N89-20291

THE STRESS RESPONSE SYSTEM OF PROTEINS: IMPLICATIONS FOR BIOREACTOR SCALEUP

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Abstract

Animal cells face a variety of environmental stresses in large-scale bioreactors, including periodic variations in shear stress and dissolved oxygen concentration. We have embarked on a major research effort to develop diagnostic techniques for identifying the particular sources of environmental stresses for animal cells in a given bioreactor configuration, and to understand the mechanisms by which cells cope with such stresses. The individual concentrations and synthesis rates of hundreds of intracellular proteins are affected by the extracellular environment (e.g. medium composition, dissolved oxygen concentration, pH, and level of surface shear stress). We are currently developing techniques for quantifying the synthesis rates and concentrations of the intracellular proteins which are most sensitive to environmental stress. Previous research has demonstrated that a particular set of "stress response proteins" are synthesized by mammalian cells in response to temperature fluctuations, dissolved oxygen deprivation, and glucose deprivation. Recently, we have demonstrated that exposure of human kidney cells to high shear stress results in expression of a completely distinct set of intracellular proteins.

This research can be expected to have a significant impact on research at NASA, and on the developing U.S. biotechnology industry. The techniques developed in this project can be utilized to assess the sources of environmental stress in any bioreactor configuration for any type of animal cell. The techniques will therefore be of significant diagnostic value in the design and operation of animal cell bioreactors. In addition, an increased understanding of the response of cells to environmental stress can be expected to ultimately lead to genetic modifications which will lead to improved growth characteristics of animal cells in large-scale culture. We are currently collaborating with Cetus Corporation to assess these industrial implications.

Introduction

There are several potential sources of stress for cells grown in suspension culture. A sudden change of environment may occur upon innoculation. High levels of shear may exist in the vicinity of an impellor. Significant hydrostatic pressure variation may exist between the top and bottom of the fermentor. Significant gradients in dissolved oxygen, pH and other environmental variables may exist in the culture vessel due to a combination of the finite circulation time in the vessel and the metabolic activities of the cells (e.g. consumption of oxygen, and production of acid or base). It will be particularly difficult to maintain a homogeneous growth environment for cells in large-scale, high-density or viscous cell cultures. As a consequence of this environmental heterogeneity, microbial, plant and animal cells will inevitably face a variety of environmental transients as they circulate in a culture vessel.

There are limits to the amount of environmental stress (e.g. periodic shear stress or variation in dissolved oxygen concentration) which can be readily accomodated by a particular cell type. Techniques for assessing these limits at the bench scale would be of obvious value in the design and scale-up of bioreactors and in the selection of host cell systems for large-scale production.

An understanding at the molecular level of the intracellular mechanisms by which cells respond to environmental stress would be even more valuable. With this knowledge one could begin to experiment with genetic modifications to improve the growth characteristics of the host system in large-scale culture.

We have embarked on a major research effort to understand the ability of cells to respond to environmental stress. In the first section of this report the scientific background of this research program will be presented. This will be followed by a brief statement of our research objectives and presentation of preliminary results. This report will conclude with a summary of the expected benefits of this research program.

The Stress Response System of Proteins

Recent developments have provided a framework for the examination of the response of

cells to environmental transients. It has been recognized that all cell types, from bacterial to plant and mammalian cells, possess a network of proteins which are expressed in response to environmental stress (Schlesinger et al., 1982; Neidardt et al., 1984; Atkinson and Walden, 1985). The expression of these proteins, variously known as the "stress response proteins" or the "heat shock proteins", was first observed in response to sudden environmental temperature changes. It is now clear that there are numerous inducers of the stress response proteins, including changes in dissolved oxygen concentration, exposure to ethanol or amino acid analogues, stimulation with serum and infection by adenovirus 5 or simian virus 40 (summarized by Anathan et al., 1986 and Wu et al, 1986).

The stress response proteins appear to play a role in protecting cells from damage in adverse situations. In general, it has been observed that pretreatment of cells to elicit synthesis of the stress proteins results in protection of those cells from subsequent severe stress. For example, pretreatment of cells with ethanol or hypoxia results in increased thermotolerance. (Li and Werb, 1982). The specific roles of the stress response proteins are an area of current investigation. Recently, it has been noted that brief severe heat shock blocks the processing (i.e. removal) of intervening sequences in mRNA. The stress response proteins act to counteract this effect (Yost and Lindquist, 1986). The stress response genes are conspicuously free of introns, permitting synthesis of the stress response proteins under otherwise restrictive conditions.

The stress response proteins are by definition those intracellular proteins whose rates of synthesis increase following sudden environmental change. Eighteen major stress response proteins have been identified in E. coli. The number of major stress response proteins identified to date in insect and mammalian cells is on the order of eight to ten. Major stress response proteins with molecular weights of approximately 70 and 85 kDa have been noted in all species so far examined.

The 70 and 85 kDa stress response proteins have been highly conserved in evolution. Antibodies raised against the 70 and 85 kDa stress response protein from chicken cells cross-react with the equivalent proteins in yeast, Drosophila, corn, mouse and human cells (Kelley and Schlesinger, 1982). Recently, it was noted that the amino acid sequence of the human 70 kDa protein is 75 to 80% homologous to both the yeast and Drosophila 70 kDa stress response proteins and 55.5% homologous to the equivalent protein from E. coli (Voellmy et al., 1985).

intracellular proteins which triggers the transcription of the stress response genes. In this regard ubiquitin would play a regulatory role in the stress response system somewhat analogous to the role of cyclic AMP in the catabolite repression system. The existence of a unique promotor sequence upstream of the stress response genes suggests the existence of a specialized transcription factor, analogous to the catabolite gene-activator (CAP) protein of the catabolite repression system. Munro and Pelham have postulated that this transcription factor is a constant candidate for ubiquitin attachment. The transcription factor is proposed to be inactive in its "ubiquitinized" form and active only when it is "non-ubiquitinied. Therefore, when the level of free ubiquitin falls following an increase in abnormal protein, the transcription factor is active and the stress response genes are transcribed. Recently, it has been noted that ubiquitin is, itself, a stress response protein (Bond and Schlesinger, 1985).

The recent identification of multiple promotor sites for the stress response genes (Wu et al., 1986) raises the possibility that multiple mechanisms may exist which trigger expression of one or more of the stress response genes.

Research Objectives

Our initial technical efforts are now focussed on the implementation of two-dimensional electrophoresis technology (O'Farrell gel electrophoresis) for analysis of the synthesis rates of intracellular proteins. A computerized, image analysis system is used for the quantitative analysis of autoradiographs produced using this technology. The utilization of 2D-PAGE for the qualitative and quantitative analysis of intracellular protein synthesis has been extensively examined over the past decade(O'Farrel, 1975; Anderson and Anderson, 1977; Cellis and Bravo, 1984). The development of computerized, image analysis systems has markedly enhanced the analysis of autoradiographic images produced by 2D-PAGE(Garrels,1979; Miller et al., 1984) and other techniques (Goochee et al., 1980).

As an additional analytical tool, an immunoassay based on the important 70,000 MW "heat shock" protein (Welch and Feramisco, 1984) is being developed in conjunction with Dr. William Welch (Cold Spring Harbor Laboratory) and the University of Texas (Dr. Lynne Rutzky). The concentration of this protein appears to be a particularly clear indicator of the magnitude of the stress response in mammalian cells. The concentration of this protein is very low in unstressed cells. Following imposition of temperature stress, the rate of synthesis of this protein has been noted to increase 100-fold. The 70-kDa stress response protein has been

The regulatory mechanism for the production of the stress response proteins has also been conserved to a remarkable degree throughout evolution. The gene for the Drosophila 70 kDa stress response protein, including its native promotor region, is expressed in a normal, temperature-regulated fashion in frog, mouse and monkey cells (see references in Voellmy et al., 1985).

The wide spectrum of stimuli which promote the coordinated expression of this system of proteins suggest that the stress response is a "global regulatory response", comparable to the catabolite repression system and bacterial SOS and stringent response systems. A unique, common promotor sequence (upstream of the normal, eukaryotic TATA box) has recently been identified for the eukaryotic stress response genes (Pelham, 1982). More recent data suggest that there may be additional, alternative promotor sites upstream of the TATA box which respond to different stimuli (e.g. one promotor sequence related to temperature shock, and another related to serum stimulation) (Wu et al., 1986).

What is the specific mechanism which couples a sudden environmental change to the enhanced transcription of the stress response genes? Several hypotheses have been presented in the past few years to explain this coupling. The most compelling current hypothesis is that the stress response is triggered by the accumulation of abnormal or misfolded protein within the cell . Goff and Goldberg (1985) noted that the synthesis of large amounts of misfolded, human tissue plasminogen activator in E. coli led to the induction of the stress response system. Recently, it has been demonstrated that injection of denatured bovine serum albumin or bovine beta-lactoglobulin into Xenopus laevis (frog) oocytes led to the induction of the stress response system, while the injection of the native bovine proteins did not lead to induction (Ananthan et al., 1986). A reexamination of all of the other known inducers of the stress response suggests that most of these agents (i.e. temperature change, ethanol, etc.) could also be expected to result in the accumulation of denatured or damaged proteins within the cell.

A very specific hypothesis has recently emerged to explain how a sudden accumulation of abnormal intracellular protein could result in the expression of the stress response proteins (Munro and Pelham, 1985). In eukaryotic cells, aberrant proteins are recognized and tagged for proteolysis by the "ubiquitin-dependent degradation system" (i.e. the ubiquitin system). Ubiquitin is a 76-amino acid protein which is covalently attached to the N-termininal and/or lysine residues of proteins marked for degradation. It has been been postulated that it is the decline in free ubiquitin concentration within the cell following an increase in abnormal

extremely conserved throughout evolution. This suggests that an antibody raised to the particular 70-kDa protein of one mammalian cell type can be utilized as a quantitative assay for the comparable protein in other mammalian, as well as for eukaryotic microorganisms, insect cells and plant cells.

Our initial research efforts will focus on mammalian and insect cell cultures, where the most significant industrial scale-up problems are perceived to exist. Two model mammalian cell culture systems are now under consideration: primary human embryonic kidney cells and mouse hybridomas (provided by Cetus Corporation). Insect cell culture scale-up studies are currently being initiated in collaboration with Max Summers of Texas A&M University.

Following the refinement of identification and quantification techniques we will examine several important questions with respect to the response of cells to stress:

1. Assessment of the effects of different types of environmental stress on intracellular protein synthesis. Temperature and dissolved oxygen transients have been clearly identified as inducers of the "stress response" system of proteins in most eukaryotic cell types which have been examined. The effects of pH and hydrostatic pressure transients and shear stress have not been examined. Our preliminary results indicate that sudden exposure to environmental shear leads to synthesis of a unique set of intracellular proteins (discussed below).

2. Assessment of the relationship between the duration and magnitude of a particular stress and the magnitude of the intracellular response. Previous studies of the stress response have focussed on rather large step changes in environmental variables (e.g. temperature step changes of 10 ^{o}C). We will examine the effect of smaller step, pulse and periodic environmental changes on intracellular protein synthesis. These are the types of environmental stress most likely to be experienced in mammalian cell bioreactors.

3. Assessment of the relationship between environmental stress and cell growth and product formation. This issue has not been directly addressed in the literature. It is our hypothesis that cell growth and the formation of protein products will be significantly curtailed during the stress response due to the preferred transcription and translation of the stress response protein mRNA.

4. Assessment of differences between alternative mammalian and insect cell host systems with respect to their ability to cope with environmental stress. One of the major issues in scale-up

is the capacity of the host cell to cope with environmental stress. It is our hypothesis that this capacity relates directly to the intracellular regulation of the stress response system of proteins.

Preliminary Results

Preliminary experiments have been conducted to assess the potential of the approach which we have outlined above. Primary human embryonic kidney (HEK) cells were subjected to sudden changes in either temperature or shear stress, and the resulting patterns of intracellular protein synthesis were compared with the pattern for unstressed HEK cells. The results are presented in figures 1, 2 and 3. Cells were labelled for two hours with [S-35] methionine and were then separated by 2D-PAGE technology. Proteins were separated in the vertical dimension on the basis of molecular weight and in the horizonal dimension on the basis of isoelectric point. In figure 1, the pattern of protein synthesis is presented for the unstressed control cells.

The response of HEK cells to a sudden temperature increase from 37 ^{o}C to 42 ^{o}C is illustrated in figure 2. The synthesis rates of many intracellular proteins are observed to remain constant or decline, while the synthesis rates of at least eleven intracellular proteins are noted to increase dramatically. These "heat-shock/stress response" proteins are indicated by arrows in figure 2. Of particular note is the dramatic increase in protein synthesis of several proteins in the vicinity of molecular weight 70,000. These proteins represent the well-characterized 70-kDa family of stress response proteins.

The response of primary HEK cells to a sudden change in the level of hydrodynamic stress was next examined. This was accomplished in collaboration with Dennis Morrison and Errol Kalmez at Johnson Space Center (Houston). HEK cells were subjected for two hours to 12 dynes per square centimeter of shear stress in a laminar flow chamber, and were then labelled for two hours as previously described. The response of these cells to this sudden increase in shear is illustrated in figure 3. A dramatic increase in the synthesis of at least fifteen intracellular proteins is observed. A comparison of figures 2 and 3 reveals that the responses of cells to temperature and shear transients are substantially different. Of particular note, is the absence of increased synthesis of the 70kDa stress response proteins in figure 3.

The preliminary results for the response of HEK cells to shear stress are intriguing. It has been widely hypothesized that the "heat shock" system of proteins represents the fundamental

intracellular stress response mechanism. But, these proteins were not expressed by HEK cells in response to a sudden increase in environmental shear stress (figure 3). Evidently, the response of cells to environmental stress is more complex than might have previously been imagined. The precise identities of the intracellular proteins which experienced increased synthesis as a result of environmental shear are not known at this time. One of these proteins has a molecular weight and isoelectric point characteristic of the cytoskeletal protein tubulin. Increased synthesis of cytoskeletal proteins in response to environmental stress would not be surprising.

Expected Benefits

This research can be expected to have a significant impact on research at NASA, and on the developing U.S. biotechnology industry. This methodology developed in this project will represent a valuable diagnostic tool in the analysis of large-scale bioreactors. The preliminary results illustrate the potential of 2D-PAGE to discriminate between different sources of environmental stress for a single cell type. Ultimately, it will be possible to discern the specific source(s) of stress for mammalian cells in any bioreactor configuration based on analysis of the profile of intracellular protein synthesis. The measurement of substantial stress for cells in a particular reactor assembly will provide justification for design changes which could be expected to lead to improvements in cell growth and product formation.

It is quite possible that the methodology developed in this study will also have an impact on the bench-scale evaluation of host systems for large-scale culture. One of the major issues in the scale-up of genetically-engineered mammalian cells is the capacity of the host cell to cope with environmental stress. The techniques developed in this study will permit rapid, side-by-side, bench-scale comparisons of alternative host systems in response to imposed environmental stress of the type which will be encountered in large-scale bioreactors. A comparison of the resulting profiles of intracellular protein synthesis may provide guidance in the selection of the best candidates for scale-up.

General guidelines will be developed with regard to the levels of environmental shear, pH, dissolved oxygen, hydrostatic pressure, and temperature stress which can be accomodated by mammalian cells in large-scale culture. These data will prove valuable in the subsequent design of mammalian cell bioreactors.

Finally, this research project can be expected to lead to an increased understanding of the mechanisms by which mammalian cells respond to environmental stress. This can be expected to ultimately suggest genetic modifications to improve the growth characteristics of mammalian cell systems in large-scale culture.

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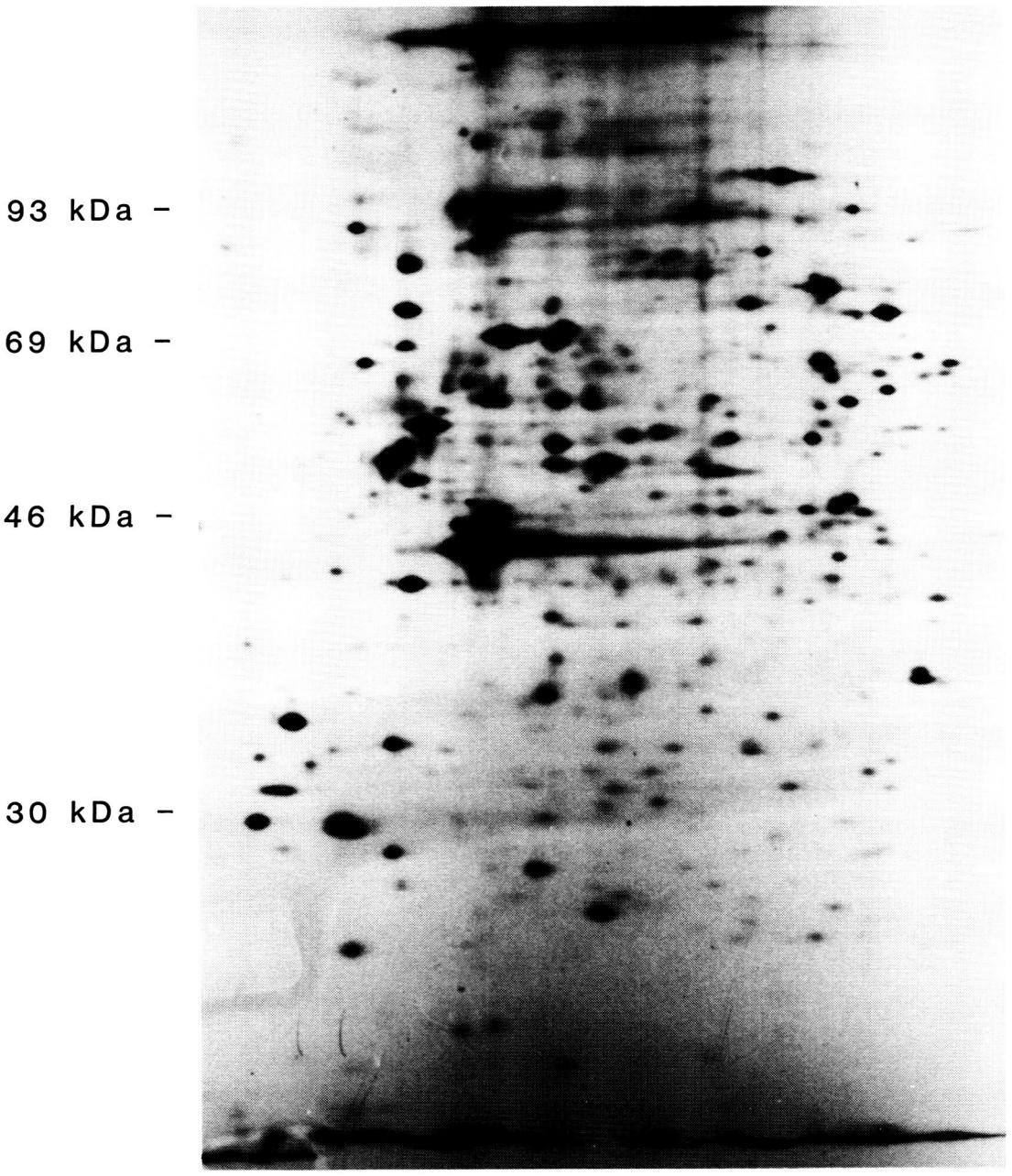

Figure 1. Unstressed Control. Primary HEK cells were labelled for two hours with [S-35] methionine and were then separated by 2D-PAGE technology. Proteins were separated in the vertical dimension on the basis of molecular weight (the location of molecular weight standards is indicated in the figure) and in the horizonal dimension on the basis of isoelectric point (pH 5 to 7 from left to right).

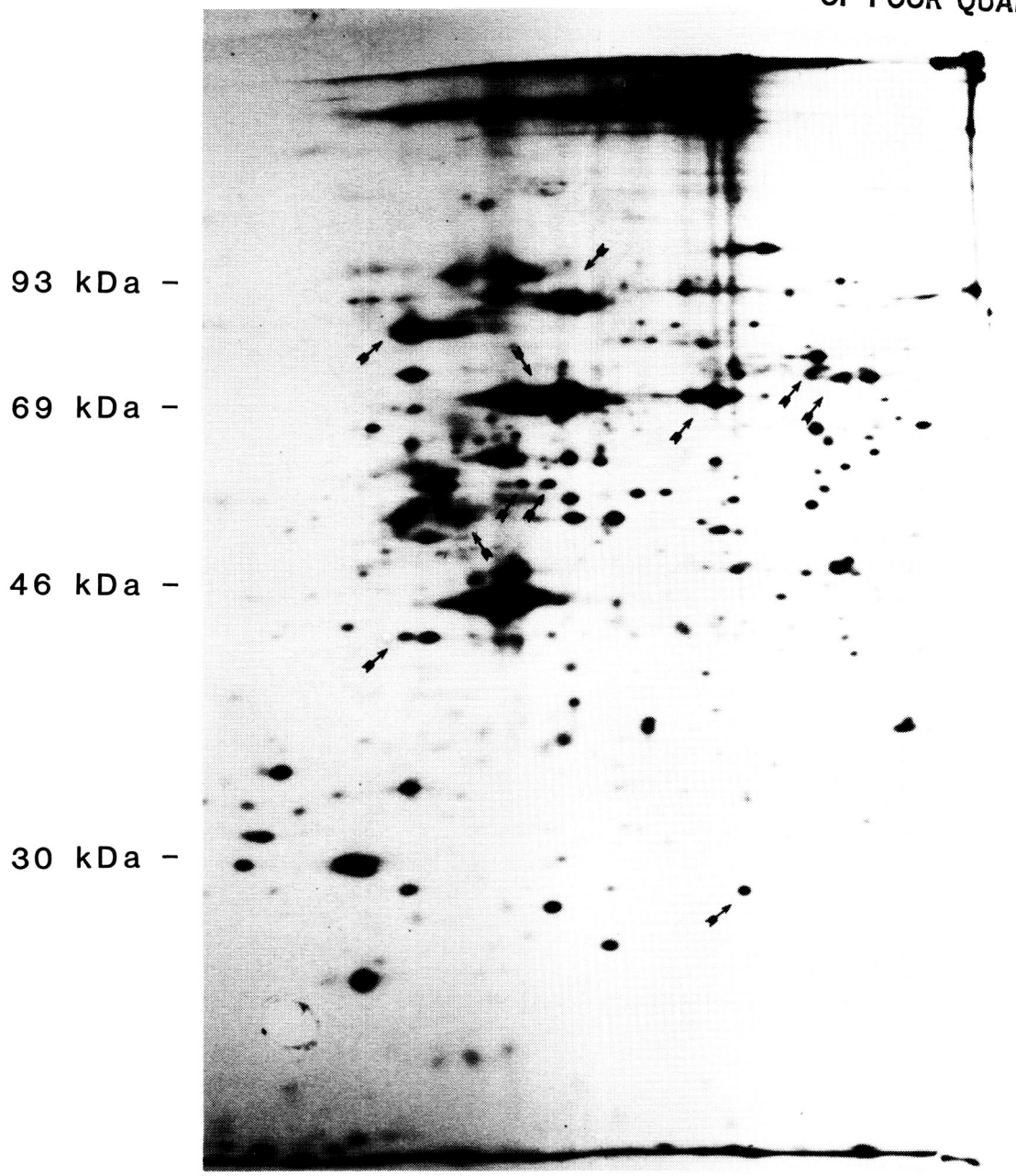

Figure 2. Response of Cells to Temperature Stress. HEK cells were subjected to a sudden temperature increase from 37 ^{o}C to 42 ^{o}C. Cells were labelled for two hours with [S-35] methionine, and were then separated by 2D-PAGE technology under conditions identical to the control cells in figure 1. The locations of proteins whose synthesis rates increase significantly are indicated by arrows. Of particular note is the dramatic increase in protein synthesis of several proteins in the vicinity of molecular weight 70,000. These proteins represent the well-characterized 70-kDa family of heat shock proteins.

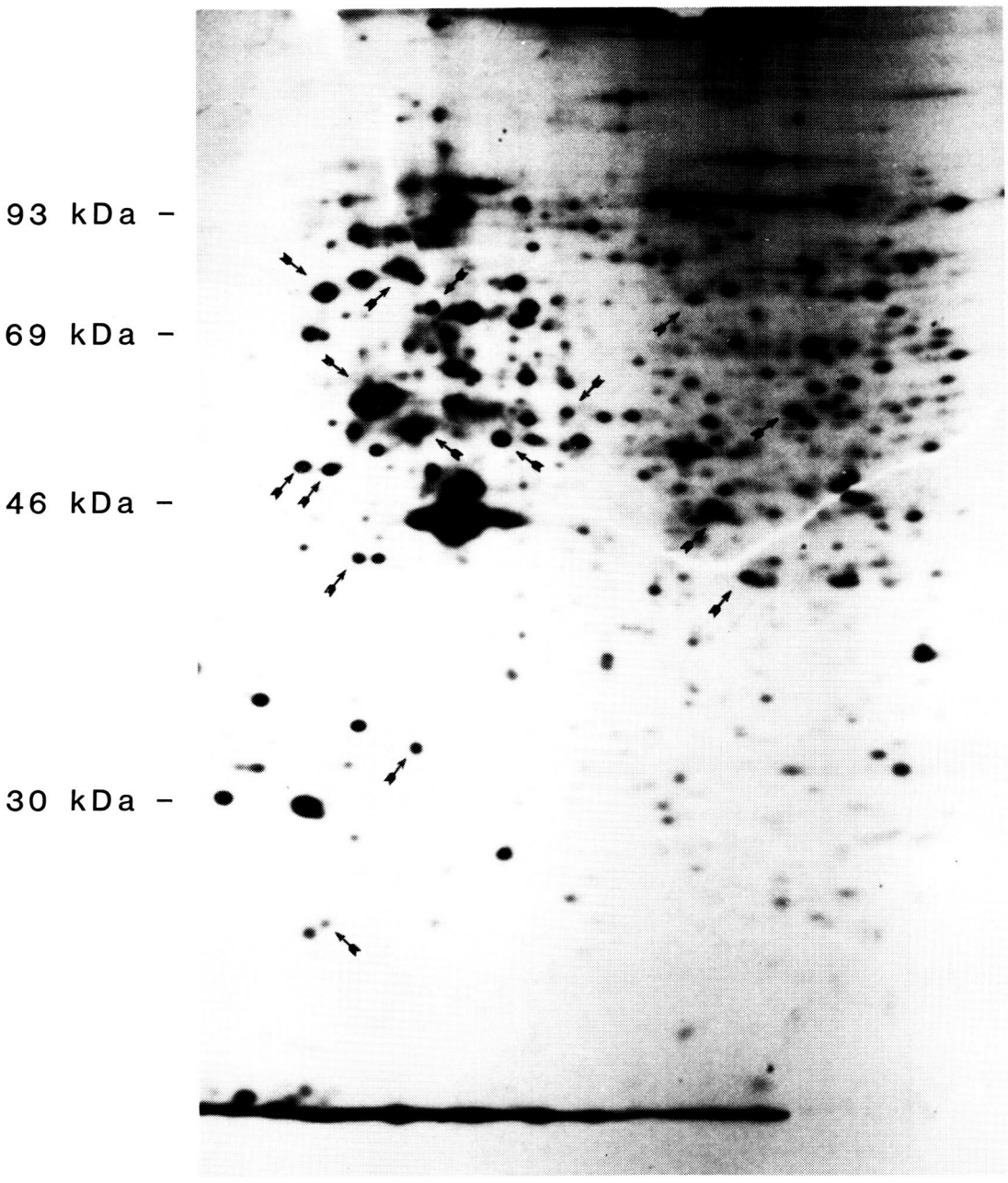

Figure 3. Response of Cells to Shear Stress. HEK cells were exposed to a sudden increase in the level of hydrodynamic stress was next examined. HEK cells were subjected for two hours to 12 dynes per square centimeter of shear stress in a laminar flow chamber. The cells were then labelled for two hours with [S-35] methionine and were separated by 2D-PAGE technology under conditions identical to the control cells in figure 1. The locations of proteins whose synthesis rates increase significantly (in comparison with the unstressed cells of figure 1) are indicated by arrows. Of particular note, is the absence of increased synthesis of the 70kDa stress response proteins.