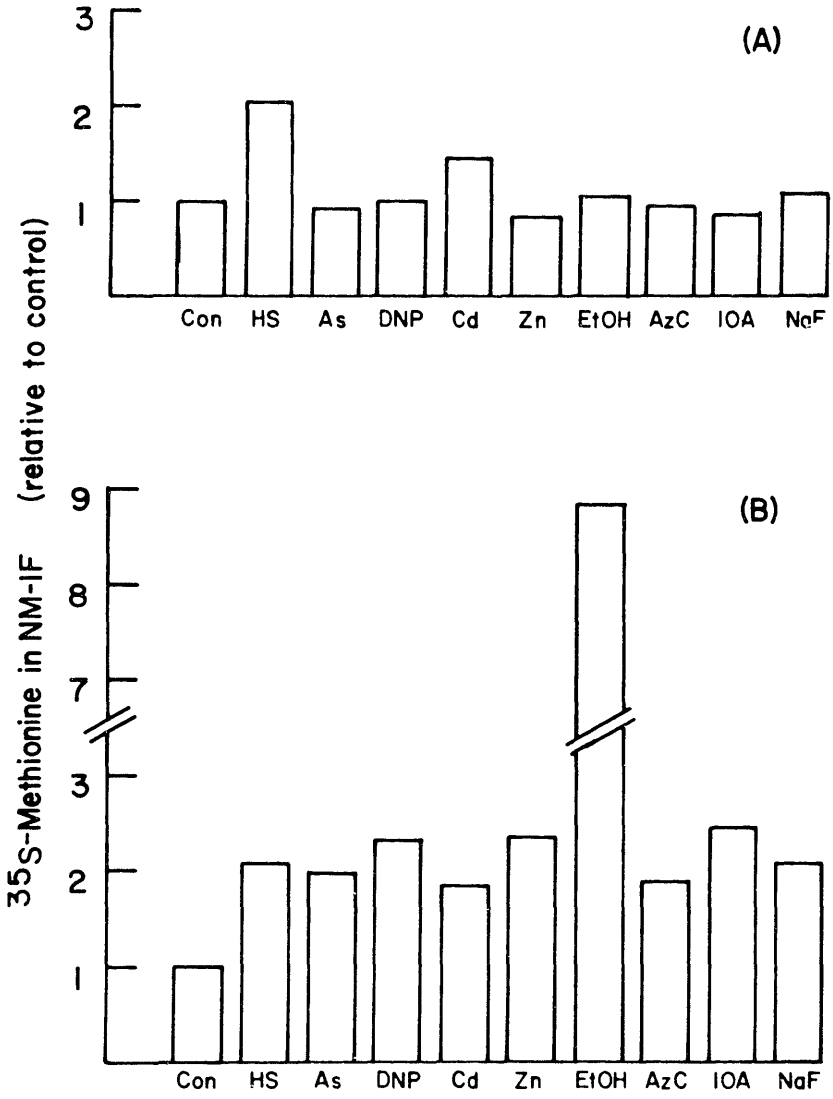
prolonged exposure to cadmium has previously been observed in cultured invertebrate cells (Heikkila et al, 1982).

As shown in Figure 33, and reported by others (Roti Roti and Wilson, 1984; Warters et al, 1986), hyperthermia causes an increase in the mass of protein associated with the NM-IF. The increase in NM-IF protein is dependent on the temperature of the HS. At 43.5°C, a 2.2-fold increase was observed and at 45°C, a 6-fold increase was seen. The increase in protein, though rapid, is not instantaneous. These additional proteins complicate the identification and study of the prompt HS proteins. The results of Figure 32 suggest that arsenite may be suitable long term induction of prompt HS proteins in the absence of newly synthesized HShift proteins. Consequently, the effect of chemical stress agents on the intracellular distribution of existing proteins was tested and these results are shown in Figure 34A.

Preliminary results using high concentrations of arsenite (1 mM) and cadmium (5 mM) revealed that cadmium induced a shift of existing protein into the NM-IF while arsenite did not. These preliminary findings suggested that the shift may be an energy-dependent process. Therefore, the stress agents and additional non-inducing poisons were also tested for an ability to prevent the 43.5°C-induced shift. These results are shown in Figure 34B. The results of a single pair of experiments is presented in Figure 34 although the results are representative of repeated determinations. These studies show that cadmium was the only stress agent tested to induce an increase in the amount of protein associated with the NM-IF. Furthermore, none of the drugs tested prevented the heat-induced shift and only one, ethanol, changed the amount of protein shifted to the NM-IF. The synergistic effect of ethanol and heat was note by Windward and Roti Roti who suggested that alcohol potentiates the heat-induced cell killing by increasing the amount of protein associated with the nuclear matrix and chromatin (Roti Roti and Wilson, 1984).

Figure 34. Effect of chemical stress on the amount of [^{35}S]methionine-labeled protein in the HeLa NM-IF at normal and elevated (43.5^{o}C) temperatures. Cells were labeled at normal temperature (37^{o}C) for 30 min with [^{35}S]methionine. The radioactive label was removed, and the culture split into aliquots of equal cell number before appropriate treatment and isolation of the NM-IF. In (A), cells were subject to the indicated treatment for 1 hour at 37^{o}C before fractionation. In both (A) and (B), the Con value reflects the amount of label in the NM-IF of the prelabeled cells that were chased at 37^{o}C for 60 min before fractionation; the HS value reflects the amount of label in the NM-IF isolated from prelabeled cells that were shifted to 43.5^{o}C for 60 min prior to fractionation. In (B), cells were exposed to the drug for 10 min at 37^{o}C then shifted to 43.5^{o}C for 1 hour before isolating the NM-IF. The concentrations used were: Ars, 100 μM sodium arsenite; DNP, 1 mM 2,4-DNP; Cd, 100 μM cadmium sulfate; Zn, 100 μM zinc sulfate; EtOH, 4% or 0.8 M ethanol; IOA, 1 mM iodoacetamide; AzC, 1 mM of the proline analog, L-azetidine-2-carboxylic acid; and NaF, 100 μM sodium fluoride.

Figure 34


(A)
3
2
1
Con
HS
As
DNP
Cd
Zn
EtOH
AzC
IOA
NaF
(B)
9
8
7
3
2
1
35S-Methionine in NM-IF (relative to control)
Con
HS
As
DNP
Cd
Zn
EtOH
AzC
IOA
NaF

DISCUSSION

The conventional HS proteins are a well characterized set of HS proteins whose synthesis is enhanced after at the level of transcription. Elevated temperature also induces a new set of proteins termed the prompt HS proteins, that, in contrast to the conventional HS proteins, appear to be made from preexisting but normally untranslated messenger RNA (Chapter 4, Reiter and Penman, 1983). The prompt HS response differs in several other aspects from the conventional HS response. The prompt HS proteins appear to associate exclusively with the NM-IF in both Drosophila and HeLa cells while the conventional HS proteins are distributed among the subcellular compartments (reviewed in Schlesinger, 1986; Tanguay, 1985) The conditions of temperature induction are also dissimilar. In Drosophila, higher temperatures were necessary for induction of the prompt HS proteins than for the conventional HS proteins; the complete set of conventional HS proteins were induced by a moderately elevated temperature HS ($33^{o}C$) while only two of fourteen Drosophila prompt HS appeared at this temperature. Also, none of the prompt HS proteins could be detected during infection with an RNA virus that also induced the conventional HS proteins.

The studies presented here show that the prompt HS response of Drosophila and human (HeLa) cells differ with respect to the induction by agents other than heat. In Drosophila cells, the prompt HS proteins were induced only by relatively high temperature. Other stress conditions, which induced the conventional HS proteins, did not provoke synthesis of the prompt HS proteins. Together, these results suggest that the prompt HS response of Drosophila may not be a general stress reaction but may be a specific response to moderately severe HS.

The situation in mammalian cells appears more complex. In contrast to Drosophila, alternative forms of stress

induced in Hela cells at least a subset of the 15 prompt HS proteins. Agents that are strong inducers of the conventional HS proteins were also efficient in evoking the complete set of prompt HS proteins while weaker inducers of the conventional response resulted in fewer of the prompt HS proteins. With the exception of one protein (HeLa pHS:9 was not induced by cadmium), arsenite and cadmium induced all of the prompt HS proteins. These two agents are also potent inducers of the conventional HS proteins in HeLa cells (Burdon, 1986; Burdon et al, 1982). Another transition series IIb metal, zinc, was found to be a weaker inducer of the conventional HS proteins than cadmium (Burdon et al, 1982). Coincidentally, zinc induced the synthesis of only 8 prompt HS proteins. Ethanol, which induced ten prompt HS proteins, has not been shown to induce the conventional HS proteins in HeLa cells (Craig, 1985; Schlesinger, 1986). However, ethanol does induce the conventional HS proteins in Chinese hamster ovary cells (Li and Werb, 1982). Finally the metabolic poison DNP induced only 4 of the prompt HS proteins. DNP is not recognized as an inducer of the stress response in HeLa cells (Craig, 1985; Schlesinger, 1986) although DNP does induce the conventional HS proteins in several insect cell lines (Nover, 1984).

The mechanism by which environmental insults triggers the stress response is not understood (see in particular the discussions in Bonner, 1985; Munro and Pelham, 1985; and Pelham, 1985). However, many of the inducing conditions, other than heat, have in common particular features such as the ability to interfere with energy metabolism or a reactivity towards sulfhydryl groups. It has been postulated that the agents or conditions that trigger the stress response lead to an excess of denatured or otherwise defective proteins which serve as the common stress stimulus (Finley et al, 1984; Pelham, 1985). Among the agents examined in these experiments, the prompt HS proteins were most strongly induced by compounds that are reactive with

sulfhydryl groups. Both the cadmium and zinc ion can crosslink proteins through cysteine resides (Webb, 1979). As a mitochondrial poison, arsenite inhibits ATP synthesis by crosslinking the vicinal sulfhydryl residues of acetyl CoA (Reiss and Hellerman, 1958). In contrast, DNP, the other mitochondrial poison examined, induced only 4 of the prompt HS proteins. Unlike arsenite, DNP is believed to act as an ionophore, inhibiting ATP synthesis by dispersing the charge gradient across the mitochondrial inner membrane (Kraayenhof and van Dam, 1969).

In HeLa cells, both the conventional and the prompt HS responses are induced by agents other than heat. The synthesis of individual proteins in each response display unique temporal behaviors. After three hours of exposure to either arsenite or cadmium, the synthesis of 5 prompt HS proteins had apparently stopped. After 16 hours, 11 prompt HS proteins could no longer be detected. In a similar manner, synthesis of the major 70 kD group of conventional HS proteins in HeLa dropped sharply after 1 hour while the synthesis of the 80 and 27 kD HS proteins did not diminish until 4 to 6 hours of continued exposure to moderately elevated temperature (Hickey and Weber, 1982). This reduction in conventional HS protein synthesis was found to correlate with decreases in the concentration of translatable mRNA encoding the HS proteins. The reason for the cessation of prompt HS protein labeling is not known.

Chemical and thermal stress both increase the number of newly synthesized proteins that associate with the NM-IF. The heat-induced increase occurs in the form of HShift proteins in both Drosophila and HeLa cells. The chemically-induced increase is reflected in the increased complexity of the electrophoretic patterns of HeLa NM-IF protein (Figure 29), and to a lesser extent, of Drosophila NM-IF protein (Figure 27). Some, but not all, of the additional protein that appeared in the NM-IF during exposure to the drugs corresponded to HShift proteins. The identity or subcellular

distribution of the remaining new protein in the NM-IF was not investigated. The increase in newly synthesized NM-IF protein is also seen in the increased proportion of [^{35}S]methionine-labeled protein isolated in the NM-IF (Table 8). For example, cells that were labeled after 1 h exposure to cadmium or zinc had approximately 20 per cent of the newly labeled protein associated with the NM-IF compared to the control value of 5 per cent. However, the temporal behavior of prompt HS proteins in the NM-IF is very different from that of the chemically-induced HShift proteins. After three hours exposure to cadmium, the proportion newly labeled protein in the NM-IF had dropped to 8.5 per cent while 10 of the 15 prompt HS proteins were still synthesized. After 16 h exposure to cadmium (or arsenite) the proportion of pulse-labeled protein in the NM-IF had decreased to the control level while 4 prompt HS proteins continued to be synthesized and incorporated into the NM-IF.

Chemically induced stress also differed from heat induced stress with regards to an effect on the intracellular distribution of preexisting proteins. Specifically, HS increased the amount of preexisting protein that associated with the NM-IF. With one exception, the chemical stressors did not change the amount of prelabeled protein isolated with the NM-IF. The one exception, cadmium, increased the amount of prelabeled protein in the NM-IF by only 50 per cent, in contrast to the increase of 240 per cent induced by elevated temperature (43.5^{o}C). The mechanism involved in the heat or cadmium induced shift is not understood although cadmium has been shown to increase the association of membrane proteins with the red cell cytoskeleton, presumably through the formation of intramolecular crosslinks (Kunimoto and Miura, 1985).

The number of prompt HS proteins identified here is substantially fewer than in the initial report (Reiter and Penman, 1983) which did not completely account for proteins shifted by heat to the HeLa NM-IF from the soluble fraction.

The number of proteins identified here as prompt HS is conservative and may actually be an underestimation. Candidate proteins which only appeared in the NM-IF during HS but could not be positively excluded as originating from the SOL fraction were not recorded as prompt HS proteins. The complexity of the SOL fraction made such exclusion difficult in a few cases. A complete identification of the prompt HS proteins would require higher resolution analysis of the SOL fraction.

The results of a different experimental approach suggest that the identification of HeLa prompt HS proteins in this chapter may be reasonably complete. Young and associates have identified 11 HS proteins in rat thymocytes that are synthesized under translational control, that is, their induction at elevated temperature was not affected by the inhibition of mRNA processing (Maytin et al, 1985). The identification of these proteins did not depend on a biochemical fractionation to partially purify the proteins as was done here. Rather, the HS-labeled proteins from whole-cell extracts were analyzed with very high resolution gel electrophoresis (Young et al, 1983). Although the 11 proteins are not identical by size and charge comparison with the prompt HS proteins, most are of low molecular weight (less than 45 kD) and slightly more basic than β-actin. The HeLa prompt HS proteins are also characterized by a similar size and charge distribution. This distribution is quite distinct from that of the conventional HS proteins; all of the methionine-containing conventional HS proteins are larger than 68 kD and are (with one exception) more acidic than β-actin (Thomas et al, 1982). If the translationally-regulated response to HS in rat thymocytes is similar to that of HeLa cells, the number of HeLa prompt HS proteins (containing metabolically stable methionine) is likely to be close to 15.

Other investigators have described additional heat-induced proteins of low abundance that are distinct from the

conventional HS proteins. The use of very high resolution two-dimensional gel electrophoresis revealed the rapid and transient induction of minor HS proteins in human lymphocytes (Anderson et al, 1982). The apparent mobility of one of these proteins very closely resembles that of the 65 kD HeLa pHS:2 (see Figure 3I in reference Anderson et al, 1982). It was not determined whether the induction of this protein was under transcriptional or translational control. Examples of translationally-regulated HS proteins have been described in the cells of organisms as diverse as hamsters (CHO cells) (Caizergues-Ferrer et al, 1984) and tomato plants (Nover and Scharf, 1984). In both of these reports, the newly synthesized proteins appeared in a background of heat-translocated proteins and were associated salt-resistant subcellular structures. Finally, a recent report analyzing the HS proteins of Tetrahymena pyriformis found both transcriptionally-controlled and translationally-controlled HS proteins (Galego and Rodrigues-Pousada, 1985). The translationally-regulated HS proteins of T. pyriformis were also of low molecular weight (25-29 kD).

In contrast to the conventional HS proteins, the prompt HS proteins appear to be localized strictly to the NM-IF. Many of the conventional, transcriptionally-controlled HS proteins have been shown to associate with the nucleus (summarized in Tanguay, 1985) or salt-resistant structures of the nucleus, (Levinger and Varshavsky, 1981; Sinibaldi and Morris, 1981). However, the nuclear localization of the conventional HS proteins is dependent upon the conditions used to induce synthesis of the HS proteins and on the extracellular environment (Lewis and Pelham, 1985; Pelham, 1984; Velazquez and Lindquist, 1984; Vincent and Tanguay, 1982; Welch and Feramisco, 1984). Hp70 was not found in the nucleus when Drosophila cells were stressed with chemical agents or low temperature heat shocks (Vincent and Tanguay, 1982). Nevertheless, hsp70 synthesized under these conditions associated with the nucleus when the cells were

challenged with more severe conditions such as high temperatures or prolonged anoxia (Velazquez and Lindquist, 1984). The Drosophila hsp70 was introduced to mammalian (mouse L and monkey COS) cells using a plasmid expression vector. The foreign hsp70, which was found primarily in the nucleus in intact cells, associated strongly with the nucleolus upon HS (Lewis and Pelham, 1985; Pelham, 1984). In a similar manner, the mammalian hsp72/73 proteins was found associated with the nucleus and nucleoli under conditions of severe stress but was distributed throughout the cytoplasm under less harsh conditions (Welch and Feramisco, 1984). These results raise similar questions with regard to the intracellular distribution of the prompt HS proteins induced by chemical agents. Because the cytoplasmic fractions were not analyzed under all conditions tested here, these findings cannot exclude the possibility that in some cases the prompt HS proteins were synthesized and failed to associate with the NM-IF. However, when proteins of the other subcellular fractions were examined by two-dimensional gel electrophoresis, the prompt HS proteins were not detected whether or not they were found in the NM-IF. However, a more accurate determination of the intracellular distribution of the prompt HS proteins induced by means other than heat requires a more specific and sensitive assays. Within these limitations, the results at present support the hypothesis that the prompt HS proteins associate with the NM-IF regardless of inducing conditions.

Currently, the function of the prompt HS proteins is unknown. Despite the large amount of phenomenological data acquired on the conventional HS proteins, functions for these proteins in eukaryotic cells have only recently been proposed and are now being tested (see especially Munro and Pelham, 1985; Pelham, 1985; Schlesinger, 1986). Nonetheless, one possible role for the prompt HS proteins can probably be ruled out. Because the conventional HS response, as measured by the activation and increased transcription of the HS

genes, is not affected by the inhibition of protein synthesis, it seems likely that prompt HS protein synthesis is not required for the activation of the conventional HS response (DiDomenico et al, 1982). The alternative inducers of the prompt HS proteins may provide means by which the function of the prompt HS proteins can be discerned.

CHAPTER 6

Effects of Elevated Temperature (43^{o}C) on the Morphology of the Cytoskeletal-Framework and Nuclear Matrix of HeLa Cells.

INTRODUCTION

Early studies on the morphologic changes provoked by elevated temperatures focused on the alterations in RNA- and DNA-containing structures of the cell. Among the first systematic studies, Simard and Bernhard reported that the granular component of the nucleolus disappeared shortly after heat shock and that the intranucleolar chromatin retracted towards the margins of the nucleolus (Simard and Bernhard, 1967). The retraction of the intranucleolar chromatin was observed to coincide with the failure of [^{3}H]uridine to be incorporated into the nucleolus but not into the nucleoplasm suggesting that ribosomal RNA synthesis was specifically inhibited by heat shock. Analysis of the RNA being synthesized during heat shock above 42oC in HeLa cells confirmed this observation. It was found that synthesis of the 45S ribosomal precursor was reduced to less than 10 per cent of the control during a sufficiently severe heat shock while hnRNA and tRNA synthesis remained at 85 and 105 per cent, respectively, of the control (Warocquier and Scherrer, 1969; Zieve et al, 1977). Subsequent studies suggested that the nucleolar degranulation was related to the cessation of ribosomal RNA processing (Welch and Suhan, 1986; Neumann et al, 1984).

The inhibition of protein synthesis observed in HeLa cells at temperatures above 42^{o}C (McCormick and Penman, 1969) was found to correlate with a change in the organization of polysomes (Heine et al, 1971). Heine et al found that the polysomes were no longer intact and that the monosomes had dispersed throughout the cytoplasm after heat shock (Heine et al, 1971). Subsequent studies have found that the monosomes no longer engaged in translation may have been released from the cytoskeletal framework (Cervera et al, 1981; Howe and Hershey, 1984; Chapter 7).

More recent investigations on the changes in morphology of the heat shocked cell have centered on the alterations in

the major cytoskeletal filaments or on the localization of the conventional HS proteins within the cell. The vimentin-containing intermediate filament network of mammalian cells (Thomas et al, 1982; Welch and Feramisco, 1985b; Welch and Suhan, 1985), avian cells (Collier and Schlesinger, 1986), as well as the analogous filament network of Drosophila cells (Biessman et al, 1982) has been observed to collapse about the nucleus upon heat shock. Similar changes were not observed for the actin and tubulin networks during heat shock although it was reported that after heat shock, densely packed, filamentous aggregates of actin could be detected within the nucleus (Welch and Suhan, 1985; Iida et al, 1986). The intracellular location of the conventional HS proteins has been found to depend on the conditions of induction and the extracellular environment. Shortly after a heat shock or severe environmental stress, the major 70,000 dalton heat shock protein (72,000 dalton in mammals) has been found primarily in the nucleus (reviewed in Tanguay, 1985) and often in the nucleolus (Welch and Feramisco, 1984; Lewis and Pelham, 1985; Pelham, 1984; Welch and Suhan, 1986). The 72 kD HS protein has been found in close association with the pre-ribosomal, granular compartment of the nucleolus as well as with dense clusters of ribosomes in the cytoplasm of heat shocked cells (Welch and Suhan, 1986). This observation, together with the affinity of hsp72 for nucleotides and RNA has lead to the hypothesis that hsp72 functions in the nucleolus to promote the disassembly of heat-induced aggregates (Lewis and Pelham, 1985; Pelham, 1984). Along these lines, Pelham has shown that Drosophila hsp70 can promote the restoration of normal nucleolar morphology in heat shocked mammalian cells (Pelham, 1984).

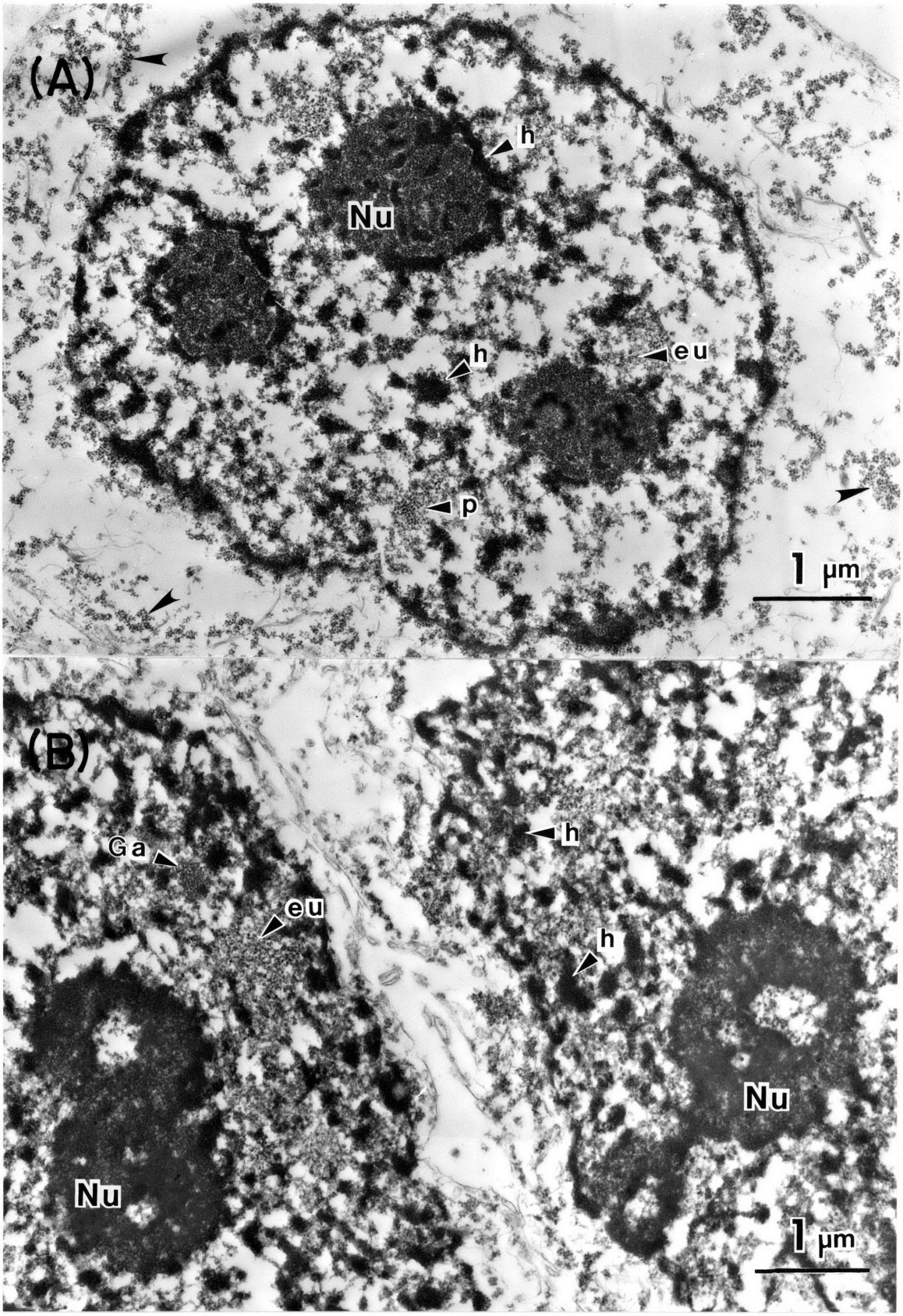
The techniques of cell fractionation, based on the selective solubilization and release of cellular constituents with non-ionic detergents and various ionic conditions, have revealed that there is a substantial increase in the amount of protein associated with high salt-resistant substructures

of the cell after heat shock. Some investigators have interpreted this as an indication of an increase in protein associated with the nucleus and nuclear substructures (Caizergues-Ferrer et al, 1984; Murname and Li, 1985; Rabilloud et al, 1985; Warters et al, 1986; Roti Roti and Wilson, 1984). It has been suggested that the additional protein associated with the nucleus and nuclear substructures serves to modulate or shut off nucleolar function, alter chromatin structure and potentiate the chromatin to thermal damage (Falkner and Biessmann, 1980; Roti Roti and Wilson, 1984; Warters et al, 1986). Others have suggested that the association of many proteins with the nucleus and nuclear matrix is an artifact of heating and fractionation (Evan and Hancock, 1985) or that the increased protein in the nuclear fraction occurs as particles of the same density and solubility as the isolated nucleus (Nover and Scharf, 1984). The results of Chapter 4 suggest that the excess protein associated with the Drosophila NM-IF may occur, in part, as a fibrous aggregate within the nucleus. In HeLa cells, studies have suggested that some of the excess protein in the nucleus after heating is found in intimate contact with the DNA (Roti Roti and Winward, 1978). These investigators suggested that the additional protein was derived from both the nucleoplasm as well as the cytoplasm. In summary, the nature of the heat-induced changes in the protein composition of the nucleus remains uncertain and warrants further investigation.

The studies presented in this chapter examine the changes in HeLa cell morphology after heat shock with the method of resinless section electron microscopy. This technique has been shown to be well-suited for the analysis of protein fiber networks (Capco et al, 1984; Wolosewick, 1980). In contrast, previous studies on the morphology of heat shocked cell have largely focused on the membranous organelles, the nucleic acid-containing structures, or the well-defined, cytoskeletal filaments. As a consequence, the disposition of the additional protein associated with the

nucleus and nuclear matrix in these studies may have escaped notice. Many of the changes described in this chapter extend or corroborate the observations of other investigators, including the heat-induced disruption of chromatin structure and nucleolar organization as well as the increase in granular particles reminiscent of the ribonucleoprotein particle associated with hnRNA. A novel finding is that the complexity and connectivity of the fiber network of the nuclear matrix apparently increased after heat shock. Changes in this fiber network is discussed in relation to the changes observed in the protein composition of the same structures studied biochemically after cell fractionation.

Figure 35. The HeLa cell cytoskeletal framework from normal and heat shocked cells visualized in resin-containing sections. The triton-extracted control cell (A) displays a well-defined nuclear-cytoplasmic border. Numerous polysomes are visible in the cytoplasmic space (solid, unlabeled arrows). The nucleoli (Nu) are prominent within the nucleus as are the dispersed clumps of heterochromatin (h). Less dense fibrillar-granular areas may include strands of euchromatin (eu) and electron dense granules surrounded by halos of electron transparent material (p). The cytoskeletal framework from cells heat shocked for 1 h at 43.5^{o}C is analyzed in (B). (Parts of two cells are visible.) Polysomes are no longer numerous and the nuclear-cytoplasmic border is less regular. The nucleoli (Nu) have less granular matter and appear devoid of electron dense material in the center. Clumps of heterochromatin (h) and euchromatin (eu) are visible. Granular aggregates (Ga) of electron dense granules with narrow, electron-transparent halos are visible in the heat shocked cells.


(A)
Nu
h
h
eu
p
1 μm
(B)
Ga
eu
h
h
Nu
Nu
1 μm

RESULTS

Heat shock-induced changes in the chromatin-containing cytoskeletal framework of HeLa cells viewed in Epon-embedded thin sections. HeLa cells, grown in suspension, were heat shocked (43.5^{o}C) for 1 hour before isolation and extraction with Triton X-100 in CSK buffer. The cytoskeletal framework (CSKF) was processed as described in the Materials and Methods for embeddment in the removable medium, diethelylene glycol diestearate (DGD). A portion of the sample was embedded in resin and processed for transmission electron microscopy by standard methods. The structures shown in Figure 35 reveal the changes in morphology that can be readily visualized in the standard, resin-containing thin section. Although essentially all of the phospholipids have been removed, the triton-extracted control cell (Figure 35A) displays a well-defined nuclear lamina. Numerous polysomes are visible in the cytoplasmic space which are not apparent in the heat shocked CSKF (Figure 35B). The failure to detect polysomes (or monosomes) in the cytoplasmic space of the heat-shocked cell reflects the release of the ribosomes from the CSKF induced by the heat shock (Howe and Hershey, 1984; Chapter 7). Characteristic features of the nucleus, including the condensed heterochromatin and less dense euchromatinic regions are retained in the CSKF preparation.

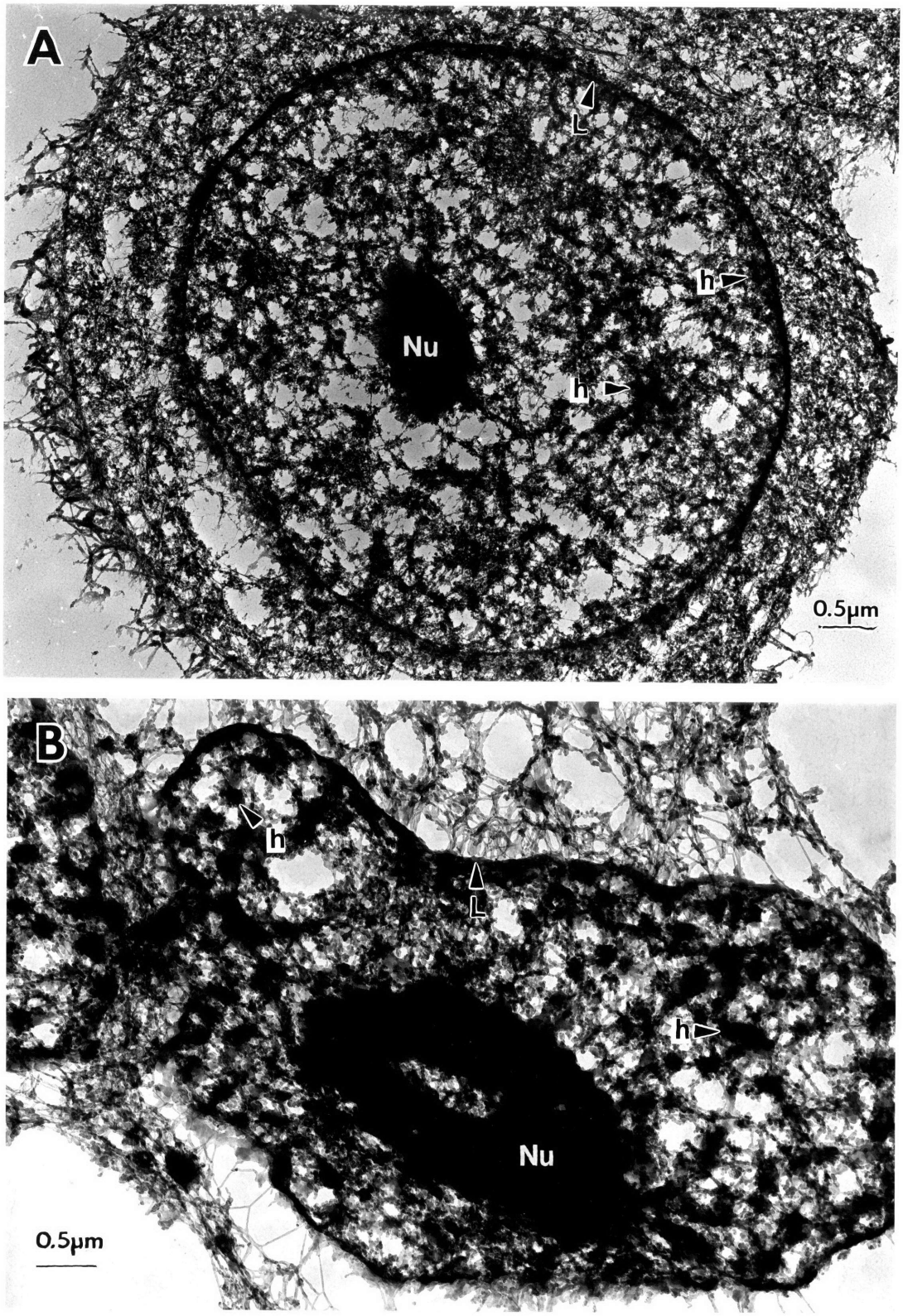
Another change in the heat-shocked cell that is readily apparent in the resin-containing sections is seen in nucleolar morphology. The nucleoli of the heat-shocked cell appeared larger and display a central region devoid of electron dense material. Under higher magnification (not shown), the empty regions appear to result from the loss of the granular aspect of the nucleolus, in agreement with other investigators who reported similar changes in the unextracted cell (Cervera, 1978; Heine et al, 1971; Simard and Bernhard, 1967; Welch and Suhan, 1986). In addition, aggregates of granular, electron-dense particles occurred in the

perichromatinic space of the heat-shocked nucleus (indicated by Ga in Figure 35B). These aggregates were composed of particles possessing an electron dense core 30 to 35 nm surrounded by a halo of electron lucent material approximately 5 to 10 nm wide. These compact granules were observed in addition to the granules seen in both normal and heat-shocked cells that had the appearance of perichromatin granules (Daskal, 1981). The perichromatin granules (identified by 'p' in Figure 35A) were also composed of an electron dense core of 30 nm but were surrounded by a clear halo of 20 nm.

Heat-shock induced changes in the CSKF of HeLa cells viewed in the absence of embedding medium. In the absence of the embedding medium, additional ultrastructural features were apparent. The micrographs of Figure 36 reveal many of the same changes noted in the resin-containing sections of Figure 35. In addition, the fiber networks of the nucleus and cytoplasm, which were essentially invisible in Figure 35, appeared to be quite extensive. Although the differences in the two methods of imaging biological material has been discussed at length by others (Capco et al, 1984; Wolosewick, 1980), the dramatic differences seen with the two methods warrants discussion before proceeding. In brief, the images obtained in the absence of embedding material differ primarily because of two features. In the absence of material possessing similar diffractive properties towards electrons, protein fibers and similar macromolecules <u>in vacuo</u> form images without the need of an electron dense stain by scattering electrons directly. In addition, because the image-forming structures are not limited to the macromolecules along the surface of the section, structures throughout the depth of the (three-dimensional) section contribute to the two-dimensional image recorded on film.

At the magnification shown in Figure 36, changes induced by heat shock could be detected in heterochromatin, the nucleolus, the shape of the nucleus, and the cytoplasmic

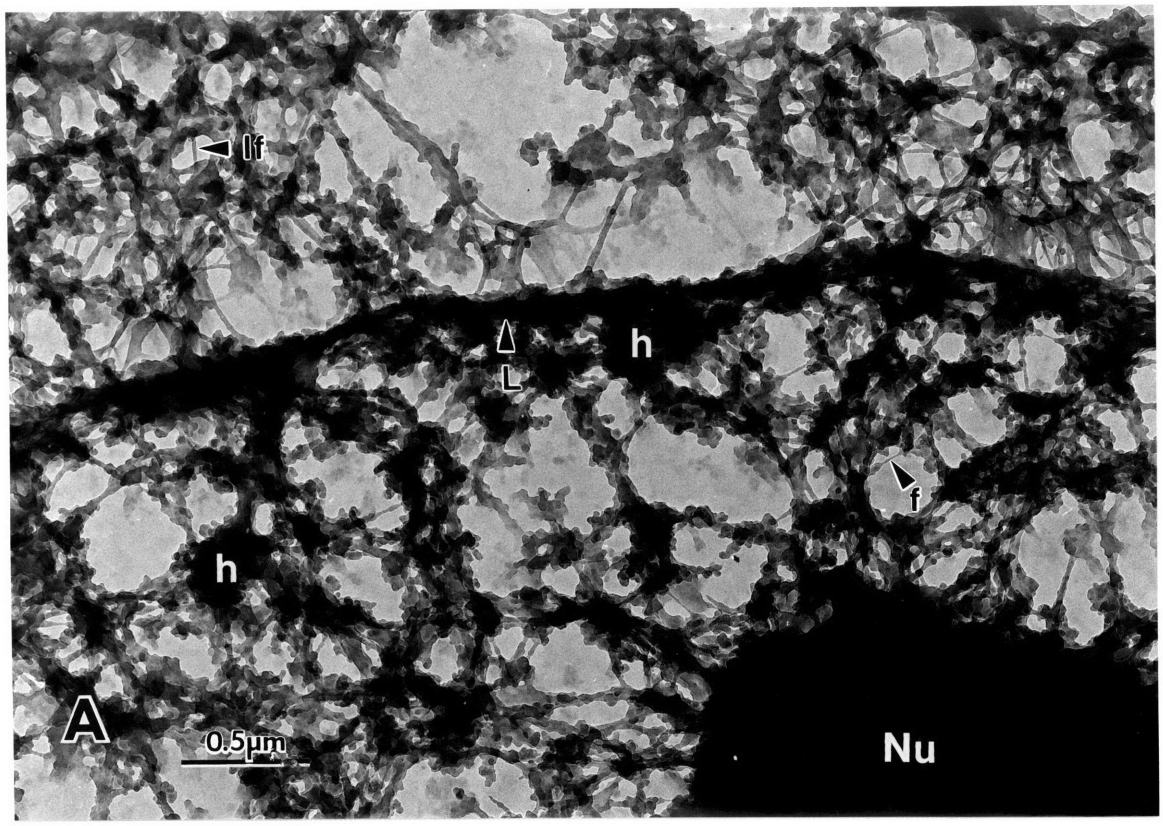
Figure 36. The cytoskeletal framework from control and heat shocked cells visualized by resinless-section transmission electron microscopy. The CSKF of control cells is seen in (A). The nuclear-cytoplasmic boundary is demarcated by the nuclear lamina (L). Finely dispersed clumps of heterochromatin (h) are seen within the nuclear space and along the periphery, against the nuclear lamina. The nucleolus (Nu) appears enmeshed in fibers of the chromatin-containing nucleus. The CSKF obtained from cells heat shocked for 1 h at 43.5^{o}C is seen in (B). The nuclear lamina (L) is less regular but distinct. The heterochromatin appears more condensed and occurs in more prominent clumps throughout the nucleus. The nucleolus (Nu) appears more prominent than in the control cells.


A
L
h
Nu
h
0.5μm
B
h
L
h
Nu
0.5μm

filament network. The heterochromatin appeared more dense and clumped following heat shock although dispersed throughout the nuclear interior in both heat shocked and control cells. The heat-induced changes in nucleolar morphology was particularly striking in the resinless sections. In both control and heat shocked cells, the nucleoli appeared suspended in the fiber network of the chromatin-containing nucleus. After heat shock, the nucleoli appeared larger and many displayed an apparently empty region in the center. These changes were further studied at higher magnification and are discussed below. The nuclear lamina, which was more distinct in the absence of embedding resin, was well-defined although it no longer appeared as a section of a spherical shell. Because intact, unextracted cells were not examined, it was not determined if the change in nuclear shape occurred in the intact cell or during isolation of the CSKF. The cytoplasmic filament network of the heat-shocked cell displayed a rarified, possibly clumped appearance. These changes in the cytoplasmic filament system were also further analyzed under greater magnification and are discussed below.

Heat-induced changes in the cytoplasmic and nuclear fibers of the chromatin-containing CSKF. The cytoplasmic fibers of the control cell (Figure 37A) occur as part of an anastomosing network. The width of many fibers varies along its length although distinct filaments of uniform dimension can be seen, presumably representing the well characterized intermediate and microfilaments (data not shown). Few microtubules were present because the initial extraction was performed at low temperature (4^{o}C) without special precautions to preserve microtubule integrity. An anastomosing fiber network was also seen within the chromatin-containing nucleus of the control cell. The nuclear fibers were generally more electron dense than the cytoplasmic fibers. In addition, thick nuclear fibers were often associated with granular particles. Many of the

Figure 37. The nuclear-cytoplasmic border visualized in the CSKF of control and heat shocked HeLa cells. The control CSKF is seen in (A). The nuclear lamina (L) and heterochromatin clumps (h) are indicated. Numerous thick fibers studded with granular particles are seen within the nuclear space and appear between the nucleolus (Nu) and the nuclear lamina. Thin fibers (f) can also be seen within the nucleus. The cytoplasmic space contains fibers of non-uniform appearance that lack the granular aspect of the nuclear fibers. Filaments in the cytoplasm of approximately 10 - 15 nm can be seen (If). In the heat shocked cell, (B), the fibers extending from the nucleolus (Nu) appear less regular and more electron dense than those of the control. Material of the same electron density of heterochromatin (h) appears collapsed about the fibers extending from the nucleolus. Aggregates of fibrillar-granular material can be seen in the heat shocked nucleus (Ga). A complex network of cytoplasmic filaments (If) of 10 - 20 nm in width is seen in proximity to the nuclear lamina.


lf
h
L
f
h
A
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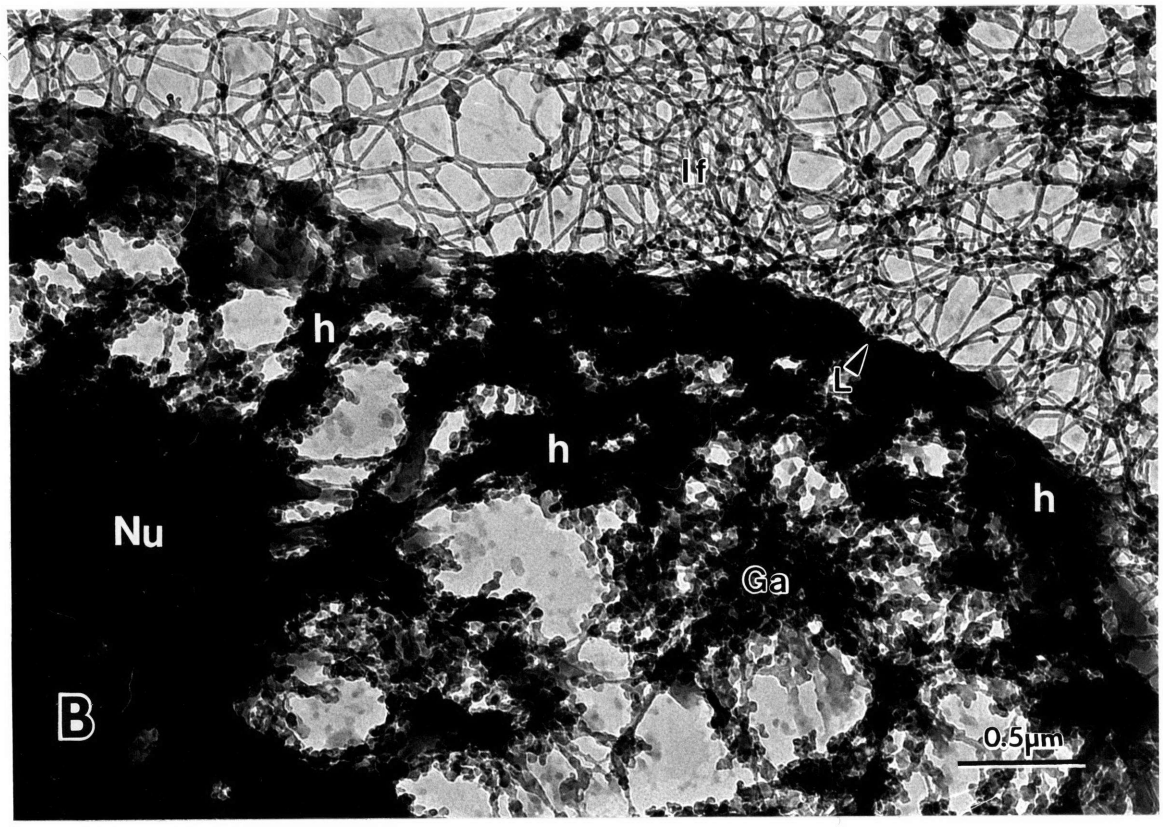
Nu


lf
h
L
h
h
Nu
Ga
B
0.5μm

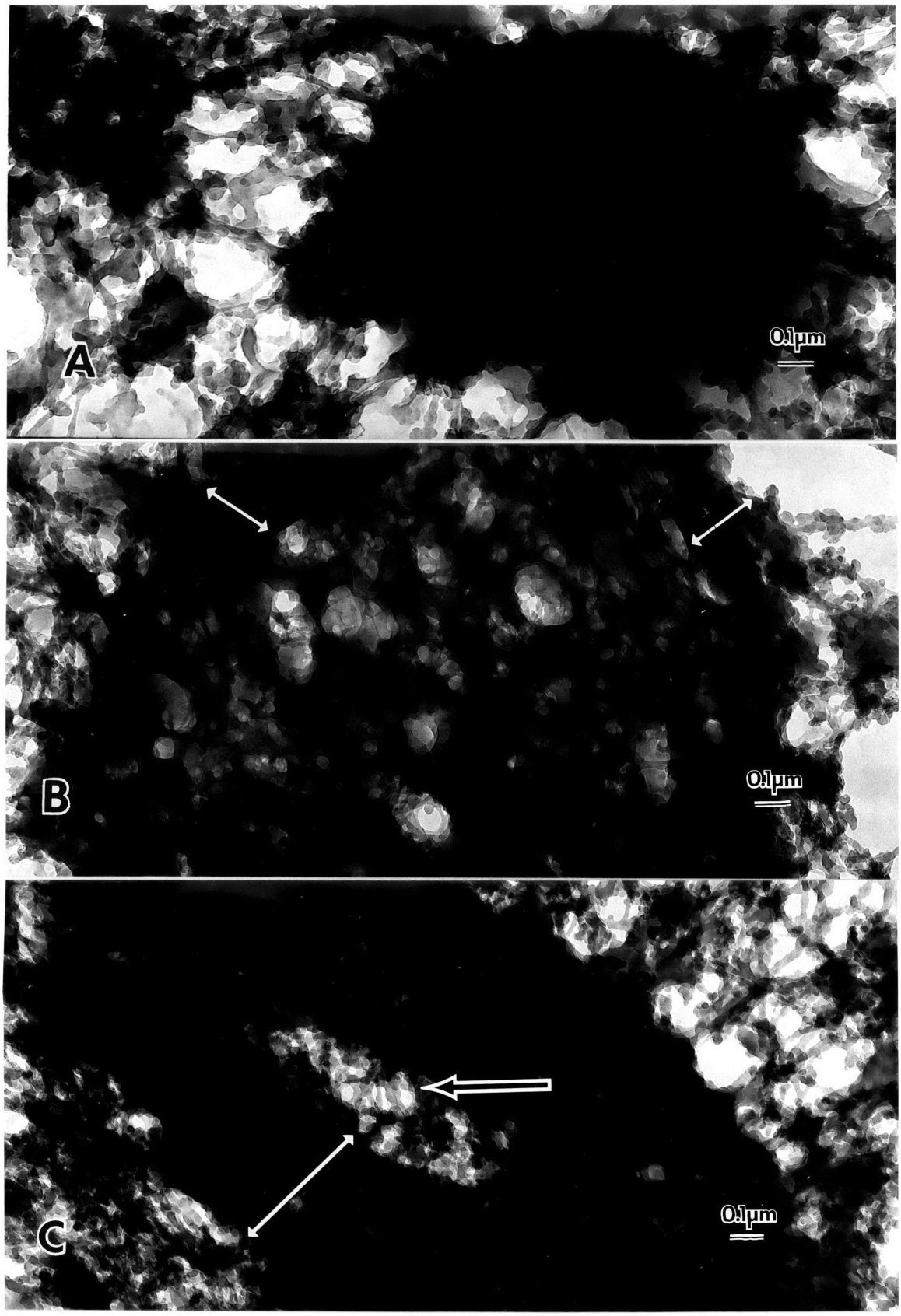
nuclear fibers appeared to connect the nucleoli with the nuclear lamina (Figure 37A).

After heat shock, approximately 10 per cent of the cells displayed a tangled network of cytoplasmic fibers in close contact with the nuclear lamina (Figure 37B). In contrast to the typical, polymorphic fibers of the cytoplasm, the fibers in the nuclear cap appeared more uniform over the length of any individual fiber. The width of these fibers (10 - 14 nm) was close to that expected for the cytokeratin- or vimentin-containing intermediate filaments (Franke et al, 1979). The composition of these fibers was not determined although similar structures containing vimentin have been observed in rat fibroblasts (Thomas et al, 1982; Welch and Feramisco, 1985b; Welch and Suhan, 1985) and chicken embryo fibroblasts (Collier and Schlesinger, 1986;) or containing a homologous protein to vimentin in Drosophila cells (Biessman et al, 1982).

The collapse of the fiber network seen in the cytoplasm after heat shock was not observed within the nucleus. Rather, the thick fibers of the chromatin-containing nuclear matrix appeared to be more heavily decorated with electron dense material after heat shock. In particular, material of the same electron density and appearance of heterochromatin appeared collapsed about the fibers extending from the nucleolus. The outline of the nucleolus was more poorly defined after heat shock. Aggregates of fibrillar-granular material were common in the heat shocked nucleus (indicated by "Ga" in Figure 37B). The equivalence of the aggregate seen in the embedment-free section to that identified in the Epon-containing sections could not be established. However, where they could be measured in relatively sparse areas, the electron-dense particles in the granular aggregates were approximately 35 nm in diameter as were the particles seen in the resin-containing sections.

Heat shock elicits an expansion and possible relaxation of the nucleolus. The nucleoli from an untreated HeLa cell,

Figure 38. Morphology of chromatin- and RNP-containing nucleoli from control and heat shocked cells. The nucleoli from an untreated HeLa cell, visualized in the CSKF, is shown in (A). The nucleoli is compact and dense, showing little fine structure in the sections used to analyze the CSKF. Under the same imaging conditions, nucleoli from heat shocked cells appear vesiculated (B) or appear annular and expanded (C). The nucleoli from heat shocked cells display variable margins of electron dense material, indicated by the doubled-headed arrows. In many cases, the margination appears complete and the fiber network viewed within the nucleoli (outline arrow in panel C) resembles the fiber network of the nuclear matrix. The micrograph shown in panel C is a higher magnification of the cell shown in Figure 36B.)


A
0.1μm
B
0.1μm
C
0.1μm

visualized in the chromatin-containing CSKF, is seen in Figure 38A. In the 0.1 μm sections analyzed here, the nucleoli appears compact and dense. In some circumstances, the fine structure seen in the nucleoli was of a densely-packed, reticular nature. Typical nucleoli from heat-shocked cells are seen in Figures 38B and 38C. In Figure 38B, the nucleoli appears larger and vesiculated. Rather than appearing as a tightly packed network of fibers (reticular), the structure appears to be more homogenous in density but penetrated with tortuous channels. The periphery of the nucleoli appears more electron dense than the center. The margin of dense material appears more extensive in many nucleoli, apparently having been drawn away from the center of the nucleolus, giving rise to the annular structure shown in Figure 38C. The fiber network seen within the empty region of the heat-shocked nucleolus resembled that of the nuclear matrix proper. However, the exact nature of this region was not determined. Similar observations were made by several investigators studying the effects of hyperthermia on nuclear structure (Welch and Suhan, 1985; Welch and Suhan, 1986; Simard and Bernhard, 1967; Reviewed in Simard et al, 1974).

Heat shock induces an increase in the apparent complexity of the RNP-containing nuclear matrix. Recent work of this laboratory has shown that the morphological integrity of the nuclear matrix may be maintained in part by RNP-containing structures (Fey et al, 1986). Therefore, the RNA component was retain in the nuclear matrix preparations analyzed by electron microscopy in the remainder of this chapter. This RNP-containing nuclear matrix (RNP-NM) was isolated from the CSKF by cutting DNA in the presence of the core histones with purified DNase I, then eluting the DNA and associated chromatin proteins with ammonium sulfate. RNA integrity was preserved by the presence of vanadyl adenosine, an inhibitor of RNase, during the initial lysis and subsequent fractionation. It should be noted that the

previous studies on the prompt HS proteins analyzed the nuclear matrix that was depleted of DNA and RNA (NM-IF). Consequently, it was necessary to assess the contribution of the additional RNA-associated protein to the nuclear matrix in normal and heat-shocked cells.

These results, summarized in Table 9, indicate that the additional RNA-associated protein in the chromatin-depleted nucleus was small compared to the amount of additional protein found in the NM-IF after heat shock. For this experiment, cells were prelabeled with [^{35}S]methionine, heat shocked, and the amount of labeled protein released during each step of the sequential fractionation determined. In the unheated cell, the protein released from the chromatin-depleted nucleus after RNase treatment represented 0.8 % of the total. After heat shock, the same treatment released protein that amounted to 1.3 % of the total. In contrast, the amount of labeled protein that remained associated with the NM-IF (after DNase and RNase treatment) was 4.2 % and 25 % of the total in the control and heat shocked cell, respectively.

The RNP-NM isolated from control cells is seen in Figure 39A. Characteristic features of this structure include the fibers extending from the nuclear lamina to the electron dense, remnant nucleoli. Previous work has suggested that the 20 - 30 nm particles associated with these fibers may contain hnRNA (Fey et al, 1986). After heat shock, the appearance of the nuclear matrix changed dramatically. As seen in Figure 39B, the density or interconnectedness of the fiber network within the nucleus increased over all regions of the nucleus. The extended fibers, studded with the 20 - 30 nm particles, were still present although less apparent in the complex network. There did not appear be a marked increase in the number of these fibers, rather, the reticular nature of the fiber network appeared to have increased through the presence of greater numbers of interconnected fibers. In addition, the large, amorphous aggregate seen in

Table 9. [^{35}S]Methionine-containing proteins released during sequential extraction of normal and heat-shocked HeLa cells.[a]

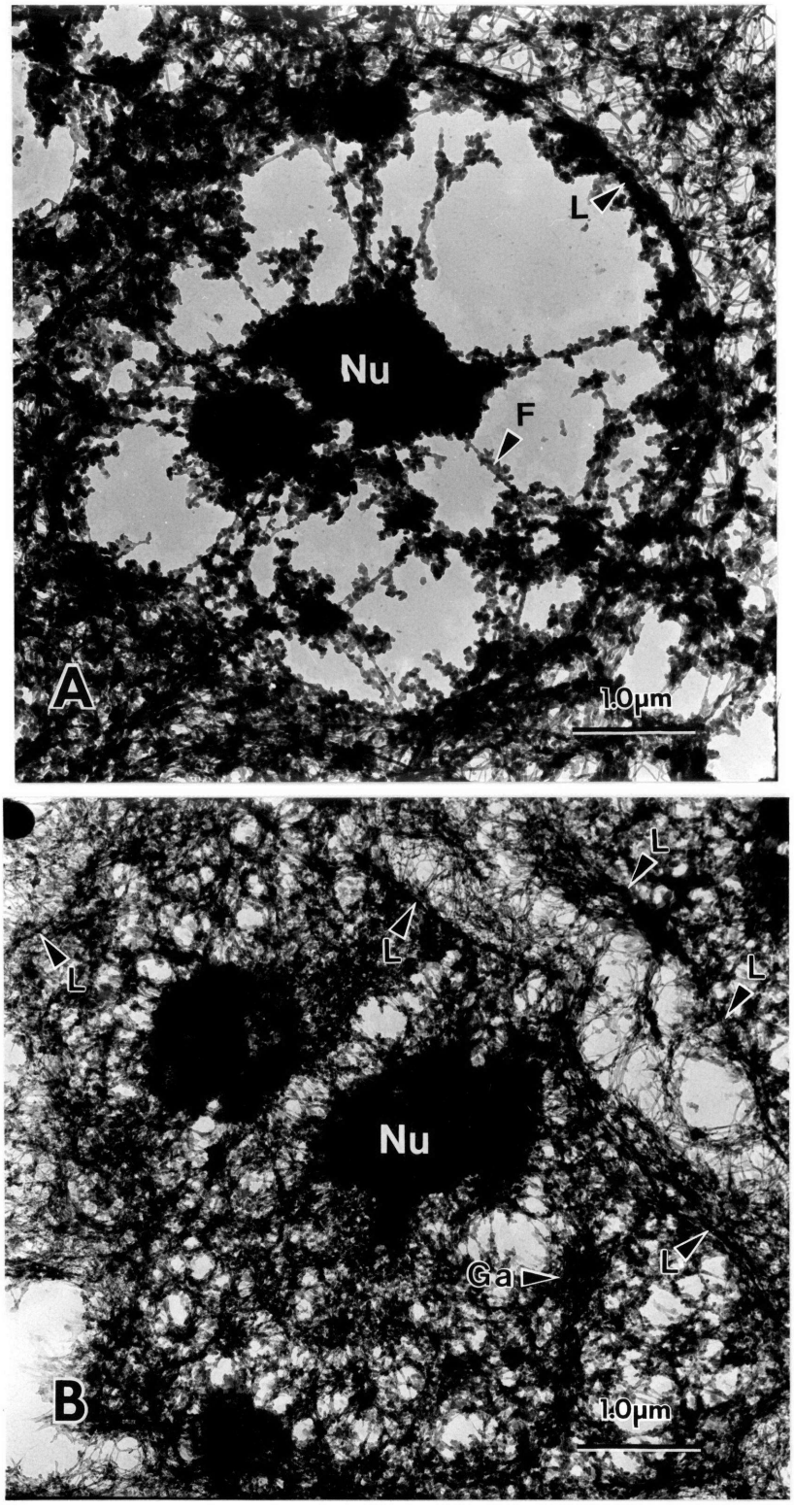
Subcellular fraction	Control	Heat shock
SOL	533 (67)	394 (49)
CSK	149 (19)	133 (17)
DNase-released[b]	69 (8.8)	79 (9.8)
RNase-released[c]	6.6 (0.8)	11 (1.3)
NM-IF	33 (4.2)	187 (23)
TOTAL	792	806

a Cells were prelabeled with [^{35}S]methionine for 1 h at 37°C, chased with complete medium and transferred to 37°C (Control) or 45°C (Heat shock) for 30 min before fractionation. The values are expressed as acid-precipitable [^{35}S]methionine CPM per 1000 cells. The values in parentheses indicate the per cent of the total label found in that fraction.

b DNase released proteins were released from the cytoskeletal framework after DNase digestion (100 μg/ml for 20 min) followed by extraction with 0.25 M (NH4)2SO4 for 5 min at 23°C.

c RNase released proteins were released from the DNase-extracted structure by RNase digestion (100 μg/ml for 10 min) followed by extraction with 0.25 M (NH4)2SO4 for 5 min at 23°C.

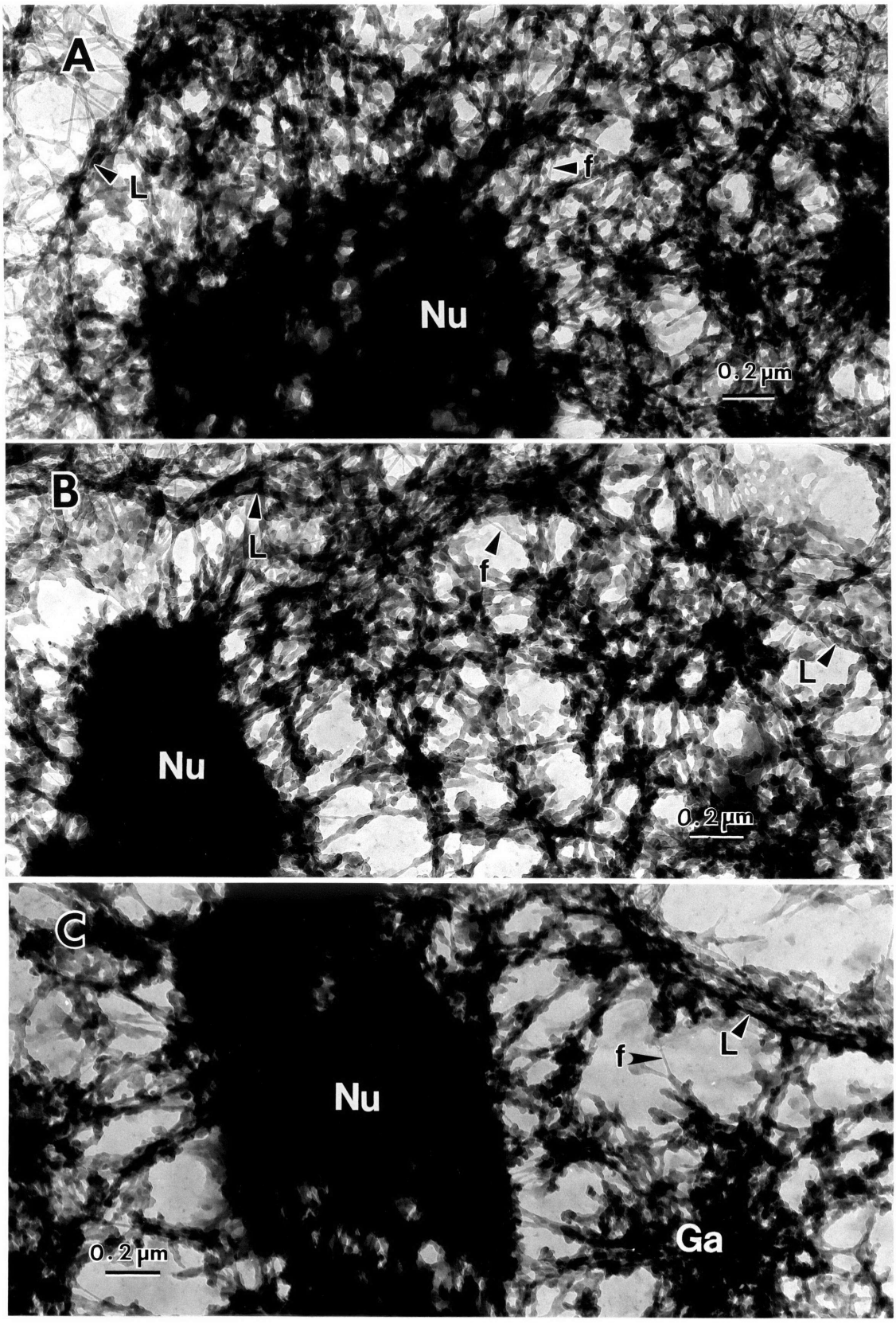
Figure 39. The chromatin-depleted, RNP-containing nuclear matrix of control and heat shocked cells. The nuclear lamina (L), nucleoli (Nu) and thick fibers, studded with particles are prominent features of the control RNP-nuclear matrix (A). The RNP-nuclear matrix from two heat shocked cells is seen in (B). A portion of the second cell enters the field in the upper right corner of the micrograph. The nuclear lamina is much less regular (L) but can be identified. The fiber network of the nuclear matrix of heat shocked cells appears more elaborate and interconnected than the control cell. Granular aggregates (Ga) can be identified in the chromatin-depleted nuclei of heat shocked cells.


L
Nu
F
A
1.0μm
L
L
L
L
Nu
Ga
L
B
1.0μm

the nuclear matrix of Drosophila cells after heat shock (Chapter 4, Figure 16) was not observed in the HeLa cells. However, structures that resembled the granular aggregates seen in the chromatin-containing nuclear matrix were seen in the heat shock RNP-NM. Again, the equivalence of these structures seen in the RNP-NM with those seen in the CSKF preparation could not be determined except on the basis of similar appearance.

Although most of the cells (> 65 %) examined after heat shock displayed the dense, reticular fiber network shown in Figure 39B, variability in the apparent density of the fiber network between individual cells was observed. The micrographs of Figure 40 illustrate three variations in the fiber density observed in the RNP-NM after heat shock. The RNP-NM shown in Figure 40A typifies that seen after heat shock. The majority of the additional fibers seen in the dense nuclear matrix were of a non-uniform appearance, between 10 and 40 nm in width. In contrast, the number or frequency of thin fibers (6 - 8 nm in diameter) did not appear to vary with the density of the fiber network although the complexity of the images make this quantitative determination difficult. Approximately 10 % of the cells examined displayed an RNP-NM of similar density and connectivity to that of the control (Figure 40C). These structures could be readily identified as heat shock-derived because of the irregular path of the nuclear lamina and by the presence of the characteristic fibrillar-granular aggregate. The remaining cells (25 %) displayed an RNP-NM of intermediate density, as shown in Figure 40B. While the separation of the structures into one of three categories was strictly a qualitative judgement, there did not appear to be a smooth continuum between the three types of filament networks described. In addition, this variability in appearance of the heat shocked RNP-NM as well as the approximate frequency of each type was consistently observed

Figure 40. Variations in the fiber network of the RNP-containing nuclear matrix of heat shocked cells. Representative nuclear matrices from heat shocked cells are shown in each panel. Approximately 65 % of the cells displayed the dense, interconnected nuclear matrix in panel (A), 25 % contained a fiber network of intermediate density as shown in (B), and 10 % displayed a fiber network of similar appearance to the control (C). The nucleoli (Nu) and nuclear lamina (L) are marked. Thin fibers are seen in each form of the nuclear matrix (f). Granular aggregates (Ga) can be found in all heat shocked matrices, included those resembling the control nuclear matrix (panel C).


A
L
f
Nu
0.2 μm
B
L
f
L
Nu
0.2 μm
C
f
L
Nu
Ga
0.2 μm

between experimental preparations. The basis for this observation was not understood.

The remnant nucleoli seen in the chromatin-depleted RNP-NM from heat-shocked cells appeared less expanded than in the chromatin-containing nucleus. Because the sections used to study the nuclear matrix were twice as thick (0.2 μm) as those used to study the CSKF, a direct comparison of the images obtained is not accurate. Nonetheless, the nucleoli visualized in the RNP-NM displayed a relaxed, vesiculated appearance when compared to the control. In addition, no annular nucleoli were observed in the RNP-NM preparation and the margin of electron dense material was no longer apparent. This observation would be consistent with the suggestion of other investigators that the intranucleolar chromatin condenses and migrates to the margins of the nucleoli during heat shock (Simard and Bernhard, 1967; Welch and Suhan, 1985; Welch and Suhan, 1986). The reduced density seen in the periphery most probably reflects the absence of condensed chromatin in these structures after DNase treatment.

DISCUSSION

In this chapter, resinless-section electron microscopy was used to study the changes in HeLa cell architecture provoked by exposure to elevated temperatures. The heat shock regime, which was a 1 hour exposure to 43.5^{o}C, was sufficient to inhibit overall protein synthesis by approximately 60 % while permitting prompt HS protein synthesis. Under these conditions, approximately twice as much protein was associated with the NM-IF than in the untreated cell. Previous work has suggested that this additional protein is normally released in the initial extraction with Triton X-100. The disposition of this additional protein, identified by cell fractionation techniques, is surrounded by some controversy and so formed a focal point in these studies. Several investigators have suggested that hyperthermia leads to the synthesis of abnormal proteins and the partial denaturation of existing proteins. Under this hypothesis, the conventional heat shock proteins serve to disassemble the aggregates of abnormal protein in an ATP-dependent manner (Lewis and Pelham, 1985; Bond and Schlesinger, 1985; Finley et al, 1984). In a related vein, it was suggested that nucleolar structure is similarly disrupted and that hsp70 serves to disassemble the incorrectly processed ribosomal RNP complexes (Pelham, 1984; Lewis and Pelham, 1985; Welch and Suhan, 1986).

The results of the studies reported here suggest that the additional protein in the nuclease-, salt-resistant nuclear matrix reinforces or forms part of the internal fiber meshwork of the nuclear matrix. Amorphous aggregates were not observed in the cytoplasm or the nucleus, nor was there any evidence of extracellular particles or debris associated with the nuclear fractions. Nonetheless, heat-induced changes were observed in both the cytoplasmic and nuclear filament networks. The principle change in the cytoplasmic filament system is best described as a rarefaction of the

meshwork. The fibers in the cytoplasm appears to have been pulled apart in some regions; as a consequence, the reticular network appears to have coalesced in adjacent areas, forming loosely aggregated bundles.

Another cytoplasmic change observed in roughly 10 % of the sections examined, was the formation of a loosely connected tangle of regular filaments, closely associated with the nuclear lamina. The fibers in this nuclear cap appeared to have the same width of intermediate filaments (Figure 37B). Previous investigators have observed a similar collapse and bundling of the vimentin-containing intermediate filaments in rat embryo fibroblasts (Welch and Suhan, 1985; Welch and Feramisco, 1985b; Thomas et al, 1982) and chicken embryo fibroblasts (Collier and Schlesinger, 1986) as well as the collapse of intermediate filaments in Drosophila cells containing a homologous protein to vimentin (Biessman et al, 1982). The nuclear cap of vimentin filaments observed by these investigators occurred as a closely packed, parallel array of filaments, in contrast to the loosely associated tangle of filaments seen in these experiments. Although the composition of the of the filaments seen in Figure 37B was not determined, Franke and associates observed that in HeLa cells exposed to colcemid for 24 hours, the cytokeratin-containing filaments formed tightly packed, parallel arrays while the vimentin-containing the filaments formed loosely aggregated bundles (Franke et al, 1979).

The alterations in the fiber meshwork of the nuclear matrix differed markedly from those seen in the cytoplasm. In the chromatin-containing nucleus of the extracted cell, hyperthermia appears to have caused an increase in the density of the thick fibers of the nucleus. The change in fiber appearance may have arisen, in part, from the condensation or clumping of heterochromatin about the fibers that extend from the nucleolus and nuclear lamina. The heat-induced changes in the nuclear matrix were even more striking after the removal of chromatin. Under these conditions, the

nuclear matrix of most cells (approximately 65 %) was composed of a much more interconnected or reticular network than was seen in the control (Figure 39). The increased connectivity did not appear to result from an increase in the type of fibers observed in the control nuclear matrix, rather, there appeared many more shorter, polymorphic fibers that were largely not represented in the control. Because there was no obvious increase in cytoplasmic fiber density or number of cytoplasmic fibers after heat shock, nor were any cytoplasmic aggregates observed that could account for the additional protein isolated with the NM-IF, it seems likely that the additional fibrous network seen in the heat shock nuclear matrix was due to this additional protein. It is not clear whether the additional protein occurs in the form of new filaments or whether the additional protein somehow stabilized an existing filament network that is lost in the control cells during some stage of sample preparation. In this regards, the embedment-free samples suffer from the limitation that the visualized structures must be self-supporting and retain some connections with the semi-thin section once the embedding material is removed.

The composition of the additional fibers in the nuclear matrix was not determined. However, by two-dimensional gel analysis, approximately 50 new polypeptides were associated with the NM-IF after heat shock (Chapter 5). The complex and variable morphology seen in the fibers of the heat-shocked nuclear matrix would suggest that the fibers are composed of a complex set of proteins. However, it may be significant to note that an example of a specific protein (actin) that becomes incorporated into filamentous or fibrillar structures in the nucleus after heat-shock or exposure to DMSO has been well-documented. This remains the only example of a cytoplasmic protein that has been shown to enter the nucleus and form a filamentous structure. Using conventional epon-embedded sections and indirect, immunofluorescent microscopy, Welch and Suhan and Iida and associates have identified

closely-packed, paracrystalline arrays of actin filaments in the nucleus of several mammalian cell lines after heat shock (Iida et al, 1986; Welch and Suhan, 1985). Similar bundles of 4 nm filaments were detected in the nucleus of the fungi, Acyla after heat shock (Pekkala et al, 1984). In all of these reports, the methodology did not allow the investigators to determine if finely dispersed actin filaments were present in addition to the closely-packed arrays. At least when induced by DMSO, the actin in the nuclear rods was clearly derived from a cytoplasmic pool (Sanger et al, 1980).

The occurrence of actin and related proteins in the nucleus is not a novel observation although the significance of the intranuclear actin is not understood (reviewed in LeStourgeon, 1978). However, Scheer et al. have provided indirect evidence for the involvement of nuclear actin in modulating transcription of lampbrush chromosomes obtained from the salamander, Pleurodeles waltii (Scheer et al, 1984). These investigators also noted that when transcription was abruptly inhibited by actinomycin D or the injection of RNase, the isolated lampbrush chromosomes were found in association with an elaborate meshwork of actin microfilaments. These authors suggest that actin plays a role in modulating the transcription of genes by polymerase II. Other investigators have provided evidence for the association of nascent RNA with actin (Nakayasu and Ueda, 1985) and have suggested that actin may function as an RNA polymerase initiation factor (Egly et al, 1984). Because actin is a major protein of the HeLa NM-IF (Capco et al, 1982; Staufenbiel and Deppert, 1984; Verheijen et al, 1986), further studies on the nature of the fibrillar network seen in the nucleus after heat shock should perhaps focus on a role for actin or actin-like proteins.

The remaining heat-induced changes in HeLa cell morphology that were recorded in these experiments included changes in nucleolar morphology and the appearance of

electron-dense nuclear particles after heat-shock. These observations largely corroborate the findings of others.

After heat shock, the nucleoli were observed to change from a compact, dense form to a more vesiculated, "relaxed" appearance. In many cases, a fiber network similar in appearance to that of the nuclear matrix proper could be seen through the nucleoli. It was not determined whether this fiber network was part of the nucleolus or was viewed through the empty nucleolus. However, it has been reported that a distinct, fibrous nucleolar matrix composed, in part, of preribosomal particles exists (Olson et al, 1986) In Chinese hamster ovary cells, the nucleolar matrix could be distinguished from the nuclear matrix by a distinct set of proteins acquired after heat shock (Caizergues-Ferrer et al, 1984). The empty regions in the nucleoli may have arisen from the loss of the granular compartment, which has been shown to be composed largely of preribosomal particles (Welch and Suhan, 1986; Neumann et al, 1984; Fakan and Bernhard, 1971; Das et al, 1970). The empty region in the center of the nucleolus may also have arisen from the condensation and margination of intranucleolar chromatin.

The nucleus of heat-shocked cells contained a greater number of electron-dense granules approximately 30 to 35 nm in diameter than the control nucleus. The granules were observed to occur generally as a loosely connected, fibrillar-granular array. In the resin-containing sections, an electron-transparent halo around these particles suggested the appearance of perichromatin granules. The work of several investigators suggest that perichromatin granules are supramolecular structures involved in the processing or transport of hnRNA (reviewed in Daskal, 1981). Although the equivalence of these particles and the perichromatin granules was not established, such particles of hnRNP would be expected to associate with the RNP-containing nuclear matrix (Fey et al, 1986). The increased occurrence of perichromatin granules after heat shock in HeLa cells has given rise to the

suggestion that messenger RNA processing was interrupted during heat shock (Heine et al, 1971; Cervera, 1979). Subsequent studies, showing that messenger RNA transport but not hnRNA synthesis is inhibited by heat shock have confirmed this prediction and demonstrated that the hnRNA synthesized during heat shock is associated with a different set of proteins (Mayrand and Pederson, 1983). Although the morphology of the altered hnRNP particles was not determined, the particles were found to have an altered RNA-protein ratio.

The results of these studies, taken together with the observations of other investigators, suggest that one line of investigation that should be pursued is on the correlates of the structural changes seen in the fiber network of the RNP-containing nuclear matrix and the alterations of transcription and RNA processing that is known to occur during heat shock.

CHAPTER 7

Post-polysomal mRNA in Control Cells Recruited into the Large Polysomal Region in Heat Shocked cells: Possible Relationship to Translationally-Activated Prompt HS Proteins.

INTRODUCTION

The heat shock response is characterized by marked changes in transcription and translation. The induction of heat shock gene transcription seen at elevated temperatures is often accompanied by a reduction in transcription and processing of normal cellular genes (reviewed in Pelham, 1985; Lindquist, 1986; Craig, 1986). In a parallel manner, heat shock or stress-induced mRNA is translated at high efficiency during and immediately following the stress while normal mRNA is translated very poorly (see especially Lindquist, 1981; Storti et al, 1980; Thomas and Mathews, 1984).

The synthesis of the prompt HS proteins is not affected by inhibitors of transcription and thus appears to be a translationally-controlled response to heat shock. Other examples of translationally-controlled heat shock proteins have recently been described. Generally, these proteins are also of low abundance. Such proteins have been described in rat thymocytes (Maytin et al, 1985), Chinese hamster ovary cells (Caizergues-Ferrer et al, 1984), cultured plant cells (Nover and Scharf, 1984), and Tetrahymena (Galego and Rodrigues-Pousada, 1985). A unique example of the strict translational induction of a conventional heat shock protein was described in xenopus oocytes. The heat-induced synthesis of hsp70 in the large xenopus oocyte appeared to result from the recruitment and translation of preformed, maternal mRNA rather than from the synthesis of new mRNA (Bienz and Gurdon, 1982). However, this finding has recently been challenged by King and Davis who suggested that the observed induction of hsp70 resulted from the transcription and synthesis of hsp70 in contaminating follicle cells (King and Davis, 1987). Other mRNA that escape the general heat-induced inhibition of protein synthesis include viral mRNA for the structural proteins of the small RNA virus of Drosophila, HPS-1 (Scott et al, 1982) and mRNA for the N and NS protein of VSV (Scott

and Pardue, 1981). The mRNA for ubiquitin (Bond and Schlessinger, 1984) and histone H2B (Sanders, 1981; Tanguay et al, 1983) have also been shown to escape the heat-induced inhibition of protein synthesis.

As a model, the synthesis of prompt HS proteins from normally untranslated mRNA is certainly not the only mechanism that could account for the appearance of the prompt HS proteins during heat shock. For example, the prompt HS proteins could be normally short-lived, nuclear matrix-specific proteins that continue to be synthesized but are not degraded at the elevated temperature or in the presence of chemical stress. The cyclins and PCNA are nuclear proteins that are normally degraded rapidly at the onset of mitosis. The failure to degrade these proteins has been associated with the failure to progress through the cell cycle (Finley and Varshavsky, 1985). Alternatively, the prompt HS proteins could be aberrantly processed proteins that are not degraded at elevated temperature. The ubiquitin-mediated proteolytic pathway is particularly thermal-labile (Finley et al, 1984). However, in HeLa cells, the aberration that produces these products must be remarkably specific in generating polypeptides that only associate with the NM-IF when elicited by a variety of drugs as well as heat. As a final example, the Drosophila hsp70 has been shown to undergo autoproteolysis, yielding a spectrum of well-defined products (DiDomenico and Lindquist, 1985; Mitchell et al, 1985). Because the synthesis of the prompt HS proteins is unaffected by the presence or absence of the conventional HS proteins, they are clearly not breakdown products of the major HS proteins. It is, however, not impossible that they are proteolytically related to other proteins that continue to be synthesized to a significant extent during heat shock or stress.

Without a specific probe or assay for the prompt HS proteins, many alternative models for the synthesis and appearance of the prompt HS proteins are difficult to

exclude. However, if the prompt HS proteins are synthesized from preformed, normally untranslated mRNA, it may be possible to identify the prompt HS mRNA in normal cells and observe an enrichment of the mRNA in the polyribosomes of stressed cells. Numerous examples of the activation of once translationally silent or repressed mRNA have been characterized. In the oocytes of many organisms, a large scale mobilization of maternal mRNA occurs upon fertilization. In most organisms, this is reflected in an increase in the number of mRNA engaged in translation (reviewed in Raff, 1980). However, the translational repression of certain mRNA in the oocyte was found to be mediated, in part, by the associated proteins (Rosenthal et al, 1980). Other changes upon fertilization associated with the release from repression have been documented. Some of these changes, including an increase in the intracellular pH and the modification of initiation factors (Winkler et al, 1985) may also occur during heat shock. The translational induction of a protein is characteristically accompanied by a shift of its mRNA into a polysomal association. The rapid induction of insulin biosynthesis by glucose was found to occur under translational control (Ashcroft et al, 1978). The mRNA for insulin could be driven from a free state into an association with membrane-bound ribosomes by exposing pancreatic cells to moderate increases in glucose (Welsh et al, 1986). Such behavior affords a strategy by which translationally induced mRNA could be enriched and identified by fractionating and separating "free" and "bound" polyribosomes and the free mRNP.

A related approach was employed in the limited search for the prompt HS mRNA described in this chapter. During a severe heat shock, polyribosomes rapidly disaggregate and normal mRNA presumably no longer associate with rapidly sedimenting structures. However, during such a heat shock ($45^{o}C$) in HeLa cells, the synthesis of the prompt HS proteins can still be detected (Reiter and Penman, 1983). These

conditions were selected to enrich for the presumptive prompt HS mRNA and to demonstrate a shift of the mRNA from a nontranslated state into an association with ribosomes. The only means of identifying the prompt heat shock proteins was by the mobility of these proteins during two-dimensional gel electrophoresis. Such an identification can only be characterized as tentative. Furthermore, the difficulty of identifying rare proteins among a complex mixture has been been addressed by other investigators (Young et al, 1983; Klose and Zeindl, 1984) and may have some significance to the limited results obtained here.

Briefly, a paradox arises when two-dimensional gel electrophoresis is applied to separate the entire spectrum of proteins synthesized by a particular cell type. In HeLa cells, studies using nucleic acid hybridization to measure sequence complexities suggest the equivalent of 25,000 to 55,000 distinct mRNA (Bishop et al, 1974; Holland et al, 1980). However, the separative procedures of O'Farrell (1975) applied by Duncan and McConkey (1982) lead these investigators to conclude that HeLa cells contained "no more than 2000 physiologically significant polypeptides." As electrophoretic systems of greater resolution were used, it became apparent that one could resolve approximately 10,000 distinct polypeptides. Evidently, the visualization of rare proteins can often be precluded by the presence of more common proteins focusing in adjacent, though not necessarily overlapping, regions of the gel. The identification of the prompt HS proteins in whole-cell lysates or among the <u>in vitro</u> translation products of total polysomal mRNA may hampered by such a phenomenon.

Even the simple two-step enrichment provided by detergent-extraction of whole cells has revealed rare or modified proteins that were not readily detected in the whole cell extract (Bravo et al, 1982). With respect to such limitations, this approach did provide evidence suggesting that at least some prompt HS proteins are synthesized from

mRNA that are translationally induced during heat shock. These mRNA, which were associated with the cytoskeletal framework, directed the synthesis *in vitro* of polypeptides with the same relative mobility in two-dimensional gel electrophoresis as three HeLa prompt HS proteins. These mRNA appeared to shift from the untranslated, postpolysomal fraction of untreated cells into the polysomal fraction of heat shocked cells. At least two other mRNA exhibiting the same behavior could be indirectly identified although the proteins encoded by these mRNA did not coincide with any of the prompt heat shock proteins.

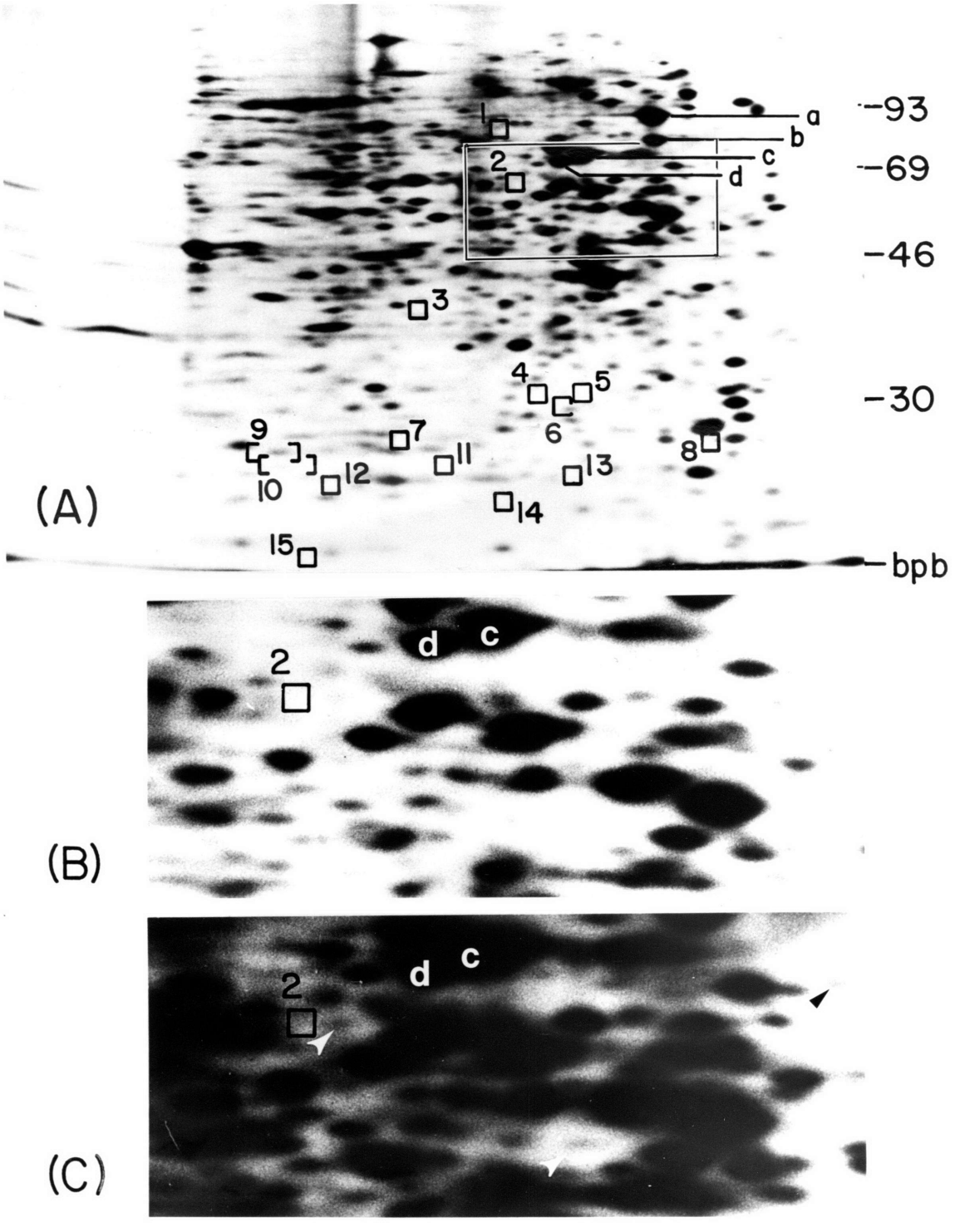
RESULTS

The experimental approach employed in this chapter was to indirectly identify mRNA for the prompt HS proteins by identifying the prompt HS proteins among the in vitro translation products of polysomal and non-polysomal mRNA. In the absence of specific probes to identify the prompt HS proteins and without any purification schemes for these proteins, this identification was based on the mobility of the proteins during two-dimensional gel electrophoresis. The limitations and difficulties in identifying rare proteins by two-dimensional gel eletrophoresis of a complex mixture has been addressed in the Introduction.

Predicted mobility of the prompt HS proteins in a two-dimensional gel electropherogram of the SOL proteins. The expected resolution of the two-dimensional gel system employed in these experiments is illustrated by the results shown in Figure 41. Figure 41A shows the expected location of the fifteen HeLa prompt HS proteins in the SOL fraction of normal HeLa cells. The limitations of the two-dimensional gel system is illustrated by the inset, repeated in Figures 41B and 41C. As indicated previously, the prompt HS proteins are not apparent in the SOL fraction. However, the predicted positions of the basic prompt HS proteins, pHS:9 and pHS:10 and the low-molecular-weight prompt HS protein, pHS:15, in the SOL fraction are approximate because of the absence of well-defined reference proteins in that region of the gel. With regards to pHS:9, repeated determinations suggest that this protein migrates slightly slower and is more basic than the poorly-focused band at that location.

The inset in Figure 41A includes approximately 15 per cent of the spots that can be identified in the electropherogram by the acetate-transfer method described in the Materials and Methods. With this method, the location of spots from exposures of the fluorogram developed after 12, 24 and 96 hours were recorded on a clear acetate overlay. 76

Figure 11. Expected location of the HeLa prompt HS proteins in the SOL fraction of normal HeLa cells. Cells that were maintained at 37°C were labeled with [^{35}S]methionine for 30 min, fractionated, and the SOL proteins analyzed by two-dimensional gel electrophoresis. The numbered squares in panel (A) mark the expected position of the prompt HS proteins. The spots marked a, b, c and d migrate with the same mobility as the constitutive HS proteins, hsp90, hsp80, hsp73 and hsp72, respectively. The position of molecular weight standards (M_r x 10^{-3}) is indicated on the right. The region of the electropherogram enclosed by the rectangle in (A) is enlarged in (B). The expected position of prompt HS protein pHS:2 is indicated as are the proteins labeled c and d. The same area of the electropherogram exposed for 4 x the length of time (96 hours) as in (B) is shown in (C). Additional spots detected after the increased exposure are marked by arrowheads.


(A)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
a
b
c
d
-93
-69
-46
-30
bpb
(B)
d
c
2
(C)
d
c
2

distinct spots could be identified in the inset using the 12 and 24 hour exposure (Figure 41B). After 96 hours of exposure, three additional spots could be identified in that region (Figure 41C). Such increases in exposure typically allow the identification of 5 to 15 per cent more proteins than by a single, optimal exposure. Nonetheless, the total number of polypeptides identified by this method, many of which are posttranslational modifications of the same gene product, is substantially below that which can be identified by other recently developed systems and is substantially below the 25,000 to 55,000 unique mRNAs predicted to be translated in a HeLa cell (Bishop et al, 1974; Holland et al, 1980).

The conventional HS proteins are synthesized in normal HeLa cells. The results of Figure 41A also indicate the relative abundance of the constitutive HS proteins in the unstressed cell. Based on the mobility of these proteins in the two-dimensional gel (Mathews et al, 1982; Welch and Feramisco, 1984; Welch et al, 1983) and their enhanced synthesis after heat shock, the spots labeled a, b, c and d appear to be hsp90, hsp80, hsp73 and hsp72 respectively. From the results of Figure 30 (Chapter 4) and in agreement with other investigators, all of hsp90 and hsp80 were released in the SOL fraction and most of hsp72/73 was released into the SOL fraction of the unstressed cell (Thomas et al, 1982; Welch et al, 1983). Consequently, the conventional HS protein seen in the SOL fraction approximates that which is synthesized by the unstressed cell. This should be compared with the amount of heat shock proteins synthesized <u>in vitro</u> by the translation of polysomal mRNA seen in Figure 43.

If previously untranslated mRNA (prompt HS mRNA) were selected for translation at the onset of the stress, it should be possible to detect this transfer of the mRNA into an association with polyribosomes. This would be characterized in sucrose density gradient separations as a

shift of the mRNA from the post-polysomal or mRNP region of control cells to the polysomal region of heat-shocked cells. From previous work (Nielson et al, 1983; op. cit.) and the results of Chapter 3, it was known that polyribosomes are found only in association with the CSKF. However, approximately 25% of the poly(A)+ RNA in the cytoplasm was isolated in the SOL fraction. These mRNA-like molecules in the SOL fraction of control cells were not translatable *in vitro* (Chapter 3). An intriguing possibility was that heat shock somehow activated these molecules and allowed them enter translation. However, the poly(A)+ RNA from the SOL fraction of heat-shocked cells did not translate *in vitro* with any greater efficiency, suggesting that this was not the case. Therefore, it seemed likely that the activated prompt HS mRNA would be derived from the untranslated pool of CSKF-bound poly(A)+ RNA and only CSKF-bound RNA was further analyzed.

Separation and *in vitro* translation of polysomal and non-polysomal RNA. Figure 42 illustrates the scheme used to obtain polysomal (>80S) and non-polysomal (<80S) RNA from the CSK fraction of control and heat-shocked cells (Perry and Kelley, 1968). The polysome profile shown was obtained from cells maintained at 37°C; an identical separation was used for the fractionation of polysomes obtained from cells exposed to 45°C for 20 min. Under these heat shock conditions, normal protein synthesis was substantially reduced and disaggregation of the polyribosomes was observed (see Figure 44). Nonetheless, prompt HS protein synthesis could still be detected (Reiter and Penman, 1983). The RNA present in the high salt-sucrose gradient was precipitated, phenol-purified, and used to program the cell-free translation system derived from reticulocytes as described in the Materials and Methods. Approximately equal amounts of acid-precipitable radioactivity synthesized from a subsaturating dilution of RNA was analyzed in the two-dimensional gel electropherograms of Figure 43. Initially,

Figure 42. Fractionation of sucrose density gradients to yield polysomal and non-polysomal RNA. Approximately 2×10^7 HeLa cells were fractionated and the polyribosomes of the CSK fraction separated by centrifugation through sucrose density gradients in HSB. For preparative purposes, the sample was centrifuged for a longer period of time than for analytical purposes. The optical density at 260 nm was monitored as the gradient was continuously pumped from the bottom. Material migrating faster than the expected position of the monoribosome was collected as the polysomal region (area 1). The remaining material comprised the non-polysomal region (area 2). Before further purification, the RNA was ethanol precipitated from the sucrose solution in the presence of purified carrier tRNA. The polysome profile shown was obtained from cells maintained at $37^{o}C$. Identical break points were used to fractionate the polyribosomes from heat shocked cells. The direction of sedimentation is indicated by the arrow along the ordinate.

Figure 42

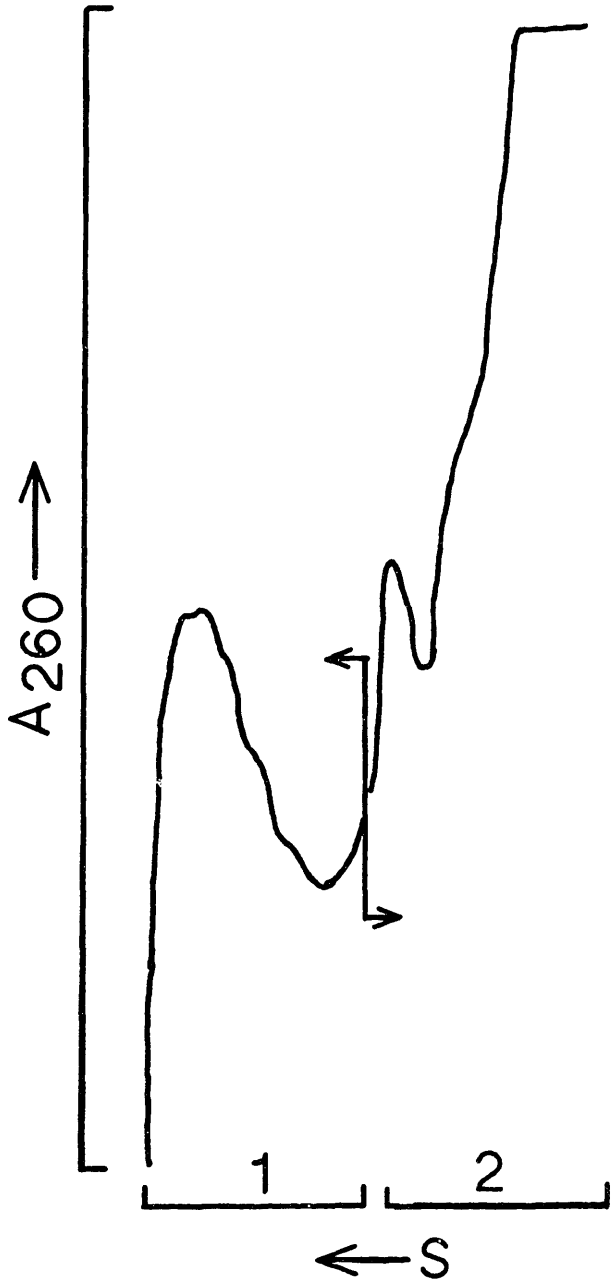
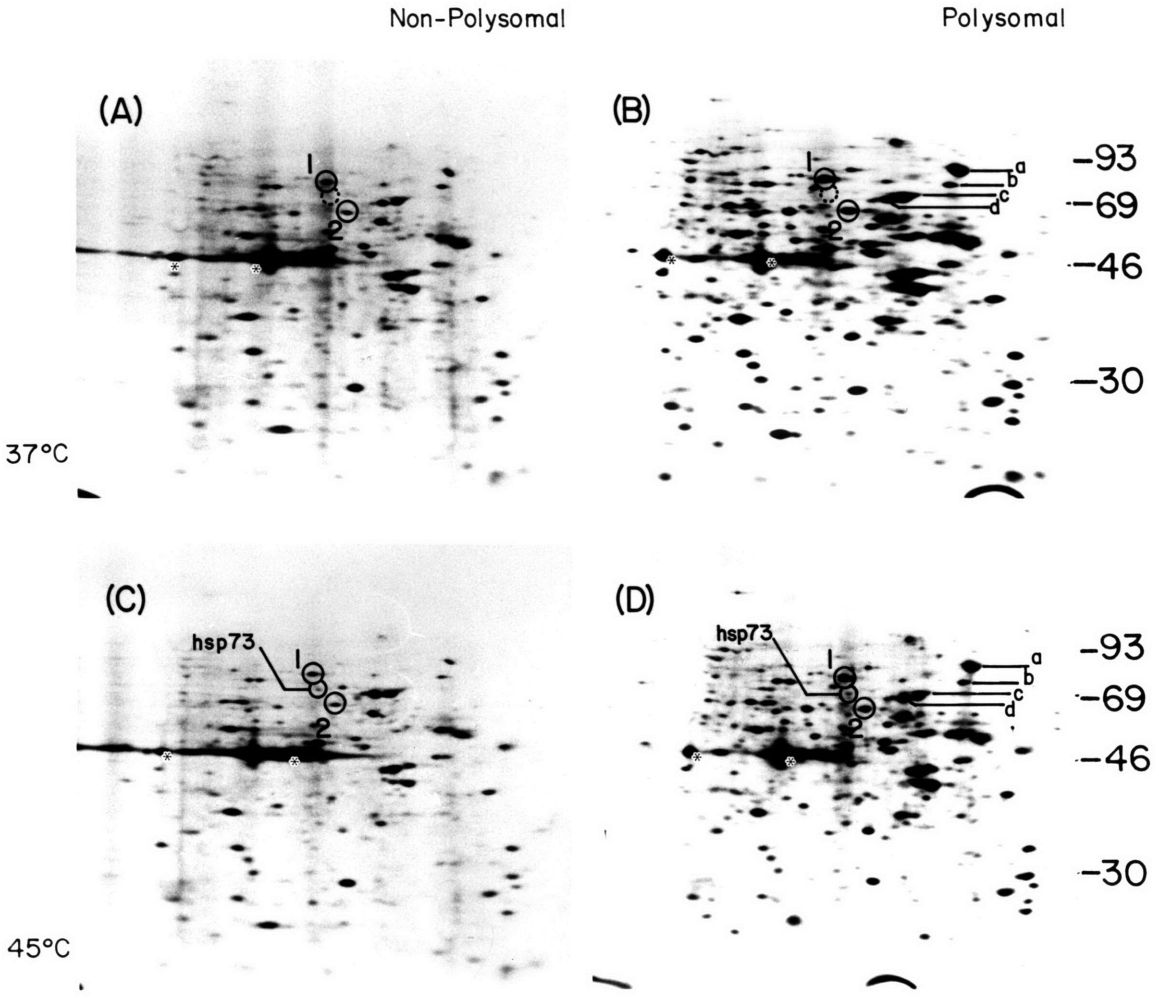


Figure 43. [^{35}S]methionine-labeled polypeptides synthesized from polysomal and non-polysomal RNA in cell-free lysates. CSK-associated polyribosomes of control (37^{o}C) and heat shocked (45^{o}C) cells were separated by sucrose density gradient centrifugation. RNA was isolated from the polysomal (> 50 S) and non-polysomal regions of the gradient as illustrated in Figure 42. The [^{35}S]methionine-labeled products from the <u>in vitro</u> translation of the poly(A) containing-RNA from untreated cells are seen in (A) and (B). Products derived from the RNA of heat shocked cells are seen in (C) and (D). Circled spots marked 1 and 2 in all panels migrate with the same relative mobility of the HeLa prompt HS proteins pHS:1 and pHS:2, respectively. The circled spot marked hsp73 in (C) and (D) appears to correspond to the inducible form of the conventional HS protein, hsp73. The same protein is not seen among the translation products of control cells; the expected position is indicated by the broken circle in panels (A) and (B). Proteins migrating with the same relative mobility of the constitutive HS proteins hsp90, hsp80, hsp73 and hsp72 are marked a, b, c, and d, respectively in the polysomal-derived translation products, panels (B) and (D). Smaller amounts of the same polypeptides can be seen in the products directed by the non-polysomal RNA but are not indicated. The asterisk (*) indicates the 45 kD endogenous protein of the reticulocyte labeled by breakdown products of the [^{35}S]methionine. Equal amounts of acid-precipitable radioactivity was loaded on the two-dimensional gels. The approximate position of molecular weight standards (M_r x 10^{-3}) is indicated on the right.

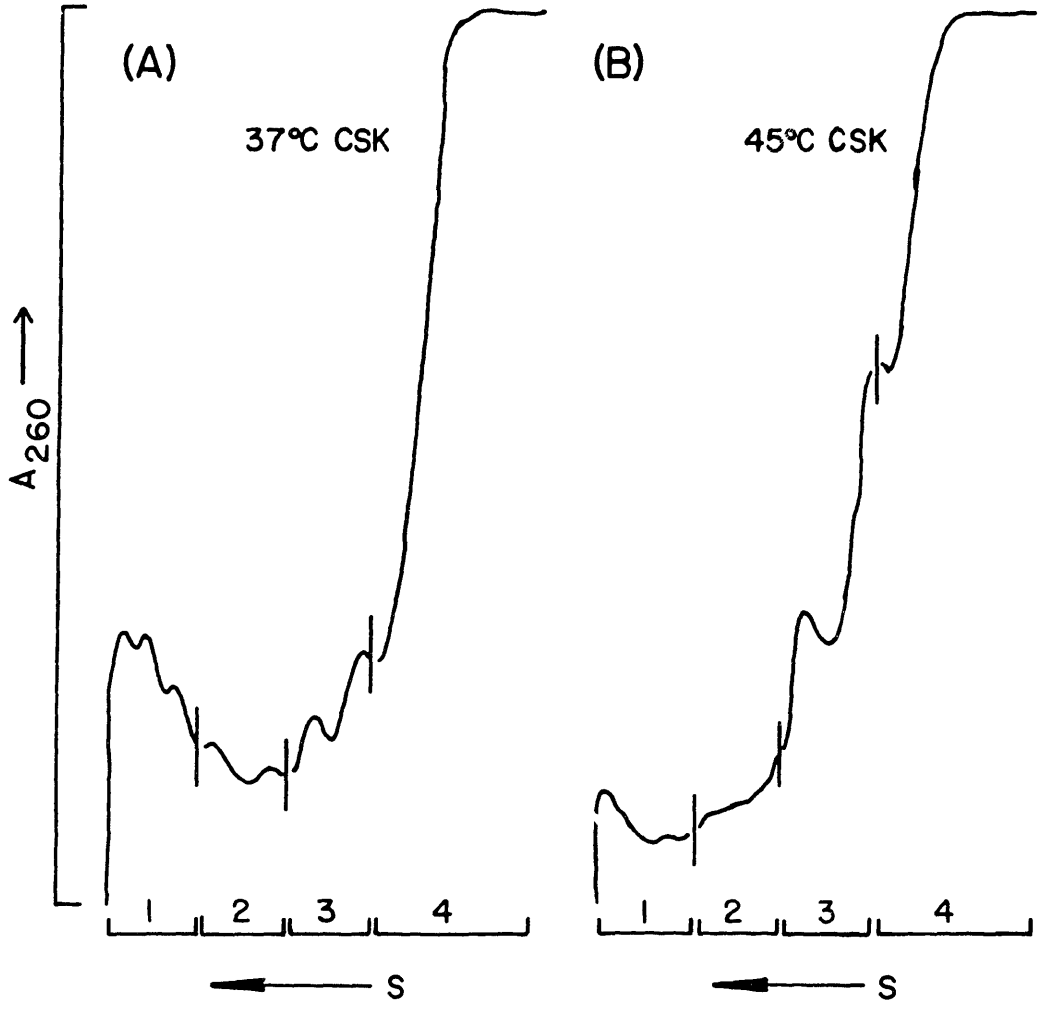

Non-Polysomal
Polysomal
(A)
(B)
(C)
(D)
37°C
45°C
hsp73
1
2
a
b
c
d
-93
-69
-46
-30

only the poly(A)+ RNA was used to program the cell-free lysate. However, it was suspected that several classes of normal mRNA as well as some of the conventional HS mRNA Drosophila cells have shorter poly(A) tracts after heat shock and these molecules are often not retained by selection with oligo(dT) chromatography (Zimmerman et al, 1980; Lindquist and DiDomenico, 1985). In addition, the HeLa conventional HS mRNA were shown to exist in both polyadenylated and non-polyadenylated forms (Kioussis et al, 1981). Therefore, total RNA was translated in vitro to avoid the loss of any unusual species of prompt HS mRNA. Although detailed comparisons were not made, it appeared that total RNA provided the same spectrum of proteins and translated at comparable or better efficiencies (per equivalent mass of poly(A)) as the poly(A) selected RNA. Perhaps previous reports detailing the poor translatability of total or post-polysomal RNA reflected the presence of the poorly translatable poly(A)+ RNA from the SOL fraction (Oulette et al, 1982; Croall and Morrison, 1980).

The similarity between the proteins synthesized from polysomal RNA obtained from control and heat shocked cells seen in Figure 43 was unexpected. From both control and heat-shocked cells, polysomal RNA directed the synthesis of approximately 4 x as much radioactive protein as did non-polysomal RNA isolated from a comparable number of cells. This value probably reflects the greater amount of CSK-bound, poly(A)+ RNA that was associated with polyribosomes (Chapter 3, Table 3). However, the amount of labeled protein directed by the translation of RNA from comparable fractions was roughly the same. Despite the large reduction in the mass of polysomes from the heat-shocked cells, a large scale shift of mRNA between the polysomal and non-polysomal regions defined in Figure 42 did not occur. Indeed, the only difference between the translation of heat shock-derived RNA and control RNA was the appearance of the inducible HS protein, labeled hsp73 in Figures 43C and 43D (Mathews et al, 1982). The

Figure 44. Fractionation of polyribosomes from control and heat shocked cells to isolate RNA from heavy polysomes, light polysomes, the subunit region and the post-polysomal supernatant. The polyribosomes from the CSK fraction of 1×10^7 control (A, 37°C) and heat shocked (B, 45°C) cells were separated by sucrose density gradient centrifugation in sucrose-containing HSB. The tracings were obtained by continuously recording the optical density at 260 nm as fractions were collected from the bottom of the gradient. Four separate fractions of approximately equal volume were collected. These are indicated by the numbered regions and were termed (1), heavy polysomal; (2), light polysomal; (3), subunits; (4) post-polysomal. Before further purification, the RNA was ethanol precipitated from the sucrose-salt solution in the presence of purified carrier tRNA. The direction of sedimentation is indicated by the arrow along the ordinate.

Figure 44

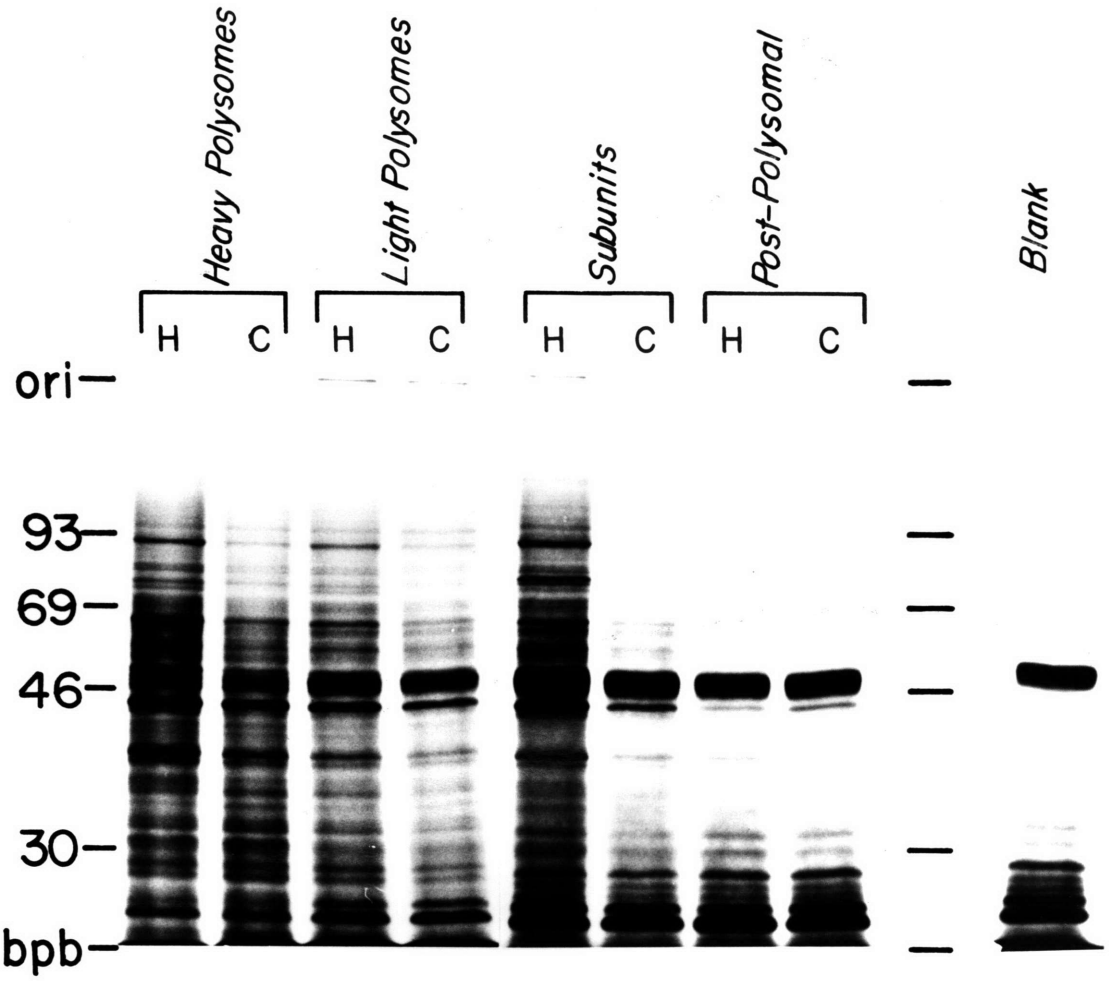


appearance of hsp73 suggested that at least scme of the inducible HS mRNA could be synthesized and transported to the cytoplasm at the high temperature. The constitutive HS proteins, labeled a through d in Figures 43B and 43D were conspicuous among the translation products of both heat-shocked and control cells. In addition, two prominent polypeptides (labeled 1 and 2) displayed the same mobility as the comparably numbered prompt HS proteins. However, the mRNA for these proteins appeared in both the polysomal and non-polysomal fractions of control and heat-shocked cells.

mRNA released from heat-disaggregated polyribosomes sediments with light polyribosomes as well as with free mRNP. The polysome gradients were further subdivided as indicated in Figure 44 to yield fractions enriched in heavy polysomes, light polysomes, ribosomal subunits and the slowly sedimenting material at the top of the gradient, termed post-polysomal. All sucrose gradients were prepared in sufficiently high salt concentrations to disassemble free ribosomes (Zybler and Penman, 1970). The expected upper limit for the sedimentation of a free mRNP would be at the extreme end of the subunit region of the gradient, corresponding to approximately 80S (area 3). As described previously, the RNA from each region of the polysome gradients prepared from control and heat-shocked cells was precipitated and purified. Serial dilutions of the RNA were translated <u>in vitro</u>, using RNA from an equivalent number of cells in each reaction. These results, shown in the one-dimensional electropherogram of Figure 45, indicate that the fractionation of the polysome gradient was sufficient to show the disaggregation of the polysomes in the heat-shocked cells and the associated shift of mRNA from the heavy polysomes to lighter regions of the sucrose gradient.

The resolution of the one-dimensional separation did not allow identification of the prompt HS proteins. However, additional information was obtained from this experiment. The appearance of a doublet at 72/73 kD in the heat-shocked

Figure 45. [^{35}S]methionine-labeled polypeptides synthesized in cell-free lysates programmed with RNA from size-fractionated polyribosomes isolated from control and heat shocked HeLa cells. CSK-associated polyribosomes from control (37^{o}C) and heat shocked (45^{o}C) cells were size fractionated as illustrated in Figure E and the RNA was purified. Serial dilutions of RNA from equivalent numbers of cells were translated in the reticulocyte cell-free system. With the exception of the heavy polysomal sample from control cells, all of the [^{35}S]methionine-labeled products derived from a subsaturating dilution of mRNA was analyzed by the one-dimensional gel electropherogram shown. One half of translation products from the heavy polysomal, control reaction was analyzed because of the large amount of radioactivity in that sample. The translation products from control cells are indicated by a C above the lane, those from heat shocked cells marked with an H. The position of molecular weight standards (M_r x 10^{-3}) is indicated at the left. The lane marked blank reflects the endogenous incorporation of [^{35}S]methionine in a reaction mixture that was primed only with purified yeast tRNA used as a carrier for precipitations.


Heavy Polysomes
Light Polysomes
Subunits
Post-Polysomal
Blank
H
C
H
C
H
C
H
C
ori
93
69
46
30
bpb

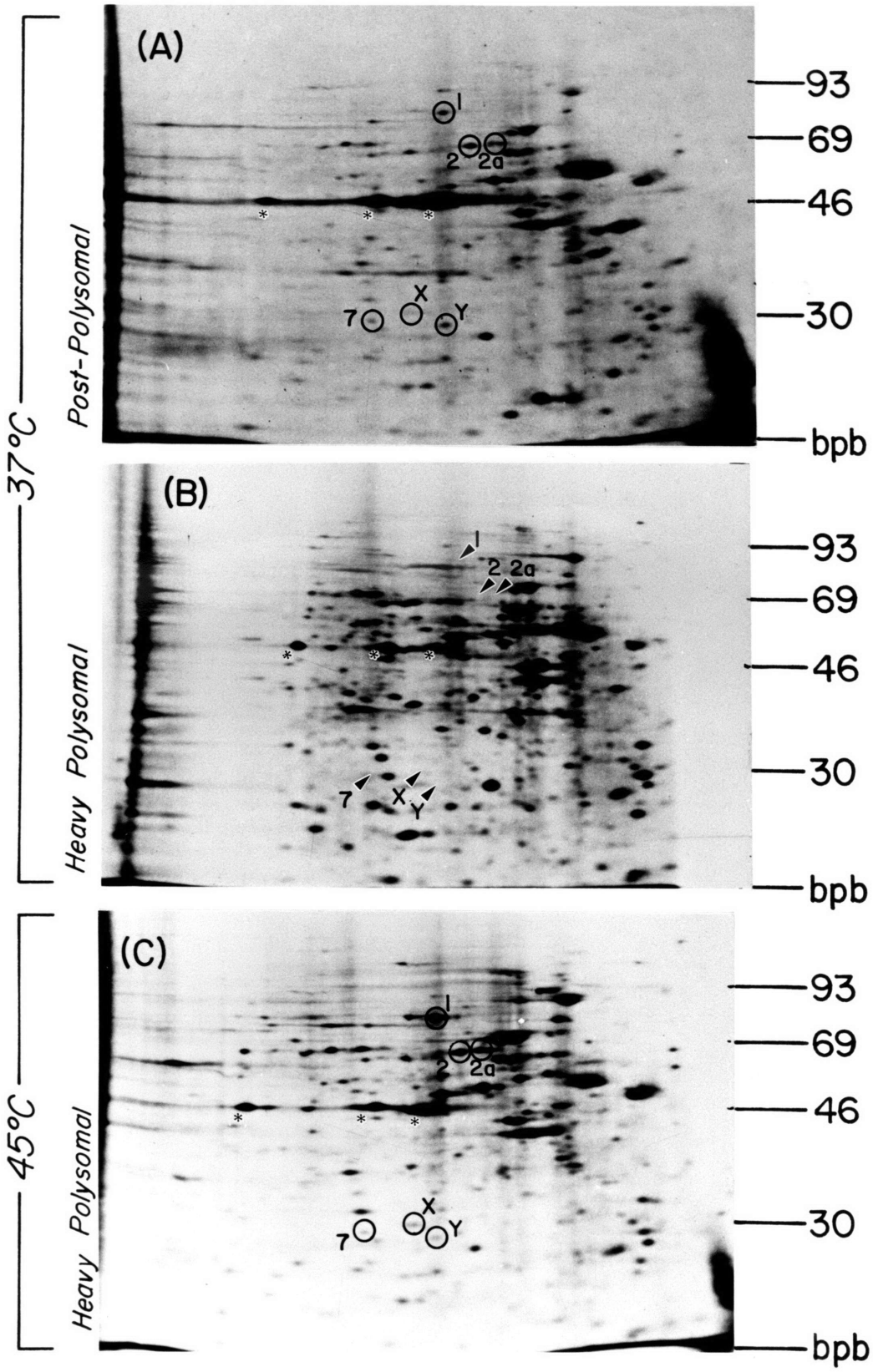
polysomal fractions probably corresponded to the slight induction of hsp73, as noted previously. The intensity of the band at 90 kD appeared greater in all heat-shocked fractions. This observation could also reflect the enhanced synthesis of the mRNA for hsp90 or stabilization of the mRNA at elevated temperature. More importantly, the data of Figure 45 also indicated that the mRNA released from polyribosomes upon heat shock sedimented in the vicinity of the ribosomal subunits and in the light polysomal regions of the sucrose gradient. Finally, although the products obtained from the translation of post-polysomal RNA appears sparse, translatable RNA was present in that region of the gradient (compare the post-polysomal lanes with the blank). The limited amount of material synthesized under the direction of post-polysomal RNA probably was due to the limited amount of poly(A)+ RNA found in that region rather than, for example, an inhibitory agent in that fraction. As seen in Figure 46, greater amounts of labeled protein was obtained when more post-polysomal RNA was translated in the cell-free system.

Untranslated mRNA in the slowly-sedimenting, post-polysomal fraction of normal cells shift to the heavy polysomes of heat shocked cells. The translation products obtained from the greatest concentration of subsaturating RNA added to the <u>in vitro</u> reaction was analyzed by two-dimensional gel electrophoresis. For these analyses, equal amounts of acid-precipitable, [^{35}S]methionine-labeled material were utilized. The translation products using RNA obtained from the light polysomes and the subunit region typically gave rise to patterns similar to those obtained by translating the total polysomal RNA (Figure 43). However, heat-induced changes in the mRNA present in the heavy polysomal and post-polysomal regions of the gradients were detected. In particular, examples of mRNA shifting from the post-polysomal region of control cells to the heavy polysomal region of heat-shocked cells were observed. This behavior

would be expected for pre-existing mRNA that became activated and translated at the high temperature such as the prompt HS mRNA. This result is shown in Figure 46.

The [^{35}S]methionine labeled products of the <u>in vitro</u> translation of post-polysomal RNA from control cells (Figure 46A) were compared to the products obtained from translation of heavy polysomal RNA from control (Figure 46B) and heat-shocked (Figure 46C) cells. To summarize these results, five distinct polypeptides could be detected after translation of control post-polysomal and heat-shocked, heavy polysomal RNA that were not seen after translation of control, heavy polysomal RNA. Two of the products, labeled 1 and 2, appeared to correspond to the prompt HS proteins pHS:1 and pHS:2. The spot labeled 2a was not consistently observed and appeared to represent a more acidic form of polypeptide 2. The apparent "shift" of the mRNA for polypeptides 1 and 2 was observed in three repetitions of the fractionation and translation. The mRNA for the polypeptides labeled X and Y also appeared to shift from the post-polysomal fraction of control cells to the heavy polysomal fraction of heat-shocked cells. The polypeptides X and Y were not detected among the translation products of total polysomal RNA. These proteins did not appear to correspond to any of the 15 prompt HS proteins. However, polypeptides X and Y appeared to be 3000 daltons smaller and 0.2 pH units more basic than the prompt HS proteins pHS:4 and pHS:6. Finally, the polypeptide labeled 7 appeared to correspond to the prompt HS protein of the same number. This protein was also not observed in the translation of total polysomal RNA. The apparent shift of the mRNA for this protein into the heavy polysome region was observed only in the experiment presented.

Figure 46. The apparent migration of mRNA from the post-polysomal compartment in control HeLa cells to the heavy polysomes of heat shocked (45°C) cells revealed by <u>in vitro</u> translation of purified RNA. Approximately equivalent amounts of acid-precipitable radioactive protein derived from a repitition of the experiment presented in Figure 45 were analyzed by two-dimensional gel electrophoresis. The electropherograms of proteins translated from: post-polysomal RNA from <u>control</u> cells is shown in panel (A), from heavy polysomal RNA from <u>control</u> cells in panel (B), and from the heavy polysomal RNA of <u>heat shocked</u> cells in panel (C). The circled proteins (in A and C) are found only among the translation products of control post-polysomal <u>or</u> heat shock heavy polysomal mRNA. The expected positions of these polypeptides are indicated in (B) by the labeled arrowheads. The number proteins display the same mobility in the two-dimensional gels as the correspondingly numbered prompt HS protein. The proteins labeled X and Y do not correspond to any of the prompt HS proteins. The [^{35}S]methionine-labeled endogenous 45 kD artifact of the reticulocyte translation system is indicated by the asterisk (*). The approximate position of radioactive molecular weight standards (M_r x 10^{-3}) is indicated at the right of each panel.


(A)
1
2
2a
7
X
Y
93
69
46
30
bpb
37°C
Post-Polysomal
(B)
1
2
2a
7
X
Y
93
69
46
30
bpb
Heavy Polysomal
(C)
1
2
2a
7
X
Y
93
69
46
30
bpb
45°C
Heavy Polysomal

DISCUSSION

The results presented in this chapter demonstrate that some mRNAs that are enriched among the untranslated, slowly-sedimenting RNP particles are shifted into a rapidly-sedimenting form shortly after heat shock. The apparent shift of these mRNAs was not representative of the behavior of most mRNA during the HS. The bulk of the mRNA was observed to shift from a rapidly-sedimenting form, presumably in polyribosomes, to a more slowly-sedimenting form, comparable to the ribosomal subunits and free mRNP. This behavior was consistent with the severe inhibition of protein synthesis observed in vivo. The absence of large polyribosomes was expected from previous work that demonstrated a heat-induced defect in initiation (McCormick and Penman, 1969; Hickey and Weber, 1982).

One interpretation for the "contrary" shift of the few mRNA from the untranslated pool of mRNP to the polysomal region of the sucrose gradient is that these mRNA were translationally activated upon heat shock. Such behavior is predicted for the prompt HS mRNA and three of apparently five mRNA that displayed such behavior did appear to code for identical proteins to three prompt HS proteins. However, the work presented in this chapter was of a preliminary nature and there is not sufficient evidence to fully support these contentions. In particular, the activated mRNA were not formally demonstrated to be actively engaged in translation in the heat shocked cells. In addition, the identification of the polypeptides synthesized in vitro as prompt HS proteins was based solely on the similar mobility displayed during two-dimensional gel electrophoresis. Within these limitations, the data are at least consistent with the hypothesis that some mRNA, distinct from the mRNA for the conventional HS proteins, are translationally activated upon heat shock. Perhaps more significantly, these findings suggest specific prompt HS proteins that may be suitable for

further study with a coordinate analysis of protein and presumptive mRNA.

These findings were based on the in vitro translation of RNA that was purified after sucrose density gradient separation of the CSK fraction. Two levels of purification were represented in this scheme. Previous work (Chapter 3) revealed that 25 per cent of the cytoplasmic poly(A)+ RNA was released by extraction with Triton X-100. This RNA was not translated in the cell (not polysome-associated) and was not translated efficiently in vitro. Heat shock did not change the distribution of mRNA between the SOL and CSK fractions nor did heat shock change the activity of the SOL mRNA in vitro (Cervera et al, 1981; Howe and Hershey, 1984; unpublished results). Consequently, the first step in purification was to remove the inactive SOL poly(A)+ RNA from the CSK-associated RNA. In a sucrose gradient separation of total cytoplasmic constituents, the postpolysomal region would normally contain these poly(A)+ RNA molecules of low translatability. It seems likely that this SOL RNA contributed to previous reports on the inefficient translation of such non-polysomal mRNA (Croall and Morrison. 1980; Oulette et al, 1982).

The second step in the enrichment separated mRNA based on its sedimentation velocity in a sucrose density gradient. Presumably, the translationally activated (prompt HS) mRNA would shift from the non-polysomal region into the polysomes upon heat shock. Because a severe heat shock inhibits initiation on most normal mRNA, such a heat shock would further enrich for the prompt HS mRNA by driving normal mRNA from the polysomes into the mRNP or light polysomal region of the gradients. The recruitment of specific mRNA from a non-polysomal state into the polysomes has been demonstrated in numerous examples of translational activation (Alton and Lodish, 1977; Rosenthal et al, 1980; Raff, 1980). One of the first systematic studies of the sequence content of polysomal and non-polysomal mRNA of an established cell line found some

non-polysomal sequences that were not represented in the polysomes. The non-polysomal location of these mRNA could not be attributed to poor initiation efficiency (as assayed in vitro) or a lack of polyadenylation (Croall and Morrison, 1980).

In the experiments reported here, differences between polysomal and non-polysomal mRNA were most apparent when sequences were compared between the more slowly-sedimenting mRNP and the more rapidly sedimenting, larger polysomes. The data of Figures 46A and 46B indicate that the non-polysomal sequences are largely a subset of polysomal sequences. However, translation in vitro of the RNA obtained from three experiments revealed approximately 10 mRNA species that were enriched in the post-polysomal region. Five of these sequences were detected in the heavy polysomal region of heat shocked cells and were indicated in Figure 46. (The other sequences remained in the post-polysomal region of control and heat shocked cells and were not further considered.)

The polypeptides labeled 1 and 2, which appeared to correspond to the prompt HS proteins pHS:1 and pHS:2, were heavily labeled in vitro. This is quite distinct from the labeling of pHS:1 and pHS:2 observed in vivo. These two proteins were not the most strongly labeled prompt HS proteins after induction by heat or by chemical stress (Chapter 5, Figures 28 and 29). In addition, polypeptides 1 and 2 were unusual among the major products of the in vitro translation in that they did not correspond to any proteins seen in the control cell. Most of the other major proteins synthesized in vitro corresponded to proteins labeled in vivo. Most could be seen in the SOL fraction (Figure 41A). The reason for this particular property was not understood. However, it should be noted that many of the posttranslational modifications that occur in the cell (including rapid turnover) are not reproduced in the reticulocyte-derived, cell-free translation system. Also, some of the non-translated, postpolysomal mRNA has bee found

associated with proteins that preclude its translation as an RNP; this same RNA was fully translatable once phenol-purified (Rosenthal et al, 1980). Polypeptide 7 appeared to correspond to the prompt HS proteins, pHS:7. This protein was detected in only one of the three sets of fractionations and translations and was also not as strongly labeled as polypeptides 1 and 2.

The remaining polypeptides encoded on mRNA that appeared to shift from the post-polysomal region into the heavy polysomes upon heat shock were labeled X and Y. Although these proteins did not directly correspond to any of the prompt HS proteins, the relative mobility of polypeptides X and Y in the two-dimensional gel most closely resembled that of pHS:4 and pHS:6, respectively. Both polypeptides X and Y were 3,000 daltons smaller and appeared to be more basic by 0.2 pH units. An interesting, though purely speculative observation, is that the addition of a single ubiquitin residue on an exposed epsilon amino residue on polypeptides X and Y could account for such a difference in mass and possibly charge. The work of other investigators suggests that further investigation into the identity and nature of polypeptides X and Y may be warranted. Using a wheat germ-derived, cell-free translation system, Croall and Morrison identified several acidic proteins whose mRNA were more heavily represented in the non-polysomal compartment of mouse neuroblastoma cells. Although direct comparison with the two-dimensional gels presented by these investigators and those analyzed here is difficult, two of the acidic proteins identified by Croall and Morrison appeared to migrate with very similar mobility to polypetides X and Y. These investigators noted that the mRNA for these two proteins were unusual among the non-polysomal mRNA in that they did initiate efficiently and were not translated <u>in vitro</u> under conditions of excess mRNA. It would be interesting to determine if the mRNA for polypeptides X and Y displayed similar properties to the murine mRNA.

Finally, the apparent absence of the remaining prompt HS proteins from the cell-free translation products is not understood, although not entirely unexpected. If the putative mRNA for these prompt HS proteins were as rare as the proteins appear to be, it could be possible that the translation products of these mRNA would not be detected among the products of the more common mRNA. Clearly, more work is required to address this problem. The results presented here were not intended to address such issues and any further speculation based on the data presented is not justified. However, these results do suggest that some examples of translationally-activated mRNA in the heat shocked HeLa cell may exist. A coordinated study of these mRNA and a possible relationship to the prompt HS proteins, particular those discussed above may be productive.

CONCLUSION

In summary, the hypothesis that messenger RNA association with the cytoskeletal framework is necessary for translation, but not sufficient, was supported by the results presented here. Cytochalasin D forced a portion of the messenger RNA molecules into the Triton X-100 extractable compartment of the cell. In parallel, a dose-dependent inhibition of protein synthesis was observed; the nature of the inhibition was unusual but was most directly explained by postulating that cytochalasin D lead to the release of messenger RNA molecules from cytostructure *in situ*, where they no longer function.

A largely separate body of work in this thesis described the prompt heat shock proteins, a class of proteins that appeared to be induced by heat alone in *Drosophila*, and by a variety of stress agents in HeLa cells. These proteins possess unusual properties among the various stress proteins that have been described. The prompt heat shock proteins are apparently not synthesized in the unstressed cell. Their synthesis during stress appears to result from a translational activation, although this was not strictly established. Finally, these proteins were found only in association with the residual core structure of the cell composed of the nuclear matrix, nuclear lamina and intermediate filaments. The function of the prompt heat shock proteins and the significance of their strict association with the NM-IF scaffold remains unknown. It is clear that during high temperature induction, the prompt heat shock proteins are part of a much larger response that leads to substantial changes in the protein composition of NM-IF. Again, the significance of these changes are not understood.

The identification of the prompt heat shock proteins was facilitated by the enrichment of these proteins through their association with a cellular structure containing less than five per cent of total cell protein. The preliminary results of the final chapter suggest that the messenger RNA for the prompt heat shock proteins may be identified by exploiting the obligatory association of messenger RNA with the cytoskeletal framework. Further understanding of the significance of the prompt heat shock response will necessarily require and provide greater understanding of interactions between cytostructure and the control of protein synthesis.

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