Arsenate Induces Stress Proteins in Cultured Rat Myoblasts

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ABSTRACT The induction of stress proteins was examined in rat myoblast cultures by two-dimensional gel electrophoresis. Data obtained by this analysis led to the following observations. (*a*) Arsenate, which behaves as a phosphate analogue in cellular phosphate-transfer reactions, stresses cultured rat cells and induces the synthesis of a unique set of proteins. (*b*) Most of the proteins synthesized after the addition of arsenate are identical to proteins synthesized in rat myoblasts in response to heat shock or arsenite stress. (*c*) However, both arsenic salts induce the synthesis of two unique proteins not induced by heat shock. (*d*) Five 25–30-kdalton stress proteins of rat cells do not contain methionine residues. (*e*) A majority of the proteins synthesized in stressed myogenic cells are also induced by stress in other rat cells such as hepatoma cells, pituitary tumor cells, and fibroblasts. The 25–30-kdalton stress-related proteins identified in myogenic cells, on the other hand, are induced in fibroblasts but not hepatoma or pituitary cells.

Many organisms respond to threatening physiological situations by inducing the synthesis of specific messenger RNAs that code for a small number of stress-related proteins (1, 30). Although the exact functions of these proteins have not yet been elucidated, the following characteristics of the response have been defined. The response has been elicited in both vertebrate and invertebrate organisms including protozoans (5), slime molds (21), higher plants (14), insects (1, 17), birds (2, 13, 33), humans (3, 6, 26, 30), and rodents (3, 7, 12, 16, 30). A wide range of very different effectors initiate the response. These include heat shock (2, 30), amino acid analogues (12, 13, 30), sulfhydryl reagents such as arsenite (11, 18), subgroup Ib and IIb metals such as Cd^{++}, Zn^{++}, Hg^{++}, and Cu^{++} (18, 20), metal chelators (18, 19, 20), and mitochondrial poisons (1, 17, 30). The induction of stress-related proteins is blocked by treating cells with cycloheximide or Actinomycin D during the stress period (1, 13, 30). Thus, the formation of new messenger RNA is required for the synthesis of these proteins. Furthermore, it has been shown that heat shock-induced proteins appear concurrently with the acquisition, maintenance, and decay of thermotolerance in stressed cells (16, 30). These kinds of observations suggest that these proteins may play a role in developing heat tolerance in eukaryotic cells (30), and that stress proteins in general may function to maintain cellular homeostasis during cellular trauma.

The regulatory aspects of the stress response have remained as baffling as the role of the stress proteins themselves. In particular, interest has focused on determining which aspect of cellular function initially responds to the stress stimulus. Thomas et al. (30) suggest that there may be many targets for the stimuli including disruption of protein structure and function, the cellular ionic milieu, or terminal respiration in the mitochondria. Characterization of this aspect of the stress response in mammalian cells, however, is limited and further studies with specific cellular inhibitors are needed to delineate initial targets.

At least one mitochondrial poison, arsenite, has been studied to a considerable degree in avian and mammalian cells (11, 20). This anion rapidly induces the synthesis of characteristic heat shock proteins as well as the synthesis of an additional stress protein in both avian (11) and mammalian cells (11, 30). Arsenite disrupts mitochondrial function by binding vicinal sulfhydryl groups, but the metal binding properties of arsenite also alter the ionic balance of the cell. It is thus impossible to distinguish the primary target of this anion or to conclude that mitochondrial disruption in avian and mammalian cells induces stress protein synthesis.

Arsenate is a phosphate analogue and interferes with cellular phospho-transfer reactions in the mitochondrion as well as in other cellular compartments. Although it has been reported that arsenate will induce the synthesis of stress proteins in avian cells (18), this ion has not been reported to induce stress proteins in mammalian cells. We have studied the induction of stress proteins by arsenate as a means to further explicate the initial regulatory mechanism of the response.

Hightower and White (7) examined the induction of stress proteins in incubated slices of seven rat tissues and found that at least two of the stress-induced proteins of rat cells are induced in all rat tissues. Many others have compared the stress proteins of widely divergent species (reference 13 for example),

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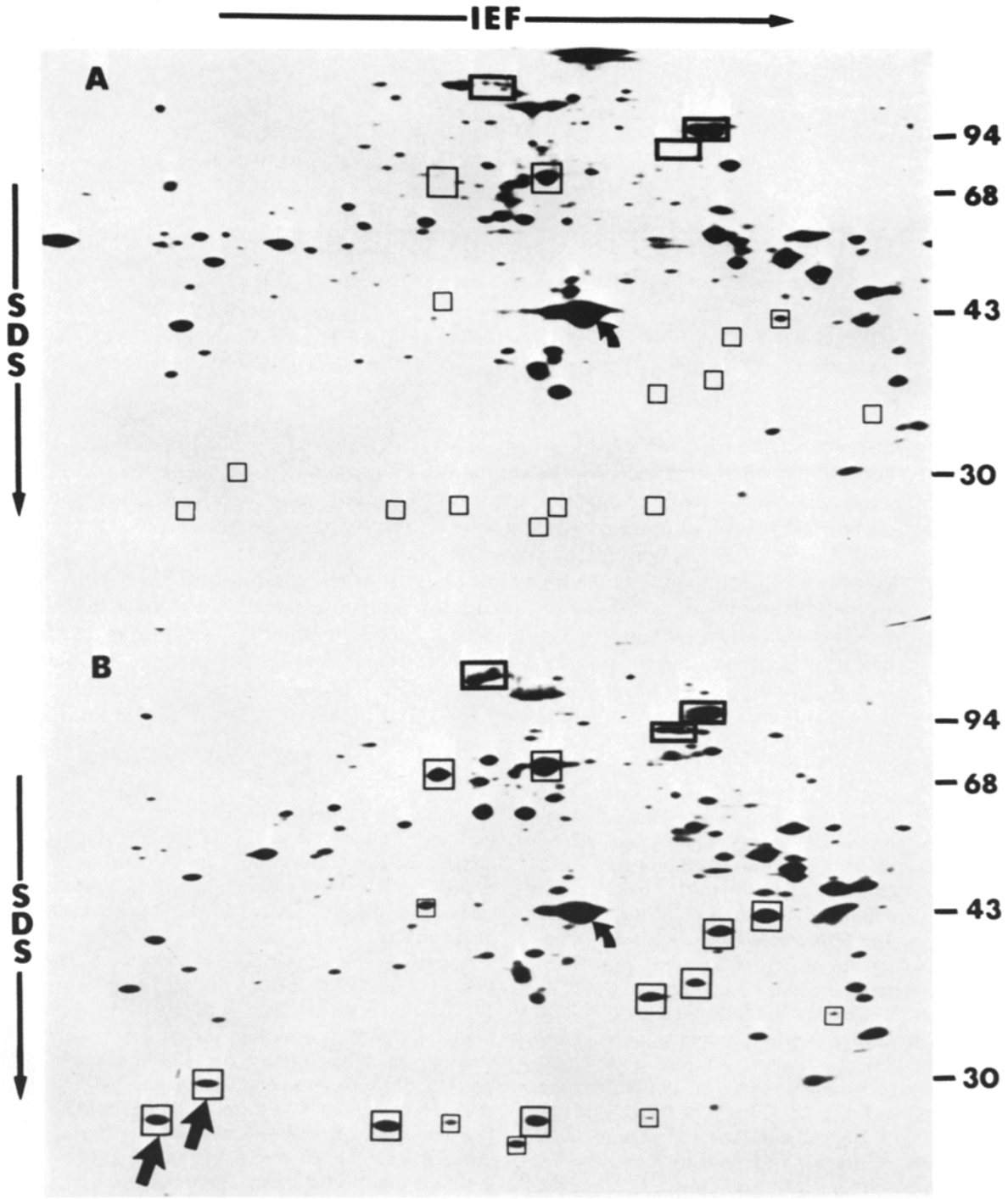

FIGURE 1 Effects of arsenate on polypeptide synthesis in rat myoblasts. Control cells (*A*) and cells treated with 150 μM arsenate for 4 h (*B*) were labeled for 1 h with [^{3}H]-leucine as described in Materials and Methods. The polypeptides synthesized under these conditions were separated by two-dimensional PAGE and detected by fluorography (2×10^5 cpm of sample was applied to each gel). Proteins whose synthesis was induced by arsenate are indicated by squares in *B*, whereas the absence of these proteins in control cells is indicated by squares placed in appropriate positions in *A*. Some arsenate-induced protein are synthesized to varying degrees in control cells (*A*). Actin is identified in this figure and all subsequent figures by the curved arrow in the center of each gel and serves to indicate a mass of 42 kdaltons as well as to orient subsequent figures for comparative purposes. Numbers are in kdaltons. The two large arrows in *B* identify two proteins whose synthesis was not induced by heat shock. These proteins are characterized further in Fig. 2. Marker proteins used to indicate relative mass in our gel system were soybean trypsin inhibitor, 21 kdaltons; bovine erythrocyte carbonic anhydrase, 30 kdaltons; chicken ovalbumin, 43 kdaltons; bovine serum albumin, 68 kdaltons; and phosphorylase a, 94 kdaltons.

but have not done a tissue survey in a single species. In this paper we have also examined the induction of stress proteins in four rat cell types and describe tissue-specific differences in the response.

MATERIALS AND METHODS

Cells and Growth Conditions: Rat myoblasts (MP102) are a clonal derivative of Yaffe's L6 cells (33) and they are grown in Dulbecco's

Modified Eagle's Medium (DME-Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with 20% fetal calf serum (Sterile Systems, Logan, Utah). HTC_4 cells are a subclone (10) of the original rat hepatoma tissue culture line established in culture in 1966 (31) and they are grown as monolayers in DME supplemented with 10% horse serum (Sterile Systems). GH_3D_6 cells are a subclone (9) of the original isolate (29) and they are grown in DME supplemented with 10% fetal calf serum. Rat skin fibroblasts were obtained from the American Type Culture Collection (#CRL1213; Rockville, MD) and they are grown in DME 10% fetal calf serum.

Primary cultures of rat fibroblasts were a gift of Drs. Dennis Campion and Jan Novakofski (Richard B. Russel Agricultural Research Center, USDA-ARS, Athens, GA) and were grown in DME 10% fetal calf serum. Chicken muscle minces are obtained from the breast muscle of 13-d chick embryos and they are kept in DME 10% horse serum until used.

Stress Conditions: Rat myoblast monolayer cultures are heat shocked by floating culture dishes in a 45°C water bath for 20 min. Following the 20-min stress period, cells are returned to a 37°C incubator for 1 h before labeling. Cell cultures are stressed with arsenite or arsenate by adjusting the culture medium to 100 μM $NaAsO_2$ or 150 μM Na_2HAsO_4 for 4 h. Following the stress period, medium is removed from the culture dishes, and cells are washed with phosphate-buffered saline solution and immediately assayed for protein synthesis.

Labeling Conditions: After stress treatment, cells in 30-mm culture dishes are labeled by incubating them in 0.5 ml of medium containing MEM (Gibco Laboratories), 5% horse serum, and 125 μCi of [^{3}H]-leucine (≥ 110 Ci/mM; New England Nuclear, Boston, MA) or [^{35}S]-methionine (≥ 600 Ci/mM; Amersham Corp., Arlington Heights, IL) for 1 h. Incorporation is terminated by rinsing the cells several times in cold Balanced Salt Solution (BSS, Gibco Laboratories). The cells are immediately placed in 0.2 ml of lysis buffer (22).

Electrophoresis: Two-dimensional polyacrylamide gel electrophoresis is performed as described by O'Farrell (22) and Ivarie and O'Farrell (10). Cells in sample buffer are frozen and thawed several times to insure lysis before isoelectric focusing. For the second dimension, 10% acrylamide gels are used and the gels are fixed and destained as described by O'Farrell. The formaldehyde procedure of Steck et al. (27) is also used to fix gels before fluorography. Gels are processed for fluorography (4), dried, and exposed to Kodak X-AR5 film.

RNA Isolation and Cell-free Protein Synthesis: RNA is isolated from rat myoblast cell cultures with guanidine hydrochloride as described previously (28) except that the extractions are performed at 4°C rather than −20°C.

Messenger RNA-dependent reticulocyte cell-free lysate is prepared as described by Pelham and Jackson (23). Translation reaction mixtures contain: 25 μl of freshly thawed lysate, 25 μM concentrations of 19 amino acids with either leucine or methionine omitted, 100 μCi of [^{35}S]-methionine (≥ 600 Ci/mM; Amersham Corp.) or [^{3}H]-leucine (≥ 110 Ci/mM; New England Nuclear), 80 mM KCl, 1 mM $Mg(CH_3CO_2)_2$, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 10 μg/ml creatine kinase (Worthington Biochemical Corp., Freehold, NJ), 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM HEPES buffer, pH 7.6. All assays contained a final volume of 62 μl and were incubated for 90 min at 25°C (24).

RESULTS

Arsenate Induction of Proteins in Rat Myoblasts

Treating cultured rat myoblasts (L6 cells) with a sublethal dose of sodium arsenate (150 μM for 4 h) induced the synthesis of several polypeptides (Fig. 1). 18 proteins whose synthesis is most dramatically and consistently induced by arsenate are noted in the figure. However, other smaller and more variable inductions are also found in response to arsenate. Two-dimensional gel analyses only measure the synthesis of proteins coded for by abundant and moderately abundant mRNA molecules and the synthesis of other stress proteins may go undetected. The synthesis of the vast majority of myoblast proteins remains constant during sublethal stress and only begins to decline as lethal levels of arsenate are reached. At lethal concentrations, the synthesis of arsenate-induced proteins is also inhibited. In marked contrast, *Drosophila* (1), plants (14), and avian cells (11, 12) exhibit an inhibition of cellular protein synthesis concurrent with an induction in the synthesis of stress-related proteins.

Arsenate-induced Proteins are Identical to Heat Shock-induced Proteins

The proteins synthesized by rat myoblasts in response to arsenate are similar in size and charge to proteins induced in other mammalian cells by heat shock (3, 6, 12), sodium arsenite (18, 30), and other chemical and physiological stresses (7, 12). Mixing experiments were performed to compare the proteins synthesized by rat cells in response to arsenate with those induced by heat shock. Two-dimensional gel electrophoresis after heat shock treatment (Fig. 2 *A*) revealed the presence of a set of proteins that were very similar to those induced by sodium arsenate (Fig. 1 *B*). When equal amounts of radioactively labeled proteins from heat shock- and arsenate-treated myoblasts were analyzed in a single two-dimensional gel (Fig. 2 *B*), the proteins synthesized in response to both stresses migrated identically. Similar mixing experiments employing

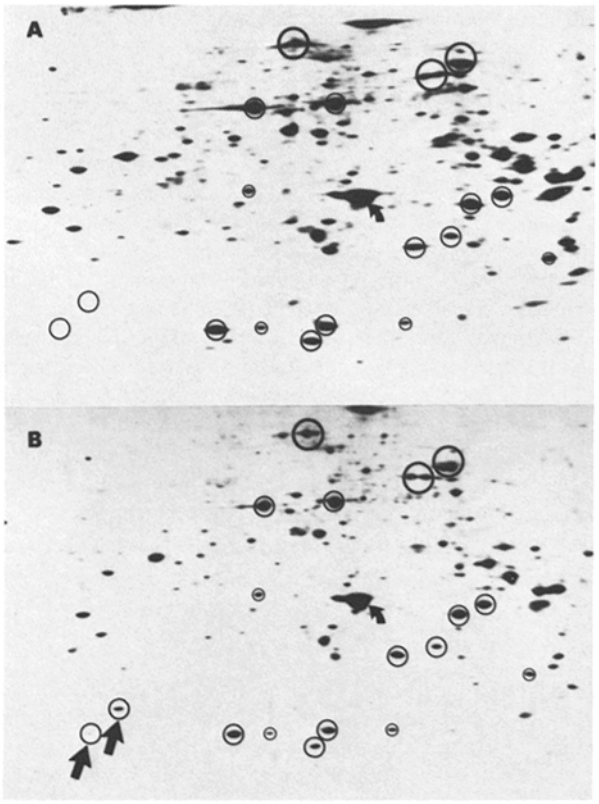

FIGURE 2 Comparison of arsenate-induced proteins to heat shock-induced proteins. Proteins synthesized by rat myoblasts that had been heat shocked at 45°C for 20 min, allowed to recover at normal growth temperature (37°C) for 60 min, and labeled for 60 min with [^{3}H]-leucine were analyzed by two-dimensional gel electrophoresis (*A*). Although the fluorographs are depicted at a lower magnification than in Fig. 1, the dimensions of the original gel were identical to those of the gels presented in Fig. 1, and the relative mobilities of proteins can be compared. Circles indicate proteins whose synthesis is induced in rat myoblasts by heat shock. Equal amounts of protein from arsenate-stressed and heat-stressed myoblasts (2×10^5 cpm each of the proteins used in Figs. 1 *B* and 2 *A*) were mixed and analyzed by two-dimensional gel electrophoresis (*B*). Circles in *B* indicate all proteins induced by both heat shock and arsenate. The straight arrows (*A* and *B*) show the only proteins not induced by heat shock (*A*) that are found in a mixture or proteins induced by heat shock and arsenate (*B*). The curved arrow identifies actin.

proteins induced by arsenite showed that all three stresses induce the same set of proteins (data not shown).

Arsenate, however, induces the synthesis of two additional proteins in rat myoblasts. Neither of these proteins is induced by heat shock (Fig. 2*A*) and both are present in mixtures of heat shock- and arsenate-induced proteins (large arrows, Fig. 2*B*). The smaller of the two proteins is difficult to identify in this figure. The isoelectric point of this protein is at the limit of resolution for our ampholine gradient. Consequently, the protein often fails to enter the focusing gel and is not observed. We are still able to identify the protein at the edge of our second-dimension gel to certify its induction. Both of these proteins appear to be induced to high levels in the experiment shown in Fig. 1 (two large arrows), as in this case both proteins were successfully focused. The synthesis of both of these 25–30-kdalton proteins was also induced in myoblasts by sodium arsenite (data not shown).

In Vitro Translation of RNA from Arsenate-induced Myoblasts

We next attempted to determine whether the two specific proteins induced by arsenate and arsenite were the result of degradation of other stress-related proteins or perhaps a different posttranslational modification. In vitro translation of total cellular RNA from myoblasts stressed with arsenate was carried out in a mRNA-dependent rabbit reticulocyte translation system. The synthesis of several stress-induced proteins is directed by this RNA (Fig. 3). It is clear that a 30-kdalton protein (large arrow) is coded for by RNA isolated from arsenate-stressed cells. This protein co-migrates in mixing experiments (data not shown) with the arsenate-induced protein shown in Fig. 2. As posttranslational modifications are not taking place in the lysate, it is most likely that the arsenate-induced protein of rat myoblasts is not a degradation product or a modified protein. As shown in Fig. 3, this specific protein induced by arsenate can be labeled with either [^{3}H]-leucine or [^{35}S]-methionine in the reticulocyte translation system. The synthesis of two of the other 25–30-kdalton proteins was not observed when [^{35}S]-methionine was used as a tracer.

Some 25–30-kdalton Stress-induced Rat Proteins Lack Methionine

Since in vitro translation experiments indicated that some of the 25–30-kdalton stress-induced proteins lacked methionine residues, we tested this finding in vivo. When methionine tracers were used to monitor protein synthesis in heat shock- or arsenate-stressed myoblast cultures, all stress proteins were labeled except for the 25–30-kdalton proteins (data not shown). These results corroborate the findings of the in vitro translation experiments.

Tissue-specific Induction of Protein Synthesis by Arsenate

In order to determine whether arsenate induced a common set of proteins in all rat tissues, we subjected rat fibroblast (FR), rat hepatoma (HTC), and rat pituitary tumor (GH_3) cell lines to arsenate stress and characterized the induced proteins by two-dimensional electrophoresis. Arsenate induced the synthesis of several proteins in all of the cell types tested (Fig. 4). In all three cell types, the induction of proteins larger than 30 kdaltons appeared to exactly parallel the induction of proteins in myoblasts (Fig. 1). Induction of the synthesis of the 25–30 kdalton-proteins, however, was quite variable.

Four proteins of 25–30 kdaltons were induced by arsenate in

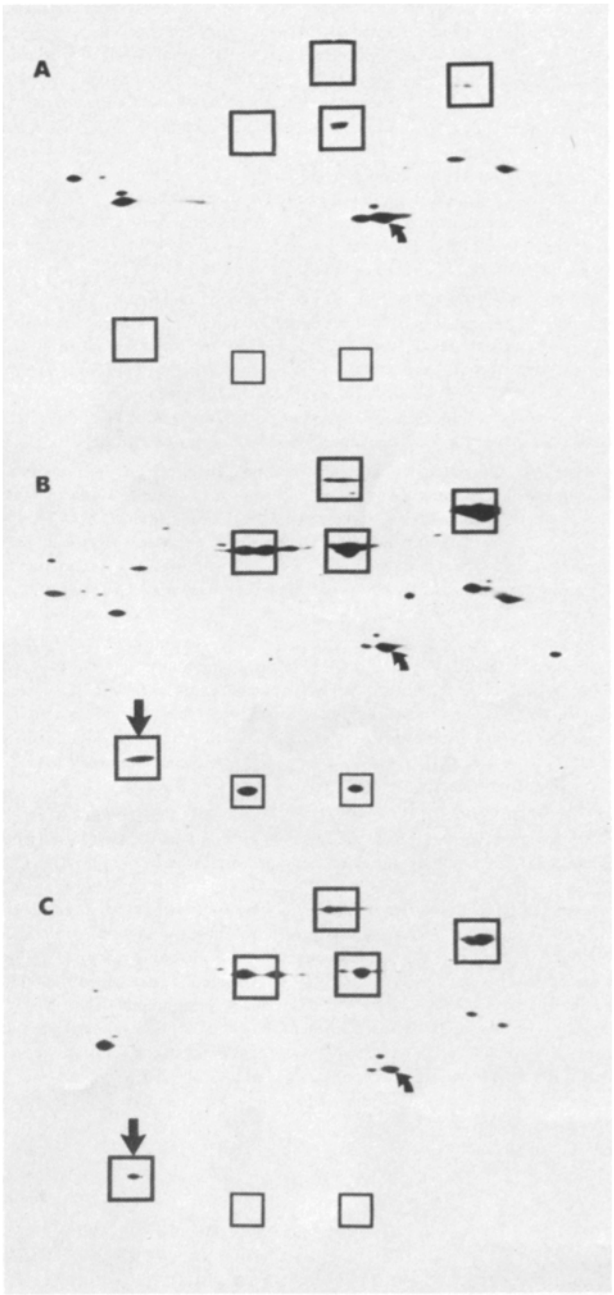

FIGURE 3 In vitro translation of total cellular RNAs from control and arsenate-stressed rat myoblasts. Proteins synthesized in mRNA-dependent rabbit reticulocyte cell-free translation lysates in response to 20 μg of total RNA isolated from control cells (*A*) or cells treated with 150 μM arsenate for 4 h (*B* and *C*) were separated in two-dimensional gels. The in vitro-synthesized proteins in *A* and *B* were labeled with [^{3}H]-leucine, whereas those in *C* were labeled with [^{35}S]-methionine. The squares indicate those proteins whose synthesis is coded for by RNAs isolated from arsenate-stressed cells (*B* and *C*) but not coded for or coded for at reduced levels by RNAs from control cells (*A*). All of the in vitro synthesized proteins coded for by arsenate-induced mRNA (squares in *B*) co-migrated with in vivo-labeled proteins in mixing experiments (data not shown). The straight arrow indicates a 30-kdalton protein that is coded for by mRNA from arsenate-induced cells and co-migrated in mixing gels (data not shown) with the larger arsenate-induced protein indicated by the straight arrow in Fig. 1.

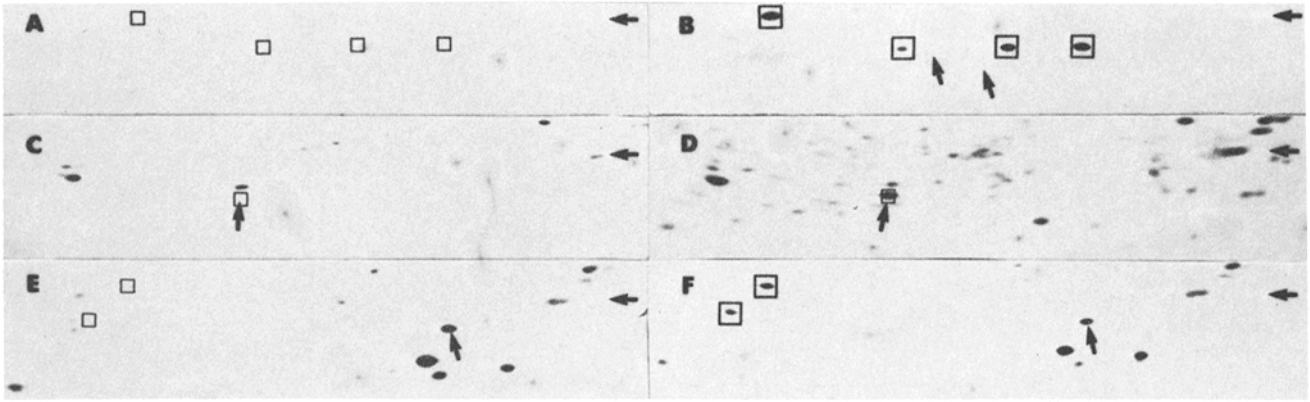

FIGURE 4 Effects of arsenate on polypeptide synthesis in rat fibroblasts (*A* and *B*), hepatoma cells (*C* and *D*), and pituitary tumor cells (*E* and *F*). Control cells (*A*, *C*, and *E*) and cells treated with 150 μM arsenate for 4 h (*B*, *D*, and *F*) were labeled for 1 h with [^{3}H]-leucine as described in Materials and Methods. Cells were immediately lysed in isoelectric focusing buffer and the polypeptides synthesized under these conditions were fractionated by two-dimensional PAGE. The gels and electrophoretic conditions used were identical to those in Fig. 1. The autoradiographs presented in Fig. 4 are portions of the original two-dimensional gels pertinent to discussions in the text (the 20–30-kdalton region). The horizontal arrows indicate the migration of carbonic anhydrase at 30 kdaltons. The squares indicate proteins synthesized in arsenate-treated cells (*B*, *D*, and *F*) that were not synthesized in the respective control cells (*A*, *C*, and *E*). The arrows in *B* show where two arsenate-induced myoblast proteins migrated in identical gels (see Fig. 1 *B*). These polypeptides are not synthesized in arsenate-induced rat fibroblasts. The arrows in *C* and *D* identify a protein whose synthesis in induced by arsenate in rat hepatoma cells but not rat myoblasts (further data is presented). The arrows in *E* and *F* indicate a protein that is synthesized in both control and arsenate-induced pituitary tumor cells and that appears to comigrate with one of the stress proteins of rat myoblasts. Further data demonstrate that this protein is unique.

rat fibroblasts (Fig. 4 *A* and *B*). One of these proteins had an apparent size and charge that was identical to that of the arsenate-specific myoblastic protein. Two 25–30-kdalton proteins induced by arsenate in rat myoblasts were not induced in rat fibroblasts (straight arrows) or the other rat cells tested and thus are myoblast-specific arsenate inductions.

Only one protein of 25–30 kdaltons was induced by arsenate in rat hepatoma cells (Fig. 4 *C* and *D*). Using various concentrations of arsenate or increasing the duration of the stress period failed to induce the synthesis of additional proteins of this size class. In other experiments, we have further established that both heat shock and arsenite induce the synthesis of a single protein of this size in HTC cells. The single hepatoma protein of 25–30 kdaltons (straight arrow) appears to have a size and charge similar to that of one of the myoblast stress proteins. Induction of the arsenate-specific rat stress protein was difficult to demonstrate in hepatoma cells. This is the only cell type tested in which this protein is poorly induced.

Two proteins of 25–30 kdaltons are synthesized in response to arsenate in rat pituitary tumor cells (Fig. 4 *E* and *F*), but they are not induced by heat shock, suggesting that they correspond to the arsenate-induced myoblast proteins (Fig. 2). One 25–30-kdalton protein synthesized in both control and stressed pituitary cells has a size and isoelectric point similar to that of one of the myoblast arsenate-induced proteins (arrows Fig. 4 *E* and *F*).

Identity of Arsenate-induced Proteins in Different Rat Cell Types

Many of the proteins induced in different rat cell types by arsenate and other stresses appeared very similar. Mixing experiments were performed to determine the identity or uniqueness of the arsenate-induced proteins of different rat cell types. Leucine-labeled protein from arsenate-induced myoblasts was mixed with each of three samples containing an equal amount of leucine-labeled protein from one of the other three cell types. The mixed samples were analyzed by two-dimensional gel electrophoresis and the results are presented in Fig. 5. In all cases the proteins larger than 30 kdaltons whose synthesis was induced by arsenate in all four cell types co-migrated in two-dimensional polyacrylamide gels, indicating that these proteins have an identical size and isoelectric point. However, significant differences were observed in the small 25–30-kdalton proteins. These differences were generally similar to those observed in the experiments described in Fig. 4. The four 25–30-kdalton proteins induced in rat fibroblast cell lines by arsenate were identical in size and isoelectric point to proteins found in stressed myoblasts. The two myoblast specific proteins can also be seen in this mixture (straight arrows, Fig. 5 *A*). Thus, arsenate induced the synthesis of identical proteins in rat myoblasts and fibroblasts except for two proteins that were myoblast-specific.

The single 25–30-kdalton protein induced in rat hepatoma cells (straight arrow Fig. 6 *B*) had an isoelectric point identical to that of one of the proteins induced in myoblasts, but it had a lower molecular weight and is thus a unique liver protein induced by arsenate stress. In a similar fashion, we concluded that the 25–30-kdalton protein synthesized in normal and arsenate-stressed rat pituitary cells (straight arrows Fig. 4 *E* and *F*) had a slightly lower isoelectric point and a slightly lower molecular weight (straight arrow Fig. 5 *C*) than a similar arsenate-induced myoblast stress protein and that this protein is unique to the pituitary cell.

We conclude that arsenate-induced synthesis of 25–30-kdalton proteins in the rat shows tissue-specific differences. In proteins larger than 30 kdaltons there are some tissue-specific differences with regards to the level of synthesis of various stress proteins, but the set of arsenate-induced proteins is identical in all cell types we have tested.

Arsenate-specific Protein Induction in Chicken Myoblasts

The specific induction of stress proteins by arsenite and arsenate has been best studied in chicken cells (11, 18, 32). We

compared arsenate-induced proteins of chick and rat cells to establish their relatedness. When 13-d chick embryo muscle was subjected to a 45°C heat shock for 1 h, the proteins induced (Fig. 6 *A*) were identical to those previously reported for chick muscle (32) and chick fibroblasts (12, 13). When

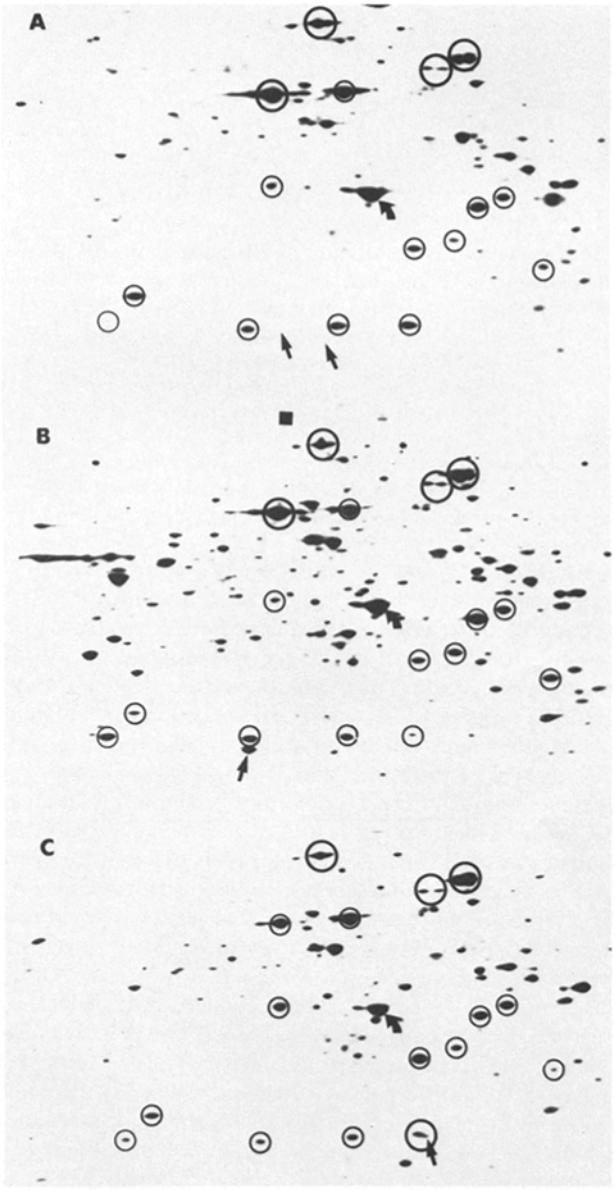

FIGURE 5 Direct comparison of arsenate-induced rat myoblast proteins and arsenate-induced proteins from rat fibroblasts (*A*), rat hepatoma cells (*B*), and rat pituitary tumor cells (*C*). An aliquot of leucine-labeled proteins from arsenate-induced rat myoblasts was mixed with each of three samples containing an equal amount of protein (2×10^5 cpm each) from one of the other three cell types. The polypeptides in the three mixtures were separated by two-dimensional gel electrophoresis, using conditions identical to those in previous experiments. The curved arrow indicates actin and the straight arrows in *A*, *B*, and *C* correspond to the arrows in Fig. 4. The two myoblast-specific proteins (arrows in *A*) are barely visible but are clearly seen in longer fluorographs of the gel. The arrow in *B* identifies the hepatoma-specific protein (see Fig. 5 *D*) as being unique from the myoblast protein in the circle above it. The arrow in *C* points to the pituitary cell protein that does not co-migrate with the muscle stress protein (notice the different intensities of the two proteins in this circle).

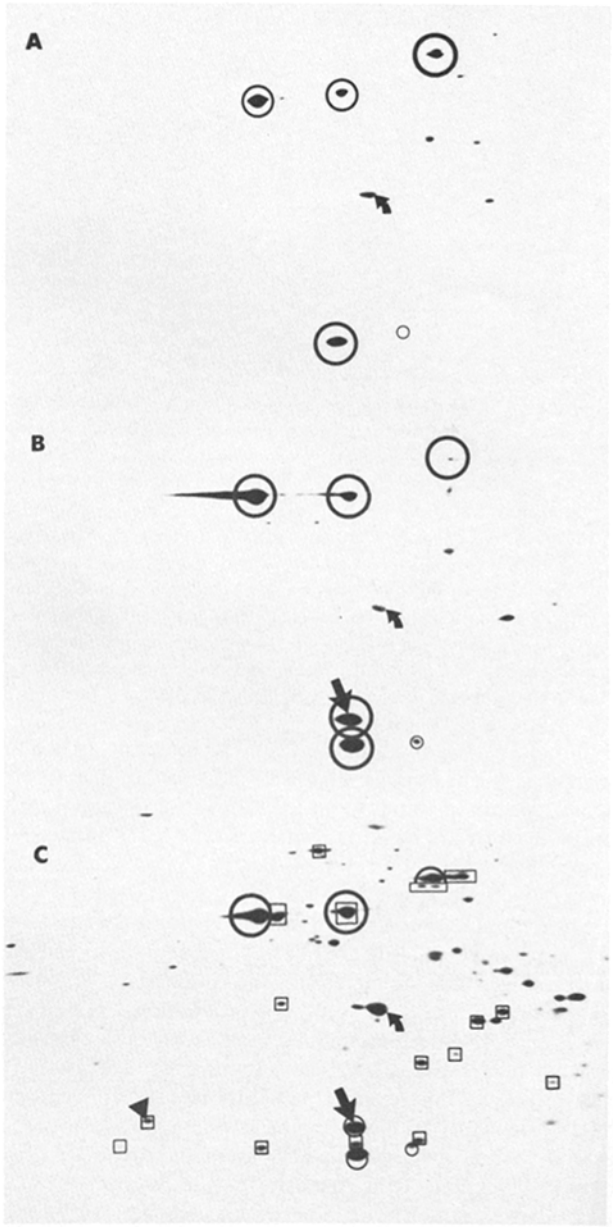

FIGURE 6 Comparison of arsenate-specific proteins from chicken and rat myoblasts. (*A*) Breast muscle from 13-d chick embryos was minced, incubated at 45°C for 1 h, equilibrated at 37°C for 1 h, and then incubated with [^{3}H]-leucine for 1 h as described in Materials and Methods. The tissue was removed from the labeling medium by centrifugation and immediately disrupted in isoelectric focusing buffer. Polypeptides synthesized during the pulse were separated by two-dimensional electrophoresis in gels identical to those in previous experiments. The curved arrow indicates actin and the circles indicate chick myoblast heat shock proteins. (*B*) Breast muscle from 13-d chick embryos was minced, incubated for 4 h with 150 μM arsenate, and labeled for 1 h with [^{3}H]-leucine. The proteins were fractionated as in *A*, and circles indicate the arsenate-induced proteins. The large arrow in *B* identifies the synthesis of a protein in response to arsenate that is not synthesized in response to heat shock (compare *A* and *B*). (*C*) [^{3}H]-leucine-labeled proteins from arsenate-stressed chick and rat myoblasts were mixed and subjected to two-dimensional gel electrophoresis. The circles indicate the chicken proteins as shown in *B*. The squares indicate the rat proteins as shown in Fig. 1 *B*. The large arrow and arrowhead point to arsenate-induced chicken and rat proteins, respectively.

muscle from the same-age embryos was incubated in 150 μM arsenate for 4 h, synthesis of the heat shock proteins and one additional 30-kdalton protein was induced (Fig. 6 *B*). The 30-kdalton arsenate-specific protein was very similar in size to the 30 kdalton arsenate-induced protein described for rat cells, but the isoelectric point of the chicken protein was different. We further examined the similarity of arsenate-induced rat and chicken proteins by analyzing a mixture of the two sets of proteins by two-dimensional gel electrophoresis. The results indicated that, although the stress proteins induced in both animals fall into similar size groups, all but one differ by either size or isoelectric point (Fig. 6 *C*). In both organisms, arsenate induced the synthesis of a protein not induced by heat shock, but the physical properties of these proteins are different.

DISCUSSION

The results obtained in the present studies show that a given rat cell type responds to different stresses in a similar manner. Arsenate, arsenite, and heat shock for the most part induced the same group of proteins in muscle cells. However, treatment with arsenate or arsenite induces the synthesis of two proteins in rat cells that are not found after heat shock. The arsenic-specific proteins identified by two-dimensional gel electrophoresis are cellular products since RNA isolated from arsenic-induced cells directs the in vitro synthesis of these proteins. Treatment of embryonic chicken muscle with arsenate or arsenite also results in the synthesis of a protein that is not induced by heat shock. Thus the induction of arsenic-specific proteins is not a phenomenon peculiar to rat cells. Recently, Kothary and Candido (15) have identified a 100-kdalton protein whose synthesis is induced in cultured trout fibroblast by sodium arsenite but not by heat shock. Their analysis was performed with one-dimensional gel electrophoresis, and therefore additional arsenite-specific inductions might have been obscured.

The induction of stress-related proteins in response to arsenite has been described (11, 32). This anion cross-links vicinal sulfhydryl groups such as those found in lipoic acid. Arsenite therefore inhibits mitochondrial oxidation by binding lipoic acid, which in turn prevents acetyl-CoA formation and causes pyruvate and other α-keto acids to accumulate. Arsenite certainly is not mitochondrion-specific, however, and must elicit a pleiotropic response by cross-linking other sulfhydryls in the cell. Due to its behavior as a sulfhydryl group ligand, arsenite also interferes with the cellular ionic milieu (30) potentially activating the stress response at this presumed target site. Thus it is not possible to definitively conclude that the initial site of interaction of the external inducer (arsenite) occurs in the mitochondria.

The interactions of arsenate with cellular components does not occur through sulfhydryl interactions. This arsenical is a phosphate analogue readily replacing phosphate in phosphorolytic reactions. Like arsenite, it is an efficient mitochondrial poison, but works by inhibiting phosphorylation of ADP at the ATPase complex. As is the case with arsenite, arsenate must cause a pleiotropic response in cells by inhibiting other phosphorolytic reactions, thus altering the modification, structure, and function of cellular proteins. Although arsenate does not behave as an ionophore, it is also possible that it could bring about changes in the ionic milieu of the cell. Arsenate toxicity is not due to its conversion to arsenite, however, as the reduction does not occur (25). This is consistent with the finding that excess phosphate can relieve arsenate toxicity but has no effect on arsenite toxicity (25). Thus initiation of the cellular stress response by arsenate could take place by disruption of protein function, ionic milieu, or mitochondrial function. Once again, it is not possible to establish a causal relationship between the stress response and a primary cellular target for the initial stimulus.

Evidence to date, however, does not preclude the possibility that the induction of the 30-kdalton protein in mammalian cells (19, 30) requires that the stress agent interact with mitochondria and disrupt cellular respiration. Heat shock and amino acid analogues are presumably targeted to the disruption of protein structure or function, and these stresses do not induce the synthesis of the 30-kdalton protein. The induction of the 30-kdalton protein could thus require the interaction of the effector with the mitochondria.

Analysis by two-dimensional gel electrophoresis also showed the induction of the synthesis of five 25–30-kdalton proteins in rat myoblasts by both arsenicals as well as heat stock. Three proteins of corresponding sizes and isoelectric points are also induced in rat fibroblasts. Other investigators have reported the induction of similar proteins in mammalian cells (3) while some laboratories have not observed inductions of proteins in this size range (13, 25). We have observed that in rat cells the 25–30-kdalton proteins lack methionine and suggest that in some cases the identification of these proteins may have been overlooked by choice of tracer for labeling studies. Hickey and Weber (6) also described the expression of two 27-kdalton methionine-free, heat-shock proteins in HeLa cells.

Our studies also revealed, however, that synthesis of the 25–30-kdalton proteins is not induced in rat liver hepatoma or rat pituitary tumor cells by either arsenic anion or heat shock. This finding implies that induction of this group of heat shock-like proteins might be cell-specific. Atkinson and Pollock (3) have made a similar observation. In their studies, two-dimensional gel electrophoresis of hamster fibroblast heat shock proteins revealed that heat shock induced the synthesis of three 25-kdalton proteins with isoelectric points similar to those of the proteins we have observed. These authors, on the other hand, found that human epidermoid carcinoma cells (KB cells) failed to express these 25-kdalton proteins when subjected to heat shock. It is thus evident from our studies and others that the expression of the 25–30-kdalton stress-related proteins in mammalian cells is variable with respect to cell type. The functional significance of this expression pattern remains unclear at this time.

Comparative studies with the rat 25–30-kdalton stress-related proteins and a chicken 24-kdalton stress-related protein indicate that these proteins are not homologous. Although the isoelectric point of the chicken protein is identical to that of one of the rat 25–30-kdalton proteins, their sizes are significantly different. More conclusive evidence of the uniqueness of these proteins was recently presented by Kelley and Schlesinger (13), who demonstrated that antibodies elicited by the 24-kdalton chicken heat shock protein did not cross-react with small stress proteins of cells of several other organisms including mouse cells. In contrast, antibodies prepared against the three larger chicken heat shock proteins showed extensive cross reaction with heat shock proteins from mammalian cells. Thus both interspecific and intraspecific differences in the stress response of eukaryotic cells appear to be focused on the group of small 25–30-kdalton stress proteins.

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REFERENCES

1. Ashburner, M., and J. J. Bonner. 1979. The induction of gene activity in Drosophila by heat shock. *Cell.* 17:241–254.
2. Atkinson, B. G. 1981. Synthesis of heat-shock proteins by cells undergoing myogenesis. *J. Cell. Biol.* 89:666–673.
3. Atkinson, B. G., and M. Pollock. 1982. Effect of heat shock on gene expression in human epidermoid carcinoma cells (strain KB) and in primary cultures of mammalian and avian cells. *Can. J. Biochem.* 60:316–327.
4. Bonner, W. M., and A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
5. Fink, K., and E. Zeuthen. 1980. Heat shock proteins in tetrahymena studied under growth conditions. *Exp. Cell. Res.* 128:23–30.
6. Hickey, E. D., and L. A. Weber. 1982. Modulation of heat-shock polypeptide synthesis in HeLa cells during hypothermia and recovery. *Biochemistry.* 21:1513–1521.
7. Hightower, L., and F. P. White. 1981. Cellular responses to stress: comparison of a family of 71–73 kilodalton proteins rapidly synthesized in rat tissue slices and canavanine-treated cells in culture. *J. Cell. Physiol.* 108:261–275.
8. Ingolia, T. D., and E. A. Craig. 1982. Four small Drosophila heat shock proteins are related to each other and to mammalian α-crystallin. *Proc. Natl. Acad. Sci. USA.* 79:2360–2364.
9. Ivarie, R. D., J. D. Baxter, and J. A. Morris. 1981. Interaction of thyroid and glucocorticoid hormones in rat pituitary tumor cells: specificity and diversity of the responses analyzed by two-dimensional gel electrophoresis. *J. Biol. Chem.* 256:4250–4528.
10. Ivarie, R. D., and P. H. O'Farrell. 1978. The glucocorticoid domain: steroid-mediated changes in the rate of synthesis of rat hepatoma proteins. *Cell.* 13:41–55.
11. Johnston, D., H. Oppermann, J. Jackson, and W. Levinson. 1980. Induction of four proteins in chick embryo cells by sodium arsenite. *J. Biol. Chem.* 255:6975–6980.
12. Kelley, P. M., and M. J. Schlesinger. 1978. The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. *Cell.* 15:1277–1286.
13. Kelley, P. M., and M. J. Schlesinger. 1982. Antibodies to two major chicken heat shock proteins cross-react with similar proteins in widely divergent species. *Mol. Cell. Biol.* 2:267–274.
14. Key, J. L., C. Y. Lin, and Y. M. Chen. 1981. Heat shock proteins of higher plants. *Proc. Natl. Acad. Sci. USA.* 78:3526–3530.
15. Kothary, R. K., and E. P. M. Candido. 1982. Induction of a novel set of polypeptides by heat shock or sodium arsenite in cultured cells of rainbow trout, *Salmo gairdnerii. Can. J. Biochem.* 60:347–355.
16. Landry, J., D. Bernier, P. Chretien, L. M. Nicole, R. Tanguay, and N. Marceau. 1982. Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. *Cancer Res.* 42:2457–2461.
17. Leenders, H. J., and H. D. Berendes. 1972. The effect of changes in the respiratory metabolism upon genome activity in Drosophila. I. The induction of gene activity. *Chromosoma (Berl.)* 37:433–444.
18. Levinson, W., J. Idriss, and J. Jackson. 1979. Metal binding drugs induce synthesis of four proteins in normal cells. *Biol. Trace Elem. Res.* 1:15–23.
19. Levinson, W., H. Oppermann, and J. Jackson. 1978. Induction of four proteins in eukaryotic cells by kethoxal Bis(thiosemicarbazone). *Biochem. Biophys. Acta.* 518:401–412.
20. Levinson, W., H. Oppermann, and J. Jackson. 1980. Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. *Biochim. Biophys. Acta.* 606:170–180.
21. Loomis, W., and S. Wheeler. 1980. Heat shock response of Dictyostelium. *Dev. Biol.* 79:399–408.
22. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
23. Pelham, H., and R. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–256.
24. Przybyla, A., R. MacDonald, J. Harding, R. Pictet, and W. J. Rutter. 1979. Accumulation of the predominant pancreatic mRNAs during embryonic development. *J. Biol. Chem.* 254:2154–2159.
25. Silver, S., K. Budd, K. Leahy, W. Shaw, D. Hammond, R. Novick, G. Willsky, M. Malamy, and A. Rosenberg. 1981. Inducible plasmid-determined resistance to arsenate, arsenite and antimony (III) in *E. coli* and *S. aureus. J. Bacteriol.* 146:983–996.
26. Slater, A., A. Cato, G. Sillar, J. Kioussis, and R. Burdon. 1981. The pattern of protein synthesis induced by heat shock of HeLa cells. *Eur. J. Biochem.* 117:341–346.
27. Steck, G., P. Leuthard, and R. R. Burk. 1980. Detection of basic proteins and low molecular weight peptides in polyacrylamide gels by formaldehyde fixation. *Anal. Biochem.* 107:21–24.
28. Strohman, R. C., P. Moss, J. Eastwood, D. Spector, A. Przybyla, and B. Paterson. 1977. Messenger RNA for myosin polypeptides: isolation from single myogenic cell cultures. *Cell.* 10:265–273.
29. Tashjian, A. H., Jr., Y. Yasumura, L. Levine, G. H. Sato, and M. L. Parker. 1968. Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. *Endocrinology.* 82:342–352.
30. Thomas, G. P., W. J. Welch, M. B. Mathews, and J. R. Feramisco. 1981. Molecular and cellular effects of heat-shock and related treatments of mammalian tissue-culture cells. *Cold Spring Harbor Symp. Quant. Biol.* 46:985–996.
31. Thompson, E. B., G. M. Tomkins, and J. F. Curran. 1966. Induction of tyrosine α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. *Proc. Natl. Acad. Sci. USA.* 56:296–303.
32. Wang, C., R. H. Gomer, and E. Lazarides. 1981. Heat shock proteins are methylated in avian and mammalian cells. *Proc. Natl. Acad. Sci. USA.* 78:3531–3535.
33. Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA.* 61:477–483.