INDUCTION AND METHYLATION OF HEAT SHOCK PROTEINS IN CULTURED VERTEBRATE CELLS

Thesis by

Chung Wang

In Partial Fullfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1983

(Submitted February 3, 1983)

Acknowledgements

Special thanks to my thesis advisor, Dr. Elias Lazarides, for his support, encouragement and giving me the freedom to pursue what I am interested in. I am particularly grateful to Dr. Steven Clarke at UCLA for his kind help and valuable suggestions during the course of my studies. I would also like to express my appreciation to Dr. Jean-Paul Revel for his advice and guidance during my first two years at Caltech. I thank all the members of this and Dr. Clarke's laboratories, particularly Clare O'Connor, David Gard and John Ngai, for many valuable discussions. I thank Ilga Lielausis for her cell culture work, Dr. Michael Raftery for providing the amino acid analyzer facilities, and Drs. Jean-Paul Revel, Charles Brokaw, Giuseppe Attardi and Steven Clarke for serving as members of my thesis committee.

Many thanks to Connie Katz for her excellent typing and the Jean Weigle Fund for supporting the preparation of this thesis. During the five plus years at Caltech, I have been supported by the fellowships provided by the Division of Biology.

Abstract

When vertebrate cells are exposed to sodium arsenite, they respond by increased synthesis of a small number of polypeptides similar to the heat shock proteins, which are induced by exposure to 40-45°C. The 70,000 dalton inducible protein (hsp70) is the most commonly induced species and this protein is methylated at both lysyl and arginyl residues. In chicken fibroblasts, the hsp70 is composed of two major distinct isoelectric variants, as well as several minor components. The more acidic one (hsp70A; pI 5.6) is highly conserved and found in all vertebrate tissues and cultured cells examined, while the more basic one (hsp70B; pI 6.0) at present has only been found in avian cells.

The hsp70 is a prominent cytoplasmic constituent under normal growth conditions. In chicken fibroblasts, the induction by arsenite not only increases the synthesis of hsp70 but also results in an accumulation of this protein. In contrast, the induction in 3T3 and SR-RSV 3T3 cells results in increased synthesis, but no accumulation of this polypeptide.

In chicken cells, ε-N-trimethyl-lysine has been identified as the major component of methyl-lysine species in hsp70, but the contribution of ε-N-monomethyl-lysine and ε-N-dimethyl-lysine is evident. The methyl-arginine of hsp70 is exclusively N^G-monomethyl-arginine; these methylations appear to be stoichiometric. Furthermore, these methylations can be modulated by arsenite. In particular, in hsp70A the amount of ε-N-trimethyl-lysine decreases and the ε-N-dimethyl-lysine significantly increases, while in hsp70B the quantity of N^G-monomethyl-arginine is reduce fivefold in the presence of sodium arsenite. In 3T3 and SR-RSV 3T3 cells, ε-N-trimethyl-lysine appears to be the only methylated lysine species; both N^G-monomethyl-arginine and N^G,N^G-dimethyl-arginine have been identified as the methylated arginyl residues. Similar to the homologous polypeptide in chicken fibroblasts, the level of arginyl methylation of hsp70 of 3T3 cells can be reduced in the presence of arsenite. This

arsenite-induced reduction in arginyl methylation of hsp70 appears to be restricted to the polypeptides synthesized during the arsenite incubation. However, the arginyl methylation level of hsp70 of SR-RSV 3T3 is constitutively lower than their untransformed counterpart (3T3 cells), and the methylation cannot be reduced further by arsenite. In addition, the level of lysyl methylation of hsp70 remains the same after arsenite treatment and transformation. Since the basic amino acid methylation in total cellular proteins remains unchanged after arsenite incubation and Rous sarcoma virus transformation, the reduction in arginyl methylation of hsp70 in chicken fibroblasts and 3T3 cells by these treatments appears to be specific.

Table of Contents

Chapter 1

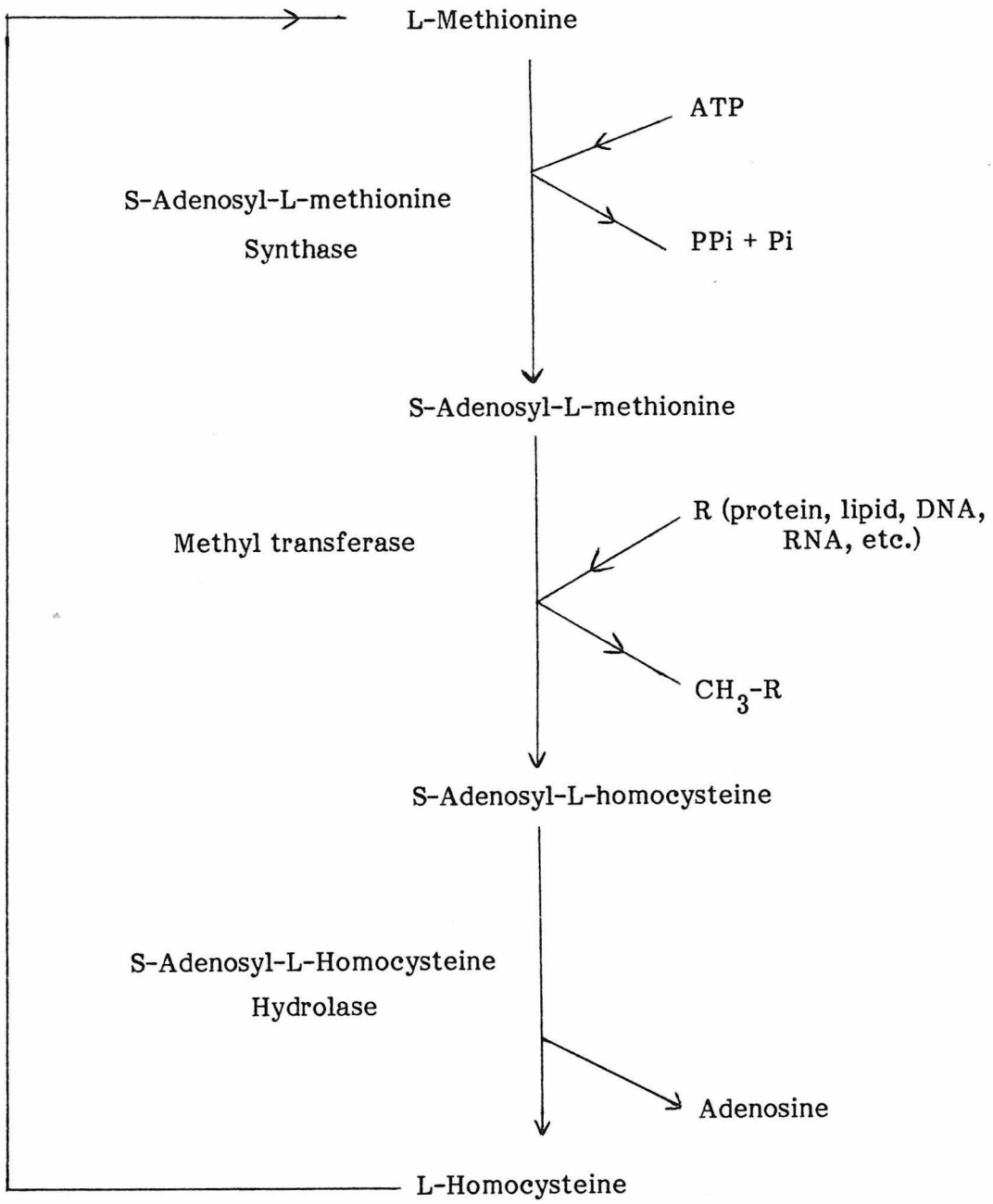
Introduction

The 70,000 Dalton Heat Shock Protein of Cultured Chicken Embryo Fibroblasts and Mouse 3T3 Cells Is Methylated

(1) Post-translational Methylation of Amino Acids in Proteins

S-Adenosyl-L-methionine (AdoMet) may be the sole methyl donor for a number of methylation reactions, with the exception of those involved in the biosynthesis of methionine (1). The demethylated product of the transmethylation reaction, S-adenosyl-L-homocysteine (AdoHcy), is a potent inhibitor of AdoMet-dependent methyl transferases (2). In eukaryotes, the intracellular methylation can be regulated by changing the concentration ratio of AdoMet/AdoHcy (3). The tissue distribution and the factors affecting the accumulation of these two compounds have been studied (4-9). Substrates for AdoMet-dependent methyl transferases may be small molecules, lipids, nucleic acids, or proteins (for a methylation pathway, see Scheme I). The methylation of amino acids in proteins has been documented (10); in some cases, the methylated amino acids have been identified and a functional role has been suggested, as will be discussed in the following paragraphs.

The motile bacteria E. coli and Salmonella typhimurium are capable of responding to environmental changes by altering swimming behavior such that they can migrate toward attractants or away from repellents; this behavior is known as bacterial chemotaxis (for a review, see 11, 12). The presence of attractants or repellents causes an immediate response (excitation), followed by a slower adaptation process. Adler and Dahl (13) discovered some years ago that L-methionine is required for chemotaxis but not for motility. This observation eventually led to a series of experiments revealing that AdoMet-dependent methylation of a set of bacterial membrane proteins, known as methyl-accepting chemotaxis proteins (MCPs), is involved in the adaptation phase of chemotaxis (14-16). The methylation sites on MCPs have been identified to be glutamic acid residues (17, 18). The specific methyl transferase and esterase also have been purified (19, 20). In addition, the level of methylation of MCPs can be modulated by


L-Methionine
ATP
S-Adenosyl-L-methionine
Synthase
PPi + Pi
S-Adenosyl-L-methionine
R (protein, lipid, DNA,
RNA, etc.)
Methyl transferase
CH_3-R
S-Adenosyl-L-homocysteine
S-Adenosyl-L-Homocysteine
Hydrolase
Adenosine
L-Homocysteine

Scheme I

the level of attractant and repellent, being increased by increasing the attractant level and decreased after adding repellents (16, 21, 22). MCPs are integral membrane proteins with apparent molecular weights of 60,000 ± 5,000 (23). In E. coli three different classes of MCPs have been identified, and each MCP appears to mediate responsiveness to a different set of chemicals (24-26). Since MCPs also may be receptors for some stimulating agents (27, 28), signal transduction possibly results from the direct binding of chemicals to MCPs. Recent experimental results clearly demonstrate that each MCP can be multiply methylated (29-32), and this sequential methylation may occur in an ordered manner (33). Hence, it is evident that the formation and hydrolysis of glutamic acid γ-methyl esters on MCPs is involved in the adaptation response of bacterial chemotaxis.

Evidence has been presented that protein carboxyl methylation also exists in mammalian cells (34-36); this type of methylation has been associated with leucocyte chemotaxis (37). It was suggested that the methyl esterification of polypeptides in mammalian cells may occur at glutamic acid residues as well (38). However, aspartic acid residues instead were found to be the methylation sites in human erythrocyte membrane proteins (39). The major methylated species include band 2.1 (ankyrin) and band 3 (anion transporter). Furthermore, the β-aspartic acid methyl esterification of the erythrocyte membrane proteins appears to be substoichiometric and reversible (40, 41). Recent experimental results indicate that the methylated aspartyl residues in human erythrocytes are always in the D-form instead of the commonly found L-form (42). The appearance of D-aspartic acid residues in mammalian proteins may be a consequence of racemization in aging; for example, the accumulation of D-aspartyl residues in lens proteins has been documented (43). Hence, McFadden and Clarke proposed that a similar racemization process may occur in aging erythrocytes, and this carboxyl methylation may be involved in a repair process for aging protein (42).

3-N-methyl-histidine was first found in human urine (44), but the source of this methylated amino acid initially was unclear. Subsequently, it was discovered that 3-N-methyl-histidine is a constituent of actin isolated from skeletal muscle (45, 46). It also exists in some other polypeptides, e.g., skeletal muscle myosin (46, 47) and duck erythrocyte histones (48). The stoichiometry of 3-N-methyl-histidine in α-actin was found to be one per molecule and the methylated histidinyl residue is localized at a specific position (49, 50). The β-/γ-actin of mouse L cells and chicken embryonic fibroblasts are also methylated at histidine stoichiometrically (51; Wang and Lazarides, unpublished observations). The methylation at a histidinyl residue on actin appears to be irreversible (52). Furthermore, methylation of actin in the presence of protein synthesis inhibitors could not be detected (53). Thus, the methylation of actin perhaps occurs only on nascent polypeptide chains (54). Attempts have been made, but have failed to elucidate the function of histidinyl methylation in actin and myosin. For example, the results obtained by photo-oxidation reactions suggest that 3-N-methyl-histidine is not essential for Ca^{2+}-ATPase activity and actin binding activity of myosin (47). It also was observed that actins isolated from chicken embryo are deficient in 3-N-methyl-histidine, but they are capable of undergoing polymerization (55). In contrast, actin isolated from fetal rabbit skeletal muscle contains the same amount of 3-N-methyl-histidine as from adult (46).

Post-translational methylation of lysyl residues in proteins has been found in a number of polypeptides. The methylated lysine species may be ε-N-monomethyl-lysine, ε-N-dimethyl-lysine or ε-N-trimethyl-lysine (for a review, see 10). It has been shown that the methylation of basic amino acids (including lysine) of rat heart proteins (56), cultured chicken muscle proteins (56) and chicken fibroblast cellular proteins (53, see also Chapter 5) is strongly inhibited by cycloheximide and therefore is coupled to their synthesis. Specifically, the methylation of lysyl residues of yeast cytochrome c (57) and chicken fibroblast heat shock proteins (58) is known to be coupled to synthesis. However, the methylation of lysyl residues on these proteins

can also occur after the polypeptide chains are completed (58, 59). In most cases, the methylation of lysyl residues in protein is irreversible. For example, by double-labeling the polypeptide backbone and incorporated methyl groups followed by a 12-20 hour chase, little turnover of methyl groups on lysyl residues was observed on yeast cytochrome c (57) and the 70,000 dalton heat shock proteins (58); in addition, histone methylation is thought to be irreversible, since the half-life of methyl groups is identical to that of the polypeptides (60).

Several different AdoMet-dependent lysyl methyl transferases with different substrate specificities have been purified. A single purified enzyme has been purified from the cytosol fraction of yeast (61) and Neurospora (62) which is specific for cytochrome c. Although ε-N-trimethyl-lysine is the only methyl-lysine species found in cytochrome c in vivo, the in vitro methylation product includes a small but constant amount of ε-N-monomethyl-lysine and ε-N-dimethyl-lysine (61, 62). It has been proposed that these lower methylated forms serve as substrates for higher methylated forms, and the methylation occurs in a stepwise fashion (62). Nuclear enzymes that catalyze the methylation of histone lysyl methylation have also been purified (63, 64). Initially it was not clear if a single enzyme was responsible for the methylation of both histone H3 and H4 (63). However, a later study demonstrated that the lysyl methyl transferases for histone H3 and H4 may be distinct (64). Recently, a methyl transferase was purified from brain cytosol which is specific for calmodulin (65). ε-N-trimethyl-lysine is the predominant methylated lysine species, but the presence of ε-N-monomethyl-lysine and ε-N-dimethyl-lysine is also observed (65).

In several cases, changes in methylation at lysyl residues in proteins have been observed under specific conditions, but the functional role of such changes is still obscure. For example, the methylation level of fungal elongation factor EF-1α changes during morphogenesis, but its enzymatic activity does not parallel these changes (66). Similarly, histone methylation occurs mainly near the end of the DNA replication phase of the cell cycle (67), but both methylated and unmethylated histone H4

can assemble with equal efficiency into nucleosomes (64). Perhaps cytochrome c is the only example where a functional role(s) has been assigned to its lysyl methylation. It has been suggested that this may facilitate the binding of cytochrome c to mitochondria (57, 59); it may also have a protective effect in in vivo proteolysis in yeast (68). However, methylation itself is not required for electron transport (57).

Three different methylated arginine derivatives have been identified in proteins, which include N^G-monomethyl-arginine, N^G,N^G-dimethyl-arginine, and N^G,N'^G-dimethyl-arginine (for a review, see 10). In many respects, the nature of the methylation at arginyl residues in proteins is similar to that of lysyl residues. For example, it is coupled to synthesis, but can also occur after the polypeptide chains have been completed (54, 56, 58); the methylation is essentially irreversible (58, 60).

Compared with the enzymatic activities of the adult rat brain, the fetal arginyl methyl transferase activities are much higher, but the specific substrates have not been identified (69). Furthermore, the histone arginyl methyl transferase activity has been found to be higher in regenerating liver (70). Thus, it has been speculated that arginyl methylation in proteins may be related to cell proliferation. An arginyl methyl transferase has been purified from calf brain cytosol fraction; it preferentially methylates histone H2, but it is not specific (71). Since the relative amount of N^G-monomethyl-arginine, N^G,N^G-dimethyl-arginine and N^G,N'^G-dimethyl-arginine produced by this enzyme remains constant at different stages of purification, it was suggested that a single enzyme may be responsible for the methylation of these three methyl-arginine derivatives (71).

Similar to the methylation of lysyl residues in proteins, the level of methylation at arginyl residues can be modulated. The methylation at arginine in the 70,000 dalton heat shock protein is reduced after sodium arsenite treatment and Rous sarcoma virus transformation (Chapters 5 and 6). The functional significance of the arginyl methylation of this polypeptide and other proteins is unclear.

(2) Induction of Protein Synthesis by Heat Shock and Sodium Arsenite Treatment

It was first discovered that a unique set of puffs indicative of increased transcription was induced in polytene chromosomes of Drosophila shortly after shifting the organism to 37°C, which is higher than the normal growth temperature (25°C) (72, 73). Subsequently, a class of polypeptides known as heat shock proteins was found to be synthesized at a higher rate in Drosophila tissue or cultured cells after heat shock; at the same time, the synthesis of other cellular proteins is reduced (74, 75, for a review see 76). The induction of heat shock proteins in Drosophila is mainly due to the increased synthesis of the corresponding mRNAs (74, 77-80). Thus, the induction of heat shock proteins in Drosophila can be regulated at the transcriptional level. At present, it is also clear that the heat shock response in Drosophila is also regulated at the translational level (see below).

McKenzie, Henikoff and Meselson (78) first demonstrated that the pre-existing polysomes in Drosophila break down shortly after heat shock, and a new class of polysomes specific for heat shock mRNA was formed. Furthermore, the existence of heat shock-specific mRNA is not required for the initial breakdown of the pre-existing polysomes (81). The pre-existing mRNA is retained during heat shock and can be reutilized after bringing the tissue or cells back to normal growth conditions (82-85). Recently, several laboratories demonstrated that a lysate prepared from heat shocked Drosophila Kc cells can selectively translate the heat shock mRNA isolated from control cells grown at 25°C (82, 83, 86). It was proposed that ribosomes may be the responsible factor. Indeed, the phosphorylation of a particular ribosomal protein in Drosophila can be rapidly reduced by heat shock (87). Nevertheless, further experiments are required to verify if the phosphorylation of this ribosomal protein is responsible for the translational control.

The heat shock response is also common to a number of organisms and tissue culture cells ranging from protozoans to mammals; for example, T. pyriformis (88), yeast (89), chicken fibroblasts (90), BHK cells (53, 90), quail myogenic cells (91).

Furthermore, recent experimental results demonstrate that a similar set of polypeptides also can be induced by certain chemicals, including sodium arsenite (53, 90, 92, see also Chapter 6).

Even after intensive studies of heat shock proteins at the DNA level (77), the functional role of these polypeptides is largely unknown. Because a non-lethal treatment, which is sufficient to induce the heat shock proteins, increases the chance of survival upon further lethal treatment (89, 93), it was suggested that heat shock proteins may be important for protecting the cells.

Evidence has been presented that the heat shock proteins are expressed under normal growth conditions (53, 91, 92, 94, 95; also see Chapters 2 and 6), and that heat shock may increase the level of these polypeptides. The possible structural and functional relationship of these heat shock proteins has been studied. The low molecular weight heat shock proteins of Drosophila have been shown to be related to one another and they may be homologous to mammalian lens α-crystallins (96). After Rous sarcoma virus transformation, the 89,000 dalton heat shock protein of chicken fibroblasts was found to be associated with the product of transforming gene, $pp60^{src}$ (97, 98); and it has been proposed that this polypeptide is important for the transport of $pp60^{src}$ to the plasma membrane (98). The intracellular localization of the 70,000 dalton heat shock protein (hsp70) in a variety of different systems has been studied. In Drosophila, most heat shock proteins, including hsp70, are located in nuclei shortly after heat shock (99, 100), and hsp70 may be associated with the nuclear cytoskeleton (101, 102). However, it is mainly a cytosolic constituent (95); a portion of it was found in a Triton X-100/0.6 M KCl insoluble pellet in a number of cultured cells (103). A fraction of it also may be associated with a membrane surface glycoprotein (104) or microtubules (105) in cultured mammalian cells. These different localizations reported may be due to the particular systems utilized in these studies. These differences may also be due to the post-translational modifications of these polyepttides, which will be discussed below.

(3) Scope of This Thesis

The 70,000 dalton heat shock protein (hsp70) is the most commonly induced species after heat shock and sodium arsenite treatment. In chicken fibroblasts, hsp70 is composed of two major distinct isoelectric variants (53, 91, 92, 103). The more acidic one (hsp70A) is highly conserved and exists in both avian and mammalian cultured cells, while the hsp70B is not expressed in mammalian cultured cells. A 68,000 dalton polypeptide also is induced in mammalian cells (53, 90, 104, see also Chapters 3 and 6). Other laboratories and I have demonstrated that hsp70 exists in most tissues and cultured cells prior to heat shock induction (53, 91, 92, 95, 103-105). It was reported that hsp70 is phosphorylated (95). I found that this polypeptide is methylated at lysyl and arginyl residues before and after sodium arsenite treatment (Chapters 3-6). In believing that a characterization of the methylation may provide valuable information to help elucidate the function of hsp70, I carried out a series of experiments to study the nature of the methylation of hsp70 in chicken fibroblasts and 3T3 cells. The results are described below.

Incubating chicken embryonic fibroblasts and some mammalian cultured cells such as BHK, 3T3, and WI38/VA13 with [methyl-^{3}H]-L-methionine in the presence of protein synthesis inhibitors resulted in the incorporation of [^{3}H]-radioactivity into hsp70 and some other polypeptides (53, Chapters 2 and 3). Because labeling the cells with [^{35}S]-L-methionine under the same conditions gave a different protein pattern, this [^{3}H]-radioactivity incorporation cannot possibly be due to leaky protein synthesis. Since this incorporation is inhibited by S-adenosyl-L-methionine-dependent methyl transferase inhibitors, we suggested that the incorporation is the consequence of methylation (53). Because nothing was known previously about the methylation of heat shock proteins, I decided to proceed to study the nature of the methylation of hsp70, such as the chemistry and reversibility of this methylation.

In order to determine the half-life of hps70 methylation, cells were double-labeled with [^{35}S]-L-methionine and [methyl-^{3}H]-L-methionine, and then chased for

0-20 hours; the ratio of [^{3}H]/[^{35}S]-radioactivity of hsp70 was calculated to determine the half-life of methyl groups. The results I obtained indicate that the methylation of hsp70 is irreversible (58, see also Chapter 4).

Carboxyl methylation has been known to be labile to base treatment. Hence, I prepared samples and performed gel electrophoresis under acidic conditions (40, 41). After immersing in 0.1 N NaOH for half an hour, the gels were dried down for autoradiography. However, no difference in [^{3}H]-radioactivity is detected on hsp70 after base treatment; by the same procedures, the [^{3}H]-radioactivity associated with human erythrocyte membrane protein is reduced dramatically. It appears that little β-aspartic acid methyl ester exists in hsp70, but the possible existence of γ-glutamic acid methyl ester cannot be ruled out completely. I could recover 80%-92% of the [^{3}H]-radioactivity in the dried residues of hsp70 samples after acid hydrolysis (58); the [^{3}H]-radioactivity contributed by glutamyl γ-methyl ester is less than 20%. Because the majority of the [^{3}H]-radioactivity may be incorporated into basic amino acids, I continued to analyze the basic amino acid methylation in hsp70.

An ion exchange chromatography procedure, which can resolve all the known methylated basic amino acids, was adopted for amino acid analysis of hsp70 hydrolysate from chicken fibroblasts. I discovered that lysyl and arginyl residues are the methylation sites (Chapter 4). The ε-N-trimethyl-lysine is the predominant methyl-lysine species; however, the contribution of ε-N-monomethyl-lysine and ε-N-dimethyl-lysine is apparent. Furthermore, N^G-monomethyl-arginine is the only methyl-arginine species in chicken fibroblast hsp70.

The incorporation of [^{3}H]-radioactivity into hsp70 remains linear for at least six hours in the presence of protein synthesis inhibitors. I estimated that the stoichiometry of methyl group incorporated into hsp70 under these conditions is one per one thousand polypeptides or less (Chapter 4). One of the simplest and most straightforward explanations for this low stoichiometry is that hsp70 methylation is closely coupled

to protein synthesis. To verify this assumption, I labeled the cells with [^{3}H]-leucine and [methyl-^{3}H]-L-methionine, or [methyl-^{3}H]-L-methionine alone, without protein synthesis inhibitors, and then the radioactively-labeled hsp70 was isolated, hydrolyzed and chromatographed. I discovered that under these conditions the methylation of hsp70 is stoichiometric (Chapters 4 and 5). At this point, it became evident that if the methylation of hsp70 can be modulated, this would be an important issue.

I labeled chicken fibroblasts with [methyl-^{3}H]-L-methionine in the presence of arsenite, then analyzed the methylation of hsp70. No difference in lysyl residue methylation was found in hsp70B, but the distribution of three different methyl-lysine species in hsp70A is altered by arsenite. On the other hand, the methylation level of N^G-monomethyl-arginine in hsp70B is reduced significantly, although it remains unchanged in hsp70A. Since the steady state level of S-adenosyl-L-methionine and the level of total cellular methylation remain the same after arsenite treatment, the difference in methylation in hsp70 from chicken fibroblasts appears to be specific (Chapter 5).

A slightly different procedure was utilized to study the methylation of hsp70 isolated from 3T3 and a Rous sarcoma virus transformed derivative, SR-RSV3T3. As with hps70 from chicken fibroblasts, I found that the polypeptide is methylated at lysyl and arginyl residues. However, major differences also were observed, which include: 1) the existence of N^G,N^G-dimethyl-arginine, and 2) the methylated lysine species is exclusively ε-N-trimethyl-lysine, i.e. , no contribution of ε-N-monomethyl-lysine and ε-N-dimethyl-lysine was detected. The change in methylation of hsp70 isolated from 3T3 cells in the presence of arsenite also was observed. The level of methylation of N^G-monomethyl-arginine and N^G,N^G-dimethyl-arginine is reduced, while no change in methyl-lysine is detected. This response is qualitatively identical to the arsenite-induced response of hsp70B from chicken fibroblasts. In addition, the methylation level of arginyl residues of hsp70 isolated from SR-RSV3T3 is lower than the counterpart from 3T3 cells; this level cannot be further reduced by arsenite.

References

1. Cantoni, G. L. (1975) Ann. Rev. Biochem. **44**, 435-451.
2. Borchardt, R. T. (1977) The Biochemistry of S-adenosyl-L-methionine (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, J. G. and Schlenk, F., eds.) Columbia University, New York, pp. 151-171.
3. Cantoni, G. L. and Chiagn, P. K. (1980) Natural Sulfur Compounds (Cavallini, D., Gaull, G. E. and Zappia, V., eds.) Plenum Press, New York, pp. 67-80.
4. Eloranta, T. O. (1977) Biochem. J. **166**, 521-529.
5. Hoffman, D. R., Marion, D. W., Cornatzer, W. E. and Duerre, J. A. (1980) J. Biol. Chem. **255**, 10822-10827.
6. Mudd, S. H. (1973) Enzymes, 3rd Ed. **8**, 121-154, Academic Press, New York.
7. Chiang, P. K., Richards, H. H. and Cantoni, G. L. (1977) Mol. Pharmacol. **13**, 937-947.
8. Frieden, C., Kurz, L. C. and Gilbert, H. R. (1980) Biochemistry **19**, 5303-5309.
9. Guranowski, A., Montgomery, J. A., Cantoni, G. L. and Chiang, P. K. (1981) Biochemistry **20**, 110-115.
10. Paik, W. K. and Kim, S. (1980) Protein Methylation, John Wiley and Sons, New York.
11. Springer, M. S., Goy, M. F. and Adler, J. (1979) Nature **280**, 279-284.
12. Koshland, D. E. Jr. (1981) Ann. Rev. Biochem. **50**, 765-782.
13. Adler, J. and Dahl, M. M. (1967) J. Gen. Microbiol. **46**, 161-173.
14. Larsen, S. H., Adler, J., Gargus, J. J. and Hogg, R. W. (1974) Proc. Nat. Acad. Sci. USA **71**, 1238-1243.
15. Aswad, D. W. and Koshland, D. E. Jr. (1975) J. Mol. Biol. **97**, 207-223.
16. Kort, E. N., Goy, M. F., Larsen, S. H. and Adler, J. (1977) Proc. Natl. Acad. Sci. USA **72**, 3939-3943.
17. Van der Werf, P. and Koshland, D. E. Jr. (1977) J. Biol. Chem. **252**, 2793-2795.

18. Kleene, S. J., Toews, M. L. and Adler, J. (1977) J. Biol. Chem. **252**, 3214-3218.

19. Springe,r W. R. and Koshland, D. E. Jr. (1977) Proc. Nat. Acad. Sci. USA **74**, 533-537.

20. Stock, J. B. and Koshland, D. E. Jr. (1978) Proc. Nat. Acad. Sci. USA **75**, 3659-3663.

21. Goy, M. F., Springer, M. S. and Adler, J. (1977) Proc. Nat. Acad. Sci. USA **74**, 4964-4968.

22. Stock, J. B. and Koshland, D. E. Jr. (1981) J. Biol. Chem. **256**, 10826-10833.

23. Ridgway, H. F., Silverman, M. and Simon, M. I. (1977) J. Bacteriol. **132**, 657-655.

24. Springer, M. S., Goy, M. F. and Adler, J. (1977) Proc. Nat. Acad. Sci. USA **74**, 3312-3316.

25. Silverman, M. and Simon, M. (1977) Proc. Nat. Acad. Sci. USA **74**, 3317-3321.

26. Kondoh, H., Ball, C. B. and Adler, J. (1979) Proc. Nat. Acad. Sci. USA **76**, 260-264.

27. Clarke, S. and Koshland, D. E. Jr. (1979) J. Biol. Chem. **254**, 9695-9702.

28. Wang, E. and Koshland, D. E. Jr. (1980) Proc. Natl. Acad. Sci. USA **77**, 7157-7161.

29. Boyd, A. and Simon, M. (1980) J. Bacteriol. **143**, 809-815.

30. DeFranco, A. L. and Koshland, D. E. Jr. (1980) Proc. Nat. Acad. Sci. USA **77**, 2429-2433.

31. Engstrom, P. and Hazelbauer, G. L. (1980) Cell **20**, 165-171.

32. Chelsky, D. and Dahlquist, F. W. (1981) Biochemistry **20**, 977-982.

33. Springer, M. S. Zanolari, B. and Pierzchala, P. A. (1982) J. Biol. Chem. **257**, 6861-6866.

34. Liss, M. and Edelstein, L. M. (1967) Biochem. Biophys. Res. Commun. **26**, 497-504.

35. Kim, S. and Paik, W. K. (1971) Biochemistry **10**, 3141-3145.

36. Quick, D. P., Orchard, P. J. and Duerre, J. A. (1981) Biochemistry **20**, 4724-4729.

37. O'Dea, R. F., Viveros, O. H., Axelrod, J., Aswanikumar, S., Schiffmann, E. and Corcoran, B. A. (1978) Nature **272**, 462-464.

38. Kim, S. and Li, C. H. (1979) Proc. Nat. Acad. Sci. USA **76**, 4255-4257.

39. Janson, C. A. and Clarke, S. (1980) J. Biol. Chem. **255**, 11640-11643.

40. Terwillinger, T. C. and Clarke, S. (1981) J. Biol. Chem. **256**, 3067-3076.

41. Freitag, C. and Clarke, S. (1981) J. Biol. Chem. **256**, 6102-6108.

42. McFadden, P. N. and Clarke, S. (1982) Proc. Nat. Acad. Sci. USA **79**, 2460-2464.

43. Masters, P. M., Bada, J. L. and Zigler, J. S. Jr. (1978) Proc. Nat. Acad. Sci. USA **75**, 1204-1208.

44. Tallan, H. H., Stein, W. H. and Moore, S. (1954) J. Biol. Chem. **206**, 825-834.

45. Asatoor, A. M. and Armstrong, M. D. (1967) Biochem. Biophys. Res. Commun. **26**, 168-174.

46. Johnson, P., Harris, C. I. and Perry, S. V. (1967) Biochem. J. **105**, 361-370.

47. Johnson, P. and Perry, V. (1970) Biochem. J. **119**, 392-298.

48. Gershey, E. L., Haslett, G. W., Vidali, G. and Allfrey, V. G. (1969) J. Biol. Chem. **244**, 4871-4877.

49. Adelstein, R. S. and Kuehl, W. M. (1970) Biochemistry **9**, 1355-1364.

50. Elzinga, M. (1971) Biochemistry **10**, 224-228.

51. Garrels, J. I. and Gibson, W. (1976) Cell **9**, 793-805.

52. Reporter, M. (1969) Biochemistry **8**, 3489-3496.

53. Wang, C., Gomer, R. H. and Lazarides, E. (1981) Proc. Nat. Acad. Sci. USA **78**, 3531-3535.

54. Morse, R. K., Vergnes, J.-P., Malloy, J. and McManus, I. R. (1975) Biochemistry **14**, 4316-4325.

55. Krzysik, B., Vergnes, J. P. and McManus, I. R. (1971) Arch. Biochem. Biophys. **146**, 34-45.

56. Watkins, C. A. and Morgan, H. E. (1979) J. Biol. Chem. **254**, 693-701.

57. Farooqui, J., Kim, S. and Paik, W. K. (1980) J. Biol. Chem. **255**, 4468-4473.

58. Wang, C., Lazarides, E., O'Connor, C. M. and Clarke, S. (1982) J. Biol. Chem. **257**, 8356-8362.

59. Scott, W. A. and Mitchell, H. K. (1969) Biochemistry **8**, 4282-4289.

60. Byzoet, P., Shepherd, G. R., Hardin, J. M. and Noland, B. J. (1972) Arch. Biochem. Biophys. **148**, 558-567.

61. DiMaria, P., Polastro, E., DeLange, R. J., Kim, S. and Paik, W. K. (1979) J. Biol. Chem. **254**, 4645-4654.

62. Durban, E., Nochumson, S., Kim, S. and Paik, W. K. (1978) J. Biol. Chem. **253**, 1427-1435.

63. Wallwork, J. C., Quick, D. P. and Duerre, J. A. (1977) J. Biol. Chem. **252**, 5977-5980.

64. Sarnow, P., Basched, I. and Knippers, R. (1981) Biochim, Biophys. Acta **655**, 349-358.

65. Sitaramayya, A., Wright, L. S. and Siegel, F. L. (1980) J. Biol. Chem. **255**, 8894-8900.

66. Hiatt, W. R., Garcia, R., Merrick, W. C. and Sypherd, P. S. (1982) Proc. Nat. Acad. Sci. USA **79**, 3434-3437.

67. Tidwell, T., Allfrey, V. G. and Mirsky, A. E. (1968) J. Biol. Chem. **243**, 707-715.

68. Farooqui, J., DiMaria, P., Kim, S. and Paik, W. K. (1981) J. Biol. Chem. **256**, 5041-5045.

69. Paik, W. K., Kim, S. and Lee, H. W. (1972) Biochem. Biophys. Res. Commun. **46**, 933-941.

70. Lee, H. W. and Paik, W. K. (1972) Biochim. Biophys. Acta **277**, 107-116.

71. Lee, H. W., Kim, S. and Paik, W. K. (1977) Biochemistry **16**, 78-85.

72. Ritossa, F. (1962) Experientia **18**, 571-573.

73. Ritossa, F. (1964) Exp. Cell Res. **35**, 601-607.

74. Tissières, A., Mitchell, H. K. and Tracy, U. M. 91974) J. Mol. Biol. **84**, 389-398.

75. Lindquist, S. (1980) Develop. Biol. **77**, 463-479.

76. Ashburner, M. and Bonner, J. J. (1979) Cell **17**, 241-254.

77. Bonner, J. J. and Pardue, M. L. (1976) Cell **8**, 43-50.

78. McKenzie, S. L., Henikoff, S. and Meselson, M. (1975) Proc. Nat. Acad. Sci. USA **72**, 1117-1121.

79. Henikoff, S. and Meselson, M. (1977) Cell **12**, 441-451.

80. McKenzie, S. L. and Meselson, M. (1977) J. Mol. Biol. **117**, 279-283.

81. Lindquist, S. (1980) J. Mol. Biol. **137**, 151-158.

82. Storti, R. V., Scott, M. P., Rich, A. and Pardue, M. L. (1980) Cell **22**, 825-834.

83. Scott, M. P. and Pardue, M. L. (1981) Proc. Nat. Acad. Sci. USA **78**, 3353-3357.

83. Petersen, N. S. and Mitchell, H. K. (1981) Proc. Nat. Acad. Sci. UAA **78**, 1708-1711.

85. DiDomenico, B. J., Bugaisky, G. E. and Lindquist, S. (1982) Proc. Nat. Acad. Sci. USA **79**, 6181-6185.

86. Kruger, C. and Benecke, B.-J. (1981) Cell **23**, 595-603.

87. Glover, C. C. V. (1982) Proc. Nat. Acad. Sci. USA **79**, 1781-1785.

88. Guttman, S. D., Glover, C. C. V., Allis, C. D. and Gorovsky, M. A. (1980) Cell **22**, 299-307.

89. McAlister, L. and Finkelstein, D. B. (1980) Biochem. Biophys. Res. Commun. **93**, 819-824.

90. Kelley, P. M. and Schlesinger, M. J. (1978) Cell **15**, 1277-1286.

91. Atkinson, B. G. (1981) J. Cell Biol. **89**, 666-673.

92. Johnston, D., Oppermann, H., Jackson, J. and Levinson, W. (1980) J. Biol. Chem. **255**, 6975-6980.

93. Mitchell, H. K., Moller, G., Petersen, N. S. and Lipps-Sarmiento, L. (1979) Develop. Genet. **1**, 181-192.

94. Findly, R. C. and Pederson, T. (1981) J. Cell Biol. **88**, 323-328.

95. Kelley, P. M. and Schlesinger, M. J. (1982) Mol. Cell. Biol. **2**, 267-274.

96. Ingolia, T. D. and Craig, E. A. (1982) Proc. Nat. Acad. Sci. USA **79**, 2360-2364.

97. Oppermann, H., Levinson, W. and Bishop, J. M. (1981) Proc. Nat. Acad. Sci. USA **78**, 1067-1071.

98. Courtneidge, S. A. and Bishop, J. M. (1982) Proc. Nat. Acad. Sci. USA **79**, 7117-7121.

99. Mitchell, H. K. and Lipps, L. S. (1975) Biochem. Genet. **13**, 585-602.

100. Arrigo, A.-P., Fakan, S. and Tissières, A. (1980) Develop. Biol. **78**, 86-103.

101. Levinger, L. and Varshavsky, A. (1981) J. Cell Biol. **90**, 793-796.

102. Sinibaldi, R. M. and Morris, P. W. (1981) J. Biol. Chem. **256**, 10735-10738.

103. Wang, C., Asai, D. L. and Lazarides, E. (1980) Proc. Nat. Acad. Sci. USA **77**, 1541-1549.

104. Hughes, E. N. and August, J. T. (1982) Proc. Nat. Acad. Sci. USA **79**, 2305-2309.

105. Weatherbee, J. A., Luftig, R. B. and Weihing, R. R. (1980) Biochemistry **14**, 4116-4123.

Chapter 2

Arsenite-Induced Synthesis of Heat Shock Proteins in Chicken Embryo Fibroblasts

Introduction

The induced synthesis of heat shock proteins (hsps) in various tissues and cultured cells in response to thermal and chemical treatment has been documented (1-8, for a review on Drosophila, see 9). In Drosophila tissue culture cells, it is evident that the increased synthesis of heat shock proteins is dependent upon the incubation temperature in a complex way (5), and the response is controlled at both the transcriptional and translational levels (9-12). In addition, the induction of heat shock proteins in cultured quail myogenic cells has been shown to be gradually diminished during muscle differentiation (6). The function of the heat shock proteins is obscure. It was found that a brief nonlethal treatment can result in an increase in survival upon further lethal treatment, suggesting that heat shock proteins may be able to confer resistance to further thermal treatment (2, 13).

Arsenite (AsO_2^-) is one of the arsenicals which are capable of interacting with vicinal dithiols to form a covalent complex, $R\langle^{S}_{S}\rangle As = 0$ (14). It was reported recently that a set of polypeptides similar to heat shock proteins can be induced in some cultured cells by arsenite treatment (4, 7, 8), although the induced synthesis may not be a direct effect of changing the thiol status in the cells. A qualitative result of arsenite-induced synthesis of polypeptides in chicken embryonic fibroblasts was reported previously by Johnston et al. (4). However, a quantitative documentation on the induction kinetics is lacking. In this chapter, I present the quantitative results of arsenite-induced heat shock protein synthesis. In particular, I present results for the 70,000 dalton polypeptide (hsp70), which is the most commonly induced species in cultured cells.

Materials and Methods

Chicken embryonic fibroblasts were prepared as described (15). Confluent 60 mm plates with $(3.0 \pm 0.5) \times 10^6$ cells were incubated with 25 μM sodium arsenite for 0-12 hours, then rinsed with methionine-free minimal essential medium and incubated

with the same medium with 20 μCi/ml of [^{35}S]-L-methionine (New England Nuclear, specific activity: 1033.6 Ci/mmole) for 15 minutes. Alternatively, the cells were incubated with arsenite over a concentration range of 0-200 μM for three hours before a 15-minute [^{35}S]-L-methionine pulse labeling. At the end of the labeling period, the cells were rinsed three times with phosphate buffered saline, then scraped off the plates and collected by centrifugation. The pellets were solubilized by immediately boiling in sample buffer (1% SDS, 0.5% 2-mercaptoethanol, 10% glycerol and 25 mM Tris-HCl, pH 7.5) before being subjected to two-dimensional gel electrophoresis.

One-dimensional SDS polyacrylamide gel electrophoresis was performed by following the method of Laemmli (16). The stacking gel contained 5% acrylamide, 0.13% N,N'-methylene bisacrylamide, 0.125 M Tris-HCl, pH 6.8 and 0.1% SDS; the resolving gel consisted of 15% acrylamide, 0.085% bisacrylamide, 0.38 M Tris-HCl, pH 8.7 and 0.1% SDS. After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue in 50% ethanol and 10% acetic acid and destained with 12.5% ethanol and 5% acetic acid. The Coomassie-stained gels were then scanned with a densitometer before they were treated with ENHANCE (New England Nuclear) for fluorography.

To quantitate the [^{35}S]-radioactivity, a particular protein band was excised from the dried gels with scissors and placed into a scintillation vial. Half a milliliter of 0.1% SDS in NH_4HCO_3 was added and the sample was incubated overnight to elute the polypeptide from the gel slices. Then 5 ml of Aquasol-2 scintillation fluid (New England Nuclear) was added and counted with a liuqid scintillation counter. Since the number of actin molecules remain unchanged upon arsenite treatment, the ratios of [^{35}S]-radioactivity associated with a particular peptide band to the area under the densitometric trace corresponding to actin were calculated and compared to determine the induction kinetics.

Results

Incubation of chicken embryonic fibroblasts with sodium arsenite results in an increased synthesis of seven polypeptides with molecular weights of 110,000, 89,000, 78,000, 76,000, 70,000, 32,000 and 22,000 (Figure 1). Four of them with molecular weights of 89,000, 70,000, 32,000 and 22,000 are the more prominently induced species. Similar results have been reported by other laboratories with arsenite or other treatments including heat shock (3, 4). A similar induction pattern was obtained after incubation with arsenite over a concentration range of 10-200 μM (Figure 1). Quantitative results of the induction kinetics for four prominently induced polypeptides corresponding to Figure 1 are given in Figure 2. The results indicate that the rates of synthesis of most of these polypeptides increase with arsenite concentration and reach maximal levels at 50 μM arsenite, except for the 32,000 dalton polypeptide. The rate of synthesis of the 32,000 dalton polypeptide appears to be maximally induced at the lowest arsenite concentration (10 μM) and decreases with increase in concentration (Figure 2.)

The increase in rate of synthesis also is dependent upon the incubation period with arsenite (Figure 3). The rate of synthesis of these inducible polypeptides reaches the maximal level between three to six hours and then decreases after prolonged incubation, with the exception of the 22,000 dalton polypeptide; the synthesis of the 22,000 dalton polypeptide remains at a high level for at least 12 hours. The induction kinetics of the 22,000 and 70,000 dalton polypeptides are given in Figure 4. The induction kinetics for the 32,000 and 89,000 dalton polypeptides are identical to that of the 70,000 dalton polypeptide except that the maximal level is lower (data not shown).

Figure 1. Induced synthesis as a function of arsenite concentration. Cells were incubated with 0-200 μM sodium arsenite for 3 hours before labeling with [^{35}S]-L-methionine. The cells were then solubilized and analyzed by one-dimensional gel electrophoresis. (A) Coomassie blue-stained gel. P: phosphorylase (94,000); B: bovine serum albumin; V: vimentin; A: actin. (B) Autoradiogram corresponding to (A). Lane a, 0 μM; b, 10 μM; c, 25 μM; d, 50 μM; e, 100 μM; f, 150 μM; g, 200 μM sodium arsenite.

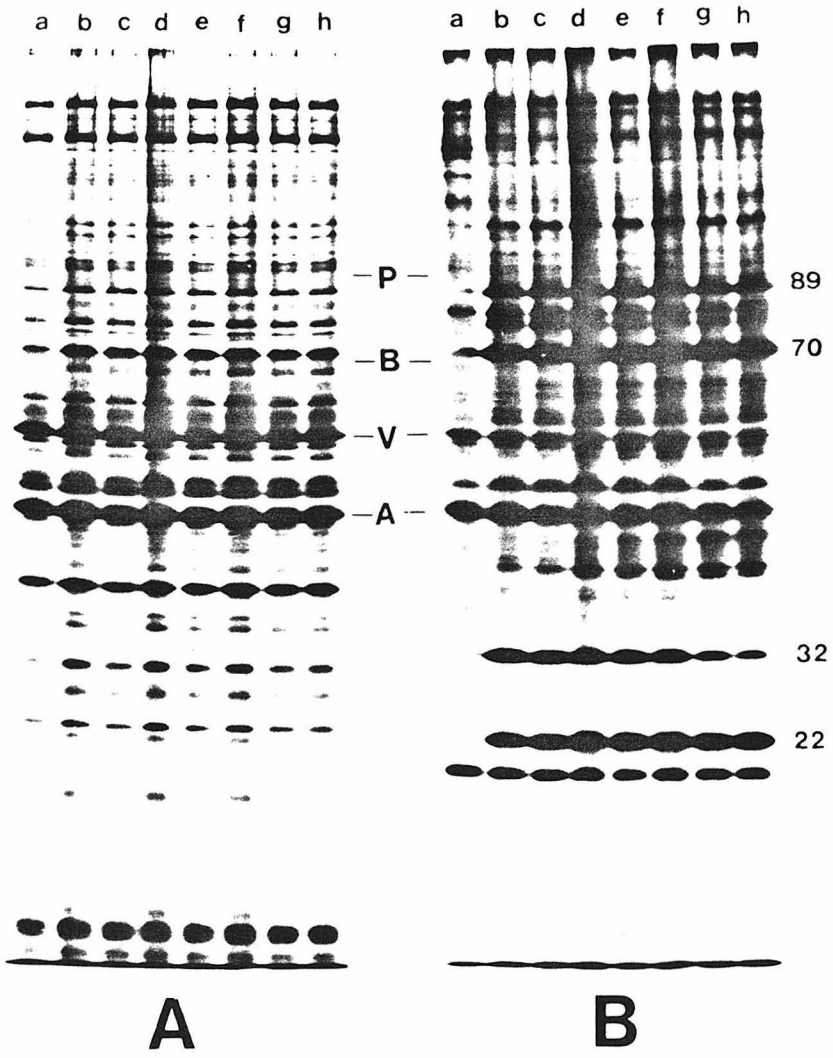

a b c d e f g h
P
B
V
A
a b c d e f g h
89
70
32
22
A
B

Figure 2. Quantitation of induction versus arsenite concentration. Induction of the four most prominent inducible polypeptides corresponding to Figure 1 are shown. The [^{35}S]-radioactivity of each band is quantitated as described in "Materials and Methods." Since the number of copies of actin molecules per cell remains unchanged after arsenite treatment, the loadings of each lane were normalized to the area of the actin band which is determined by scanning each lane of Coomassie blue-stained gel with a densitometer. The control is arbitrarily assigned as 1. Top to bottom corresponding to 89,000, 70,000, 32,000 and 22,000 dalton polypeptides.

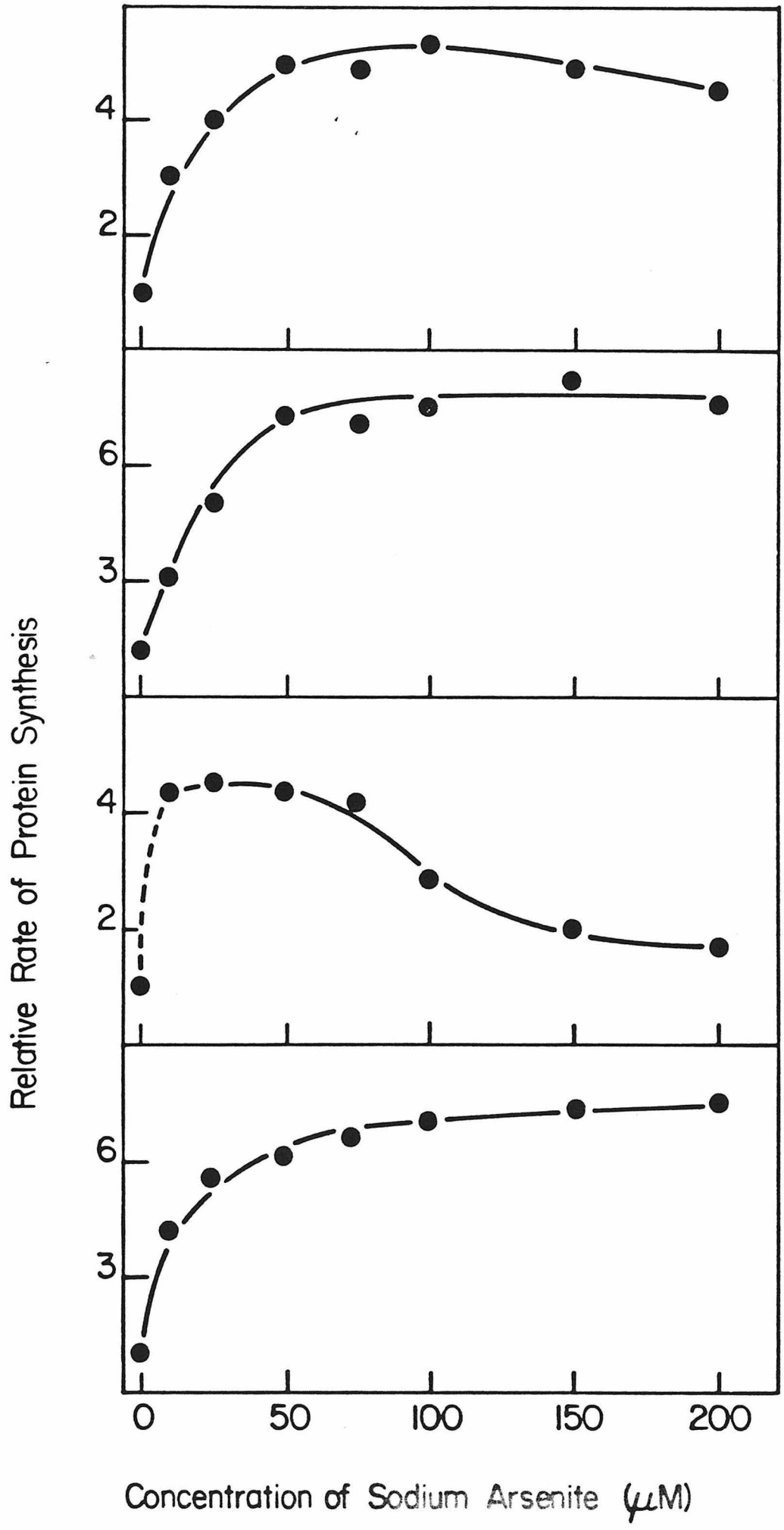

Relative Rate of Protein Synthesis
4
2
6
3
4
2
6
3
0
50
100
150
200
Concentration of Sodium Arsenite (μM)

Figure 3. Kinetics of induction. Cells were exposed to 25 μM sodium arsenite for 0-12 hours before labeling, then solubilized and analyzed by one-dimensional gel electrophoresis. (A) Coomassie blue-stained gels. P: phosphorylase; B: bovine serum albumin; V: vimentin; A: actin. (B) Autoradiogram corresponding to (A). Lane a, 0 hr; b, 1 hr; c, 2 hr; d, 3 hr; e, 4 hr; f, 6 hr; g, 7 hr; h, 8.5 hr; i, 10 hr; j, 12 hr of arsenite incubation.

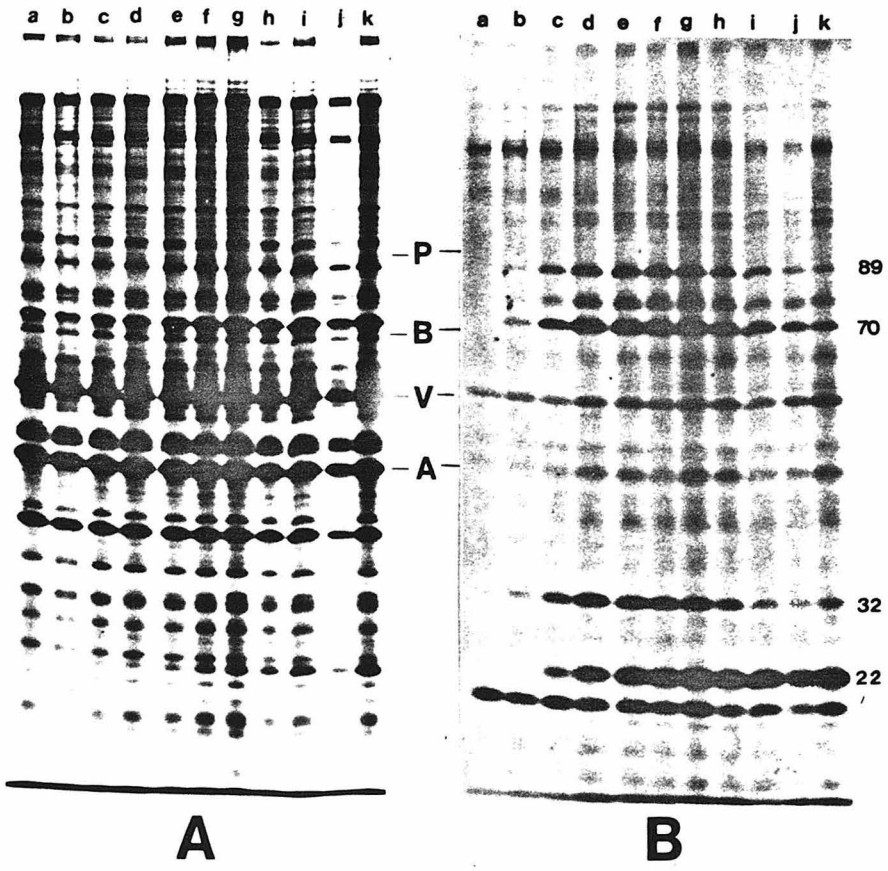

a b c d e f g h i j k
P
B
V
A
A
a b c d e f g h i j k
89
70
32
22
B

Figure 4. Quantitation of induction kinetics. Two polypeptides with different induction-kinetic behaviors are shown. The quantitation procedure is similar to that of Figure 2. Closed circle (●): 22,000 dalton polypeptide; open circle (O): 70,000 dalton polypeptide. The control level is assigned as 1.

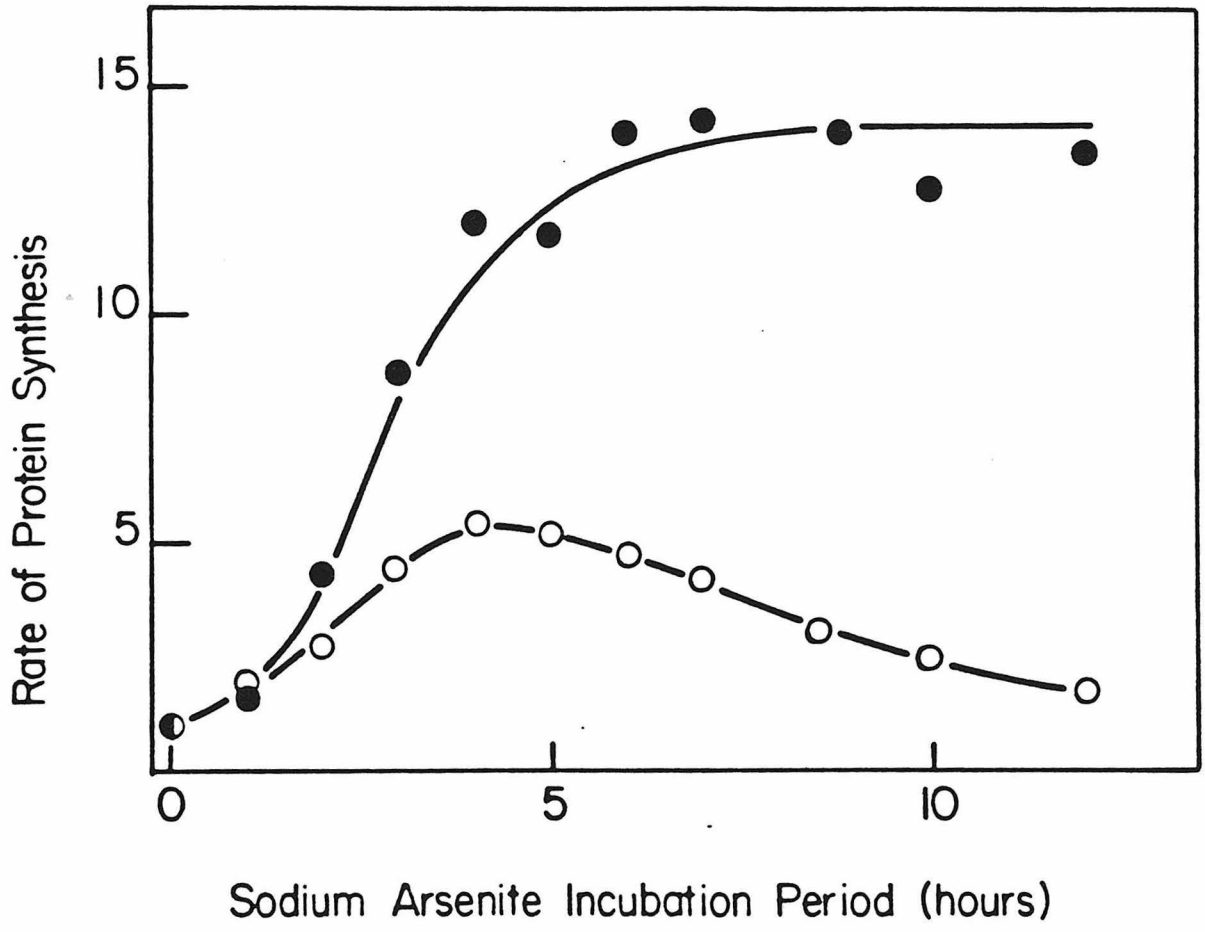

15
10
5
0
5
10
Rate of Protein Synthesis
Sodium Arsenite Incubation Period (hours)

Figure 4

It is also clear from the Coomassie-stained gels that the heat shock proteins are accumulated in the cells (Figure 3). Although the increased amount of the 22,000 and 32,000 dalton polypeptides is evident, it is difficult to obtain a quantitative result, because these two polypeptides are in very low abundance and hence obscured by the background before arsenite treatment. The accumulation of the 70,000 and 89,000 dalton polypeptides in chicken embryonic fibroblasts is given in Figure 5. The results indicate that the total quantities of the 70,000 and 89,000 dalton polypeptides are tripled and doubled, respectively, after a six-hour arsenite treatment.

The 70,000 dalton polypeptide can be resolved into two distinct major variants by two-dimensional gel electrophoresis (4, 7); the more acidic one has a pI of 5.6 while the more basic one has a pI of 6.0. The accumulation of the 70,000 dalton polypeptide after arsenite treatment is due to a comparable increase of both species. It is also apparent from Figures 1 and 3 that arsenite does not significantly alter the rate of synthesis of most other cellular proteins.

I also labeled the cells with [methyl-^{3}H]-L-methionine in the presence of protein synthesis inhibitors. Apparently, a very different pattern of radioactive incorporation was obtained (Figure 6); the 70,000 and 78,000 dalton polypeptides are among the major polypeptides labeled. There is a significant increase in [^{3}H]-radioactivity incorporated into the 70,000 dalton polypeptide after arsenite treatment, whereas no difference is observed on 78,000 dalton polypeptide (Figure 7). Furthermore, the incorporation increases linearly with incubation time for at least 6 hours (Figure 7). We have previously shown that the [^{3}H]-radioactivity incorporated into these two arsenite-inducible polypeptides is due to methylation, presumably by S-adenysyl-L-methionine-dependent methyl transferases (7, 17).

Figure 5. Accumulation of the 89,000 and 70,000 dalton polypeptides after arsenite treatment. Each lane of Coomassie blue-stained gel (Figure 3A) was scanned by a densitometer. Since the level of actin per cell remains unchanged after arsenite treatment, the loading was standardized by the area corresponding to the actin peak. The control is assigned as 1.

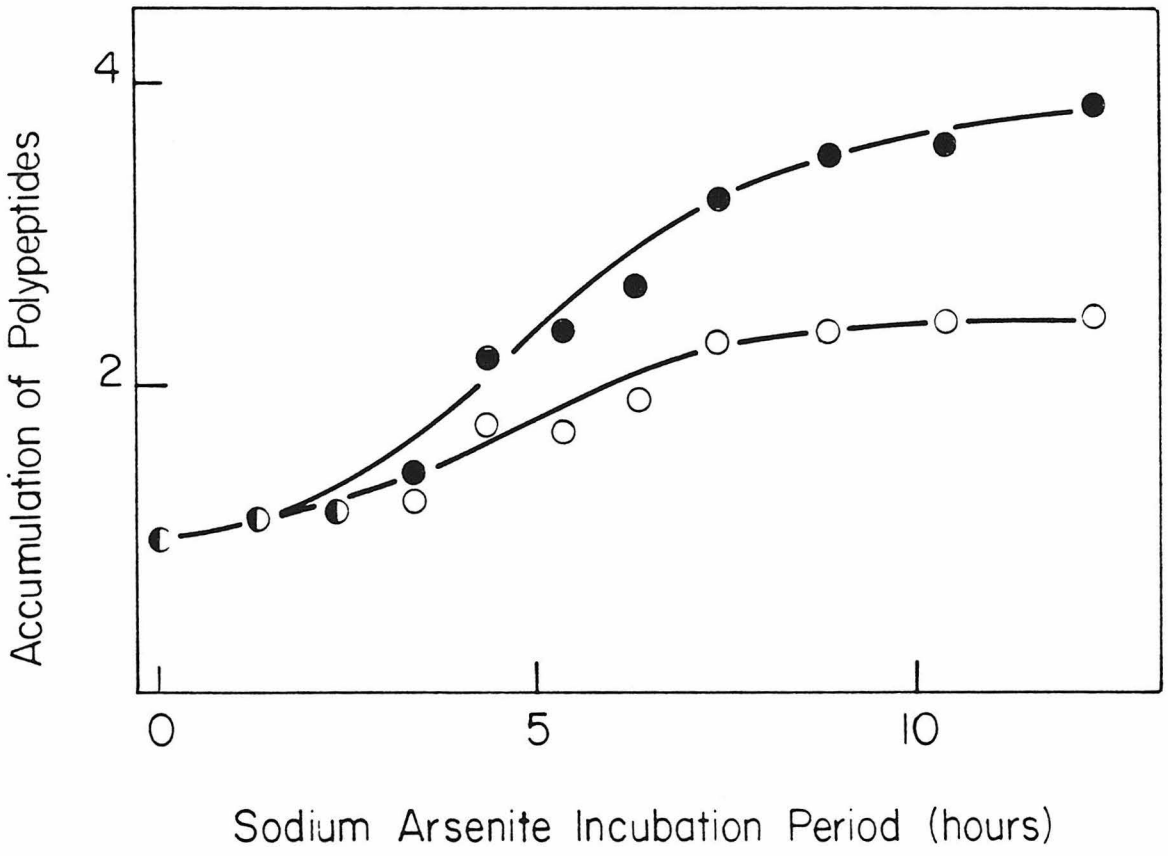

Accumulation of Polypeptides
4
2
0
5
10
Sodium Arsenite Incubation Period (hours)

Figure 5

Figure 6. Labeling with [methyl-^{3}H]-L-methionine. The cells were incubated with or without 25 μM sodium arsenite for four hours, then rinsed with methionine-free minimal essential medium with 100 μg/ml cycloheximide for 45 minutes. They were subsequently labeled with [methyl-^{3}H]-L-methionine for 0-6 hr. Thereafter, the gels were treated with ENHANCE, dried and fluorographed with preflashed film. (A) Control; (B) Arsenite-treated cells. Lane 1, 1 hr; b, 2 hr; c, 3 hr; d, 4 hr; e, 5 hr, f, 6 hr of labeling.

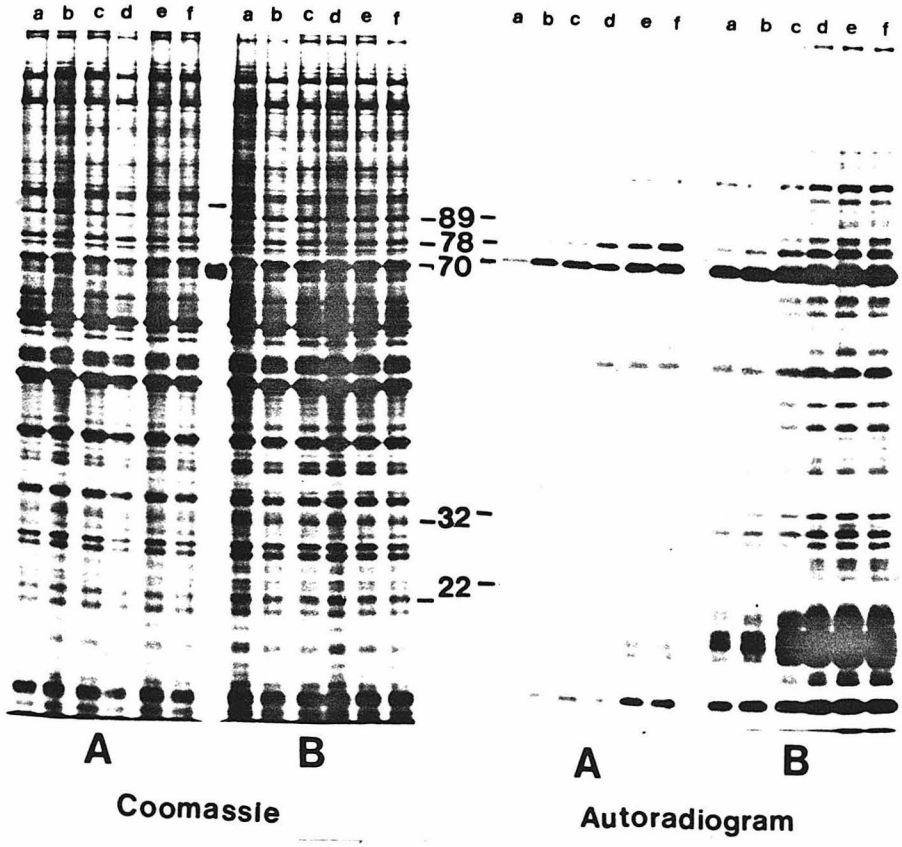

a b c d e f
a b c d e f
89
78
70
32
22
A
B
Coomassie
a b c d e f
a b c d e f
A
B
Autoradiogram

Figure 7. Relative [^{3}H]-radioactivity incorporation. The Coomassie blue-stained gels and autoradiograms corresponding to Figure 6 were scanned by a densitometer. The ratios of the areas of autoradiogram to Coomassie blue gel peaks corresponding to 78,000 and 70,000 dalton polypeptides were calculated. Open circle (O): 78,000, control; Closed circle (●): 78,000, arsenite; Open triangle (Δ): 70,000, control; Closed triangle (▲): 70,000, arsenite.

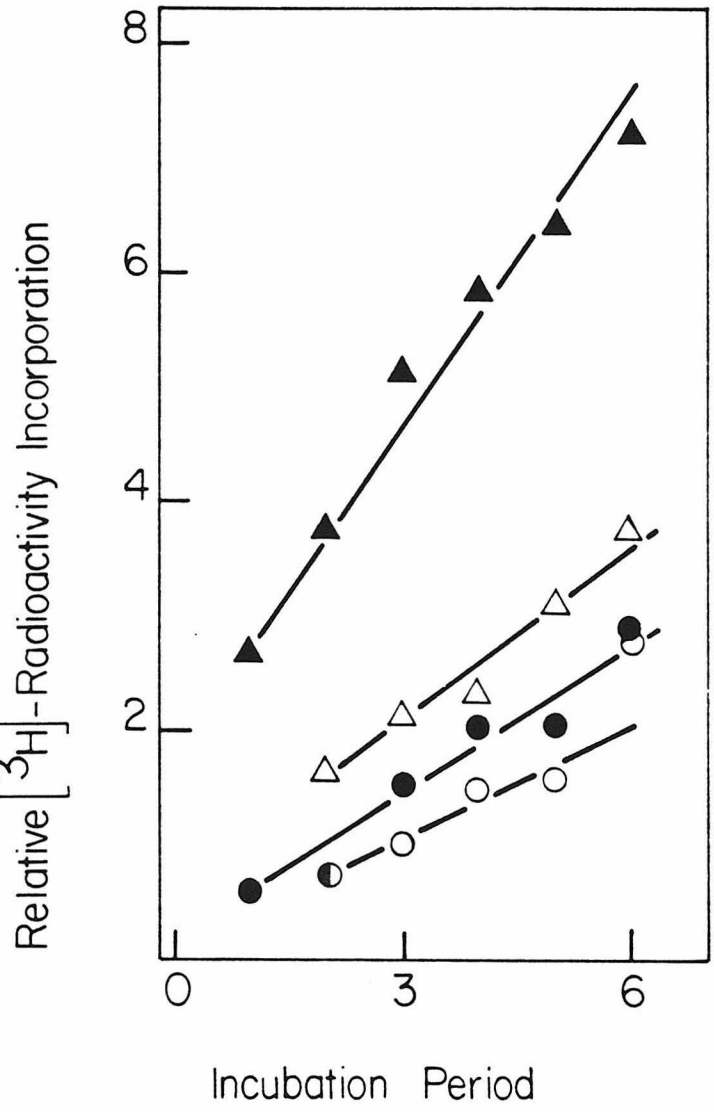

8
6
4
2
0
3
6
Relative [3H]-Radioactivity Incorporation
Incubation Period

Figure 7

Discussion

The induction of heat shock proteins by elevating growth temperature and chemical compounds has been found to be common to many eukaryotes. The number of inducible polypeptides varies among species. The 70,000 dalton polypeptide is one of the most prominently inducible species in chicken embryonic fibroblasts. This polypeptide also may be the most common inducible species in different tissues and cultured cells. Comparison of the pattern of proteins induced with arsenite or heat shock indicates that the 70,000 dalton polypeptide described here, the 73,000 dalton protein described by Johnston et al. (4), the p76 described by Kelley and Schlesinger (3) and the 68,000 dalton polypeptide (7, 17) are the same protein. The disparity in molecular weight may be due to differences in gel systems and molecular weight standards utilized. The polypeptides also appear to comigrate with the hsp70 of Drosophila (4). However, differences in hsp70 among different species exist. For example, the 70,000 dalton polypeptide of chicken embryonic fibroblasts is composed of two different isoelectric variants (4, 7), but only the acidic one (with pI 5.6) exists in some mammalian cultured cells such as BHK, 3T3, HeLa and WI38 (7, 26).

The induction kinetics of heat shock proteins of chicken embryonic fibroblasts by arsenite were presented here. The relative rate of synthesis of these polypeptides was calculated by assuming that the [^{35}S]-radioactivity associated with a heat shock protein band is solely contributed by that protein. Since different polypeptides with the same apparent molecular weights will comigrate, the rate of synthesis obtained here should represent the lower limit. However, the kinetic properties given in Figures 3 and 4 should still be valid, although the true maximal level of induction may be higher than I report here.

The quantitative results on induced heat shock protein synthesis as a function of arsenite concentration obtained (Figures 1 and 2) are similar to the qualitative results reported previously by Johnston et al. (4). After incubating the cells with

25 μM sodium arsenite for different time periods, I found that the maximal level of induction was reached between four and six hours of incubation, except for the 22,000 dalton polypeptide, which remains at a high level for at least six more hours (Figures 3 and 4). Qualitatively, this is similar to the results obtained by Johnston et al. (4) after the cells were incubated with 50 μM sodium sodium arsenite for various time periods. In addition, the induction kinetics of hsp70 of chicken fibroblasts is similar to the homologous polypeptide of 3T3 cells.

The hsp70 and 78,000 dalton arsenite-inducible polypeptides, previously reported as 83,000 dalton polypeptides (7, 17), are methylated. The methylation sites of hsp70 have been identified as lysyl and arginyl residues (17), while the hsp78 is methylated exclusively at lysyl residues. In addition, incubation of cells in [^{32}P]-phosphoric acid results in the incorporation of [^{32}P]-radioactivity into hsp78. This polypeptide may be the highly conserved glucose-regulated protein (27). It is also clear at present that hsp78 is not inducible by heat shock or sodium arsenite treatment in a number of mammalian cultured cells, including BHK, HeLa, 3T3 and human WI38/VA13 cells.

From the results of this work and some other reports (4, 6, 7), it is evident that the 70,000 and 89,000 dalton heat shock proteins exist in a reasonably large quantity of steady state in chicken embryonic fibroblasts. Judging from the densitometric traces of Coomassie blue-stained one-dimensional gels, the ratios of these two polypeptides to actin are approximately 1:25, which cannot be regarded as small, since actin is the most abundant polypeptide in cells. Hsp89 has been found to be associated with pp60^{src} (18), and it has been postulated that the complex formed by hsp89, pp60^{src} and/or other proteins may facilitate the transport of pp60^{src} to the plasma membrane (19).

The localization of hsp70 has been intensively studied in a number of tissues and cultured cells. In Drosophila, hsp70 is localized in the nuclei shortly after heat shock (20, 21), and probably is associated with the nuclar cytoskeleton (22). The

results of immunofluorescence suggest that hsp70 is one of the cytoplasmic constituents in chicken embryonic fibroblasts (23). The polypeptide is also associated with a Triton X-100/0.6 M KCl-insoluble pellet, which is mainly composed of intermediate filaments and nuclei (24). Hsp70 also appears to copurify with HeLa cell microtubules after two polymerization/depolymerization cycles but does not stoichiometrically copurify with tubulin through additional cycles and does not stimulate tubulin polymerization (25). Recently, it was found that the hsp70 may be associated with a membrane glycoprotein in 3T3 cells (26). These results suggest that the hsp70 may be either an important regulatory molecule (in structural complexes) with an as yet unidentified function, or the molecule may be capable of performing diverse functions by way of different post-translational modifications.

References

1. Tissières, A., Mitchell, H. K. and Tracy, V. M. (1974) J. Mol. Biol. **84**, 389-398.
2. McAlister, L. and Finkelstein, D. B. (1980) Biochem. Biophys. Res. Commun. **93**, 819-824.
3. Kelly, P. M. and Schlesinger, M. J. (1978) Cell **15**, 1277-1286.
4. Johnston, D., Oppermann, H., Jackson, J. and Levinson, W. (1980) J. Biol. Chem. **255**, 6975-6980.
5. Lindquist, S. (1980) Develop. Biol. **77**, 463-479.
6. Atkinson, B. G. (1981) J. Cell Biol. **89**, 666-673.
7. Wang, C., Gomer, R. H. and Lazarides, E. (1981) Proc. Nat. Acad. Sci. USA **78**, 3531-3535.
8. Currie, R. W. and White, F. P. (1981) Science **214**, 72-73.
9. Ashburner, M. and Bonner, J. J. (1979) Cell **17**, 241-254.
10. McKenzie, S. L., Henikoff, S. and Meselson, M. (1975) Proc. Nat. Acad. Sci. UA **72**, 1117-1121.
11. Storti, R. V., Scott, M. P., Rich, A. and Pardue, M. L. (1980) Cell **22**, 825-834.
12. DiDomenico, B. J., Bugaisky, G. E. and Lindquist, S. (1982) Proc. Nat. Acad. Sci. USA **79**, 6181-6185.
13. Mitchell, H. K., Moller, G., Petersen, N. S. and Lipps-Sarmeinto, L. (1979) Develop. Genet. **1**, 181-192.
14. Stocken, L. A. and Thompson. H. S. (1946) Biochem. J. **40**, 529-535.
15. Gard, D. L., Bell, P. B. and Lazarides, E. (1979) Proc. Nat. Acad. Sci. USA **76**, 3894-3898.
16. Laemmli, U. K. (1970) Nature **227**, 680-686.
17. Wang, C., Lazarides, E., O'Connor, C. M. and Clarke, S. (1982) J. Biol. Chem. **257**, 8356-8362.
18. Oppermann, H., Levinson, W. and Bishop, J. M. (1981) Proc. Nat. Acad. Sci. USA **78**, 1067-1071.

19. Courtneidge, S. A. and Bishop, J. M. (1982) Proc. Nat. Acad. Sci. USA **79**, 7117-7121.

20. Mitchell, H. K. and Lipps, L. S. (1975) Biochem. Genet. **13**, 585-602

21. Arrigo, A.-P., Fakan, S. and Tissières, A. (1980) Develop. Biol. **78**, 86-103.

22. Levinger, L. and Varshavsky, A. (1981) J. Cell Biol. **90**, 793-796.

23. Kelley, P. M. and Schlesinger, M. J. (1982) Mol. Cell. Biol. **2**, 267-274.

24. Wang, C., Asai, D. J. and Lazarides, E. (1980) Proc. Nat. Acad. Sci. USA **77**, 1541-1545.

25. Weatherbee, J. A., Luftig, R. B. and Weihing, R. R. (1980) Biochemistry **19**, 4116-4123.

26. Hughes, E. N. and August, J. T. (1982) Proc. Nat. Acad. Sci. USA **79**, 2305-2309.

27. Lee, A. S., Delegeane, A. and Scharff, D. (1981) Proc. Nat. Acad. Sci. UAA **78**, 4922-4925.

Chapter 3

Heat Shock Proteins Are Methylated in Avian and Mammalian Cells

Proc. Natl. Acad. Sci. USA
Vol. 78, No. 6, pp. 3531–3535, June 1981
Biochemistry

Heat shock proteins are methylated in avian and mammalian cells

(isoelectric focusing/NaDodSO$_4$/polyacrylamide gel electrophoresis/sodium arsenite/erythrocytes/skeletal muscle)

CHUNG WANG, RICHARD H. GOMER, AND ELIAS LAZARIDES*

Division of Biology, California Institute of Technology, Pasadena, California 91125

Communicated by James F. Bonner, March 19, 1981

ABSTRACT **Exposure of chicken cells grown in tissue culture to heat shock or sodium arsenite results in a dramatic increase in the synthesis of three major polypeptides with molecular weights of 83,000 (HSP 83), 68,000 (HSP 68; referred to here as "thermin"), and 25,000 (HSP 25). Incubation of BHK-21 or HeLa cells under the same conditions results in induction of HSP 68 and a 66,000-dalton polypeptide (HSP 66). Chicken thermin is resolved by isoelectric focusing into a major acidic and a more-basic component; mammalian thermin is resolved only into one major acidic component. HSP 83 and the acidic form of thermin are highly conserved in all avian and mammalian cells examined as judged by their electrophoretic mobilities, isoelectric points, and one-dimensional peptide maps. In addition, the acidic form of thermin is indistinguishable from a protein that copurifies with brain microtubules and that remains associated with the intermediate filament-enriched Triton/KCl cytoskeletons of cells grown in tissue culture. Thermin is also a component of skeletal myofibrils. HSP 83 and thermin are methylated in cells cultured under normal growth conditions. Induction of heat shock proteins by incubation of cells in the presence of sodium arsenite results in a marked methylation of the newly synthesized thermin. Under the same experimental conditions, no significant increase in methylation of the HSP 83 is observed. HSP 25 is not methylated in untreated cells or in cells treated with sodium arsenite. These results suggest that methylation of heat shock proteins may have an important role in regulating their function.**

Exposure of avian and mammalian cells grown in tissue culture to heat shock results in a dramatic change in their pattern of protein synthesis. This treatment induces the synthesis of three or four proteins with a general reduction in the overall pattern of protein synthesis. *In vivo* and *in vitro* experiments have indicated that this group of proteins is synthesized in relatively low levels in uninduced cells and that the increase in their synthesis after induction results from an increase in the amount of mRNA specific for these proteins. Various treatments including chelating agents, certain transition metal ions, thiol reagents, heat shock, and amino acid analogues can induce similar, if not identical, proteins in avian and mammalian cells (1–7). Many of the inducers capable of eliciting the heat shock response in vertebrate cells also elicit a similar response in *Drosophila melanogaster* cells, in which the phenomenon was originally described (reviewed in ref. 8). The proteins induced in vertebrate and invertebrate cells have closely similar electrophoretic mobilities, with a protein at 68,000–70,000 daltons being the most reproducibly and commonly elicited species. Despite the extensive work on the molecular details of the induction of these proteins as well as the detailed analysis of the structure of their corresponding genes in *Drosophila*, their natural inducer and their function remain unknown.

We have studied the heat shock proteins (HSPs) of mammalian and avian cells. We show that two of these proteins, HSP 83 and HSP 68 (thermin), are highly conserved and are methylated in cells grown under normal conditions. Induction of both proteins by sodium arsenite, a common inducer of HSP, is accompanied by specific methylation of the newly synthesized thermin. These observations point to the importance of methylation in the expression and function of the HSPs.

MATERIALS AND METHODS

Cells. Cultures of chicken embryonic fibroblasts, chicken embryonic myotubes, and baby hamster kidney (BHK-21) cells were prepared and grown as described (9). BHK-21 cells with a passage number between 59 and 64 were used in the experiments described. HeLa cells grown in spinner culture were obtained from G. Attardi.

Sodium Arsenite Treatment. Confluent plates of fibroblasts, myotubes, or BHK-21 cells were first rinsed three times with methionine-free minimal essential medium (Met-free ME medium) and then incubated in the same medium with 25 μM sodium arsenite for 4 hr. After the incubation [^{35}S]methionine (New England Nuclear) was added either to the medium directly to a final concentration of 3–5 μCi/ml (1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) or to a fresh medium in the absence of $NaAsO_2$ and incubation was continued for 45 min. Both methods gave the same result. The cells were then scraped off the plates and collected in a table-top clinical centrifuge at top speed (approximately 500 × *g*) for 2 min. The supernatant was decanted and the pellet was dissolved in isoelectric focusing sample buffer for two-dimensional gel electrophoresis (see below) (9).

HeLa cells were treated the same way. After the $NaAsO_2$ treatment the cells were collected at 500 × *g*, suspended in Met-free ME medium, and labeled with [^{35}S]methionine (3–5 μCi/ml) for 1 hr.

Heat Shock Treatment. Cells were placed for 1 hr inside a wet chamber floating on top of a water bath at 45°C. After a wash with Met-free ME medium, the cells were labeled with [^{35}S]methionine (3–5 μCi/ml) at 37°C for 1 hr. They were then prepared for two-dimensional gel electrophoresis as described above.

Labeling of Proteins with L-[*methyl*-^{3}H]Methionine. Confluent cultures (100-mm plates) of primary chicken embryonic myotubes were first incubated with 25 μM $NaAsO_2$ for 4 hr, rinsed with Met-free ME medium, and incubated for 40 min in Met-free ME medium in the presence of $NaAsO_2$. Cycloheximide and chloramphenicol were then added to the medium to final concentrations of 100 and 40 μg/ml, respectively, and incubation was continued for another 45 min, at which time L-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: HSP, heat shock protein; Met-free ME medium, methionine-free minimal essential medium.

* To whom correspondence should be addressed.

[*methyl*-^{3}H]methionine (8 μCi/ml; 80 Ci/mmol) was added. After an additional 2 hr the cells were washed with phosphate-buffered saline and then scraped into ice-cold phosphate-buffered saline containing cycloheximide (100 μg/ml), chloramphenicol (10 μg/ml), 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride. Under these conditions, incorporation of [^{35}S]methionine into proteins was inhibited by 98%. The cells were collected by centrifugation and dissolved either directly in two-dimensional gel electrophoresis sample buffer or first in 0.1% $NaDodSO_4$ and subsequently in the sample buffer (see below). For normal protein methylation, cells were treated as above without exposure to $NaAsO_2$. To examine whether sodium arsenite had any effect by itself on protein methylation, cells were treated as described above except that $NaAsO_2$ was added together with the protein synthesis inhibitors.

Two-Dimensional Isoelectric Focusing and $NaDodSO_4$/Polyacrylamide Gel Electrophoresis and Autoradiography. The analysis was performed as described (10, 11). After electrophoresis the gels were stained in 0.1% Coomassie brilliant blue in 47.5% ethanol/10% acetic acid and destained in 12.5% ethanol/5% acetic acid. The gels were dried and autoradiographed at −70°C on Kodak X-Omat R XR-5 film; the film was developed in Kodak x-ray developer. Gels containing proteins labeled with [^{35}S]methionine were dried for autoradiography directly after destaining. Gels containing proteins labeled with L-[*methyl*-^{3}H]methionine were treated with EN^{3}HANCE (New England Nuclear) for 1 hr after destaining and before drying for fluorography and were exposed on preflashed or normal x-ray film. To avoid proteolysis, cells were lysed in an equal volume of 0.1% $NaDodSO_4$ and immediately placed in a boiling water bath for 2 min. The samples were subsequently adjusted to 9 M in urea and 2% in Nonidet P-40 prior to isoelectric focusing.

Partial Purification of Thermin and HSP 25 From Adult Chicken Skeletal Muscle. Chicken breast muscle was excised and placed on ice. All further steps were carried out at 4°C. The muscle was homogenized in 2.5 vol of cold double-distilled water in a commercial blender at top speed for 30 sec and then centrifuged at 20,000 × *g* for 20 min. After the pellet was washed twice with distilled water the homogenate was passed through a layer of cheese cloth. The myofibrils were collected by centrifugation and were extracted with 0.6 M KCl/0.1 M sodium phosphate/1 mM $MgCl_2$/10 mM $Na_4P_2O_7$, pH. 6.5, overnight. The pellet was then reextracted with 0.6 M KI/10 mM $Na_2S_2O_3$/10 mM Tris·HCl/1 mM ethylene glycol bis(β-aminoethyl ether) *N*, *N*, *N'*, *N'*-tetraacetic acid, pH 7.5, for 8–10 hr. The supernatant was dialyzed exhaustively against distilled water, clarified by centrifugation (25,000 × *g*, 30 min), and then fractionated with solid $(NH_4)_2SO_4$. The precipitate collected between 40% and 80% ammonium sulfate saturation was resuspended in 10 mM sodium citrate buffer (pH 5.7) and dialyzed against the same buffer. The solution was centrifuged again to remove the precipitate and then applied to a DEAE-cellulose column (Whatman DE-52) that had been preequilibrated in the same buffer. The proteins were then eluted with 100 mM NaCl in the same buffer. Thermin and HSP 25 are major components of this fraction.

Purification of Thermin From Chicken Erythrocytes. Chicken blood was collected in an equal volume of ice-cold 155 mM choline chloride/5 mM Hepes, pH 7.4, containing 0.1 mg of heparin per ml. After centrifugation for 5 min at 1000 × *g*, the buffy coat and plasma were removed by aspiration. The erythrocyte pellet, minus a dark red layer at the bottom of the tube, was resuspended in choline chloride/Hepes and recentrifuged as above. The top layer of cells was again removed, and the process

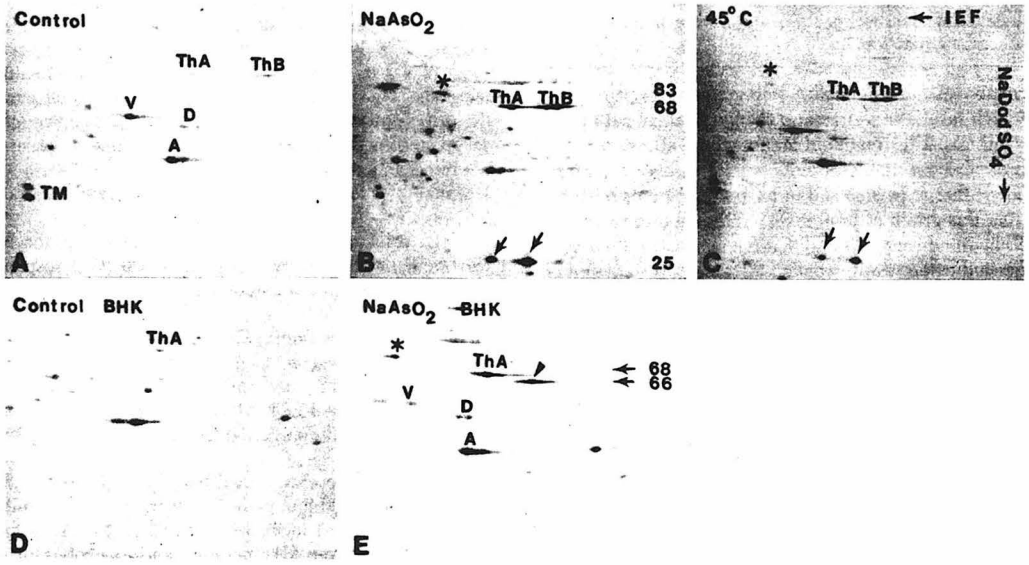


FIG. 1. Two-dimensional gel electrophoresis of avian and mammalian proteins induced by sodium arsenite and heat shock in cells grown in tissue culture showing thermins (Th A, Th B), HSP 25 (arrow), HSP 66 (▼), HSP 83 (*), actin (A), desmin (D), vimentin (V), and tropomyosin (TM). (*A*) Proteins extracted from a primary culture of chicken embryonic myotubes grown in normal medium and incubated with [^{35}S]methionine. (*B*) As *A* but incubated in the presence of 25 μM sodium arsenite for 4 hr before further incubation with [^{35}S]methionine. (*C*) Secondary cultures of 10-day chicken embryonic myotubes grown in normal medium and exposed to 45°C for 1 hr before labeling with [^{35}S]methionine. Equal amounts of protein were loaded on the gels for *A*, *B*, and *C*. (*D*) BHK-21 cells grown in normal growth medium and incubated with [^{35}S]methionine. (*E*) As *D* but incubated in the presence of 25 μM sodium arsenite for 4 hr before further incubation with [^{35}S]methionine. Equal amounts of protein were loaded on the gels for *D* and *E*. All gels were exposed for 3–4 days. IEF, isoelectric focusing; $NaDodSO_4$, $NaDodSO_4$/polyacrylamide gel electrophoresis.

was repeated six to eight times to remove most of the contaminating cells. The final erythrocyte pellet was lysed with 10–20 vol of 10 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/1 mM EDTA/1 mM ethylene glycol bis(β-aminoethyl ether) *N, N, N′, N′*-tetraacetic acid/1 mM *o*-phenanthroline/0.5 mM phenylmethylsulfonyl fluoride. The supernatant (100 ml) was lyophilized, resuspended in 30 ml of 10 mM sodium citrate (pH 5.7), and dialyzed against several changes of this buffer. Insoluble material was removed by centrifugation at 12,000 × *g*, and the supernatant was passed through a 30-ml column of DEAE-cellulose equilibrated in 10 mM sodium citrate (pH 5.7). Protein was eluted with a 120-ml gradient of 0.01–0.5 M sodium citrate (pH 5.7). Thermin eluted in two major fractions, between 10 and 30 mM and between 50 and 150 mM sodium citrate. A portion of the first fraction was dialyzed against water, lyophilized, and redissolved in urea sample buffer. Thermin exhibited higher purity in the first than the second fraction.

RESULTS

Induction of Thermin by Heat Shock and Sodium Arsenite. Exposure of primary cultures of chicken embryonic fibroblasts (a mixture of fibroblastic and myogenic cells) to sodium arsenite (25 μM, 4 hr) resulted in increased levels of three polypeptides with molecular weights of 83,000 (HSP 83), 68,000 (thermin), and 25,000 (HSP 25); the latter two polypeptides were the most prominent of the three (Fig. 1). The induction of all three polypeptides was concomitant with an increase in their synthesis as indicated by the incorporation of ^{35}S from [^{35}S]methionine into protein during and after treatment with arsenite. The same pattern of polypeptide induction was observed in chicken embryonic fibroblasts free of myogenic cells. However, fibroblast-free chicken myotubes incubated under the same conditions exhibited a specific increase in the levels and the *de novo* synthesis of thermin and HSP 25; while HSP 83 remains uninduced at the basal levels (see Fig. 3*D*). Induction of thermin and HSP 25 was maximal after 8 hr and persisted 12 hr after exposure to sodium arsenite (data not shown; see also ref. 7). Thermin was resolved into two major variants by two-dimensional analysis (isoelectric focusing and $NaDodSO_4$/polyacrylamide gel electrophoresis)—thermin A, pI 5.70; and thermin B, pI 5.95—each flanked by several minor acidic variants. Similarly, HSP 25 also was resolved into two major forms. A similar set of polypeptides was induced by sodium arsenite in BHK-21 (Fig. 1) and HeLa cells (not shown). However, a notable difference in these mammalian cells was the absence of the more basic components of thermin (thermin B) and the presence instead of HSP 66 with a pI of 5.90. In addition, the HSP 83 and HSP 25 polypeptides were not induced by arsenite under the same conditions in either BHK or HeLa cells. HSP 83 had the same pI and electrophoretic mobility in both avian and mammalian cells. Results similar to those obtained with arsenite were obtained after exposure of chicken embryo myotubes to heat shock (45°C, 1 hr) (Fig. 1*C*). Induction of thermin and HSP 25 was more efficient with arsenite than with heat shock under these conditions.

Methylation of Thermin and HSP 83. Incubation of chicken embryonic primary cells with [*methyl*-^3H]methionine in the presence of cytoplasmic and mitochondrial protein synthesis inhibitors resulted in methylation of a number of proteins, including HSP 83 and the two thermin variants A and B (Fig. 2*A*). A small amount of radioactivity was incorporated into a protein that had the same pI and electrophoretic mobility as the intermediate filament subunit desmin. Actin, tropomyosin, and vimentin did not exhibit any detectable incorporation of radioactivity. Exposure of cells to sodium arsenite 4 hr prior to the addition of protein synthesis inhibitors and [*methyl*-^3H]methionine resulted in a dramatic increase in methylation of all thermin variants but no discernible change in the level of methylation of HSP 83. HSP 25 remained unmethylated in both normal and arsenite-treated cells. In this experiment, we observed that arsenite-treated cells denatured in urea sample buffer prior to isoelectric focusing exhibited a number of methylated proteins, in addition to thermin, at the acidic side of the gel. These proteins were absent when the cells first were lysed in 0.1% $NaDodSO_4$ at 90°C prior to isoelectric focusing, suggesting that they are degradation products of thermin.

Exposure of cells to sodium arsenite and protein synthesis inhibitors simultaneously prior to the addition of [*methyl*-^3H]methionine resulted in inhibition of the increased methylation of thermin. However, both thermin and HSP 83 exhibited the level of methylation seen in untreated cells (Fig. 2*D*). These

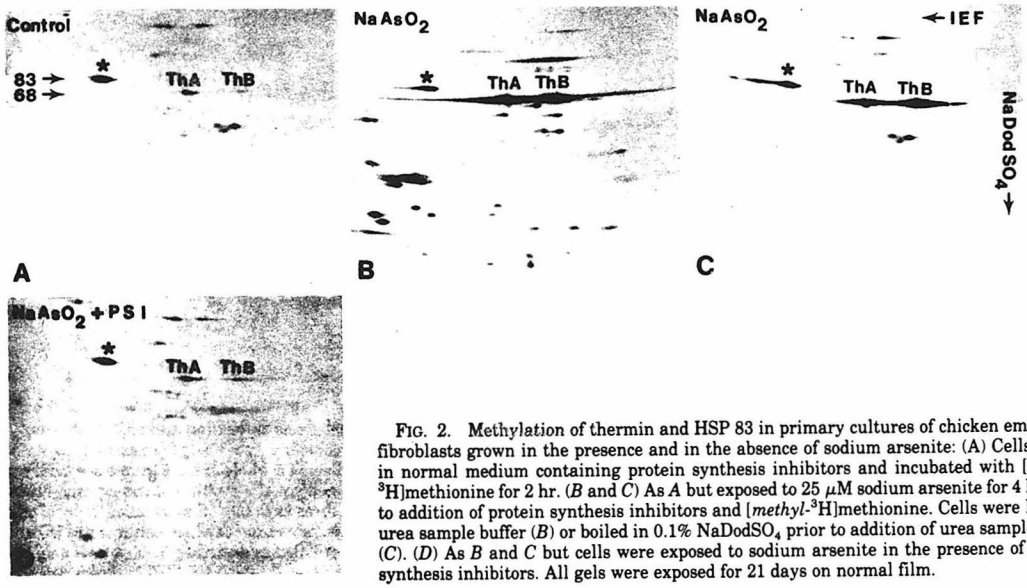


FIG. 2. Methylation of thermin and HSP 83 in primary cultures of chicken embryonic fibroblasts grown in the presence and in the absence of sodium arsenite: (A) Cells grown in normal medium containing protein synthesis inhibitors and incubated with [*methyl*-^3H]methionine for 2 hr. (*B* and *C*) As *A* but exposed to 25 μM sodium arsenite for 4 hr prior to addition of protein synthesis inhibitors and [*methyl*-^3H]methionine. Cells were lysed in urea sample buffer (*B*) or boiled in 0.1% $NaDodSO_4$ prior to addition of urea sample buffer (*C*). (*D*) As *B* and *C* but cells were exposed to sodium arsenite in the presence of protein synthesis inhibitors. All gels were exposed for 21 days on normal film.

results indicate that sodium arsenite does not activate preexisting protein methylases. Similarly, methylation of thermin and HSP 83 was observed in BHK-21 cells treated with sodium arsenite (not shown). In these cells the inducible HSP 66 remained unmethylated. Both in normal and arsenite-treated cultures grown in the presence of protein synthesis inhibitors, the incorporation of ^3H from [*methyl*-^3H]methionine was completely suppressed by the methylation inhibitors homocysteine thiolactone and adenosine (not shown). This result indicates that, under the experimental conditions used here, the incorporation of ^3H from [*methyl*-^3H]methionine into protein is mainly the result of methylation.

Partial Characterization of Thermin. Analysis of whole extracts from chicken skeletal muscle, erythrocytes, and primary cells grown in tissue culture shows the presence of two proteins, with pIs and electrophoretic mobilities indistinguishable from those of thermin A and B (12). Partially purified thermin from chicken skeletal muscle comigrated with thermin induced by arsenite in primary cultures of chicken embryonic myotubes (Fig. 3). In addition, a polypeptide with pI and electrophoretic mobility indistinguishable from those of HSP 25 copurified with chicken skeletal muscle thermin, indicating that HSP 25 is present also in substantial amounts in skeletal muscle. Thermin purified from chicken skeletal muscle and from chicken erythrocytes showed the copurification of the two major forms, A and B, each flanked by several minor acidic variants. Thermin purified from mammalian (porcine) skeletal muscle consisted of one major polypeptide whose electrophoretic mobility and pI were indistinguishable from those of avian thermin A. A protein with pI and electrophoretic mobility closely similar to those of HSP 66 also was found in this preparation.

Both avian and mammalian thermin A gave similar one-dimensional peptide maps, indicating that thermin A is highly conserved in different species (12). Comparative analysis of thermin A and B by one-dimensional peptide mapping reveals homologies and differences, suggesting that these two proteins may be evolutionally related (not shown). By coelectrophoresis and one-dimensional peptide mapping, thermin A is the same protein as the protein previously shown to copurify with brain microtubules and intermediate filaments but is distinct by pI from the 68,000-dalton neurofilament subunit protein (ref. 12; unpublished observations). Thermin also remained associated with the intermediate filament-rich cytoskeletons prepared in the presence of 0.5% Triton X-100 and 0.6 M KCl from a number of avian and mammalian cell types grown in tissue culture. Finally, both thermin A and B are components of chicken myofibrils as determined by two-dimensional analysis and immunofluorescence (12).

DISCUSSION

Distribution of HSPs in Avian and Mammalian Cells and Tissues. The inducibility of a small set of polypeptides called HSPs by higher-than-normal growth temperatures or chemical agents is common to many eukaryotic cell types (for review, see ref. 13). The number of induced HSP varies among species. In avian cells, HSP 25, HSP 68 (thermin), and HSP 83 are the most prominent induced polypeptides (3, 7). Thermin, as described here, exists as two major forms, acidic (A) and basic (B), with the same electrophoretic mobility but different pIs. One-dimensional peptide mapping has indicated that thermin A and B are not identical polypeptides. This difference is further strengthened by the fact that both forms of thermin are translated in a reticulocyte cell-free system using poly(A)$^+$mRNA obtained from chicken embryonic fibroblasts treated with so-

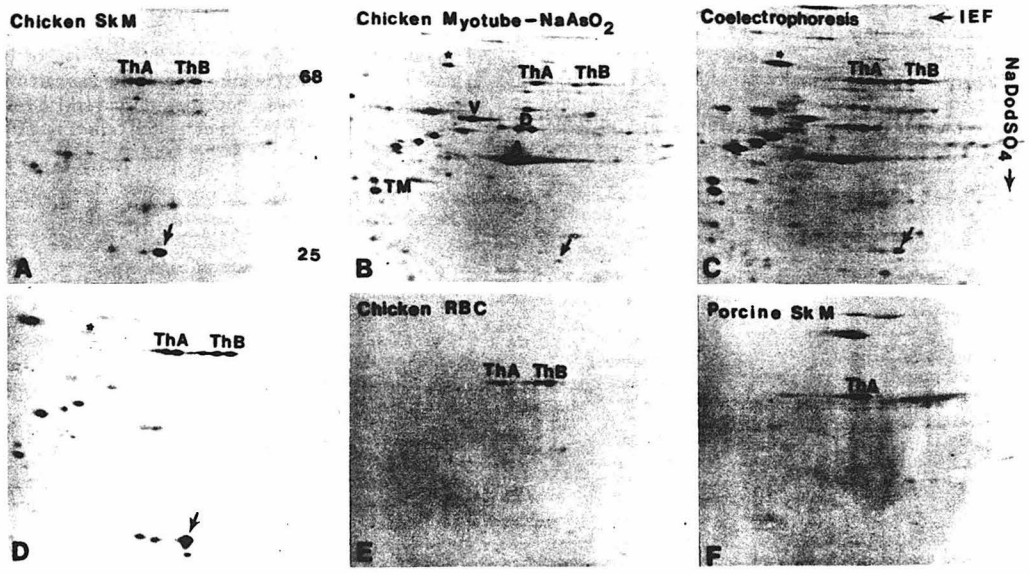


FIG. 3. Two-dimensional analysis of partially purified thermin. (*A*, *B*, *C*, and *D*) Coelectrophoresis of thermin and HSP 25 partially purified from chicken breast muscle, with thermin and HSP 25 of secondary cultures of chicken embryonic myotubes. (*A*) Coomassie blue-stained gel of partially purified thermin and HSP 25 from chicken breast muscle. (*B*) Coomassie blue-stained gel of a whole extract from a secondary culture of chicken embryonic myotubes incubated with sodium arsenite for 4 hr. (*C*) Coomassie blue-stained gel of a mixture of the chicken breast muscle preparation of thermin and HSP 25 in *A* and the whole extract from the secondary myotube cultures in *B*. (*D*) ^{35}S autoradiography of chicken embryonic myotubes in *C*. (*E* and *F*) Coomassie blue-stained gels showing the partially purified thermins from chicken eyrthrocytes (*E*) and porcine skeletal muscle (*F*). Note the absence of thermin B in mammalian tissue. Porcine thermin was purified as described in *Materials and Methods* for chicken skeletal muscle thermin.

dium arsenite (7) or from chicken smooth and skeletal muscle (14). In mammalian cells, however, the B form of thermin is absent and HSP 66 is coinduced with thermin A. A further difference between avian and mammalian cells is the failure of arsenite to induce HSP 83 and HSP 25 in the latter cells. Whether the proteins described above constitute the complete set of inducible HSPs in avian and mammalian cells is not yet known.

Comparison of the pattern of proteins induced by arsenite in chicken embryonic fibroblasts (7) and in the avian and mammalian cells and tissues (studied here) indicates that the 68,000-dalton protein (thermin) described here and the 73,000-dalton protein described by Johnston *et al.* are the same protein. Similarly, the HSP 25 and HSP 83 we observed correspond to the 27,000- and 98,000-dalton proteins described by them. The differences in molecular weight of the protein induced might be due to differences in the $NaDodSO_4$ electrophoretic systems used. Furthermore, the 70,000-dalton protein induced by heat shock in *Drosophila* cells is resolved by two-dimensional analysis into two main components (13), analogous to the A and B components of thermin in avian cells. All these results indicate that thermin is a highly conserved polypeptide in vertebrate and invertebrate cells. The genes of the 70,000-dalton polypeptide from *Drosophila* have been cloned and the primary amino acid sequence of the protein has been determined (15). Cloning and sequence analysis of thermin from avian and mammalian cells will establish unambiguously the evolutionary conservation of these proteins.

In most, if not all, cases it has been assumed that the HSPs exist in low levels in the cell (see ref. 13 for review). In this paper we have shown that thermin exists in substantial amounts in avian and mammalian cells and in particular avian erythrocytes and avian and mammalian skeletal muscle. We have also shown that HSP 25 exists in substantial amounts in skeletal muscle. We have previously described (12) a highly conserved 68,000-dalton polypeptide that copurifies with intermediate filaments from rat spinal cord and neurotubules isolated from brain by two cycles of polymerization/depolymerization but is distinct by pI from the 68,000- to 70,000-dalton neurofilament subunit protein (unpublished data). A 68,000- and a 66,000-dalton protein also appear to copurify with HeLa microtubules after two polymerization/depolymerization cycles but do not stoichiometrically copurify with tubulin through additional cycles of assembly/disassembly and do not stimulate tubulin polymerization (16). Coelectrophoresis of these proteins with mammalian thermin and HSP 66 indicates that these proteins are indistinguishable. A 68,000-dalton polypeptide is also a component of intermediate filament-enriched cytoskeletons prepared from a number of avian or mammalian cell types grown in tissue culture as well as a component of chicken skeletal myofibrils. By one-dimensional peptide mapping, pI, and electrophoretic mobility, thermin A and the 68,000-dalton polypeptide are the same protein (12). In particular, both thermin A and thermin B are components of chicken skeletal myofibrils. The association of thermin with myofibrils, microtubules, and intermediate filaments is suggestive of a cytoskeletal role for this protein.

Methylation of HSP. Under normal growth conditions, thermins A and B and HSP 83 of avian cells are methylated but HSP 25 is not. Induction of the synthesis of these proteins results in a dramatic increase in the methylation of thermins A and B. The level of methylation of HSP 83 in induced cells is similar to that found in uninduced cells. Inhibition of the induced methylation of thermin when translation of the induced proteins is inhibited argues that the methylation of thermins A and B after arsenite induction is specific for the newly synthesized polypeptides. This argument is strengthened by the observation that, under these conditions, the normal level of methylation of thermin is not inhibited. On the other hand, the high levels of methylated HSP 83 in untreated cells and the rather low induction of this protein in cells treated with arsenite may obscure the detection of the methylation of newly synthesized HSP 83. The function of protein methylation in eukaryotic cells remains largely unknown (for review, see ref. 17). In bacterial chemotaxis, methylation and demethylation of glutamyl residues of a small number of proteins has been shown to be a key factor in the process of adaptation to new chemical environments. Each methyl acceptor protein is resolved into multiple species by high-resolution one- and two-dimensional gel electrophoresis, and each species differs in the number of methyl groups per polypeptide chain (18, 19). Thermin and HSP 83 are also resolved into multiple isoelectric variants, all of which appear to be methylated. Whether this heterogeneity is due to different degrees or sites of methylation remains to be elucidated. Nevertheless, even though many aspects of this system are still unknown, the methylation of HSPs provides us with a rapidly inducible methylation system in eukaryotic cells and thus an experimental avenue for determining the function of these proteins and the regulation of their induction.

We thank Dr. Ignacio Sandoval and Dr. David L. Gard for their critical reading of the manuscript and Dr. Bruce L. Granger for providing us with the erythrocyte extracts used in the purification of thermin. We thank Dr. J. A. Weatherbee for providing us with a sample of purified 68,000-dalton protein from HeLa cells to compare with the proteins described here. This work was supported by grants from the National Institutes of Health (GM-06965), National Science Foundation, and Muscular Dystrophy Association of America and a Biomedical Research Support Grant to the Division of Biology. E.L. is a recipient of a Research Career Development Award. R.H.G. was also supported by a National Research Service Award from the National Institute of General Medical Sciences.

1. Levinson, W., Oppermann, H. & Jackson, J. (1978) *Biochim. Biophys. Acta* **518**, 401–412.
2. Levinson, W., Mikelens, P., Oppermann, H. & Jackson, J. (1978) *Biochim. Biophys. Acta* **519**, 65–75.
3. Kelley, P. M. & Schlesinger, M. J. (1978) *Cell* **15**, 1277–1286.
4. Hightower, L. E. & Smith, J. (1978) in *Negative Strand Viruses and Host Cell*, ed. Mahy, B. (Academic, New York), pp. 395–405.
5. Levinson, W., Oppermann, H. & Jackson, J. (1980) *Biochim. Biophys. Acta* **606**, 170–180.
6. Kelley, P. M., Aliperti, G. & Schlesinger, M. J. (1980) *J. Biol. Chem.* **255**, 3230–3233.
7. Johnston, D., Oppermann, H., Jackson, J. & Levinson, W. (1980) *J. Biol. Chem.* **255**, 6975–6980.
8. Ashburner, M. & Bonner, J. J. (1979) *Cell* **17**, 241–254.
9. Gard, D. L., Bell, P. B. & Lazarides, E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3894–3898.
10. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
11. Hubbard, B. D. & Lazarides, E. (1979) *J. Cell Biol.* **80**, 166–182.
12. Wang, C., Asai, D. J. & Lazarides, E. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1541–1545.
13. Storti, R. V., Scott, M. P., Rich, A. & Pardue, M. L. (1980) *Cell* **22**, 825–834.
14. O'Connor, C. M., Asai, D. J., Flytzanis, C. N. & Lazarides, E. (1981) *Mol. Cell. Biol.* **1**, 303–309.
15. Ingolia, T. D., Craig, E. A. & McCarthy, B. J. (1980) *Cell* **21**, 669–679.
16. Weatherbee, J. A., Luftig, R. B. & Weihing, R. R. (1980) *Biochemistry* **19**, 4116–4123.
17. Paik, W. K. & Kim, S. (1980) *Protein Methylation* (Wiley, New York).
18. Springer, M. S., Boy, M. F. & Adler, J. (1979) *Nature (London)* **280**, 279–284.
19. Engström, P. & Hazelbauer, G. L. (1980) *Cell* **20**, 165–171.

Chapter 4

Methylation of Chicken Fibroblast Heat Shock Proteins at Lysyl and Arginyl Residues

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 14, Issue of July 25, pp. 8356-8362, 1982
Printed in U.S.A.

50

Methylation of Chicken Fibroblast Heat Shock Proteins at Lysyl and Arginyl Residues*

(Received for publication, January 4, 1982)

Chung Wang and Elias Lazarides‡

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

Clare M. O'Connor§ and Steven Clarke¶

From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024

Exposure of chicken embryonic fibroblasts to 25-50 μM sodium arsenite or to heat shock results in the elevated synthesis of several polypeptides which can be resolved by two-dimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis. Three of these polypeptides (one with a molecular weight of 83,000 and two with a molecular weight of 68,000 but different isoelectric points) are methylated *in vivo* (Wang, C., Gomer, R. H., and Lazarides, E. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 3531-3535).

In this work, heat shock proteins were labeled *in vivo* by incubating cultured fibroblasts in L-[*methyl*-^{3}H]methionine. Using a combination of amino acid analysis and thin layer chromatography, we show that lysyl residues are the predominant methylation site(s) of the 83,000 polypeptide and that both lysyl and arginyl residues are methylated in the 68,000 polypeptides A and B. In all cases, the major methylated lysine species is ε-*N*-trimethyl lysine. The major arginine species is N^G-monomethyl arginine.

The stoichiometry of the methylation was determined in similar experiments using proteins isolated from cells cultured in the presence of both L-[^{3}H]leucine and L-[*methyl*-^{3}H]-L-methionine. It is estimated that each of the three polypeptides contain one to three methylated lysine residues per molecule. In addition, there are one to two N^G-monomethyl arginines per molecule of both 68,000 polypeptide A and 68,000 polypeptide B.

Cells labeled with [*methyl*-^{3}H]methionine in the presence of protein synthesis inhibitors incorporate less than 1% of the methyl groups incorporated by cells labeled in the absence of inhibitors. We conclude that methylation at basic amino acid residues occurs during or very soon after translation of the polypeptides. In addition, there is little turnover of the methyl groups *in vivo*. Pulse-chase experiments provide a minimum estimate of the average half-life of methyl groups of 65 h.

Brief exposure of *Drosophila* tissue or cultured cells to elevated temperature induces a profound change in their pattern of protein synthesis. The translation of normal cellular protein is suppressed, and the synthesis of a new class of proteins, known as "heat shock proteins," is greatly enhanced (Ref. 1; for a review, see Ref. 2). The major heat shock proteins fall into several size classes with molecular weights of 20,000 to 35,000, 65,000 to 70,000, and 80,000 to 100,000. Since shocked cells are less susceptible to the effect of further heat treatment, it has been suggested that the function of these proteins is to confer resistance to further lethal heat shock (3). A similar set of polypeptides is induced when many kinds of avian and mammalian cells are exposed to heat shock or incubated with various other agents, including chelators, certain transition metal ions, sodium arsenite, as well as amino acid analogues (4-7). We have presented preliminary evidence that three of these species, the 83K[1] polypeptide and the 68K polypeptides A and B, are methylated both in normal growing cells and in cells treated with sodium arsenite (7). All three polypeptides incorporate radioactivity from [*methyl*-^{3}H]methionine, but not from [^{35}S]methionine, under conditions in which protein synthesis is inhibited. Since incorporation is blocked by inhibitors of *S*-adenosyl-L-methionine-dependent methyl transferases, we postulated that all three polypeptides contained methylated amino acids. However, the chemical nature of the methylated amino acid residues was not identified, and the stoichiometries of the methylation reactions were not determined.

Post-translational methylation of several amino acid residues in proteins has been well documented, and in some cases, a well defined function has been suggested. For example, the formation and hydrolysis of glumatic acid γ-methyl esters on amino acid chemoreceptors is thought to be involved in the adaptation response of bacterial chemotaxis in *Escherichia coli* and *Salmonella typhimurium* (8, 9). Aspartic acid has been identified as an *in vivo* site of reversible methylation in human erythrocyte cytoskeletal and membrane proteins (10, 11), but the function of these reactions has not yet been established. Basic amino acid residues, such as histidine, lysine, and arginine can also be methylated *in vivo* (for a review, see Ref. 12), but in most cases the methylation is irreversible. The most well studied case involves the methylation of fungal

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of grants from the National Institute of Health (GM-06965), the National Science Foundation, and the Muscular Dystrophy Association of America in support of this work. Recipient of a Research Career Development Award from the National Institutes of Health.

§ Supported by an Advanced Research Fellowship of the American Heart Association, Greater Los Angeles Affiliate.

¶ Recipient of Grant GM-26020 from the United States Public Health Service and a grant from the American Heart Association (with funds contributed in part by the Greater Los Angeles Affiliate) in support of this work.

[1] The abbreviations used are: 83K, 68K, etc., 83,000-dalton, 68,000-dalton, etc.; SDS, sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

cytochrome *c* which may facilitate the binding of cytochrome *c* to mitochondria (13–15).

Because methylation may play a functional role in the heat shock response (7), the chemistry and reversibility of the methylation reaction may be important. In this communication we report that the sodium arsenite-inducible 83K polypeptide as well as 68K polypeptides A and B are methylated stoichiometrically at one or more lysine residue(s), while the 68K polypeptides A and B are also methylated stoichiometrically at arginyl residues. Methylation reactions occur during or soon after polypeptide synthesis and appear to be irreversible.

EXPERIMENTAL PROCEDURES

Materials—Minimal essential medium, antibiotics, and horse serum were obtained from Gibco Laboratories. L-[*Methyl*-^{3}H]methionine (80 Ci/mmol) and L-[3,4,5-^{3}H]leucine (122 Ci/mmol) were New England Nuclear products. Cycloheximide, chloramphenicol, and N^G,N'^G-dimethyl arginine were from Sigma. ϵ-*N*-Monomethyl lysine and ϵ-*N*-dimethyl lysine were obtained from Vega Biochemicals. ϵ-*N*-Trimethyl lysine oxalate and N^G-monomethyl arginine were purchased from Calbiochem. Trypsin III-TPCK was from Worthington.

Cell Cultures—Chicken embryonic fibroblasts were prepared as described (16). Fibroblasts were grown in Eagle's minimal essential medium supplemented with 10% horse serum, 2% chicken embryo extract, and antibiotics. Primary muscle cultures were first plated on collagenized Falcon dishes in minimal essential medium with 15% horse serum, 5% embryo extract, and antibiotics. They were then maintained in the same medium as fibroblast cultures.

Two-dimensional Isoelectric Focusing/SDS Polyacrylamide Gel Electrophoresis—Two-dimensional gel electrophoresis was carried out by the method of O'Farrell (17) with modifications as previously described (18). Cell pellets dissolved in a small volume of 0.1% SDS were placed briefly in a boiling water bath and then diluted in isoelectric focusing sample solution (9 M urea, 2% NP40, 1% 2-mercaptoethanol). After electrophoresis, the gels were stained overnight in 0.1% Coomassie brilliant blue, 47.5% ethanol, 10% acetic acid and destained in 12.5% ethanol and 5% acetic acid.

Sample Preparation for Analysis of Methylated Amino Acids—Confluent 100-mm plates containing 5–10 × 10^6 chicken embryonic fibroblasts or primary muscle culture cells were first incubated in 25 or 50 μM sodium arsenite for 4 h at 37 °C to induce the heat shock proteins. The plates were then rinsed three times in methionine-free minimal essential medium, and after a 30-min incubation in the same medium at 37 °C, cycloheximide, chloramphenicol, and sodium arsenite were added to final concentrations of 100 μg/ml, 40 μg/ml, and 25 μM, respectively. The incubation was allowed to proceed for 45 min at 37 °C, and then L-[*methyl*-^{3}H]methionine was added to a final concentration of 0.125 μM (10 μCi/ml) in a total volume of 3 ml. Incubation with L-[*methyl*-^{3}H]methionine was continued for 4 h.

Alternatively, after sodium arsenite treatment, the cells were washed three times with methionine- and leucine-free minimal essential medium, and then L-[*methyl*-^{3}H]methionine, L-[3,4,5-^{3}H]leucine, and sodium arsenite were added to final concentrations of 0.375 μM (30 μCi/ml), 0.25 μM (30 μCi/ml), and 25 μM, respectively, in a total volume of 3 ml. The incubation was allowed to continue for 4 h. The cells were then washed with cold phosphate-buffered saline and were collected in the same buffer with a rubber policeman. After centrifugation, the cell pellet (approximately 10 μl in volume) was mixed with 20 μl of 0.1% SDS, heated at 100 °C for 1 min, and then dissolved in isoelectric focusing sample buffer for two-dimensional gel electrophoresis. After staining and destaining, Coomassie-stained spots corresponding to the 83K polypeptide and 68K polypeptides A and B were cut out of acrylamide gels and rinsed briefly with double distilled water. The polypeptides were eluted directly from the acrylamide slices with 0.1% SDS in 100 mM NH_4HCO_3 at room temperature overnight. Alternatively, the slices were washed extensively with 10% ethanol, dehydrated with 95% ethanol, lyophilized, and the dried gel slices were digested with trypsin as described (19). After lyophilization, the eluted proteins or peptides were hydrolyzed with 6 N HCl at 108 °C for 24 h in evacuated sealed tubes. The HCl was then removed under reduced pressure at 50 °C, and the methylated amino acids in the residue were identified either by ion exchange column chromatography or by thin layer chromatography.

Ion Exchange Chromatography—The dried amino acid residue was resuspended in a small volume of 0.2 N sodium citrate buffer at pH 2.2. Radioactive samples, containing several thousand counts per min, were mixed with amino acid standards (approximately 0.4 μmol of each amino acid) and were applied to a column (0.9 × 30 cm) of sulfonated 8% cross-linked polystyrene amino acid analysis resin (Durrum type DC-6A) which was equilibrated at 28 °C with sodium citrate buffer, 0.35 N Na^+, pH 5.69. The column was eluted with the same buffer at 28 °C at a flow rate of 30 ml/h. Alternatively, the samples were applied to a short column (0.9 × 10 cm) of Beckman PA-35 amino acid analysis resin which was equilibrated with sodium citrate buffer (0.35 N Na^+, pH 5.28) and then eluted with the same buffer at 56 °C at a flow rate of 67 ml/h. Fractions were analyzed for radioactivity by liquid scintillation counting in 7 to 10 volumes of AquaMix (West Chem Products) and for amino acids by reaction with ninhydrin as described (20).

The peaks eluted from the resin were desalted prior to thin layer chromatography by passing the sample through a small column of Dowex AG 50W-X4 (Bio-Rad Laboratories, NH_4^+ form) which had been prewashed with double distilled water. After washing the column extensively with double distilled water, the ^{3}H-radioactivity was eluted with 3 N NH_4OH and was lyophilized. Alternatively, the method of Dreze *et al.* was employed (21). Briefly, the sample was applied to a Dowex AG 50W-X4 column (H^+ form), and the column was washed with 20 volumes of 0.5 N HCl. Methylated basic amino acids were eluted with 4 N HCl and lyophilized.

Thin Layer Chromatography—The salt-free dry amino acid residue was dissolved in a small volume of double distilled water, spotted on cellulose than layer chromatogram sheets (Eastman), and allowed to develop in pyridine/acetone/NH_4OH/H_2O (15:9:1.5:6). A set of amino acid standards was chromatographed as a mixture with the radioactive samples or in parallel with the samples. Amino acids were detected by spraying the sheets with 1% ninhydrin in acetone and letting them develop at room temperature for 30 min. Sections of the cellulose thin layer (0.5–0.8 cm) were removed from the plates, incubated in 0.5 to 1.0 ml of NCS tissue solubilizer (Amersham Corp.) containing 10% water, and counted in 5 ml of OCS scintillation fluid (Amersham Corp.).

RESULTS

Stability of Methylated Residues on Heat Shock Proteins—In a previous study we demonstrated that radioactivity from L-[*methyl*-^{3}H]methionine was readily incorporated by cells into heat shock proteins under conditions where protein synthesis was inhibited by cycloheximide and chloramphenicol (7). When similarly labeled proteins were hydrolyzed for 24 h at 108 °C in 6 M HCl *in vacuo* and the volatile components were removed by flash evaporation, 80–92% of the radioactivity was recovered in the residue fraction. Under these conditions, protein carboxyl [^{3}H]methyl esters hydrolyze to form volatile [^{3}H]methanol. It is thus possible to place an upper limit of 8–20% on the contribution of [^{3}H]methylated aspartyl (11) and glutamyl (22, 23) residues to this preparation. However, when fibroblast proteins were electrophoresed at pH 2.4 (10), conditions under which methyl ester hydrolysis does not occur, no base-labile radioactivity was found associated with the heat shock polypeptides, suggesting that methyl esters are not present. Since the majority of the radioactivity was acid-stable, we investigated the incorporation of the radioactivity into basic amino acids.

Identification of Methylated Amino Acids After Acid Hydrolysis—Fig. 1 shows the elution profiles of the hydrolyzed 68K polypeptides A and B and the 83K polypeptide from an ion exchange resin capable of resolving methylated basic amino acids. Three peaks (I, II, III) of ^{3}H-radioactivity were detected in the 68K polypeptides A and B, while two peaks were observed with the 83K polypeptides. Peak I coeluted with the acidic and neutral amino acid fraction and may include some L-[*methyl*-^{3}H]methionine which was incorporated into protein. Peak II was located between the elution positions of lysine and histidine, while peak III eluted shortly before arginine. Standards of mono-, di-, and tri-ϵ-*N*-methylated lysines applied to the column with the 68K polypeptide

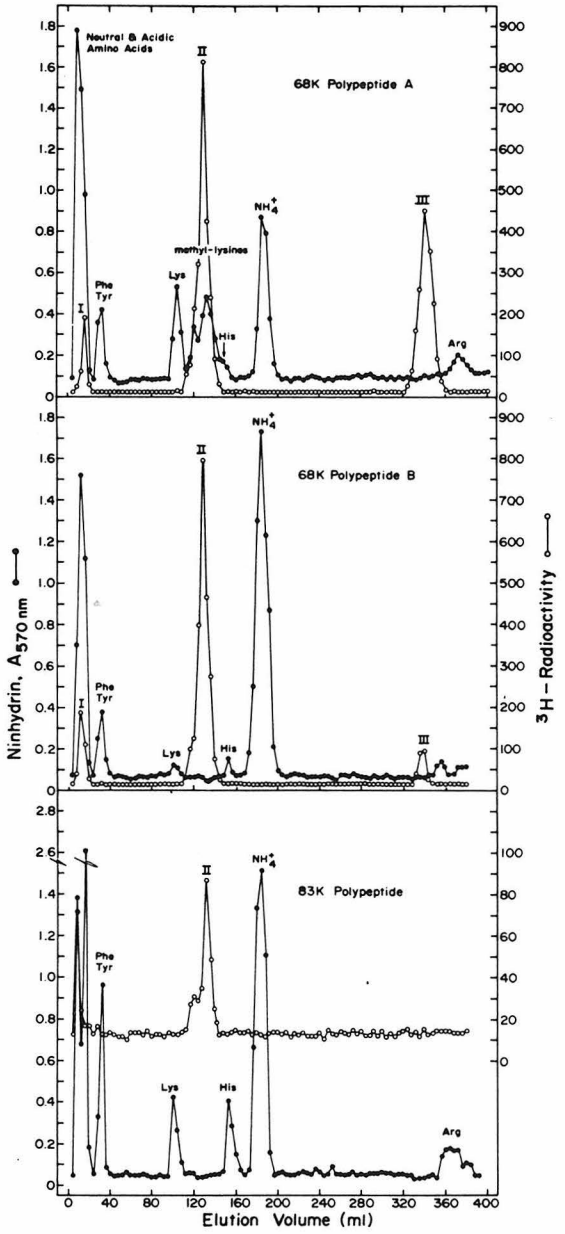


FIG. 1. **Ion exchange chromatography of hydrolyzed [^{3}H] methylated chicken fibroblast heat shock proteins.** Acid hydrolysates were prepared from trypsin-eluted polypeptides which were labeled in the presence of protein synthesis inhibitors and chromatographed on a 30-cm column as described under "Experimental Procedures." Fractions (6–10 min; 3–5 ml) were collected; an aliquot of 1.0 ml was analyzed for radioactivity, and an aliquot of 0.4 ml was analyzed for amino acid content by reaction with ninhydrin. *Top*, hydrolysate of the 68K polypeptide A was mixed with amino acid standards including ϵ-mono-, di-, and trimethyl lysines. *Middle*, hydrolysate of 68K polypeptide B without additional amino acid standards. The amount of radioactivity in the peak III region was variable but always less than 5% of the total radioactivity. This material may originate from a small amount of contaminating 68K polypeptide A in this preparation. *Bottom*, hydrolysates of the 83K polypeptide were chromatographed without additional amino acid standards.

A hydrolysate coeluted with peak II, suggesting that the radioactivity in peak II was in the form of methylated lysines. Comparison of the elution profile of ^{3}H-radioactivity obtained here with that previously shown to occur with methylated amino acids under nearly identical conditions (24, 25) suggests that the peak III may be either N^G,N'^G-dimethyl arginine or N^G-monomethyl arginine.

The total recovery into identifiable peaks of the radioactivity applied to the column varied from 50 to 90%. Similarly, the proportions of ^{3}H-radioactivity associated with each peak varied slightly in different preparations. However, peak I has never exceeded 15% of the total counts per min applied to the column. Peak II accounts for 50% of the total counts per min for 68K polypeptide A, 50 to 75% for the 68K polypeptide B, and 50–75% for the 83K polypeptide. Approximately 30% of

TABLE I

Recovery of ^{3}H-methylated residues on amino acid analysis

Peak	^{3}H-radioactivity applied to column		
	68K polypeptide A	68K polypeptide B	83K polypeptide
		%	
I (unidentified neutral and acidic amino acids)	5	6	13
II (methyl lysines)	49	47	54
III (N^G-monomethyl arginine)	33	4	0
Total recovery	87	57	67

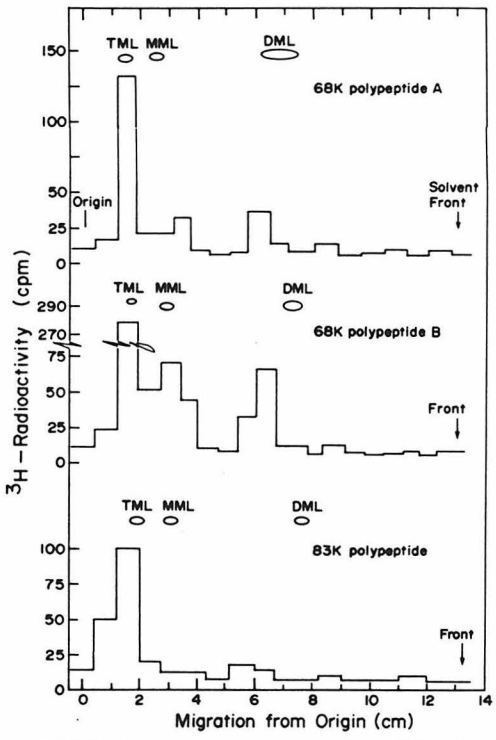


FIG. 2. **Thin layer chromatography of methylated amino acids.** The acid hydrolysates of (*top*) 68K polypeptide A, (*middle*) 68K polypeptide B, and (*bottom*) 83K polypeptide were applied to cellulose thin layer chromatogram sheets and developed in pyridine/acetone/NH_4OH/H_2O (15:9:1.5:6). ϵ-*N*-Monomethyl lysine (*MML*), ϵ-*N*-dimethyl lysine (*DML*), and ϵ-*N*-trimethyl lysine (*TML*) were run in parallel.

the total counts per min of 68K polypeptide A and less than 5% of 68K polypeptide B were recovered in peak III. Table I summarizes the calculated percentages of the various peaks corresponding to the elution profiles depicted in Fig. 1 for these polypeptides.

Confirmation of Basic Amino Acid Assignments by Thin Layer Chromatography—When the hydrolysates of the three proteins were analyzed by thin layer chromatography in 1-butanol/acetic acid/H_2O (4:1:1), a solvent system which provides good resolution of unmodified amino acids but poor resolution of the various methylated lysines and arginines, virtually all of the ^3H-radioactivity in each hydrolysate was recovered as a peak which comigrated with the methylated lysines and/or arginines (data not shown). A second solvent system of pyridine/acetone/NH_4OH/H_2O (15:9:1.5:6) was utilized to further characterize the methylated basic amino acids. This system effectively resolves the various methylated lysines, but it provides poor resolution of ϵ-*N*-monomethyl lysine from the methylated arginine species. Here, the hydrolysates of the polypeptides A and B gave similar chromatographic profiles (Fig. 2, *top* and *middle*). The major peak of radioactivity comigrated with an ϵ-*N*-trimethyl lysine standard. Only one distinct peak comigrating with ϵ-*N*-trimethyl lysine was observed with the 83K polypeptide (Fig. 2, *bottom*).

To confirm these assignments, a combination of ion exchange and thin layer chromatography was adopted. Each fraction of peaks II and III of the amino acid analysis column was separately desalted, spotted on thin layer chromatogram sheets, and developed in the pyridine solvent system. Fig. 3 shows the elution profile of the methylated lysine peak of the 68K polypeptide A hydrolysate; indentical results were obtained for 68K polypeptide B. In both cases, all three substituted lysines were present and were eluted in the order of ϵ-*N*-monomethyl-, ϵ-*N*-dimethyl-, and ϵ-*N*-trimethyl lysine. Nearly 80% of the total ^3H-radioactivity in peak II is contributed by ϵ-*N*-trimethyl lysine, while the remaining radioactivity was recovered as ϵ-*N*-monomethyl lysine and ϵ-*N*-dimethyl lysine (Table II).

When samples of peak III material were desalted and analyzed by thin layer chromatography as above, the radioactivity comigrated with a standard of N^G-monomethyl arginine and was well resolved from N^G,N'^G-dimethyl arginine (Fig. 4).

Methylation Stoichiometry in the Presence and Absence of Protein Synthesis Inhibitors—We have previously observed

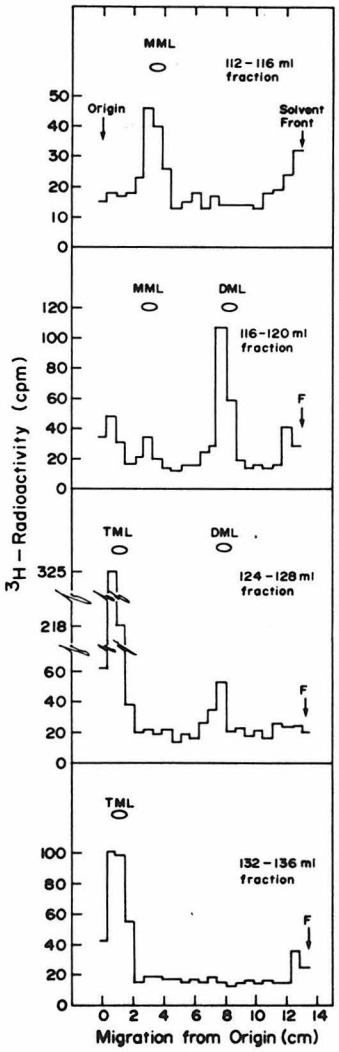


FIG. 3. **Methylated lysine species in 68K polypeptide A.** Each fraction of peak II (Fig. 1) was separately desalted and developed by cellulose thin layer chromatography as in Fig. 2. *MML*, ϵ-*N*-monomethyl lysine; *DML*, ϵ-*N*-dimethyl lysine; *TML*, ϵ-*N*-trimethyl lysine.

TABLE II

Distribution of methylated lysine residues on heat shock proteins

The percentage of each methylated lysine species was determined by thin layer chromatography of desalted peak II material (68K polypeptides A and B; see Fig. 3) or the amino acid hydrolysate (83K polypeptide; see Fig. 2).

	68K polypeptide A		68K polypeptide B		83K polypeptide	
	(+)[a]	(−)	(+)	(−)	(+)	(−)
MML[b]	3%	7%	8%	9%	6%	21%
DML[b]	17%	31%	15%	9%	13%	21%
TML[b]	80%	62%	77%	82%	81%	57%
Average methyl groups/site[c]	2.6	2.3	2.4	2.5	2.5	2.0

[a] (+), with protein synthesis inhibitors; (−), without protein synthesis inhibitors.

[b] MML, ϵ-*N*-monomethyl lysine, DML, ϵ-*N*-dimethyl lysine; TML, ϵ-*N*-trimethyl lysine.

[c] The specific activities of DML and TML are assumed to be 2 and 3 times, respectively, that of MML.

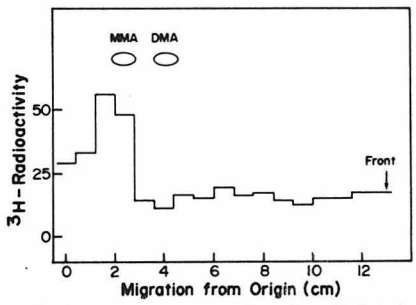


FIG. 4. **Thin layer chromatography of methylated arginine species in 68K polypeptide A.** Fractions of peak III (Fig. 1) were desalted and developed in cellulose thin layer chromatograms as in Fig. 2. The sample was mixed with both N^G,N'^G-dimethyl arginine (*DMA*) and N^G-monomethyl arginine (*MMA*) before chromatography. Radioactivity is given in counts per min; the background radioactivity of 15 cpm was not subtracted.

that both the rates of synthesis and methylation of the heat shock proteins increase upon sodium arsenite treatment of cultured fibroblasts (7), but the stoichiometries of the methylation reactions were not determined. These experiments could detect protein methylation only in the presence of protein synthesis inhibitors. Under these conditions, we calculate that less than 1% of each polypeptide became methylated during the labeling period (Table III).

On the other hand, significant error could enter into these calculations if methylation was very closely coupled to protein synthesis and the methyl groups were metabolically stable. In this case, we would be measuring methyl group incorporation into the few sites on newly synthesized proteins which remained unmodified for at least 30 min after synthesis and into unoccupied sites resulting from methyl group turnover.

Therefore, in order to calculate more accurately the stoichiometries of the methylation reactions, it was necessary to label the methyl groups in the absence of protein synthesis inhibitors. In this protocol, cultures were labeled simultaneously with [^{3}H]leucine and [*methyl*-^{3}H]methionine, and labeled amino acid extracts were prepared and chromatographed as described. The number of methylated amino acids in each polypeptide was normalized to the number of leucine residues incorporated during the culture period. Leucine was chosen as the standard since the mole fraction of leucine has been estimated for the 68K polypeptides and because it is several-fold more prevalent than methionine in proteins (26), which would also become labeled in this protocol. In addition, leucine is less susceptible to destruction during preparation of samples for chromatography. Chromatography was performed using a shorter column than in the previous experiments. Although the resolution is reduced, methylated lysines and arginines are well resolved from each other and from the neutral amino acid fraction, which contains methionine and leucine. The methylated amino acids in each peak were further characterized by thin layer chromatography using the pyridine system. Qualitatively, the chromatographic profiles obtained using this protocol are similar to those shown in Figs. 1–4. The only significant difference is that a methylated arginine residue, which has been identified as N^G-monomethyl arginine by thin layer chromatography, is now more prominent in the 68K polypeptide B. Table II compares the distribution of the methylated lysine species observed in this protocol with that obtained in the previous protocol. Again, all three variants are present, but there appears to be slightly more of the mono- and dimethyl forms.

TABLE III

Substoichiometric protein methylation in the presence of protein synthesis inhibitors

	68K polypeptides		83K polypeptides	
	Control	Induced	Control	Induced
Methyl groups incorporated per cell ($\times 10^{-4}$)[a]	0.88	6.6, 5.2[b]	0.54	0.68, 0.48
Estimated number of molecules/cell ($\times 10^{-6}$)[c]	5.2	12.0, 12.0	3.1	3.2, 2.9
Calculated methyl groups incorporated per molecule	0.0017	0.0055, 0.0043	0.0017	0.0021, 0.0017

[a] Estimated from radioactivity incorporated into the appropriate polypeptide band on one-dimensional gels after 4 h of incubation with [*methyl*-^{3}H]methionine. Corrections were made for counting efficiency (30%). It was assumed that the specific activities of the methyl donor *S*-adenosyl-[*methyl*-^{3}H]methionine were the same as that of the [*methyl*-^{3}H]methionine added (80 Ci/mmol). The number of cells was determined by direct counting in a hemacytometer after the release of the cells from the plates with trypsin.

[b] Values from two separate experiments are shown.

[c] Estimated from densitometric traces of the specific polypeptide band on Coomassie-stained one-dimensional gels, using averaged values for standards of phosphorylase and bovine serum albumin.

TABLE IV

Estimated stoichiometries of the methylation reactions

Estimates were made by assuming that (i) the mole per cent of Leu and Met equal 9.3 and 1.7% for 68K A, 3 and 1.7% for 68K B, and 7.4 and 1.5% for 83K. The amino acid compositions of the 68K polypeptides A and B were determined directly by conventional amino acid analysis. The values for 83K are average values for proteins described in reference 26. (ii) The total number of amino acid residues is 635 for the 68K polypeptides and 776 for the 83K polypeptides. (iii) The specific activity of the intracellular *S*-adenosylmethionine is the same as that of the [*methyl*-^{3}H]methionine in the incubation medium, and the specific activity of intracellular leucine is the same as that of the [^{3}H]leucine in the incubation medium.

	68K A		68K B		83K	
	Control	Induced	Control	Induced	Control	Induced
Lysyl methyl groups per polypeptide chain[a]	2.7 2.4	4.3 2.4	0.4 0.54	3.3	5.2 5.9	9.0
Arginyl methyl groups per polypeptide chain[a]	2.3	1.4 1.7	0.2 1.0	0.5	0	0

[a] Values given from two separate experiments.

Table IV shows that calculated values for the number of lysyl and arginyl methyl groups in each 68K A, 68K B, and 83K polypeptide. Since lysyl residues are methylated to mono-, di-, and trimethyl derivatives, the total number of modified lysyl residues in each polypeptide can be calculated from the data in Table IV and the frequencies of the various methyl lysine species given in Table II. On the other hand, only the monomethyl arginyl derivative is found in the 68K polypeptides. Here, the number of methylated residues is equal to the number of methyl groups in each polypeptide chain. We calculated that following heat shock, about 1.4 methylated lysyl residues and 1.6 methylated arginyl residues are present in each 68K polypeptide A. Similarly, 1.4 methylated lysyl residues and 0.5 methylated arginyl residues are present in each 68K polypeptide B. Each 83K polypeptide contains 4.5 methylated lysyl residues but no methylated arginyl residues. Comparing the stoichiometric incorporation of methyl groups under these conditions with the substoichiometric incorporation where protein synthesis is inhibited (Table III), we conclude that methylation occurs during or very shortly after the ribosomal synthesis of these proteins.

Turnover of Methyl Groups in Vivo—A number of pulse-chase experiments were conducted to determine directly the stability of methyl groups *in vivo*. In these experiments, cultures were first labeled with [^{35}S]methionine to label the polypeptide backbone, and they were then thoroughly washed with medium containing protein synthesis inhibitors before a second 4-h labeling period with L-[*methyl*-^{3}H]methionine in the presence of chloramphenicol and cyycloheximide. After several additional washings, the ratio of ^{3}H/^{35}S radioactivity was monitored as the cultures were incubated in the standard culture medium which contains 0.1 mM methionine. No change in this ratio was detected over a 10-h period. Assuming that we could detect a 10% decrease in the ^{3}H/^{35}S ratio, this indicates that the average half-life of methyl groups covalently linked to basic amino acid residues is at least 65 h.

DISCUSSION

Exposure of chicken embryonic myogenic cultures to sodium arsenite results in the increased expression of the same

group of proteins which are induced by heat shock. Although the functions of these proteins are unknown, it has been possible to obtain structural information on these species which may be relevant to their physiological role. One of these polypeptides with a molecular weight of 83,000 and two others with molecular weights of 68,000 (referred to as 68K polypeptide A and 68K polypeptide B according to their respective acidity on isoelectric focusing gels) are methylated proteins (7). Here we have shown that lysine is the predominant methylated amino acid obtained from acid hydrolysates of the 83K polypeptide and 68K polypeptides A and B. Most of the methylated lysine is in the form of ϵ-*N*-trimethyl lysine, but there are contributions from ϵ-*N*-monomethyl lysine and ϵ-*N*-dimethyl lysine as well. The 68K polypeptides A and B were also found to contain a methylated arginine species, which has been identified as N^G-monomethyl arginine. This species was not detected in the 83K polypeptide.

From the data in Table IV, it is clear that each polypeptide contains multiple methylation sites. The 68K polypeptides A and B in induced cells each contain both N^G-monomethyl arginine and methylated lysine, indicating the presence of at least two methylation sites. If the assumptions involved in our quantitation of the methylation sites are correct, both 68K polypeptides contain at least one additional lysyl site as well. Similarly, our calculations suggest that there are several lysyl methylation sites on the 83K polypeptide.

These data provide a minimum estimate of the methylation sites on the three polypeptides. In the absence of peptide analysis, it is not possible to determine the precise number of sites and their degree of saturation. An additional complication is the possibility that a specific lysine residue may be modified to mono-, di-, or trimethyl lysine. It has been shown, for example, that a single enzyme purified from *Neurospora* catalyzes the stepwise methylation of a specific lysine residue of cytochrome *c* (27). Protein lysyl methyltransferases are widely distributed in mammalian (28, 29) and avian (30) tissues. The substrate specificities of these latter enzymes appear distinct from that of the fungal one, and it is not clear if the avian enzymes catalyze the stepwise addition of methyl groups to lysine residues.

The limited extent of the methylation reactions in the presence of protein synthesis inhibitors, in contrast to the stoichiometric methylation observed in the absence of protein synthesis inhibitors, is caused by the close coupling of methylation to protein synthesis and by the absence of appreciable methyl group turnover ($t_{1/2} > 65$ h). Methylation must occur either co-translationally or very shortly after completion of the polypeptide, since the proteins are not substrates for the methyltransferase(s) 30 min after their synthesis. The reason why methylation occurs at all in the presence of protein synthesis inhibitors is unclear. Conceivably, we could be observing only the final stage of the methylation process begun during synthesis of the proteins. Alternatively, we could be measuring remethylation at residues which had become demethylated. In support of this latter possibility, we have observed that the rate of methylation in the presence of protein synthesis inhibitors is constant for at least 6 h after induction (data not shown), a finding more consistent with remethylation than with methylation representing the final stages of an initial methylation process. We find it less likely that methyl group incorporation is due to leaky protein synthesis, since we detect only small amounts of radioactive methionine in the protein hydrolysates (peak I in Fig. 1).

In several respects, the methylation of heat shock proteins resembles methylation reactions occurring at basic amino acid residues in other proteins. It has been reported that basic amino acid methylation of myosin (31), rat heart proteins (32), and yeast cytochrome *c* (15) is strongly inhibited by cycloheximide and, therefore, is closely coupled to protein synthesis. In the case of myosin, methylated amino acids are present in the nascent polypeptides present on the polysomes, but methylation can occur on completed proteins as well (31). The HeLa cell ribosomal proteins show site-specific differences in their abilities to serve as methyl acceptors in the presence of cycloheximide (33), but it is not clear if these methylations are a manifestation of methyl group turnover, which is a very slow process (34). Similarly, histone methylation is thought to be virtually irreversible, since the half-lives of the methyl lysine and methyl arginine residues in histones are the same as those of the histones themselves (35).

The functional significance of heat shock protein methylation remains elusive. From the data presented here, it is clear that methylation occurs at several sites. The extent of methylation appears to vary after sodium arsenite treatment in several instances (Table IV). With the exception of lysyl methylation on the 68K B polypeptide, these changes are small. Further studies of the kinetics of protein synthesis, methylation, and demethylation, as well as methionine utilization will be required to establish any causal link between methylation and heat shock.

REFERENCES

1. Tissieres, A., Mitchell, H. K., and Tracy, U. M. (1974) *J. Mol. Biol.* **84,** 389–398
2. Ashburner, M., and Bonner, J. J. (1979) *Cell* **17,** 241–254
3. Mitchell, H. K., Moller, G., Peterson, N. S., and Lipps-Sarmiento, L. (1979) *Dev. Genet.* **1,** 181–192
4. Johnston, D., Oppermann, H., Jackson, J., and Levinson, W. (1980) *J. Biol. Chem.* **255,** 6975–6980
5. Levinson, W., Oppermann, H., and Jackson, J. (1980) *Biochim. Biophys. Acta* **606,** 170–180
6. Kelley, P. M., and Schlesinger, M. J. (1978) *Cell* **15,** 1277–1286
7. Wang, C., Gomer, R. H., and Lazarides, E. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 3531–3535
8. Springer, M. S., Goy, M. F., and Adler, J. (1979) *Nature* **280,** 279–284
9. Wang, E. A., and Koshland, D. E., Jr. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 7157–7161
10. Freitag, C., and Clarke, S. (1981) *J. Biol. Chem.* **256,** 6102–6108
11. McFadden, P. N., and Clarke, S. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2460–2464
12. Paik, W. K., and Kim, S. (1980) *Protein Methylation*, John Wiley & Sons, New York
13. Scott, W. A., and Mitchell, H. K. (1969) *Biochemistry* **8,** 4282–4289
14. Polastro, E. T., Deconinck, M. M., Devogel, M. R., Mailier, E. L., Looze, Y. R., Schnek, A. G., and Loenis, J. (1978) *FEBS Lett.* **86,** 17–20
15. Farooqui, J., Kim, S., and Paik, W. K. (1980) *J. Biol. Chem.* **255,** 4468–4473
16. Gard, D. L., Bell, P. B., and Lazarides, E. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 3894–3898
17. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021
18. Hubbard, B., and Lazarides, E. (1979) *J. Cell Biol.* **80,** 166–182
19. O'Connor, C. M., Gard, D. L., and Lazarides, E. (1981) *Cell* **23,** 135–143
20. Moore, S. (1968) *J. Biol. Chem.* **243,** 6281–6283
21. Dreze, A., Moore, S., and Bigwood, E. J. (1954) *Anal. Chim. Acta* **11,** 554–558
22. Van Der Werf, P., and Koshland, D. E., Jr. (1977) *J. Biol. Chem.* **252,** 2793–2795
23. Kleene, S. J., Toews, M. L., and Adler, J. (1977) *J. Biol. Chem.* **252,** 2793–2795
24. Zarkadas, C. G. (1975) *Can. J. Biochem.* **53,** 96–101
25. Zarkadas, C. G. (1978) *Can. J. Biochem.* **56,** 952–957
26. Dayhoff, M. O., Hunt, L. T., and Hurst-Calderone, S. (1978) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., ed) Vol. 5, Suppl. 3, p. 363, National Biomedical Research Foundation, Washington, D. C.
27. Durban, E., Nochumson, S., Kim, S., Paik, W. K., and Chan, S.-

K. (1978) *J. Biol. Chem.* **253,** 1427–1435
28. Wallwork, J. C., Quick, D. P., and Duerre, J. A. (1977) *J. Biol. Chem.* **252,** 5977–5980
29. Paik, W. K., and Kim, S. (1970) *J. Biol. Chem.* **245,** 6010–6015
30. Greenaway, P. J., and Levine, D. (1974) *Biochim. Biophys. Acta* **350,** 374–382
31. Morse, R. K., Vergnes, J.-P., Malloy, J., and McManus, I. R. (1975) *Biochemistry* **14,** 4316–4325
32. Watkins, C. A., and Morgan, H. E. (1979) *J. Biol. Chem.* **254,** 693–701
33. Scolnik, P. A., and Eliceiri, G. L. (1979) *Eur. J. Biochem.* **101,** 93–101
34. Goldenberg, C. J., and Eliceiri, G. L. (1977) *Biochim. Biophys. Acta* **479,** 220–234
35. Byzoet, P., Shepherd, G. R., Hardin, J. M., and Noland, B. J. (1972) *Arch. Biochem. Biophys.* **148,** 558–567

Chapter 5

Arsenite-Induced Changes in the Methylation of the 70,000 Dalton Heat Shock Proteins in Chicken Embryonic Fibroblasts

Abstract

Methylated lysyl and arginyl residues are present in the two major heat shock proteins, hsp70A and hsp70B, of chicken embryonic fibroblasts (Wang, C., Lazarides, E., O'Connor, C. M., and Clarke, S. (1982) J. Biol. Chem. **257**, 8356-8362). Here, we present evidence that several methylated and unmethylated forms of hsp70 exist in chicken embryonic fibroblasts, and that the ratio of these forms can be modulated by sodium arsenite, a chemical that induces the synthesis of hsp70 at least fivefold. In particular, in hsp70A the amount of ε-N-trimethyl-lysine decreases and ε-N-dimethyl-lysine significantly increases, while in hsp70B the quantity of N^G-monomethyl-arginine is reduced by fivefold after sodium arsenite treatment. The total cellular level of methylated proteins was determined by amino acid analysis. Approximately 0.06 mole of methyl groups is incorporated into basic amino acids for every mole methionine incorporated in total cellular protein. After arsenite treatment, no significant changes in the level of lysine or arginine methylation are observed in total cellular proteins. Arsenite-induced changes in methylation, therefore, appear to be specific for the hsp70 polypeptides.

Introduction

Exposure of Drosophila melanogaster, chicken and mammalian tissue or cultured cells to temperatures higher than their normal growth temperature results in a profound change in protein synthesis (2-5; for a review, see 1). Under these conditions the translation of normal cellular proteins is suppressed, and the synthesis of a new class of proteins, known as "heat shock proteins," is enhanced. A similar set of polypeptides is induced upon exposure of cells to sodium arsenite and some other agents (4,6,7). In Drosophila, the change in protein synthesis after induction is regulated at both the transcriptional and translational level (1,8,9). Despite efforts for the analysis of the genes coding for these proteins in Drosophila, the function of heat shock proteins is not well understood. Evidence has been presented that a non-lethal pretreatment at elevated temperatures or with sodium arsenite, which is sufficient to induce the heat shock proteins, increases the chance of survival of the organism or cultured cells upon further heat challenge (3,5,10).

Among all the heat shock proteins, a polypeptide(s) of an approximate molecular weight of 70,000 (hsp70) is the most commonly induced species. In chicken fibroblasts the molecular weight of these polypeptides has been measured as 68,000[1] (7,11,12). Hsp70 of chicken embryonic fibroblasts is composed of two different major polypeptides which are synthesized under normal growth conditions and whose rate of synthesis increases five to tenfold after arsenite treatment. We have previously demonstrated that both of the hsp70 variants are methylated, presumably by a S-adenosyl-L-methionine dependent methyl transferase (7). The methylated sites have been identified as lysyl and arginyl residues, and their methylation appears to be irreversible and coupled to the synthesis of these proteins (11). In this report, we present evidence which indicates that several stably methylated forms of hsp70 exist in the cell and that the methylation of hsp70 changes after sodium arsenite treatment. The intracellular AdoMet[2] pool size and the total cellular protein methylation remain unchanged

after arsenite treatment, suggesting that the changes in hsp70 methylation are specific for these two polypeptides.

EXPERIMENTAL PROCEDURES

Cell Cultures—Chicken embryonic fibroblasts were isolated from 10-12-day-old chicken embryonic leg muscle (13). The cells were grown and maintained in Dulbecco's modified Eagle's medium (0.2 mM in L-methionine) supplemented with 10% calf serum, and subcultured at least three times before use to eliminate any contaminating muscle cells.

Isolation of [^{35}S]-adenosyl-L methionine—S-adenosyl-L-methionine (AdoMet) was isolated essentially by the method of Glazer and Peale (14). [^{35}S]-L-methionine (New England Nuclear, specific activity 1033.6 Ci/mmole) was added to 60 mm plates of confluent chicken embryonic fibroblasts ($3.0 \pm 0.5 \times 10^6$ cells) to a final concentration of 20 μci/ml in a total volume of 1.5 ml (final specific activity: 100 Ci/mole). After incubation for 2 minutes to 5 hours, the cells were rinsed three times with ice cold phosphate buffered saline (PBS), and then swollen in 1 ml of 10 mM HCl in the presence of 60 nmole of AdoMet (Sigma) added as a carrier. The cells were then scraped off the plates, an equal volume of cold 30% trichloroacetic acid (TCA) was added, and the suspension was mixed well. The supernatant was collected after centrifugation and the pellet was washed twice with 1 ml 5% TCA. All the supernatants were pooled together and TCA was subsequently extracted four times with an equal volume of ethyl ether. Thereafter, the sample was passed through a SP-Sephadex (C-25, Pharmacia) column (1x5 cm) equilibrated with 10 mM HCl. The column was washed extensively with 0.2 N HCl until both the OD_{256} and [^{35}S]-radioactivity of the effluent reached background levels. The AdoMet was eluted immediately after addition of 0.5 N HCl, and 3 ml fractions were collected. The recovery of

AdoMet was determined by OD_{256} and the [^{35}S]-radioactivity by liquid scintillation counting with 10 volumes of Aquasol-2 (New England Nuclear). The purity of the [^{35}S]-AdoMet isolated by this method was further checked by thin layer chromatography, using two different solvent systems; n-butanol:acetic acid: H_2O (12:3:5) and ethanol:acetic acid:H_2O (65:1:34).

Amino Acid Analysis of Cellular Protein After Labeling with [^{3}H]-L-methionine—Confluent chicken embyronic fibroblasts were incubated in methionine-free minimal essential medium with 0.63 µM [methyl-^{3}H]-L-methionine (50 µCi/ml, New England Nuclear, specific activity: 80 Ci/mmole) and with or without 25 µM sodium arsenite for 1.5 to 24 hours. After incubation, the plates were rinsed with ice cold PBS, the cells were scraped and 100% TCA was added to a final concentration of 15%. The pellet was collected by centrifugation, washed twice with 2 ml 5% TCA and then twice with ethyl ether. The final pellet was hydrolyzed with 6 N HCl at 108°C in an evacuated tube for 24 h. Subsequently, the HCl was removed by evaporation under reduced pressure, and the dried residue was resuspended with 0.2 N sodium citrate buffer, pH 2.5. It was then applied to an amino acid analysis column (Beckman PA-35, 0.9 x 13 cm) equilibrated with sodium citrate buffer (0.35 N in Na^+, pH 5.28). The column was operated at 38°C at a flow rate of 67 ml/h with the same buffer, and 3 ml fractions were collected. [^{3}H]-radioactivity was determined by liquid scintillation counting.

Analysis of Methylated Basic Amino Acids of hsp70——Confluent 100 mm plates of chicken embyronic fibroblasts containing 10^7 cells each were incubated in methionine-free minimal essential medium with 50 µCi/ml [methyl-^{3}H]-L-methionine and 2% calf serum for 2.5 to 14 hours with or without 25 µM sodium arsenite. The hsp70 was isolated by a modification of the two-dimensional gel electrophoresis procedure previously described (11). Briefly, the cell pellets first were boiled in 200 µl SDS sample buffer (1% SDS, 1% 2-mercaptoethanol, 25 mM Tris, pH 7.5) per plate, before

the addition of 0.15 to 0.2 g solid urea to each sample. Isoelectric focusing gels were prepared without detergent. The Coomassie spots corresponding to hsp70 were excised from the gels and washed with 10% ethanol overnight, then dehydrated with 95% ethanol. After removal of the ethanol, the polypeptides were electrophoretically eluted from the gel slices in 100 mM NH_4HCO_3 and 0.1% SDS[2]. After lyophilization, the polypeptides were hydrolyzed with 6 N HCl and the hydrolysates were analyzed by ion exchange chromatography (0.9 x 13 cm Beckman PA-35) as described in the previous paragraph.

Thin Layer Chromatography—Since the conditions adopted for ion exchange chromatography cannot resolve the three different methylated lysine species, thin layer chromatography was utilized to separate ε-N-mono, di- and tri-methyl-lysine. Samples were desalted and chromatographed on cellulose thin layer sheets (Eastman Kodak, No. 13255), using pyridine:acetone:3 M NH_4OH (10:6:5) as solvent, as described previously (11). Amino acids were detected by spraying with 1% ninhydrin in acetone. Six millimeter-wide cellulose strips were cut perpendicular to the axis of migration and scraped into scintillation vials, mixed with 0.5 N HCl (0.5 ml) and allowed to sit at room temperature for at least 20 min; finally 10 volumes (5 ml) of Aquasol-2 scintillation fluid (New England Nuclear) were added and the samples counted.

RESULTS

Determination of Cellular Pool Size of AdoMet in Control and Arsenite-treated cultures—Cells were labeled with [^{35}S]-L-methionine as described in "Experimental Procedures". S-adenosyl-L-methionine (AdoMet) was isolated from acid extracts of cells by SP-Sephadex column chromatography as described by Glazer and Peale (14). In the experiments reported here, greater than 70% of the AdoMet standard, as assayed by its absorbance at 256 nm, was recovered from the column. The results in Figure 1 indicate that [^{35}S]-AdoMet is formed rapidly ($t_{1/2}$ = 0.4 h) and the

Figure 1. Intracellular level of [^{35}S]-AdoMet. [^{35}S]-L-methionine was added extracellularly and, at a given time, [^{35}S]-AdoMet was isolated by the method described in Experimental Procedure. Each point represents the average of duplicate samples. The dpm were determined by counting a set of internal standards. The intracellular pool size (45 pmole/3 x 10^6 cells) was estimated by assuming that the specific activity of intracellular [^{35}S]-AdoMet is identical to the [^{35}S]-L-methionine of the medium (100 Ci/mole) when steady state is reached. The concentration (1.5 x 10^{-5} M or 60 nmole/g protein) is calculated by assuming the cell volume is 1 pl (the cell volume of CHO-K1 was measured as 1.5 pl (35)) and the total cellular protein of chicken fibroblasts was measured by the Lowry method as 250 pg/cell. Open circle (O), control; closed circle (●), sodium arsenite treated cells; the bar represents the range of the duplicate determinations.

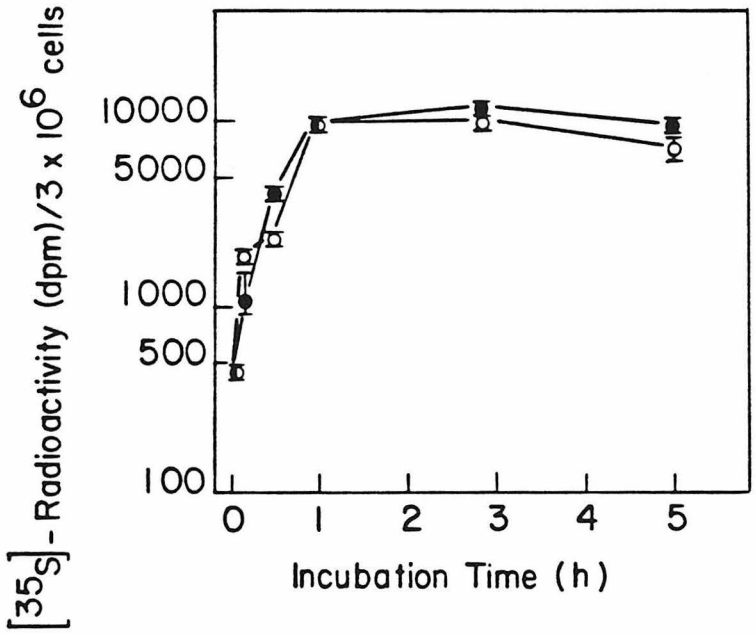

[35S]-Radioactivity (dpm)/3 x 10^6 cells
10000
5000
1000
500
100
0
1
2
3
4
5
Incubation Time (h)

Figure 1

radioactivity reaches a constant level in approximately 1 hour after [^{35}S]-L-methionine is added extracellularly. In particular, arsenite has little effect on the intracellular level of AdoMet (Fig. 1); hence, changes in methylation induced by arsenite cannot be attributed to changes in AdoMet pool size.

The purity of the [^{35}S]-AdoMet isolated from the cells was further verified by thin layer chromatography; more than 85% of the [^{35}S]-radioactivity comigrated with the AdoMet standard, although a minor peak of radioactivity also was observed, which was more prominent when the chromatogram was developed in ethanol: acetic acid:water (65:1:34) rather than in n-butanol:acetic acid:water (12:3:5) (Fig. 2).

Assuming that the specific activity of AdoMet is the same as that of the methionine, the data from Fig. 1 allow us to calculate the intracellular concentration of AdoMet. We estimate that 45 pmoles of AdoMet are present in 3×10^6 cells, corresponding to a concentration of 1.5×10^{-5} M or 60 nmole/g of protein. This value of concentration of AdoMet in chicken embryonic fibroblasts is similar to the level of AdoMet obtained from isolated rat tissue (15).

[^{3}H]-Radioactivity Incorporated into Basic Amino Acids—Chicken embryonic fibroblasts labeled metabolically with [methyl-^{3}H]-L-methionine incorporate [^{3}H]-radioactivity into polypeptides not only as methionine but also as methyl groups through [methyl-^{3}H]-S-adenosyl-L-methionine. The relative amounts of methionine and methylated basic amino acid residues can be determined by amino acid analysis of the labeled proteins. Table I shows the kinetics of incorporation of radioactivity into methyl histidinyl, methyl lysyl and methyl arginyl residues relative to the rate of incorporation into methionine. These ratios appear similar for short and long labeling times. The incorporation of methyl groups into N^G-monomethyl-arginyl residues relative to methionine may be slightly reduced after prolonged incubation. The smaller ratio at earlier time points may simply reflect the fact that the average specific activity of AdoMet is smaller than that of methionine at these time points.

Figure 2. Purity of [^{35}S]-AdoMet eluted from Sp-Sephadex. Fractions containing [^{35}S]-AdoMet were pooled and, after lyophilization, the dried residues were resuspended in double distilled water, spotted on thin layer chromatogram sheets and developed in (A) n-butanol:acetic acid:H_2O (12:3:5). (B) ethanol:acetic acid:H_2O (65:1:34). AdoMet was located by spraying with 1% ninhydrin in acetone. Background cpm have not been subtracted.

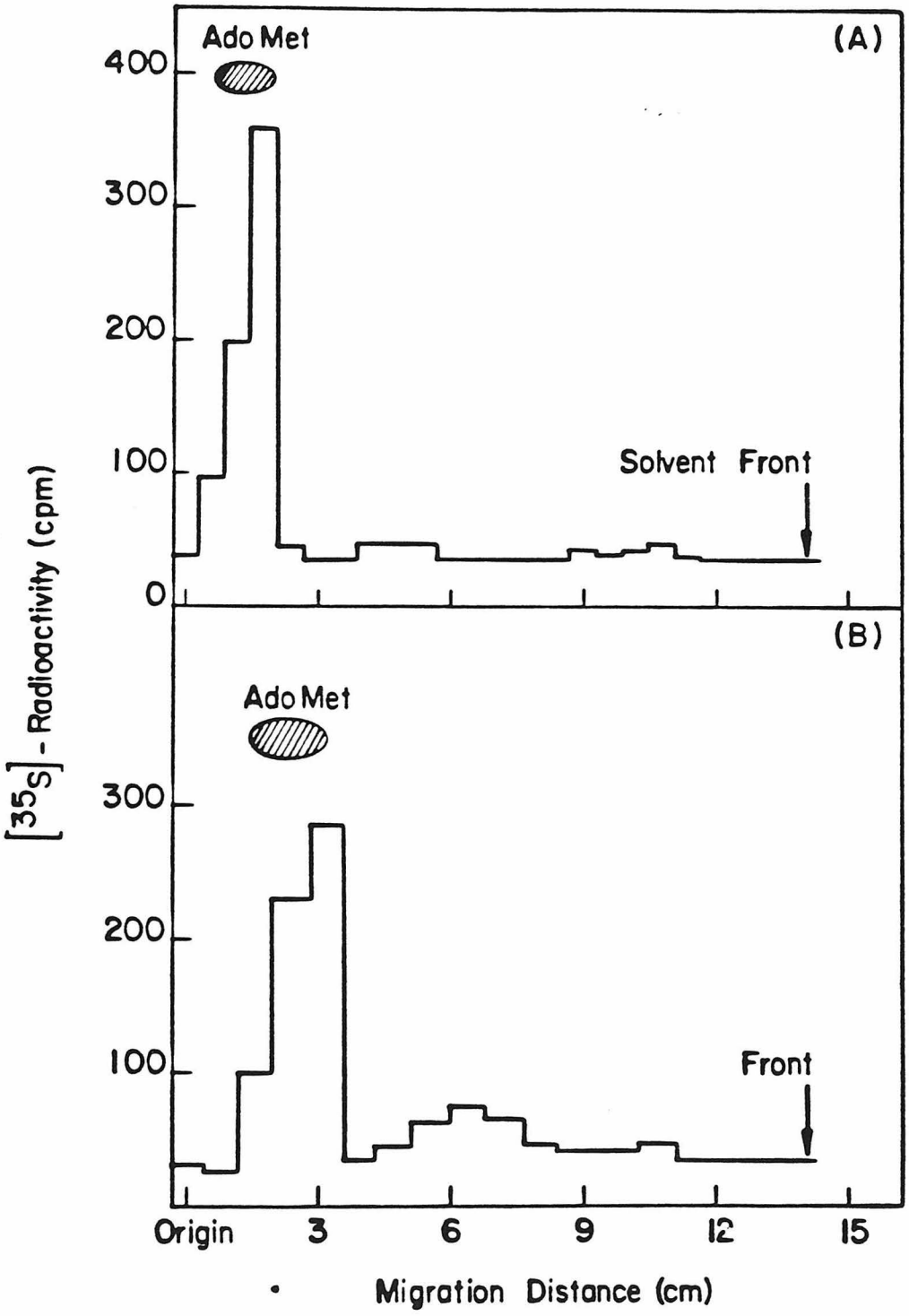

Ado Met
(A)
400
300
200
100
0
Solvent Front
(B)
Ado Met
300
200
100
Front
[35S] - Radioactivity (cpm)
Origin
3
6
9
12
15
Migration Distance (cm)

Figure 2

TABLE I

[^{3}H]-methyl groups incorporated into basic amino acids of chicken embryonic fibroblast total cellular protein[a]

incubation time	methyl lysines[b]		3-N-methyl-histidine[b]		dimethyl-arginine[b]		N^G-monomethyl-arginine[b]	
	control	arsenite	control	arsenite	control	arsenite	control	arsenite
1.5 h	1.5%	1.4%	0.37%	0.35%	2.3%	2.7%	0.33%	0.44%
3 h	1.8%	1.8%	0.38%	0.38%	3.2%	3.6%	0.42%	0.68%
6 h	2.2%	2.3%	0.45%	0.44%	3.8%	4.3%	0.31%	0.80%
12 h	2.2%	2.2%	0.39%	0.46%	3.4%	3.2%	0.24%	0.53%
24 h	2.3%	-[c]	0.46%	-[c]	3.2%	-[c]	0.20%	-[c]

[a][^{3}H]-methionine incorporated into polypeptide backbone is arbitrarily assigned as 100%. The numbers given here are the averages of two determinations.

[b]The maximum range of the measurements for methyl-lysines, 3-N-methyl-histidine, dimethyl-arginine and N^G-monomethyl-arginine is 0.2%, 0.09%, 0.2%, 0.12%, respectively.

[c]Not determined.

It is also apparent that the relative amount of the three methyl lysine species is similar for long and short labeling periods (Table II).

From the data in Tables I and II, it appears that arsenite has little effect on basic amino acid methylation in general, since the frequency of methylated amino acids in total cellular protein is not appreciably altered by sodium arsenite treatment. In both control and treated cells, approximately 60 methyl groups are incorporated into basic amino acids per 1000 methionines incorporated into polypeptide backbones; this is compatible with the results obtained by amino acid analysis of mammalian and chicken tissues (16).

Methylation of hsp70A and hsp70B—From the results presented above, we can conclude that the intracellular pool size of AdoMet and the total amount of protein methylation remain largely unchanged in the presence of sodium arsenite. Therefore, we proceeded to study whether arsenite had any effect on the methylation of the hsp70 polypeptides in chicken embryonic fibroblasts. Figure 3 shows the elution profile from an amino acid analysis column of the hydrolysate of hsp70A from a sodium arsenite-treated culture. Under these conditions, the three methylated lysine species and N^G-monomethyl-arginine are separated from the other methylated amino acids, but the three different methyl lysine species are not resolved from one another. Three different peaks of [^{3}H]-radioactivity were found: peak I coeluted with methionine, peak II with methyl-lysines and peak III with N^G-monomethyl-arginine. Virtually identical results were obtained with hsp70A isolated from control cells. The profile obtained from hydrolysates of hsp70B is similar, except that the level of N^G-monomethyl-arginine in hsp70B in control cells is slightly lower than that in hsp70A. However, the N^G-monomethyl-arginine content of hsp 70B drops sharply after sodium arsenite treatment. Quantitative analysis of these column profiles is shown in Table III. In all cases, the cells were labeled for periods long enough to minimize any differences caused by asynchrony in the kinetics of [^{3}H]-Met-tRNA^{Met} and [^{3}H]-AdoMet labeling.

TABLE II

Relative amount of methylated lysine species in total cellular proteins of chicken embryonic fibroblasts[a]

	Control cells			Aresenite treated cells		
incubation time	3 h	6 h	24 h	3 h	6 h	12 h
ε-N-monomethyl-lysine	18%	20%	31%	27%	18%	28%
ε-N-dimethyl-lysine	41%	38%	29%	28%	38%	18%
ε-N-trimethyl-lysine	41%	42%	40%	45%	44%	54%
$\frac{\text{methyl groups}}{\text{methylated lysine}}$	2.2	2.2	2.1	2.2	2.3	2.3

[a]The [^{3}H]-radioactivity corresponding to the three methyl lysine species was determined as described in Experimental Procedures, and its distribution was calculated by assuming that the ratio of the specific activity of ε-N-monomethyl-lysine:ε-N-dimethyl-lysine:ε-N-trimethyl-lysine = 1:2:3.

Figure 3. Ion exchange chromatography of hsp70A hydrolysate. Chicken embryonic fibroblast hsp70A was labeled, isolated, hydrolyzed, and chromatographed as described in Experimental Procedures. 3 ml fractions were collected and 1 ml was analyzed for radioactivity. Peaks I, II and III coelute with the amino acid standard of L-methionine, methyl-lysines and N^G-monomethyl-arginine, respectively.

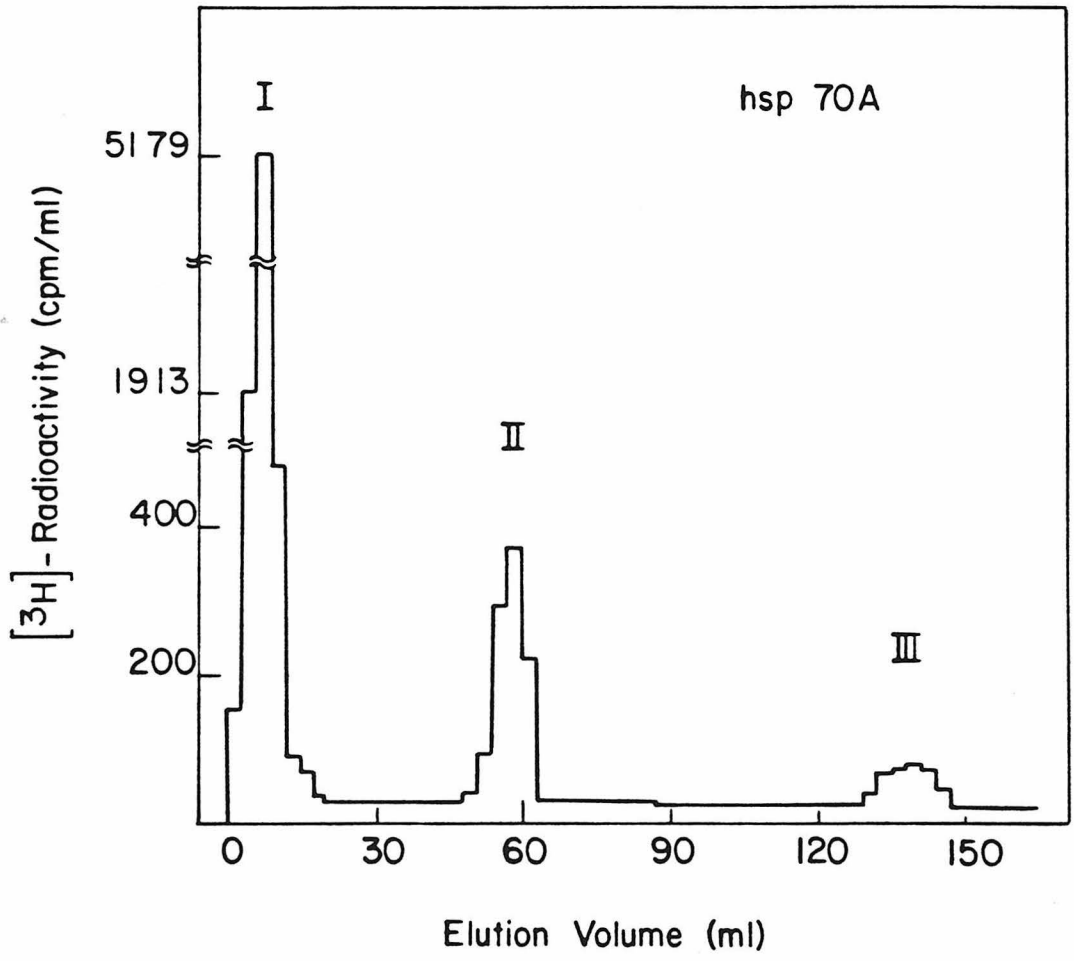

I
hsp 70A
5179
1913
400
200
II
III
[3H]- Radioactivity (cpm/ml)
0
30
60
90
120
150
Elution Volume (ml)

Figure 3

TABLE III

[^{3}H]-methyl groups incorporated into lysyl and arginyl residues of chicken embryonic fibroblast hsp70[a]

Experimental Conditions	hsp70A		hsp70B	
	MLs[b]	MMA[b]	MLs	MMA
control (6 h)[c]	9%	3.3%	11%	2.1%
control (18 h)	11%	3.4%	8%	2.5%
arsenite (6 h)	10%	3.0%	11%	0.33%
arsenite (14 h)	11%	2.7%	8%	0.58%
preincubated (8 h), then arsenite (6 h)	12%	3.3%	13%	1.0%

[a][^{3}H]-methionine incorporated into hsp70 polypeptide backbone is assigned a value of 100%.

[b]MLs: methyl-lysines, MMA: N^G-monomethyl arginine.

[c][methyl-^{3}H]-L-methionine incubation period is given in parenthesis.

To further examine any changes in the ratio of the three methylated lysine species after sodium arsenite treatment the methylated lysines were desalted and further resolved by thin layer chromatography. In both hsp70A and B, the distribution of radioactivity in the three different methyl-lysines is essentially the same for short and long labeling periods, with ε-N-trimethyl-lysine being the predominant species. No significant differences were found in the distribution of methyl-lysines in hsp70B from arsenite treated cultures (data not shown). However, in hsp70A there was a significant increase in the amount of ε-N-dimethyl-lysine after sodium arsenite treatment (Table IV). In control cells, there are on the average 2.6 methyl groups per methylated lysine residue for both hsp70A and hsp70B; it is reduced to 2.2 for hsp70A after sodium arsenite treatment.

DISCUSSION

Methylation of basic amino acids appears to occur as a post-translational event. It has been reported that the methylation of cultured chicken muscle cell protein is coupled to their synthesis, perhaps occurring on the nascent polypeptide chain (17). In the present work, experiments were performed to determine the relationship between protein synthesis and basic amino acid methylation in chicken embryonic fibroblasts. The cells were incubated with [methyl-^{3}H]-L-methionine, and then the ratio of [^{3}H]-radioactivity incorporated into methylated amino acids and methionine was calculated. The results (Table I) are consistent with the interpretation that the majority of these methylation events are coupled to protein synthesis; however, the possibility that methylation occurs shortly after completion of nascent polypeptide chains cannot be ruled out. We determined that there is one methylated lysine and one methylated arginine per 100 methionines in total cellular protein. In addition, arsenite treatment does not change the amount of the methylated lysine species in total cellular protein, even though the synthesis of the methylated lysine containing hsp70 polypeptides is increased five to tenfold. Therefore, we conclude that the methylation of basic amino acids should be common to many proteins.

TABLE IV

Distribution of methylated lysines in hsp70A[(a)]

	Control cells			Sodium arsenite treated		
Incubation time	6 h	18 h	30 h	2.5 h	6 h	14 h
ε-N-monomethyl-lysine	14%	17%	14%	30%	20%	27%
ε-N-dimethyl-lysine	11%	6%	2%	22%	27%	26%
ε-N-trimethyl-lysine	76%	77%	84%	48%	53%	47%
$\frac{\text{methyl groups}}{\text{methylated lysines}}$	2.6	2.6	2.7	2.2	2.3	2.2

(a) The [^{3}H]-radioactivity corresponding to three methyl lysine species was first determined, and its distribution was calculated by assuming that the ratio of the specific activity of ε-N-monomethyl-lysine: ε-N-dimethyl-lysine: ε -N-trimethyl-lysine = 1:2:3.

It is not clear why the level of N^G-monomethyl-arginine in total cellular protein increases after sodium arsenite treatment (Table I). This increase may be due simply to the increased synthesis of hsp 70. It is possible that the decrease in the level of N^G-monomethyl-arginine observed in hsp 70B after arsenite treatment may be compensated by the increased number of polypeptide chains synthesized. Alternatively, the increase may result from an increase in methylation of N^G-monomethyl-arginine on some yet unidentified polypeptide(s) after sodium arsenite treatment.

We found that the distribution of ε-N-mono-, di-, and tri-methyl-lysine of chicken embryonic fibroblast hsp70 remains constant with respect to incubation time and that the appearance of the lower methyl-lysine forms is not transient (Table IV). The change in the distribution among different methyl lysines of hsp70 by sodium arsenite is evident (Table IV). Arsenite treatment also results in a 15% reduction in the average number of methyl groups per methylated lysine residue. Such a small difference may escape detection with the methods used in this work, since when two identical samples were used for analysis, a 10% difference in methyl-lysine residues was obtained.

The use of larger amounts of material for the measurements reported here have allowed us to obtain a better quantitative estimate than before of the degree of protein methylation (11). The values reported here are slightly lower than those of the previous report (11). Assuming that there are 12 methionines per molecule, we calculate that there are 0.4 methylated lysines and 0.3 methylated arginines per hsp70 polypeptide.

Hsp70A and hsp70B are two distinct polypeptides, though some homology among them may exist (6). We reported previously that both polypeptides are methylated at lysyl and arginyl residues (11). Here, we have demonstrated that the amount of N^G-monomethyl-arginine in hsp70B is reduced fivefold after sodium arsenite treatment, while in hsp70A, it remains unchanged. Arsenite, which interacts with

vicinal dithiols to form a relatively non-dissociable complex (18), may alter certain enzymatic activities; for example, aldehyde dehydrogenase can be inhibited with arsenite (19). It remains to be determined whether the change in methylation after arsenite treatment is a general phenomenon for all agents which can induce the 70,000 dalton heat shock proteins.

Methylation of several different amino acid residues in proteins has been documented. The reversible methylation of chemoreceptors of bacterial cells at glutamic acid residues is thought to be involved in the adaptive response of chemotaxis (20,21). D-aspartic acid residues have been identified as the reversible methylation sites in human erythrocyte proteins (22,23), and it has been suggested that this reversible esterification may be involved in a recemization-repair reaction (23). Whereas basic amino acid residues such as histidine, lysine and arginine also can be methylated in vivo in some cases, on most occasions the methylation is irreversible and the functional significance is unknown. For example, the level of methylation of fungal elongation factor EF-1α changes during morphogenesis, but the enzymatic activity does not parallel these changes (24). Similarly, histone methylation occurs mainly near the end of DNA replication phase of cell cycle (25), but both methylated and unmethylated histone H4 can assemble equally well into nucleosomes (26). Perhaps, cytochrome c is the only case where a functional role has been assigned to its lysyl methylation. It has been suggested that methylation may facilitate the binding of cytochrome c to mitochondria (27,28), without affecting electron transport (28).

The localization of heat shock proteins has been intensively studied. In Drosophila, hsp70 is located in nuclei shortly after heat treatment (29,30,31); however, a fraction of it may also be cytosolic (30). By immunofluorescence, hsp70 is a cytoplasmic protein in chicken embryonic fibroblasts (32); a portion of it possibly may be associated with the cytoskeleton or nuclei, since it is insoluble in Triton-KCl (12). A fraction of it may be associated also with a membrane surface glycoprotein (33) and microtubules

(12,34) in cultured mammalian cells. However, one must bear in mind that different systems were used in these studies and differences in hsp70 exist as well. As we have shown in this study, hsp70 can exist in several different methylated forms; each form may possibly have a preferential intracellular localization.

Acknowledgements——We thank Drs. Steven Clarke, Clare M. O'Connor and John Ngai for their many valuable suggestions during the preparation of this manuscript. We also thank Dr. Michael Raftery for the use of his laboratory facilities in part of this work.

FOOTNOTES

This research was supported by grants from the National Institutes of Health (GM 06965), the National Science Foundation and the Muscular Dystrophy Association of America. E. L. is the recipient of a Research Career Development Award from the National Institutes of Health.

[1]In this report, the name hsp70 is used for this particular sodium arsenite inducible protein; hsp70A refers to the more acidic species (pI 5.6), while hsp70B refers to the more basic polypeptide (pI 6.0) among the two.

[2]The abbreviations used are: AdoMet, S-adenosyl-L-methionine; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PBS, phosphate buffered saline.

REFERENCES

1. Ashburner, M., and Bonner, J. J. (1979) Cell **17**, 241-254.
2. Tissieres, A., Mitchell, H. K., and Tracy, V. M. (1974) J. Mol. Biol. **84**, 389-398.
3. McAlister, L., and Finkelstein, D. B. (1980) Biochem. Biophys. Res. Commun. **93**, 819-824.
4. Kelly, P. M., and Schlesinger, M. J. (1978) Cell **15**, 1277-1298.
5. Li, G. C., and Werb, Z. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3218-3222.
6. Johnson, D., Oppermann, H., Jackson, J., and Levinson, W. (1980) J. Biol. Chem. **255**, 6975-6980.
7. Wang, C., Gomer, R. H., and Lazarides, E. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3531-3535.
8. Storti, R. V., Scott, M. P., Rich, A., and Pardue, M. L. (1980) Cell **22**, 825-834.
9. Lindquist, S. (1980) Dev. Biol. **77**, 463-479.
10. Mitchell, H. K., Moller, G., Petersen, N. S., and Lipps-Sarmiento, L. (1979) Dev. Genet. **1**, 181-192.
11. Wang, C., Lazarides, E., O'Connor, C. M., and Clarke, S. (1982) J. Biol. Chem. **257**, 8356-8362.
12. Wang, C., Asai, D. J., and Lazarides, E. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1541-1545.
13. Gard, D. L., Bell, P. B., and Lazarides, E. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 3894-3898.
14. Glazer, R. I., and Peale, A. N. (1978) Anal. Biochem. **91**, 516-520.
15. Eloranta, T. O. (1977) Biochem. J. **166**, 521-529.
16. Paik, W. K., and Kim, S. (1980) Protein Methylation, New York, John Wiley and Sons.
17. Morse, R. K., Vernges, J.-P., Malloy, J., and McManus, I. R. (1975) Biochemistry **14**, 4316-4325.

18. Stocken, L. A., and Thompson, H. S. (1946) Biochem. J. **40**, 529-535.

19. Jakoby, W. B. (1958) J. Biol. Chem. **232**, 89-97.

20. Springer, M. S., Groy, M. F., and Adler, J. (1979) Nature **280**, 279-284.

21. Wang, E. A., and Koshland, D. E. Jr. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 7157-7161.

22. Freitag, C., and Clarke, S. (1981) J. Biol. Chem. **256**, 6102-6108.

23. McFadden, P. N., and Clarke, S. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 2460-2464.

24. Hiatt, W. R., Garcia, R., Merrick, W. C., and Sypherd, P. S. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3434-3437.

25. Tidwell, T., Allfrey, V. G., and Mirsky, A. E. (1968) J. Biol. Chem. **243**, 707-715.

26. Sarnow, P., Rasched, I., and Knippers, R. (1980) Biochim. Biophys. Acta **655**, 349-358.

27. Scott, W. A., and Mitchell, H. K. (1969) Biochemistry **8**, 4282-4289.

28. Farooqui, J., Kim, S., and Paik, W. K. (1980) J. Biol. Chem. **255**, 4468-4473.

29. Mitchell, H. K., and Lipps, L. S. (1975) Biochem. Genet. **13**, 585-602.

30. Arrigo, A.-P., Fakan, S., and Tissieres, A. (1980) Dev. Biol. **78**, 86-103.

31. Levinger, L., and Varshavsky, A. (1981) J. Cell. Biol. **90**, 793-796.

32. Kelly, P. M., and Schlesinger, M. J. (1982) Mol. Cell. Biol. **2**, 267-274.

33. Hughes, E. N., and August, J. T. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 2305-2309.

34. Weatherbee, J. A., Luftig, R. B., and Weihing, R. R. (1980) Biochemistry **19**, 4116-4123.

35. Bass, R., Hedegaard, H. B., Dillehay, L., Moffett, J., and Engelsberg, E. (1981) J. Biol. Chem. **256**, 10259-10266.

Chapter 6

The Methylation of the 70,000 Dalton Heat Shock Protein In 3T3 Cells Is Altered by Arsenite Treatment and Rous Sarcoma Virus Transformation

Summary

Exposure of 3T3 cells and a Rous sarcoma virus transformed derivative of 3T3 cells (SR-RSV 3T3) to sodium arsenite, results in the induction of synthesis of several polypeptides in both cell types. One of the inducible polypeptides, with a molecular weight of 70,000, is shown to be methylated at lysyl and arginyl residues in the normal and transformed cell lines both before and after induction of its synthesis by arsenite. However, in the transformed cell line, the level of methylation of this protein at arginyl residues is lower than in its normal counterpart, while that at lysyl residues remains the same. Induction of the synthesis of the 70,000 dalton polypeptide in 3T3 cells by sodium arsenite results in a decrease in the methylation of this protein at its arginyl residues specifically in those 70,000 dalton molecules synthesized after induction, but it has no effect on its methylation at lysyl residues. In the transformed cells, on the other hand, arsenite treatment has no effect on the level of methylation of either arginyl or lysyl residues in this protein. Since the methylation level of total cellular proteins remains unchanged after arsenite treatment and transformation, the reduction of arginyl methylation in the 70,000 dalton polypeptide appears to be specific for this protein.

Introduction

It was first observed in Drosophila melanogaster that the synthesis of a particular set of polypeptides, known as heat shock proteins (hsp), is induced, and the synthesis of other cellular proteins is inhibited, when tissues are exposed to higher than normal growth temperatures (Tissières, Mitchell and Tracy, 1974). Since then, the experimental results from a number of laboratories have clearly demonstrated that the heat shock response in Drosophila may be regulated at both the transcriptional and the translational level (McKenzie, Henikoff and Meselson, 1975; Storti et al., 1980; Petersen and Mitchell, 1982; Scott and Pardue, 1982; for a review, see Ashburner and Bonner, 1979). The heat shock response was later shown to be generalized to a variety of tissue and cultured cells ranging from protozoans to mammals (Guttmann et al., 1980; McAlister and Finelstein, 1980; Kelley and Schlesinger, 1978; Atkinson, 1981; Wang, Gomer and Lazarides, 1981). However, recently, it has been shown that in addition to heat shock certain chemical compounds, including sodium arsenite, are also capable of inducing a similar, if not identical, set of polypeptides (Kelley and Schlesinger, 1978; Johnston et al., 1980; Wang, Gomer and Lazarides, 1981), suggesting that induction of heat shock proteins is a complex phenomenon.

A polypeptide with molecular weight of 70,000 is the most commonly induced species after heat shock and sodium arsenite treatment of several different cultured cells, including chicken embryo fibroblasts. We and other laboratories have reported that the 70,000 dalton polypeptide of chicken embryo fibroblasts is composed of two homologous, but not identical polypeptides, referred to as hsp70A and hsp70B, which have the same electrophoretic mobility on SDS gels but distinct isoelectric points (Wang, Gomer and Lazarides, 1981; Johnston et al., 1980). Both polypeptides are prominent cytoplasmic constituents expressed in substantial levels under normal cellular growth conditions and their synthesis can be induced by incubating cells with sodium arsenite. The more acidic variant (hsp70A) is a highly conserved polypeptide and is induced in

a number of avian and mammalian cells grown in tissue culture. On the other hand, the more basic variant (hsp70B) is not expressed in most mammalian cells; these cells synthesize instead a 68,000 dalton polypeptide in response to heat shock or sodium arsenite treatment. We have recently shown that in chicken embryo fibroblasts both hsp70A and hsp70B are irreversibly methylated at lysyl and arginyl residues before and after induction of their synthesis by sodium arsenite (Wang et al., 1982). The methylation of hsp70B at arginyl residue is reduced specifically after the induction of synthesis of this polypeptide by sodium arsenite (Wang and Lazarides, submitted).

In order to investigate if the methylation of hsp70 is also common to mammalian cultured cells, and whether arsenite treatment and viral transformation have any effect on the methylation of hsp70, we examined the level of methylation of this polypeptide in 3T3 and a Rous sarcoma virus transformed derivative of 3T3 cells (SR-RSV 3T3). We show that arsenite treatment induces the synthesis of several polypeptides in both 3T3 and SR-RSV 3T3 cells. However, the number of inducible polypeptides and the level of induction appear to be different. Transformed cells exhibit a higher level of induction of these polypeptides. In addition, hsp89, a polypeptide that is induced by arsenite in chicken fibroblasts, and a polypeptide with a molecular weight of 110,000, are induced only in the SR-RSV 3T3 cells. Similar to chicken embryo fibroblasts, hsp70 in 3T3 and SR-RSV 3T3 is methylated at lysyl and arginyl residues both before and after induction of its synthesis by arsenite. However, while the level of lysyl methylation of this polypeptide is the same in both 3T3 and SR-RSV 3T3 cells, the level of its methylation at arginyl residues is substantially reduced in SR-RSV 3T3 cells. Furthermore, the level of methylation at arginyl residues of hsp70 in 3T3 cells is reduced upon arsenite treatment specifically only to the population of molecules synthesized after induction, but it is not modulated and it remains at a low level in SR-RSV 3T3 cells both before and after arsenite treatment. These results suggest that hsp70 exists in different functional forms in these cells, which are determined by their methylation at their arginyl residues.

Normal cells appear to require a highly methylated form of hsp70; however, when transformed by Rous sarcoma virus they require additionally unmethylated forms of this protein.

Results

Arsenite Induces the Synthesis of Several Polypeptides in 3T3 and SR-RSV 3T3 Cells

Incubation of 3T3 and SR-RSV 3T3 cells with sodium arsenite increases the synthesis of several polypeptides. From the autoradiograms shown in Figure 1 and Figure 2, the increased synthesis of three polypeptides with molecular weights of 28,000, 68,000 and 70,000 is evident in both 3T3 and SR-RSV 3T3 cells. Two additional polypeptides with molecular weights of 89,000 and 110,000 are also synthesized at a higher rate specifically in SR-RSV 3T3 after sodium arsenite treatment. After coelectrophoresis of the arsenite-treated SR-RSV 3T3 cells with arsenite-treated chicken embryo fibroblasts, it appears that the 89,000 and 70,000 dalton polypeptides of 3T3 cells have identical mobilities with the corresponding induced polypeptides in chicken embryo fibroblasts. However, the 3T3 28,000 dalton polypeptide migrates between the two low molecular weight arsenite-inducible polypeptides (molecular weights 32,000 and 22,000) of chicken embryo fibroblasts (data not shown). In addition, we found that the 28,000 dalton polypeptide in 3T3 cells is not significantly induced by growing the cells at 43°C for two hours, under which conditions, the 68,000 and 70,000 dalton polypeptides are induced (Figure 1).

Figure 2 shows the results of the induced synthesis of polypeptides as a function of incubation time of SR-RSV 3T3 with sodium arsenite. Quantitation of the induction kinetics for SR-RSV 3T3 together with those of the 3T3 cells is given in Figure 3. The results suggest that 3T3 cells are less responsive to arsenite induction than SR-RSV 3T3 cells. For example, there is at least a three-fold increase in synthesis of the 70,000 dalton polypeptide in SR-RSV 3T3 after 4 to 6 hr of arsenite incubation, while the apparent increase in 3T3 cells of the same polypeptide is only approximately two-fold (Figure 3C).

Figure 1. Autoradiogram showing the arsenite and heat shock-inducible polypeptides in 3T3 cells. 3T3 cells were labeled with [^{35}S]-L-methionine as described in "Experimental Procedures". Comparable amounts of total cellular proteins were loaded onto each lane of the gel. (a) control, (b) incubated with 25 μM sodium arsenite for 4 hr, (c) grown at 43°C for 2 hr before labeling.

Figure 2. Autoradiogram showing the induction kinetics of proteins in arsenite-treated SR-RSV 3T3 cells. SR-RSV 3T3 cells were incubated with 25 μM sodium arsenite for 0-12 hr before labeling with [^{35}S]-L-methionine. Comparable quantities of protein were loaded in each lane. The times indicated below do not include the labeling period (15 min). Lane (a) 0 hr, (b) 1 hr, (c) 2 hr, (d) 4 hr, (e) 6 hr, (f) 8 hr, (g) 12 hr.

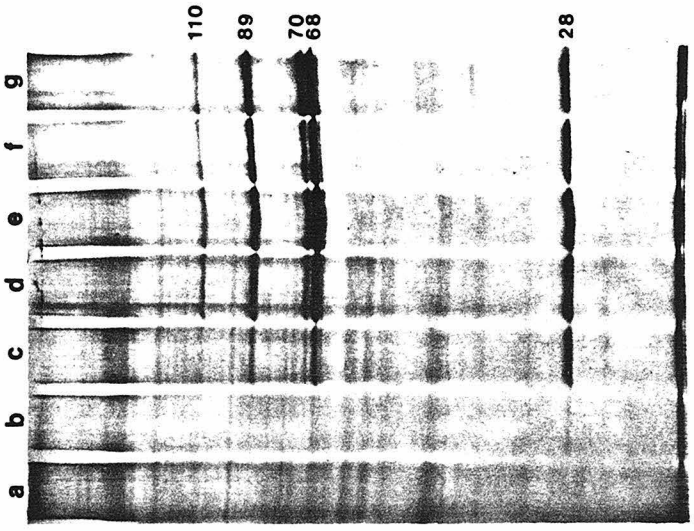


Figure 2

Figure 1

Figure 3. Induction kinetics of arsenite-inducible polypeptides. The [^{35}S]-radioactivity associated with a particular Coomassie blue-stained band was quantitated as described in "Experimental Procedures". The protein, loaded onto each lane, was estimated by scanning the Coomassie blue-stained gel with a densitometer. The rate of synthesis of control cells was arbitrarily assigned a value of 1 and the relative synthesis at other time points was obtained by taking the ratio of [^{35}S]-radioactivity per unit area to those of the control values. (A) 28,000 dalton polypeptide, (B) 68,000 dalton polypeptide, (C) 70,000 dalton polypeptide, (D) 89,000 dalton polypeptide, (E) 110,000 dalton polypeptide. Closed circles (●) and open circles (o) represent the data of SR-RSV 3T3 and 3T3 cells, respectively.

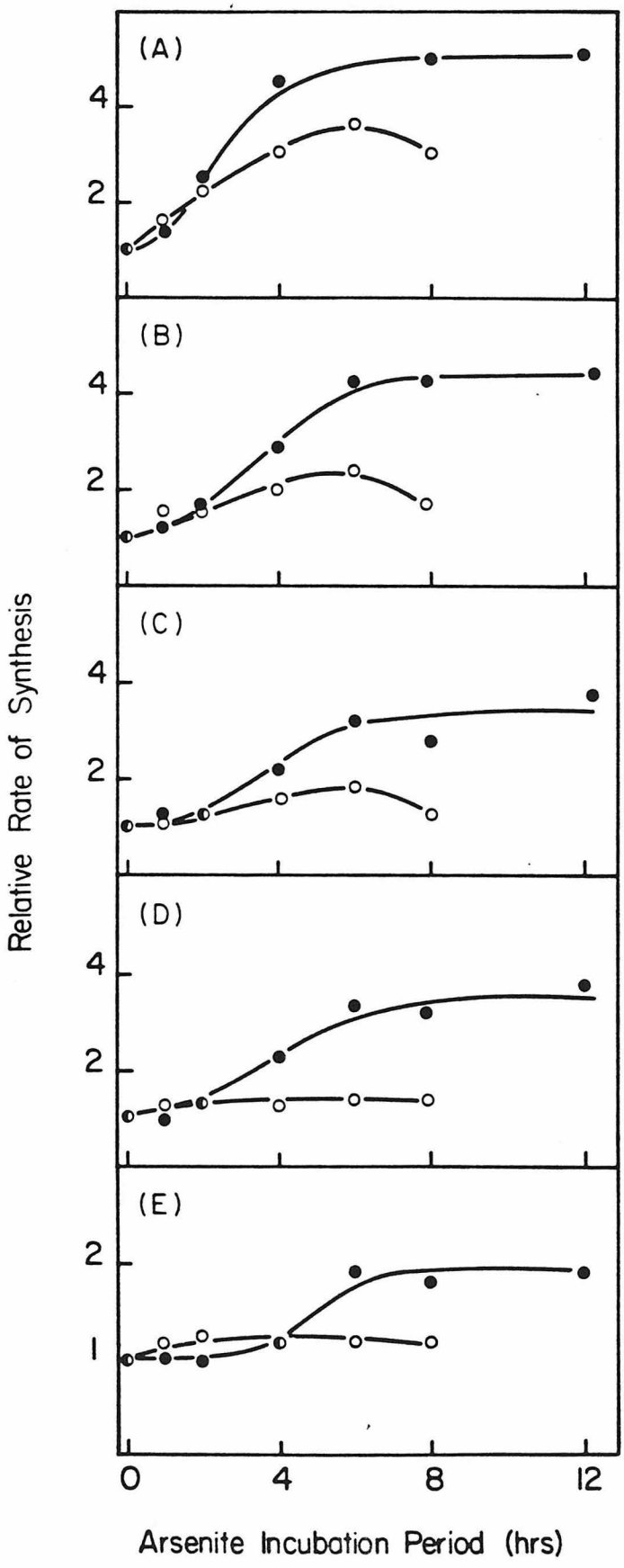

(A)
(B)
(C)
(D)
(E)
4
2
1
0
4
8
12
Relative Rate of Synthesis
Arsenite Incubation Period (hrs)

Figure 3

It is also evident that the induction of all the polypeptides follows the same kinetics in either 3T3 or SR-RSV 3T3 cells and that their rate of synthesis reaches a maximal level between 4 and 6 hr of arsenite incubation. However, while in 3T3 cells, the synthesis started to decrease after 6 to 8 hr of incubation, in SR-RSV 3T3 cells, the high rate of synthesis was maintained for at least 6 hr after reaching the maximal level. From Figure 3D and E, it is also evident that the 89,000 and 110,000 dalton polypeptides are induced only in SR-RSV 3T3 cells.

Comparison of the densitometric traces of Coomassie blue-stained gels corresponding to control cells with those of arsenite-treated cells showed no significant increase or decrease in any of the protein bands. Furthermore, quantitation of the [^{35}S]-radioactivity of many arbitrarily chosen protein bands showed that arsenite treatment does not inhibit the rate of protein synthesis in both 3T3 and SR-RSV 3T3 cells (data not shown).

Methylation of the 70,000 Dalton Arsenite-Inducible Polypeptide in 3T3 and SR-RSV 3T3 Cells

The cells (3T3 and SR-RSV 3T3) were grown and labeled in medium containing [methyl-^{3}H]-L-methionine; hence the [^{3}H]-radioactivity is incorporated into polypeptides not only as methionine but also as methyl groups, presumably by S-adenosyl-L-methionine dependent methyl transferases. The 70,000 dalton polypeptide was isolated by two-dimensional gel electrophoresis (Wang et al., 1981) and then hydrolyzed. The hydrolysates were subsequently subjected to amino acid analysis by ion exchange chromatography under conditions that only the basic amino acids could be retained and resolved by the resin. A set of elution profiles of such experiments with the 70,000 dalton polypeptides is shown in Figure 4. Besides the [^{3}H]-radioactive peaks corresponding to methionine, methyl-lysines and N^{G}-monomethyl-arginine, previously shown to be present also in hydrolysates from chicken embryo fibroblast hsp70 (Wang et al., 1982; Wang and Lazarides, submitted), an additional peak (peak III) was also observed which eluted

Figure 4. Ion exchange chromatography of hydrolyzed 70,000 dalton polypeptide labeled with [methyl-^{3}H]-L-methionine. The samples were prepared and the columns were operated as described in "Experimental Procedures". An aliquot from each fraction was analyzed for amino acids by reaction with ninhydrin and another aliquot was analyzed for [^{3}H]-radioactivity. (Top) ninhydrin reaction corresponding to a hsp70 hydrolysate from arsenite-treated SR-RSV 3T3 cells. (Middle-Top) [^{3}H]-radioactivity of a hsp70 sample from 3T3 cells. (Middle-Bottom) [^{3}H]-radioactivity of a hsp70 sample from arsenite-treated 3T3 cells. (Bottom) [^{3}H]-radioactivity of hsp70 isolated from arsenite-treated SR-RSV 3T3 cells corresponding to the ninhydrin in (Top). The results obtained for hsp70 from untreated SR-RSV 3T3 cells is similar to (Bottom). $Lys(Me)_3$: ε-N-trimethyl-lysine; $Arg(Me)_{2,S}$: N^{G},N'^{G}-dimethyl-arginine; Arg(Me): N^{G}-monomethyl-arginine.

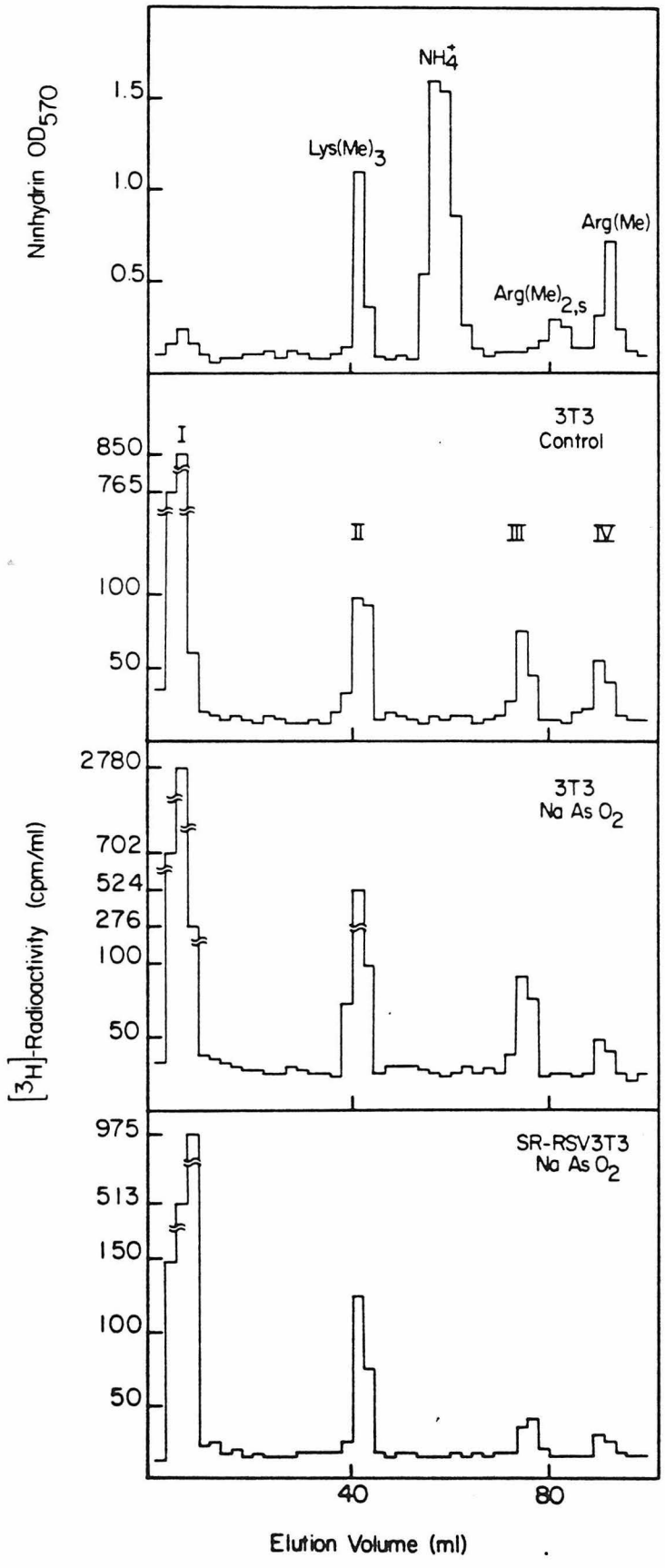

Ninhydrin OD570
1.5
1.0
0.5
NH4+
Lys(Me)3
Arg(Me)
Arg(Me)2,s
3T3
Control
I
II
III
IV
850
765
100
50
3T3
Na As O2
2780
702
524
276
100
50
SR-RSV3T3
Na As O2
975
513
150
100
50
[3H]-Radioactivity (cpm/ml)
40
80
Elution Volume (ml)

Figure 4

between NH_4^+ and N^G,N'^G-dimethyl-arginine. Since the only known methylated amino acid in proteins that elutes between NH_4^+ and N^G,N'^G-dimethyl-arginine with the procedure adopted here is N^G,N^G-dimethyl-arginine (data not shown) and since the [^{3}H]-radioactivity in peak III comigrates with the N^G,N^G-dimethyl-arginine standard by cellulose thin layer chromatography (see below), we tentatively conclude that the radioactive peak III is N^G,N^G-dimethyl-arginine.

Quantitative analysis of the ion exchange chromatography is given in Table I. Here, the level of methylation of various methylated amino acids is measured as fractions of the methionine incorporated into the same polypeptide backbone. From the data, it is evident that arsenite has little effect on the methylation of lysyl residues in the 70,000 dalton polypeptide of 3T3 cells. However, it appears that the level of methylation of N^G,N^G-dimethyl-arginine and N^G-monomethyl-arginine is reduced to 60% and 20% of the control level, respectively, in the presence of sodium arsenite. When 3T3 cells are prelabeled with [methyl-^{3}H]-L-methionine for 16 hr and then incubated with arsenite for 5 hr, the [^{3}H]-radioactivity in methylated arginyl residues relative to that of methionine is similar, if not identical, to the control cells (data not shown). We can, therefore, conclude that the reduction in the level of methylation at arginyl residues in the presence of arsenite occurs only to those 70,000 dalton polypeptides which are synthesized during the incubation period with the chemical. Furthermore, arsenite cannot remove those methyl groups which have already been incorporated into this protein prior to the treatment.

Table I also indicates that the amount of methyl-lysines in the 70,000 dalton polypeptide is practically the same in both 3T3 and SR-RSV 3T3 cells. On the other hand, the amount of [^{3}H]-radioactivity incorporated into the arginyl residues of the 70,000 dalton polypeptide from SR-RSV 3T3 is only 40% of that of 3T3 cells. In addition, arsenite treatment is not capable of further suppressing the methylation level at arginyl residues in SR-RSV 3T3 cells, as it is the case in 3T3 cells (Table I).

Table I. [^{3}H]-Radioactivity Incorporated into Basic Amino Acids of the 70,000 Dalton Arsenite-Inducible Polypeptide

	3T3 Cells		SR-RSV 3T3 Cells	
	control	arsenite-treated	control	arsenite-treated
ε-N-trimethyl-lysine	13±2%[a],[b]	14±3%	16±1%	14±3%
N^GN^G-dimethyl-arginine	5.5±0.5%	3.1±3%	2.0±0.4%	2.0±0.5%
N^G-monomethyl-arginine	4.5±0.2%	0.95%[c]	2.3±0.5%	1.8±0.7%

[a] The [^{3}H]-radioactivity of L-methionine is arbitrarily assigned as 100%.

[b] Average of duplicate determinations; the range is also given.

[c] Single determination; too little radioactivity associated with the other determination. If four times the standard deviation of background was used to calculate the percentage for that undetermined experiment, we obtained a value of 1.5% of N^G-monomethyl-arginine.

Since the elution conditions adopted above for ion exchange chromatography cannot distinguish among the ε-N-monomethyl-lysine, ε-N-dimethyl-lysine and ε-N-trimethyl-lysine species, thin layer chromatography was additionally utilized to separate these three different methyl-lysines. The methyl-lysine fractions (peak II of Figure 4) were pooled together, desalted and spotted on thin layer chromatogram sheets before developing in pyridine:acetone:3 M NH_4OH (15:9:6). From the results, it is evident that ε-N-trimethyl-lysine is the predominant, if not the only, species (Figure 5A). Furthermore, neither arsenite treatment, nor Rous sarcoma virus transformation, change the distribution of methyl-lysines (data not shown).

The same thin layer chromatography procedures have also been applied to peaks III and IV of Figure 4. The results (Figure 5B, C) demonstrate that the [^{3}H]-radioactivity in peaks III and IV comigrate with the N^G,N^G-dimethyl-arginine and N^G-monomethyl-arginine standard, respectively, which confirms the previous assignments (see above, the results of ion exchange chromatography).

In order to calculate the stoichiometry of these methylations, one has to know the relative specific activity of [^{3}H]-methionine and [^{3}H]-methyl groups incorporated into the 70,000 dalton polypeptides and the number of methionines per polypeptide. Assuming that the specific activity of [^{3}H]-methionine and [^{3}H]-methyl group incorporation is identical and that the number of methionine residues per 3T3 70,000 dalton polypeptide is the same as that which has been predicted from the DNA sequence of the Drosophila hsp70 gene (Ingolia, Craig and McCarthy, 1980), we estimate that there are 0.5 ε-N-trimethyl-lysines, 0.3 N^G,N^G-dimethyl-arginines and 0.5 N^G-monomethyl-arginines per 70,000 dalton polypeptide in 3T3 cells under normal growth conditions.

Methylation of the 68,000 Dalton Inducible Polypeptides in SR-RSV 3T3 Cells

The 68,000 dalton heat shock protein has been shown to be highly homologous to the 70,000 dalton polypeptide by limited proteolysis (Hightower and White, 1981). Therefore, we wished to find out if the 68,000 dalton polypeptide is also methylated.

Figure 5. Thin layer chromatography of methylated amino acids present in the 70,000 dalton arsenite-inducible polypeptide. (A) Methyl-lysine fractions (Peak II, Figure 4) were mixed with ε-N-monomethyl-lysine (lys(Me); Vega Biochemicals), ε-N-dimethyl-lysine ($lys(Me)_2$; Vega Biochemicals) and ε-N-trimethyl-lysine before they were desalted and developed by cellulose thin layer chromatography as described in "Experimental Procedures". The results presented here correspond to a hsp70 hydrolysate from 3T3 cells. (B) Peak III (Figure 4) was mixed with N^G,N^G-dimethyl-arginine then desalted and developed by cellulose thin layer chromatography. (C) Peak IV (Figure 4) was mixed with N^G-monomethyl-arginine, desalted and developed by cellulose thin layer chromatography. The background [^{3}H]-radioactivity was not substracted in this Figure.

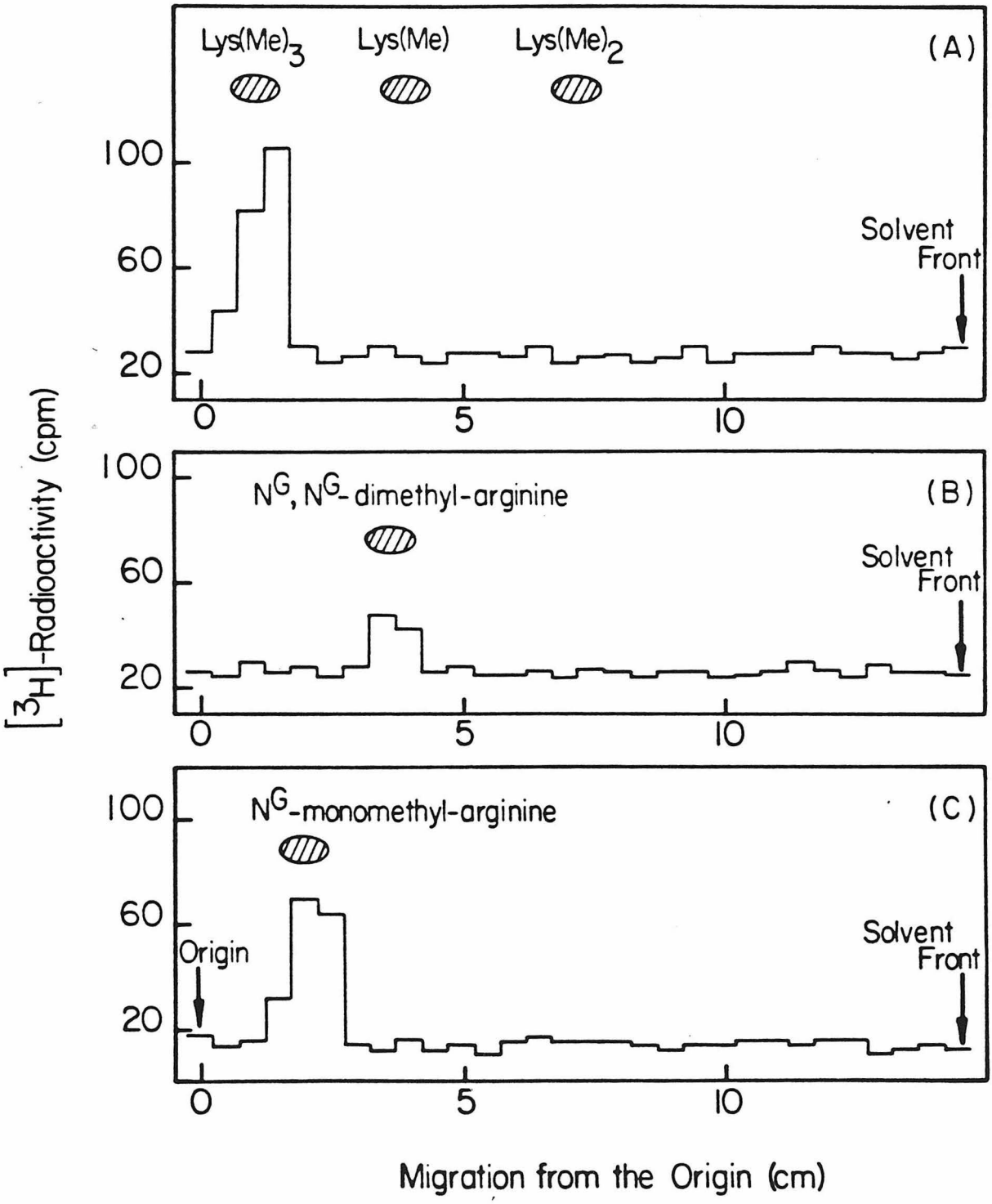

Lys(Me)3
Lys(Me)
Lys(Me)2
(A)
100
60
20
Solvent Front
0
5
10
N^G, N^G- dimethyl-arginine
(B)
N^G-monomethyl-arginine
(C)
Origin
[3H]-Radioactivity (cpm)
Migration from the Origin (cm)

Figure 5

Since the amount of this polypeptide is very low before induction (Wang, Gomer and Lazarides, 1981; Hughes and August, 1982), we analyzed only the samples isolated from arsenite treated SR-RSV 3T3 cells. The elution profile of [methyl-^{3}H]-L-methionine-labeled 68,000 dalton polypeptide from an ion exchange column is similar to those of Figure 4. It is evident that, similar to the 70,000 dalton polypeptide, the 68,000 dalton arsenite-inducible polypeptide is methylated at both lysine and arginine residues. However, we obtained a rather different stoichiometry on two samples prepared at different times (data not shown), and presently it is unclear how the level of methylation in this protein compares to that in the 70,000 dalton polypeptide.

Discussion

Arsenite-inducible Polypeptides in 3T3 and SR-RSV 3T3 Cells

In this paper we have shown that the number of sodium arsenite-inducible polypeptides and the level of increase in their rate of synthesis are different between 3T3 and SR-RSV 3T3 cells, being higher in the transformed cells (SR-RSV 3T3). One of the polypeptides, which is induced only in SR-RSV 3T3 cells, has a molecular weight of 89,000 and comigrated with the 89,000 dalton heat shock protein in chicken embryo fibroblasts. This polypeptide has been shown to be associated with the protein kinase pp60^{src} encoded by the transforming gene of Rous sarcoma virus (Oppermann, Levinson and Bishop, 1981; Courtneidge and Bishop, 1982). At present, contradictory conclusions have been drawn on whether this polypeptide is also the 95,000 dalton polypeptide whose synthesis is induced by glucose starvation (Shiu, Pouyssegur and Pastan, 1977; Kelley and Schlesinger, 1978; Lee, Delegeane and Scharff, 1981; Lanks et al., 1982).

The results of two-dimensional gel electrophoresis from this and other laboratories indicate that the 70,000 dalton arsenite-inducible polypeptide of 3T3 cells is composed of a single major species which is highly conserved in avian and mammalian cells and comigrates with the more acidic of the two major heat shock and arsenite-inducible polypeptides from chicken embryo fibroblasts (Wang, Gomer and Lazarides, 1981).

The more basic variant of hsp70 observed in chicken fibroblasts has not been found in mammalian cells. Instead in 3T3 and some other mammalian cells heat shock and sodium arsenite induce the synthesis of a 68,000 dalton polypeptide (Wang, Gomer and Lazarides, 1981; Hughes and August, 1982). However, slightly different molecular weights of the 68,000 and 70,000 dalton inducible polypeptides have been reported by different laboratories, possibly due to differences in the gel system and/or molecular weight standard utilized. For comparison, we tabulate here these different molecular weights reported in the literature and referred to in this work corresponding to the same polypeptide (Table II).

Sodium arsenite also induces in both 3T3 and SR-RSV 3T3 cells the synthesis of a 28,000 dalton polypeptide, while it does not induce a corresponding polypeptide in chicken fibroblasts. Instead, sodium arsenite induces the synthesis of two polypeptides with molecualr weights of 32,000 and 22,000 in chicken embryo fibroblasts. However, apparently neither the 32,000 dalton polypeptide in cultured chicken and quail cells nor the 28,000 dalton polypeptide in certain mammalian cultured cells can be induced by heat shock (Kelley and Schlesinger, 1978; Atkison, 1981; this work). The possibility that these polypeptides may be inducible under certain heat shock conditions cannot be completely ruled out (Johnston et al., 1980). Nevertheless, one may speculate that the 28,000 dalton arsenite-inducible polypeptide in 3T3 and SR-RSV 3T3 cells is not a heat shock-inducible protein. It is not uncommon that heat shock and arsenite treatment produce different results. For example, arsenite treatment facilitates the turn-over of phosphate on tropomyosin in chicken myogenic cells grown in tissue culture (Wang and Lazarides, 1983), but heat shock fails to produce a similar effect (unpublished observation).

We have also compared the densitometric traces of one-dimensional Coomassie blue-stained gels of total cellular proteins of 3T3, SR-RSV 3T3 and chicken embryo fibroblasts before and after treatment with sodium arsenite. They reveal that the

Table II. Reported Molecular Weights of 68,000-70,000 Dalton Heat Shock Protein of Cultured Cells

Heat Shock Proteins		cultured cells	References
1	2		
68,000	70,000	3T3, SR-RSV 3T3	this work
75,000	76,000	chicken fibroblasts, BHK	Kelley and Schlesinger, 1978
-	73,000	chicken fibroblasts	Johnston et al., 1980
-	64,000	quail myogenic cells	Atkinson, 1981
66,000	68,000	chicken fibroblasts, BHK	Wang, Gomer and Lazarides, 1981 Wang et al., 1982
70,000	72,000	3T3	Hughes and August, 1982
71,000	73,000	rat embryo cultured cells	Hightower and White, 1981
-	73,000	3T3	Lee, Delegoan and Scharff, 1981

number of polypeptide copies of the 70,000 and 89,000 dalton polypeptides in chicken fibroblasts steadily increase for at least 8 hr (data not shown). However, no accumulation of the polypeptides was observed in 3T3 and SR-RSV 3T3 cells. The reason for this difference is not clear. It may be due to the fact that the number of copies of these two polypeptides and their rate of turn-over in 3T3, SR-RSV 3T3 and chicken fibroblasts is very different. Nevertheless, it is clear that the failure to detect a significant increase in the 68,000 dalton polypeptide by one dimensional gel electrophoresis after arsenite treatment is due to the other proteins which comigrate with the 68,000 dalton polypeptide; these contaminating polypeptides can be easily resolved from the 68,000 dalton inducible polypeptide by two-dimensional gel electrophoresis.

We have quantitated the increase in the rate of synthesis of several polypeptides of arsenite-treated 3T3 and SR-RSV 3T3 cells by counting the [^{35}S]-radioactivity associated with a particular band and comparing that with the corresponding densitometric traces of Coomassie blue-stained one-dimensional gels. Since the whole cell exctracts were analyzed, it is likely that some unrelated polypeptides may comigrate with the inducible polypeptides; hence the actual control level of synthesis may be lower than what we obtained here. Thus, the numbers presented in Figure 3 should be the lower limit of increase in synthesis. Nevertheless, the kinetic properties of induction should not be affected by the higher background, although the true quantitative level of induction may be higher. From the data, it is clear that the arsenite-induced response of 3T3 polypeptides is different from that of SR-RSV 3T3. In 3T3 cells, the rate of synthesis of inducible proteins reaches a maximal level between 4 and 6 hr of incubation; then it begins to decline. However, in SR-RSV 3T3 cells, the rate of synthesis remains at the maximal level for at least 6 hr. The induction kinetics of 3T3 inducible polypeptides is identical to that of the arsenite-inducible polypeptides in chicken fibroblasts; an exception is the induction kinetics of the 22,000 dalton polypeptide in chicken fibroblasts, which is similar to the SR-RSV 3T3 indubicle polypeptides. Thus transformation of 3T3

cells by Rous sarcoma virus appears to affect not only the polypeptide species inducible by arsenite but also their kinetics of induction.

Methylation of hsp70 is Altered in 3T3 Cells by Arsenite and Rous Sarcoma Virus Transformation

In chicken embryo fibroblasts the 70,000 dalton polypeptide has been shown to be methylated at lysyl and arginyl residues. The most abundant methyl lysine species detected in this protein is ε-N-trimethyl-lysine; however, ε-N-dimethyl-lysine and ε-N-monomethyl-lysine are also detected. Furthermore, N^G-monomethyl-arginine is the only detectable methylated arginine species (Wang et al., 1982; Wang and Lazarides, submitted). However, as we have shown here, the methylation pattern of hsp70 in 3T3 cells is somewhat different. ε-N-trimethyl-lysine appears to be the only methylated lysine species and, in addition to N^G-monomethyl-arginine, N^G,N^G-dimethyl-arginine also is consistently found in hydrolysates of the 70,000 dalton polypeptide isolated from 3T3 and SR-RSV 3T3 cells.

The existence of minute amounts of ε-N-monomethyl-lysine and ε-N-dimethyl-lysine in 3T3 hsp70 cannot be entirely ruled out. Assuming that the contribution of ε-N-monomethyl-lysine and ε-N-dimethyl-lysine is equal to twice the standard deviation of the background [^{3}H]-radioactivity (the upper limit) in thin layer chromatography (Figure 5A), we estimate that the lower limit of the contributuion of ε-N-trimethyl-lysine to the total methylated lysine species is 90%. This value is still significantly higher than that of the hsp70 in chicken fibroblasts (Wang et al., 1982; Wang and Lazarides, submitted).

Arsenite treatment of chicken embryo fibroblasts causes a change in the ratio of the three methylated lysine species in hsp70A, while in hsp70B it is unaffected (Wang and Lazarides, submitted). Here we have demonstrated that in all cases ε-N-trimethyl-lysine is the only methyl-lysine species in the 70,000 dalton polypeptides of 3T3 and SR-RSV 3T3 cells and that it cannot be modulated by arsenite treatment. We also found that the level of methylation at arginyl residues in hsp70B of chicken embryo fibroblasts

was substantially reduced after arsenite treatment, while it remained unchanged in hsp70A (Wang and Lazarides, submitted). Here, we have demonstrated that the amount of methyl groups incorporated into arginyl residues in 3T3 hsp70 is reduced significantly in the presence of arsenite. Thus, the methylation behavior of the 70,000 dalton polypeptide of 3T3 cells is similar to that of the hsp70B polypeptide in chicken fibroblasts, even though the mammalian 70,000 dalton polypeptide is highly homologous to the chicken fibroblast hsp70A rather than to hsp70B. Furthermore, since basic amino acid methylation of total protein remains unchanged after arsenite treatment (Wang and Lazarides, submitted) the reduction of arginyl methylation of hsp70 observed in 3T3 cells after arsenite treatment appears to be specific for this protein.

Relatively little [^{3}H]-radioactivity was found to be incorporated into the 70,000 dalton polypeptide in the presence of protein synthesis inhibitors (data not shown), just as was the case with the homologous protein in chicken embryo fibroblasts (Wang et al., 1982). Thus, it is reasonable to assume that, with the procedures ultiized here, the majority of the methylation of the 70,000 dalton polypeptide occurs only to the polypeptides which are synthesized during the labeling period. We have estimated that the stoichiometry of methylation is 0.5 ε-N-trimethyl-lysines, 0.3 N^G,N^G-dimethyl-arginines and 0.5 N^G-monomethyl-arginines per molecule. The substoichiometry obtained here may be partly due to the assumption that the average specific activity of [^{3}H]-methionine and [^{3}H]-methyl groups incorporated into the polypeptide is identical. It is likely that methylation may be stoichiometric, i.e., one methylated lysine and one methylated arginine per molecule. Furthermore, the appearance of two different methylated arginine species in 3T3 hsp70 does not necessarily mean that two different methyl arginine sites exist in the molecule; it may be simply due to a stepwise methylation at an identical site (Lee, Kim and Paik, 1977).

Although we did not detect any difference in the level of methylation of lysyl residues in hsp70, the level of methylation of this protein at arginyl residues was clearly

reduced in SR-RSV 3T3 cells. This difference in the level of methylation of the hsp70 after virus transformation appears to be specific to this protein. Since the ratio of S-adenosyl-L-methionine to S-adenosyl-L-homocysteine remains normal after transformation (Coalson et al., 1982), presumably, the gross methylation level of proteins should not be altered (Hoffman et al., 1980). Indeed, the radioactive methyl groups incorporated into total cellular proteins and nucleic acids in chicken embryo fibroblasts does not change appreciably after Rous sarcoma virus transformation (Pierre et al., 1977).

Thus, it is clear that both the level of induction of protein synthesis by arsenite and the methylation of the 70,000 dalton arsenite-inducible polypeptide at arginyl residues are different between 3T3 and SR-RSV 3T3 cells. One may speculate that these differences are due to the transformation of 3T3 cells by Rous sarcoma virus. Nevertheless, one has to keep in mind that the 3T3 cells utilized in these experiments were all quiescent (confluent plates), and the SR-RSV 3T3 cells were still actively growing even after reaching confluency. Therefore, the difference observed here may be due to the different growth phases of the two cell populations.

The function of the 70,000 dalton arsenite-inducible polypeptide is presently unknown, even though it is a rather abundant cytoplasmic constituent, even under normal growth conditions, and highly conserved. Thus, this polypeptide should be functionally important for the cells. Similarly, the functional significance of basic amino acid methylation in a variety of polypeptides is largely unclear (Paik and Kim, 1980). Conceivably, such a chemical modification of proteins may play an as yet unidentified regulatory role. From the results presented here and previously, it is clear that the 70,000 dalton heat shock or arsenite-inducible proteins are methylated at both lysyl and arginyl residues in 3T3, SR-RSV 3T3 and chicken fibroblasts. Furthermore, its level of methylation can be altered by arsenite and by Rous sarcoma virus transformation. Thus it is not unreasonable to assume that changes in the methylation of hsp70 may either alter the functional effectiveness of the polypeptide or completely change its functional role

or cytoplasmic distribution. If this is the case then the results presented here suggest that normal cells require predominantly the methylated form of hsp70 under normal growth conditions; however, when treated with arsenite or transformed by a RNA tumor virus they required additionally unmethylated forms of this protein.

Experimental Procedures

Sodium Arsenite Treatment

3T3 and SR-RSV 3T3 (a Rous sarcoma virus transformed 3T3 cell line kindly provided by Dr. T. Hunter) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (GIBCO). The cells were incubated with 25 μM sodium arsenite for 0 to 12 hr. Then they were rinsed once with methionine-free minimal essential medium and labeled with 15 μCi/ml of [^{35}S]-L-methionine (New England Nuclear, specific activity: 1033.6 Ci/mmole) in the same medium for 20 min. At the end of the incubation, the cells were rinsed twice with phosphate buffered saline, scraped off the plates and harvested by centrifugation. The cell pellets were immediately boiled for 3 min in SDS sample buffer (2% SDS, 0.5% 2-mercaptoethanol, 25 mM Tris·HCl pH 7.5, 10% glycerol and 0.0003% Bromophenol Blue) for one-dimensional gel electrophoresis. Alternatively, the pellets were first boiled in 10 mM Tris·HCl pH 6.8 containing 1% 2-mercaptoethanol and 2% SDS (200 μl solution per 100 mm confluent plate), then 0.15 to 0.2 grams of solid urea were added, before subjecting the samples to two-dimensional IEF/SDS polyacrylamide gel electrophoresis.

Gel Electrophoresis and Autoradiography

The procedure described by Laemmli (1970) was followed for one-dimensional SDS gel electrophoresis. The stacking gel contained 5% acrylamide, 0.13% N.N'-methylene bis-acrylamide, 0.125 M Tris.HCl pH 6.8 and 0.1% SDS. The resolving gel was 12.5% acrylamide, 0.09% N,N'-methylene bisacrylamide, 0.38 M Tris·HCl pH 8.7 and 0.1% SDS. Two-dimensional IEF/SDS polyacrylamide gel electrophoresis was performed

by the method of O'Farrell (1975), except that the isoelectric focusing gels were prepared without detergent. After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue in 50% ethanol and 10% acetic acid, and destained with 12.5% ethanol, 5% acetic acid. The one-dimensional gels were then scanned with a densitometer before they were dried for autoradiography.

To quantitate the [^{35}S]-radioactivity in Coomassie blue-stained bands of interest, the stained bands were excised from the dried gels and placed into scintillation vials. Half a milliliter of 0.1% SDS in 0.15 M NH_4HCO_3 was added and allowed to incubate overnight to elute the polypeptides. Then 5 ml of Aquasol-2 scintillation fluid (New England Nuclear) was added and the samples were counted in a liquid scintillation counter.

Labeling of Cells with [Methyl-^{3}H]-L-Methionine and Basic Amino Acid Analysis

Confluent 100 mm plates of 3T3 and SR-RSV 3T3 cells were rinsed once with methionine-free minimal essential medium. Then the cells were allowed to incubate in the same medium supplemented with 1% calf serum and 50 μCi/ml [methyl-^{3}H]-L-methionine (New England Nuclear, specific activity: 80 Ci/mmole) with or without 25 μM sodium arsenite for 6 to 12 hr. The cells were subsequently harvested and subjected to two-dimensional gel electrophoresis as described above. The spot corresponding to the 70,000 dalton polypeptide was excised from the Coomassie blue-stained gel, and electrophoretically eluted from the gel slices in 0.1 M NH_4HCO_3 and 0.1% SDS. The eluted protein was then hydrolyzed with 6 N HCl in an evacuated tube for 24 hr at 108°C as described (Wang et al., 1982). Subsequently, the HCl was evaporated, and the dried residues were resuspended in 0.2 N sodium citrate sample buffer, pH 2.0 with 0.2 to 1.0 μmole of ε-N-trimethyl-lysine, N^G,N'^G-dimethyl-arginine and N^G-monomethyl-arginine as added standard (CalBiochem). The samples were loaded onto an amino acid analysis column (0.9 x 10 cm, Beckman PA-35) operated at 56°C, with a flow rate of 66 ml/hr in 0.35 N sodium citrate buffer, pH 5.28. Two min fractions were collected and the [^{3}H]-radioactivity and amino acids were analyzed by liquid scintillation

counting and ninhydrin, respectively, as described (Wang et al., 1982).

After ion exchange chromatography, the [^{3}H]-radioactivity peaks corresponding to the methylated basic amino acids were desalted and chromatographed on cellulose thin layer sheets (Eastman Kodak, No. 13255), using pyridine:acetone:3 M NH_4OH (10:6:5) as solvent, as described previously (Wang et al., 1982). Amino acids were localized by spraying the sheets with 0.2% ninhydrin in acetone. Subsequently, 5 mm wide strips of cellulose were cut perpendicular to the direction of migration and scraped into scintillation vials. Thereafter, they were digested overnight with NCS tissue solubilizer (Amersham; 1 ml/5 cm^2 of cellulose) containing 15% double distilled water; then 10 volumes of OCS scintilltion fluid (Amersham) were added for liquid scintillation counting.

Acknowledgements

We thank Dr. Steven Clarke for the use of his amino acid analyzer facilities. This work was supported by grants from the National Institutes of Health, the National Science Foundation and the Muscular Dystrophy Association of America. E.L. is a recipient of a National Institutes of Health Research Career Development Award.

References

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

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

Coalson, D. W., Mecham, J. O., Stern, P. H. and Hoffman, R. M. (1982). Reduced availability of endogenously synthesized methionine for S-adenosylmethionine formation in methionine-dependent cancer cells. Proc. Nat. Acad. Sci. USA 79, 4248-4251.

Courtneidge, S. A. and Bishop, J. M. (1982). Transit of $pp60^{v-src}$ to the plasma membrane. Proc. Nat. Acad. Sci. USA 79, 7117-7121.

Guttman, S. D., Glover, C. V. C., Allis, C. D. and Gorovsky, M. A. (1980). Heat shock, deciliation and release from anoxia induce the synthesis of the same set of polypeptides in starved T. pyriformis. Cell 22, 299-307.

Hightower, L. E. and White, F. P. (1981). Cellular response 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.

Hoffman, D. R., Marion, D. W., Cornatzer, W. E. and Duerre, J. A. (1980). S-adenosyl-L-methionine and S-adenosyl-L-homocysteine metabolism in isolated rat liver. J. Biol. Chem. 255, 10822-10827.

Hughes, E. N. and August, J. T. (1982). Coprecipitation of heat shock proteins with a cell surface glycoprotein. Proc. Nat. Acad. Sci. USA 79, 2305-2309.

Ingolia, T. D., Craig, E. A. and McCarthy, B. J. (1980). Sequence of three copies of the gene for the major Drosophila heat shock induced protein and their flanking region. Cell 21, 669-679.

Johnston, D., Oppermann, H., Jackson, J. and Levinson, W. (1980). Induction of four proteins in chicken embyro cells by sodium arsenite. J. Biol. Chem. 255, 6975-6980.

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

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lanks, K. W., Kasambalides, E. J., Chinkers, M. and Brugge, J. S. (1982). A major cytoplasmic glucose-regulated protein is associated with the Rous sarcoma virus pp60^{src} protein. J. Biol. Chem. 257, 8604-8607.

Lee, A. S., Delegeane, A. and Scharff, D. (1981). Highly conserved glucose-regulated protein in hamster and chicken cells: Preliminary characterization of its cDNA clone. Proc. Nat. Acad. Sci. USA 78, 4922-4925.

Lee, H. W., Kim, S. and Paik, W. K. (1977). S-adenosyl-L-methionine: protein-arginine methyltransferase. Purification and mechanism of the enzyme. Biochemistry 16, 78-85.

McAlister, L. and Finkelstein, D. B. (1980). Heat shock proteins and thermal resistance in yeast. Biochem. Biophys. Res. Commun. 93, 819-824.

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

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

Oppermann, H., Levinson, W. and Bishop, J. M. (1981). A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. Proc. Nat. Acad. Sci. USA 78, 1067-1071.

Paik, W. K. and Kim, S. (1980). Protein Methylation, New York, John Wiley and Sons.

Petersen, N. S. and Mitchell, H. K. (1981). Recovery of protein synthesis after heat shock: Prior heat treatment affects the ability of cells to translate mRNA. Proc. Nat. Acad. Sci. USA 78, 1708-1711.

Pierre, A., Richou, M., Lawrence, F., Robert-Gero, M. and Vigier, P. (1977). Decrease rate of S-adenosyl-L-homocysteine metabolism: An early event related to transformation in cells infected with Rous sarcoma virus. Biochem. Biophys. Res. Commun. 76, 813-819.

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

Shiu, R. P. C., Pouyssegur, J. and Pastan, I. (1977). Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chicken embryo fibroblasts. Proc. Nat. Acad. Sci. USA 74, 3840-3844.

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

Tissières, A., Mitchell, H. K. and Tracy, U. M. (1974). Protein synthesis in salivary glands of Drosophila melanogaster: relation to chormosome puffs. J. Mol. Biol. 84, 389-398.

Wang, C., Gomer, R. H. and Lazarides, E. (1981). Heat shock proteins are methylated in avian and mammalian cells. Proc. Nat. Acad. Sci. USA 78, 3531-3535.

Wang, C. and Lazarides, E. (1983). Arsenite facilitates the turn-over of phosphate on cultured chicken muscle tropomyosin. Biochem. Biophys. Res. Commun., in press.

Wang, C., Lazarides, E., O'Connor, C. M. and Clarke, S. (1982). Methylation of chicken fibroblast heat shock proteins at lysyl and arginyl residues. J. Biol. Chem. 257, 8356-8362.

Chapter 7

Conclusion

It has been documented that arsenite increases the synthesis of a set of polypeptides similar to those induced by heat shock (1, Chapters 3 and 6). The effect of heat shock and arsenite treatment does not appear to be identical; the 28,000 dalton arsenite-inducible polypeptide is not a heat shock protein (Chapter 6), and the 32,000 dalton arsenite-inducible polypeptide of chicken fibroblasts may not be inducible by heat shock (1, 2 and Chapter 2). While it is generally accepted that heat shock results in a reduction in synthesis of non-inducible cellular proteins (3), this regression of normal protein synthesis is not observed with arsenite treatment (Chapters 2 and 6). In addition, the turnover of phosphate on tropomyosin of cultured chicken muscle cells is facilitated by arsenite (Chapter 8), presumably by changing the thiol status of the enzyme(s) involved; heat shock cannot produce the same effect. Thus, it is evident that heat shock and sodium arsenite treatment are not equivalent.

I have demonstrated that similarities and differences exist in the kinetics of arsenite-induced protein synthesis among chicken fibroblasts, 3T3 and SR-RSV 3T3 cells (Chapters 2 and 6). Briefly, there are seven and three arsenite-inducible polypeptides in chicken fibroblasts and 3T3 cells, respectively. Compared to those of 3T3 cells, two additional polypeptides are inducible in a Rous sarcoma transformed derivative of 3T3 cells (SR-RSV 3T3). In all these cell systems, only the 70,000 dalton polypeptide is commonly induced. It appears that the rates of synthesis of these polypeptides reach maximal levels after four to six hours of arsenite incubation. The synthesis of inducible polypeptides of SR-RSV 3T3 and the 22,000 dalton inducible polypeptide of chicken fibroblasts remains at a high level for at least six more hours. On the other hand, the synthesis of the rest of the inducible polypeptides of chicken fibroblasts and 3T3 cells starts to decline after prolonged arsenite incubation (Chapters 2 and 6). At present, the course of this difference in induction kinetics is unclear. In the case of 3T3 and SR-RSV 3T3 cells, the difference may be a consequence of viral transformation. Alternatively, the discrepancy may be due to the given cellular state, as quiescent 3T3 cells were actively dividing (Chapter 6).

The genetics of heat shock proteins in Drosophila has been intensively studied (for a review, see 4). Despite this effort, the function of these proteins is not well defined. Although the function of heat shock proteins has been suggested to confer resistance for further lethal heat shock (5, 6), the molecular basis for this "thermotolerance" is completely unknown. Furthermore, recent experimental results on a mammalian cultured cell line suggests that the 68,000 dalton heat shock protein alone is sufficient for the protection from lethal heat shock (7), although the polypeptide is not detectable in chicken fibroblasts.

The intracellular localization of these heat shock proteins has been studied. It is generally accepted that the low molecular weight heat shock proteins are nuclear proteins and the high molecular weight proteins are cytoplasmic (8, 9, 10). It is not unreasonable to assume that these proteins are capable of performing different functional roles which may or may not be related to each other.

The 70,000 dalton heat shock protein (hsp70) in cultured chicken cells is composed of two major distinct isoelectric variants (1, Chapter 3) which may be cytoplasmic constituents both before and after arsenite treatment (10). The more acidic one (hsp70A; pI 5.6) is highly conserved and also exists in mammalian cultured cells (Chapters 3 and 6). The more basic one (hsp70B; pI 6.0) has been found only in avian tissues or cultured cells. A polypeptide of 68,000 daltons is expressed in mammalian cells after induction (Chapter 3); these three polypeptides may be homologous to one another (1, 11).

I have observed that each major polypeptide of hsp70 is flanked by minor isoelectric variants in two-dimensional gels. Originally, I thought that this heterogeneity could be the result of phosphorylation, but no [^{32}P]-phosphate was found to be incorporated into these polypeptides. Subsequently, it became evident that the existence of minor components of hps70 could be due to basic amino acid methylation (Chapters 2-6), which will be summarized below.

The basic amino acid methylation of hsp70 appears to be irreversible and closely coupled to its synthesis, but can also occur hours after the polypeptide has been completed. The methylation sites have been identified as lysyl and arginyl residues. In chicken fibroblasts, ε-N-trimethyl-lysine is the major methylated lysine species, but the contribution of ε-N-dimethyl-lysine and ε-N-monomethyl-lysine is evident. Furthermore, N^G-monomethyl-arginine is the only methylated arginine species (Chapters 4 and 5). However, in 3T3 cells, the methyl-lysine in hsp70 is exclusively ε-N-trimethyl-lysine and both N^G,N^G-dimentyl-arginine and N^G-monomethyl-arginine were found (Chapter 6). In addition, the level of methylation at arginyl residues of hsp70B of chicken fibroblasts can be modulated by arsenite (Chapter 5). A similar effect was observed for the hsp70 of 3T3 cells. However, the reduction of arginyl methylation can occur only in those polypeptides synthesized in the presence of arsenite. I also discovered that under normal growth conditions, the methylation level of arginyl residues of hsp70 isolated from SR-RSV 3T3 cells is constitutively lower than their untransformed counterparts (3T3 cells), and it cannot be reduced further by arsenite (Chapter 6).

I demonstrated in Chapter 2 that a 78,000 dalton polypeptide is induced in chicken fibroblasts by arsenite (the molecular weight has been reported as 83,000 in Chapters 3 and 4), but it is not inducible in mammalian cultured cells such as BHK, HaLa and 3T3 cells. The polypeptide is highly conserved and is methylated at lysyl residues in both chicken fibroblasts and mammalian cultured cells. The methylation of this polypeptide appears closely coupled to its synthesis and can be modulated by arsenite treatment. [^{32}P]-Radioactivity is also incorporated into this polypeptide after incubating the cells with [^{32}P]-phosphoric acid. Since the polypeptide is also ADP-ribosylated (Carlsson and Lazarides, unpublished), it is unclear whether the polypeptide is phosphorylated. Because of their similarities in molecular weights and isoelectric points, I speculate that this polypeptide is the highly conserved glucose-regulated polypeptide (12).

In the early stages of this study, [^{3}H]-methyl incorporation was carried out in the presence of protein synthesis inhibitors. I have found that this method is not adequate for the study of hsp70 methylation. Since the methylation is closely coupled to synthesis, the methyl group incorporation may be due to residual methylation occurring hours after polypeptide chain completion. Therefore, the observed increase in methylation after arsenite treatment in the presence of protein synthesis inhibitors (Chapter 2) may not be biologically significant. Rather, this may reflect the increase in substrate availability after arsenite treatment, which in turn would cause the incorporation of [^{3}H]-methyl group to increase.

It is evident that more hsp70 molecules with an unmethylated arginyl residue exist in the cells after arsenite treatment. This methylation probably has to occur on nascent polypeptide chains, since little N^G-monomethyl-arginine was found in hsp70B in the presence of protein synthesis inhibitors (Chapter 4). One may suspect that the observed lower methylation level at arginyl residues may be due to the dramatic increase in the rate of protein synthesis, such that the methyl transferase simply cannot methylate all available sites. If so, one must then derive another explanation for the rate of SR-RSV 3T3 cells, where arginyl methylation cannot be modulated by arsenite.

The major advantage of labeling cells with [methyl-^{3}H]-L-methionine to study amino acid methylation is that one can hardly miss any major methylated species. However, the intracellular specific activity of [methyl-^{3}H]-S-adenosyl-L-methionine reaches steady state much slower than [methyl-^{3}H]-L-methionine (Chapter 5). One therefore must incubate the cells in [methyl-^{3}H]-L-methionine for sufficiently long periods of time in order to obtain reliable quantitative results, especially if one wishes to measure the stoichiometry of methylation. Furthermore, if the methylation is closely coupled to synthesis and is irreversible, the method utilized here can only detect methylation of those polypeptides synthesized during the [methyl-^{3}H]-L-methionine incubation period. This also may be regarded as an advantage, since one could then

observe subtle changes in methylation which may not be otherwise detected. However, certain adjustments will make the method more usable. For example, it should be possible to radioactively label the cells or tissues with a particular amino acid(s) which has been identified as methylated and then separate the methylated and non-methylated species by HPLC or an equivalent system capable of such a separation. Under these conditions, the difference in specific activity between S-adenysyl-L-methionine and L-methionine or the stability of L-methionine upon storage are no longer of concern.

I do not know if heat shock or other treatments will produce a similar effect on the arginyl methylation of hsp70. If the effect is specific for arsenite, the resultant low level of arginyl methylation most likely is due to the change of thiol status of the specific methyl transferases. Hence, in SR-RSV 3T3 cells under normal growth conditions, the third status of this enzyme system may be changed (lowering its activity), since the level of arginyl methylation of hsp70 is constitutively low and cannot be further reduced by arsenite. Alternatively, this effect could be a general phenomenon to all physical and chemical treatments which are capable of inducing the synthesis of hsp70. One can speculate that the regulation of arginyl methylation of hsp70 may be important for the cells to cope with environmental stress, although the putative causal stress under which SR-RSV 3T3 cells exist is unknown.

I demonstrate in this thesis that the hsp70 isolated from both chicken fibroblasts and mouse 3T3 cells are methylated. It would be interesting to establish if this phenomenon is general to other species. It also may be important to verify if this methylation is tissue-specific and/or is dependent upon developmental stages. Recently, Blikstad and Lazarides (unpublished observations) discovered that hsp70 is synthesized at a high rate and is accumulated in large quantity in cells or tissues isolated from phenylhydrazine-treated chickens. Since survival is possible for several weeks after treatment, the method may provide a better means to study the methylation of

hsp70. In particular, radioactive labeling may not be required if one can properly separate the methylated amino acids from unmethylated ones.

Although the methylation of basic amino acids in proteins has been known for decades (for a review, see 13), the functional role of this post-translational modification is largely unclear. One can speculate that these methylations may play some as yet unidentified regulatory role. Similarly, although hsp70 is highly conserved and is reasonably abundant even before induction, its function is unknown.

The role of hsp70 as an important regulatory molecule is suggested by its conservation in evolution. It is evident that different methyled forms of hps70 exist; methylation may change the affinity of the molecule to different cellular components, or laternatively, hsp70 may play diverse functional roles by virtue of its variable state of methylation.

References

1. Johnston, D., Oppermann, H., Jackson, J. and Levinson, W. (1980) J. Biol. Chem. **255**, 6975-6980.
2. Kelley, P. M. and Schlesinger, M. J. (1978) Cell **15**, 1277-1286.
3. Tissières, A., Mitchell, H. K. and Tracy, U. M. (1974) J. Mol. Biol. **84**, 389-398.
4. Ashburner, M. and Bonner, J. J. (1979) Cell **17**, 241-254.
5. Mitchell, H. K., Moller, G., Petersen, N. S. and Lipps-Sarmiento, L. (1979) Develop. Genet. **1**, 181-192.
6. McAlister, L. and Finkelstein, D. B. (1980) Biochem. Biophys. Res. Commun. **93**, 819-824.
7. Morimoto, R. and Meselson, M. (1982) Cold Spring Harbor Abstracts - Induction of Heat Shock Proteins. Cold Spring Harbor, New York, p. 70a.
8. Arrigo, A.-P., Fakan, S. and Tissières, A. (1980) Develop. Biol. **78**, 86-103.
9. Sinibaldi, R. M. and Morris, P. W. (1981) J. Biol. Chem. **256**, 10735-10738.
10. Kelley, P. M. and Schlesinger, M. J. (1982) Mol. Cell. Biol. **2**, 267-274.
11. Hightower, L. E. and White, F. P. (1981) J. Cell Physiol. **108**, 261-275.
12. Lee, A. S., Delegeane, A. and Scharff, D. (1981) Proc. Nat. Acad. Sci. USA **78**, 4922-4925.
13. Paik, W. K. and Kim, S. (1980) Protein Methylation, John Wiley and Sons, New York.

Chapter 8

Appendix

(1) The 68,000 Dalton Neurofilament-Associated Polypeptide Is a Component of Nonneuronal Cells and of Skeletal Myofibril

Proc. Natl. Acad. Sci. USA
Vol. 77, No. 3, pp. 1541–1545, March 1980
Cell Biology

The 68,000-dalton neurofilament-associated polypeptide is a component of nonneuronal cells and of skeletal myofibrils

(intermediate filaments/immunofluorescence/two-dimensional isoelectric focusing/sodium dodecyl sulfate/polyacrylamide gel electrophoresis)

CHUNG WANG, DAVID J. ASAI, AND ELIAS LAZARIDES*

Division of Biology, California Institute of Technology, Pasadena, California 91125

Communicated by James F. Bonner, December 10, 1979

ABSTRACT **Purified preparations of 10-nm neurofilaments from rat spinal cord and bovine or porcine brain contain a predominant 68,000-dalton polypeptide. This polypeptide is also a major component of the neurofilaments that copurify with brain tubulin isolated by cycles of polymerization and depolymerization. A protein that has the same isoelectric point and molecular weight as the neurofilament-associated polypeptide has also been identified as a cytoskeletal protein in a variety of avian and mammalian cell types, including baby hamster kidney (BHK-21) mouse 3T3, Novikoff rat hepatoma, chicken fibroblast, and chicken muscle cells. This protein is also a component of isolated chicken skeletal myofibrils. One-dimensional peptide maps of the 68,000-dalton proteins purified by two-dimensional isoelectric focusing/NaDodSO$_4$/polyacrylamide gel electrophoresis from myofibrils, cycled tubulin, purified neurofilaments, and various cultured cell types were identical. In immunofluorescence this protein was associated with cytoplasmic intermediate filaments and myofibril Z discs. These results indicate that the neurofilament-associated polypeptide is a conserved protein that is present in many different cell types in addition to neuronal cells. Because some of these cells contain the major components of two other intermediate filament classes, desmin and vimentin, a given cell type may contain the subunits of at least three distinct intermediate filament types.**

Intermediate or 10-nm filaments have been observed in many cell types of higher vertebrates. Although these filaments share several morphological and biochemical characteristics that suggest a common origin, the major protein components of the filaments from different tissue sources are biochemically and immunologically distinct. At least five subclasses of 10-nm filaments can be identified: muscle intermediate filaments, 10-nm filaments in cells of mesenchymal origin, neurofilaments, glial filaments, and keratin filaments in cells of epithelial origin (for a review see ref. 1). One of the most intriguing observations about intermediate filaments to date is that a large number of cell types possess two distinct major filament components. Most cell types contain vimentin, the 52,000-dalton intermediate filament component in cells of mesenchymal origin. In epithelial cells, vimentin and keratin make up distinct filament populations as demonstrated by immunofluorescence and electron microscopy (2, 3). In a number of other cell types, and in particular muscle cells, vimentin coexists with desmin, the 50,000-dalton muscle-specific intermediate filament component (4–7). In this case the cytoplasmic filamentous distributions of the two proteins are indistinguishable (4–6).

Purified mammalian neurofilaments are composed of three major polypeptides of 200,000, 160,000, and 68,000 daltons, known as the neurofilament triplet (8–13). These three proteins have been assumed to be the subunit constituents of neurofilaments because they copurify with the filaments and they are not dissociated from them under a variety of chemical treatments. Recently it has been demonstrated that the 68,000-dalton polypeptide is present in conventionally purified microtubules from brain that are contaminated with 10-nm neurofilaments (14–16).

In this paper we show that the 68,000-dalton polypeptide of purified mammalian neurofilaments is highly homologous, if not identical, to the protein that copurifies with microtubule protein isolated from brain by cycles of polymerization and depolymerization. In addition, the 68,000-dalton protein is enriched in the microtubule-free cytoskeletons prepared by high salt and detergent extraction of a variety of nonneuronal cell types. Because these cytoskeletons are highly enriched in intermediate filaments, the extraction results in conjunction with immunofluorescence indicate that the 68,000-dalton polypeptide is associated with cytoplasmic intermediate filaments also in a variety of nonneuronal cell types.

MATERIALS AND METHODS

Cells. Cultures of nonmyogenic chicken embryo fibroblasts (CEF), embryonic chicken myogenic cells, and baby hamster kidney (BHK) 21 cells were prepared and grown as described (4). Chicken skeletal myofibrils were prepared from fresh or glycerol-extracted adult chicken skeletal muscle as described (5).

Preparation of Triton X-100 KCl Cytoskeletons and Isoelectric Focusing (IEF)/NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Triton X-100 KCl cytoskeletons of CEF and BHK cells were prepared as described (4). Purified skeletal myofibrils were extracted with 0.6 M KI for 10 min at room temperature as described (5). Two-dimensional IEF/NaDodSO$_4$/polyacrylamide gel electrophoresis was performed also as described (17, 18). The rat spinal cord and brain extracts were precipitated with 8 vol of acetone at 0°C and the acetone was removed under reduced pressure prior to solubilization in the urea sample buffer. This treatment was found to reduce streaking in the first IEF dimension.

One-Dimensional Peptide Mapping by Limited Proteolysis. Peptide mapping by limited proteolysis followed the procedure outlined by Cleveland *et al.* (19) as described (4). Gel slices were placed in wells atop the mapping gel and overlayed with 10–20 ng of *Staphylococcus aureus* protease V8 (Miles). Electrophoresis was at 10 mA until the dye front reached the top of the resolving gel; electrophoresis was then continued at 35 mA until the dye front reached the bottom of the gel.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: CEF, chicken embryo fibroblast(s); BHK, baby hamster kidney; IEF, isoelectric focusing; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

* To whom correspondence should be addressed.

Purification of 10-nm Filaments from Brain. The method of Runge *et al.* (15) was followed for the purification of pig brain 10-nm filaments. All procedures were carried out at 4°C. Brain was homogenized in buffer consisting of 0.1 M 1,4-piperazinediethanesulfonic acid (Pipes), 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM $MgSO_4$, and 10 mM 2-mercaptoethanol, pH 6.9. The homogenate was centrifuged first for 15 min at 7000 rpm in a Sorvall RC-2B centrifuge with an SS34 rotor. The supernatant was clarified again for 75 min at 29,000 rpm (approximately 100,000 × *g*) in a Beckman L5-75 ultracentrifuge with a type 42.1 fixed-angle rotor. The high-speed supernatant was applied to a column of Bio-Gel A-150m pre-equilibrated in the Pipes/EGTA/$MgSO_4$/2-mercaptoethanol buffer and eluted with the same buffer. The void volume was collected and centrifuged for 2 hr at 35,000 rpm as above. The high-speed pellets were either dissolved in the two-dimensional urea sample buffer or resuspended and precipitated by cold acetone (8:1). The precipitated material was collected at 15,000 rpm for 30 min in a Beckman SW50.1 rotor. The excess acetone was removed from the pellets under reduced pressure and the material was dissolved in urea sample buffer.

Purification of Rat Neurofilaments. Rat neurofilaments were isolated essentially according to the procedure of Schlaepfer (8, 10, 20). All experiments were carried out at 18–20°C. Rat spinal cords were excised and placed in isotonic saline after cardiac perfusion of etherized animals with saline to clear the vasculature of blood. The spinal cords were then minced into small pieces and subjected to hypotonic shock by immersing them into a low ionic strength buffer (1 mM sodium phosphate/2 mM EDTA/2 mM EGTA, pH 7.0). After 2 hr of slow agitation, the osmotically shocked and swollen tissue was homogenized. After the addition of 120 μl of 1 M NaCl per ml of extract, the homogenates were clarified at 12,000 rpm in a Beckman Ti50 fixed-angle rotor for 30 min. The supernatant was carefully decanted and spun again at 35,000 rpm for 2 hr. The high-speed pellets were washed once in the above buffer containing 0.1 M NaCl. The resultant pellets were either stored at −20°C or treated with acetone prior to two-dimensional electrophoresis as described above.

Purification of Brain Microtubule Protein. Bovine or porcine brain microtubule protein was purified by two cycles of polymerization-depolymerization according to the method of Shelanski *et al.* (21) and Borisy and Olmsted (22). The protein was a generous gift of W. Z. Cande (University of California, Berkeley).

Electron Microscopy. For electron microscopy, samples were applied to freshly carbon-coated grids by floating the grids upside-down on a drop of resuspended high-speed pellets from rat spinal cord or brain for 30 sec. The grids were then allowed to float on a drop of 0.5% aqueous solution of uranyl acetate for 3 min. After the excess liquid had been drawn off with a filter paper, the grids were air dried. The specimens were examined and photographed in a Phillips 201 electron microscope operated at 80 kV.

Indirect Immunofluorescence. Antibodies to the 68,000-dalton polypeptide were obtained against the antigen purified by preparative $NaDodSO_4$ gel electrophoresis from rat spinal cord. The material shown in Fig. 2*A* was electrophoresed on a preparative $NaDodSO_4$ slab gel. After staining and destaining the 68,000-dalton band was excised, neutralized with 0.1 M Tris·HCl at pH 8.7, homogenized in the same buffer, precipitated with $AlCl_3$, and emulsified for immunization as described (5). The immunization scheme was as described (5). Two-dimensional IEF of the antigen revealed the presence of no other detectable contaminants of the same molecular weight and different isoelectric point (not shown). The detailed characterization of this antiserum by two-dimensional immunoautoradiography will be presented elsewhere.

Indirect immunofluorescence on CEF was performed on cells fixed with formaldehyde in the presence of 0.6 M KCl and 1% Triton X-100 (4). Indirect immunofluorescence on myofibrils was as described (5).

RESULTS

Protein Composition of 10-nm Filaments Purified from Spinal Cord and Brain. The high-speed pellet of extracts from rat spinal cord contained numerous filaments with a diameter of approximately 10 nm as well as vesicles of various diameter (Fig. 1*A*). Analysis of this material by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis showed three prominent polypeptides of 200,000, 160,000, and 68,000 molecular weight in addition to a number of other polypeptides, consistent with the results of Schlaepfer and Freeman (8) (not shown). Two-dimensional IEF/$NaDodSO_4$/polyacrylamide gel electrophoresis revealed that the 68,000-dalton polypeptide is composed of a main isoelectric variant and a slightly more acidic minor variant (Fig. 2*A*). It is apparent from these gels that α and β tubulins are also present in the 10-nm filament preparations. The 68,000-dalton polypeptide is distinct from any rat serum albumin possibly present in these preparations, because the two proteins are clearly resolvable on these gels (Fig. 2*C*).

The high-speed supernatant from brain contained α and β tubulins as major components as well as a protein with the molecular weight and isoelectric point of the rat neurofilament

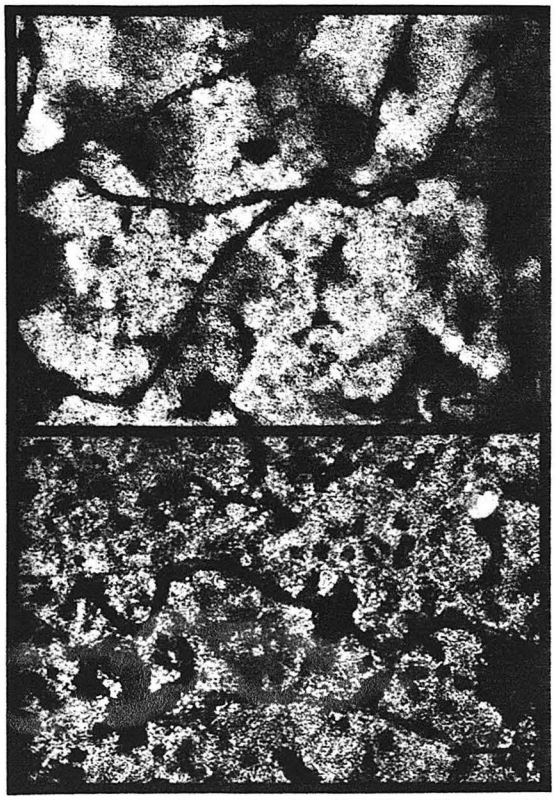


FIG. 1. Electron microscopy of purified 10-nm filaments. (*A*) High-speed pellet of rat spinal cord extracts. (*B*) High-speed pellet of pig brain extract purified by chromatography on Bio-Gel A-150m. Bars = 0.1 μm.

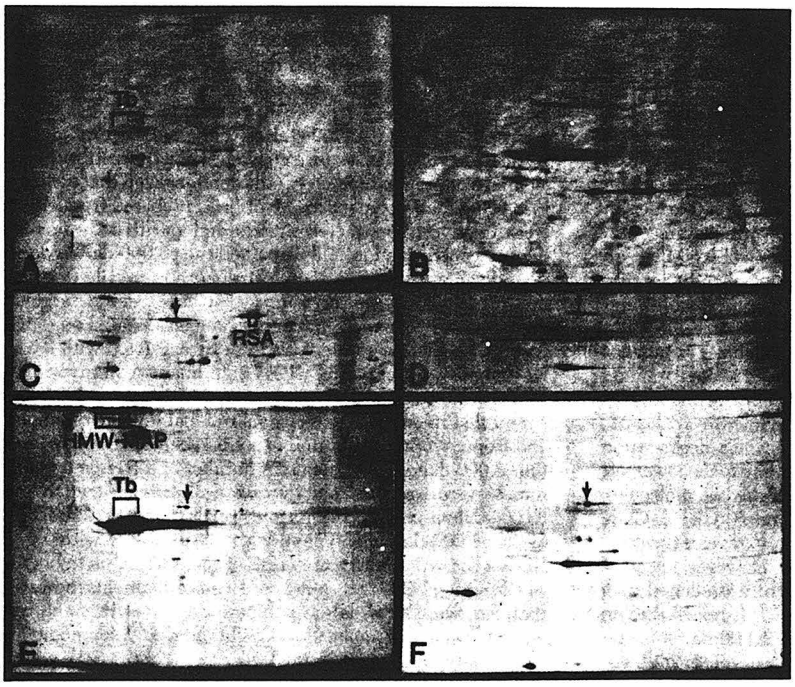


FIG. 2. Comparative two-dimensional gel electrophoresis of the 68,000-dalton polypeptide. In all electropherograms IEF was performed from right to left (acid on the left); pH range 4.5–7.0. The arrow points to the 68,000-dalton peptide. (*A*) Purified 10-nm filaments from rat spinal cord. Tb, α and β tubulins. (*B*) High-speed supernatant of a pig brain extract prior to gel filtration. (*C*) Coelectrophoresis of purified rat spinal cord 10-nm filaments and 1 μg of rat serum albumin (RSA). (*D*) High-speed pellet of the pig brain extract after gel filtration of the high-speed supernatant through a Bio-Gel A-150m column and high-speed centrifugation of the void volume material. (*E*) Microtubule protein purified by two cycles of polymerization–depolymerization from pig brain. The pellet after the second polymerization step was suspended directly in urea sample buffer. HMW-MAP, high molecular weight microtubule-associated proteins. (*F*) Coelectrophoresis of rat spinal cord 10-nm filaments and KI-extracted myofibrils (compare Figs. 2*A* and 3*D*).

68,000-dalton polypeptide (Fig. 2*B*). When this material was purified by gel filtration on Bio-Gel A-150m, the void volume contained numerous 10-nm filaments that could be pelleted by high-speed centrifugation, but no discernible 25-nm microtubules (Fig. 1*B*). Analysis of this material by two-dimensional gel electrophoresis revealed the presence of α and β tubulins as the major components and two other prominent polypeptides with molecular weights of 68,000 and 42,000 (Fig. 2*D*). Coelectrophoresis of these brain extracts with rat spinal cord purified 10-nm filaments showed that the 68,000-dalton polypeptides from the two sources have the same molecular weight and isoelectric point (not shown).

The pellets of microtubule protein purified from brain extracts by two cycles of polymerization–depolymerization contained α and β tubulins as their major component, a group of polypeptides with a very high molecular weight, corresponding to the high molecular weight microtubule-associated proteins, and another prominent polypeptide with a molecular weight of 68,000 (Fig. 2*E*). This is consistent with the results of Berkowitz *et al.* (14) and Runge *et al.* (15) [protein N4, figure

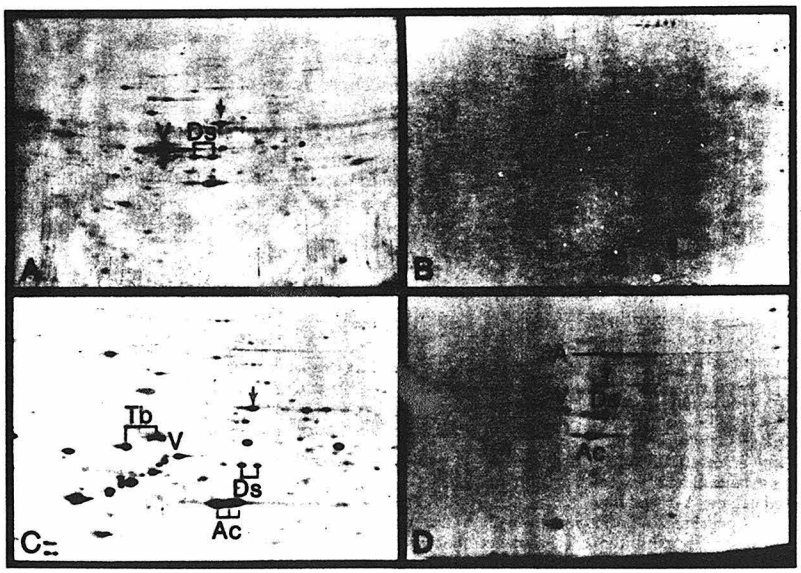


FIG. 3. Presence of the 68,000-dalton polypeptide in cytoskeletons and whole cell extracts of cells grown in tissue culture and in myofibrils. V, vimentin; Tm, tropomyosin; Ac, actin; Ds, α and β desmin; αA, α actinin. The arrow points to the 68,000-dalton polypeptide. (*A*) Cytoskeletal residues of BHK-21 cells. (*B*) Cytoskeletal residues of CEF. (*C*) Whole cell extracts of 7-day-old chicken embryonic skeletal myotubes. (*D*) Triton X-100/KI-extracted chicken pectoralis myofibrils.

2 of Berkowitz *et al.* (14)]. The microtubule-associated polypeptide is also composed of a major variant and a slightly more acidic variant. Coelectrophoresis of the microtubule protein with purified rat spinal cord 10-nm filaments (Fig. 2 *A* and *E*) showed that the two 68,000-dalton polypeptides have the same molecular weight and isoelectric point (not shown).

Presence of the 68,000-Dalton Polypeptide in Triton/KCl Cytoskeletons of Cells Grown in Tissue Culture. Extraction of BHK-21 cells and CEF with 1.0% Triton X-100 leaves an insoluble cytoskeletal residue, composed primarily of actin filaments and 10-nm filaments (23). Inclusion of 0.6 M KCl in the extraction buffer facilitates the extraction of actin, leaving predominantly 10-nm filaments. Electron microscopy and two-dimensional gel electrophoresis shows that these cytoskeletal residues contain no detectable microtubules or tubulins (4, 24). The major polypeptide species enriched in these residues have been identified by two-dimensional IEF/NaDodSO$_4$/polyacrylamide gel electrophoresis (4) as vimentin with a M_r of 52,000, desmin with a M_r of 50,000, their degradation products, which form a diagonal line extending to the lower left (acidic, lower molecular weight) of each of the two molecules, and residual actin [Fig. 3 *A* (BHK-21) and *B* (CEF)]. Close examination of these electropherograms also reveals the presence of a polypeptide with a M_r of 68,000. However, not all of the 68,000-dalton polypeptide may be tightly associated with these cytoskeletons, because a fraction of it is released upon prolonged extraction (not shown). The 68,000-dalton polypeptide has been identified in whole cell extracts or high salt detergent cytoskeletons of a number of cell types grown in tissue culture, including chicken embryonic skeletal myotubes (Fig. 3*C*), mouse 3T3, and Novikoff rat hepatoma cells (not shown). In each case this protein has the same isoelectric point and molecular weight as the rat spinal cord 10-nm filament-associated polypeptide.

Presence of the 68,000-Dalton Polypeptide in Chicken Skeletal Myofibrils. Mature chicken skeletal myofibrils give a pattern of protein spots on two-dimensional gels that includes a 68,000-dalton polypeptide. Myofibrils, prepared from either fresh or glycerol-extracted muscle and purified by sucrose density centrifugation, still contained the 68,000-dalton polypeptide. Fig. 3*D* shows the electrophoretic profile of glycerol-extracted chicken myofibrils that have been detergent-washed to remove membranous organelles, banded on a discontinuous sucrose gradient, and briefly extracted with 0.6 M KI to remove actomyosin, whose high quantity in myofibrils would otherwise obscure minor protein components. It is apparent that these myofibrils contain residual myosin, α-actinin, and tropomyosin, in addition to actin, desmin, vimentin, and the 68,000-dalton polypeptide. A number of different types of muscle were examined, and they were all shown to contain the 68,000-dalton polypeptide. As in cytoskeletons, a small fraction of the 68,000-dalton polypeptide can be extracted with 0.6 M KI (not shown). Coelectrophoresis of these KI-extracted myofibrils with purified rat spinal cord 10-nm filaments indicates that the two 68,000-dalton polypeptides have the same isoelectric point and molecular weight (Fig. 2*F*). The 68,000-dalton polypeptide has an isoelectric point of 5.7 (25), which agrees well with that estimated for this molecule by Berkowitz *et al.* (protein N4 in figure 2 and table 2 of ref. 14).

Comparative Peptide Analysis of the 68,000-Dalton Polypeptide. One-dimensional peptide analysis of the 68,000-dalton polypeptide was performed with protease V8 from *S. aureus*. In order to maximize the purity of the 68,000-dalton polypeptide, it was isolated from two-dimensional IEF/NaDodSO$_4$/polyacrylamide gels. As shown in Fig. 4, the peptide maps of this protein isolated from rat spinal cord 10-nm filament preparations (Fig. 4*C*), BHK-21 cells (Fig. 4*D*), CEF (Fig. 4*E*), brain 10-nm filament preparations (Fig. 4*F*), skeletal myofibrils (Fig. 4*G*), and cycled brain microtubule protein (Fig. 4*H*) are highly homologous. Comparison of the peptide map of this protein with the maps of rat serum albumin (Fig. 4*A*) and chicken serum albumin (Fig. 4*B*) indicates that the 68,000-dalton polypeptide is a completely distinct polypeptide; it also has no apparent homology with desmin or vimentin (not shown).

Immunofluorescence. Indirect immunofluorescence on CEF was performed on cells fixed in the presence of 0.6 M KCl/1.0% Triton X-100, which solubilizes microtubules and the majority of actin filaments but leaves intermediate filaments insoluble (4). The rat spinal cord 68,000-dalton antibodies reacted with a cytoplasmic filamentous system in these cells (Fig. 5*A*) resembling that observed with antibodies to inter-

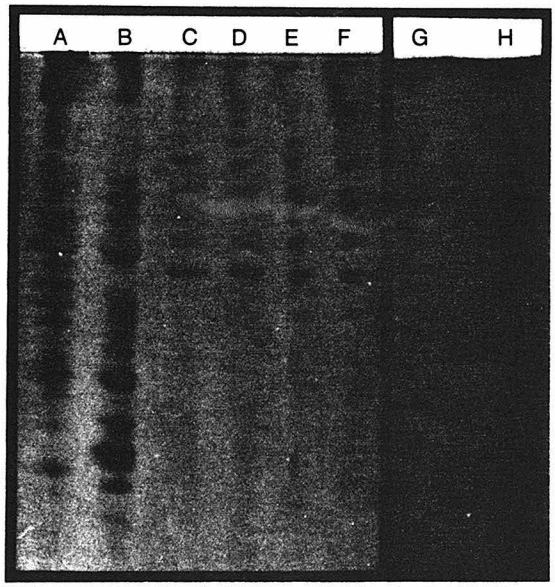


FIG. 4. One-dimensional peptide analysis of the 68,000-dalton polypeptide with *S. aureus* protease V8. The proteins were purified in each case by two-dimensional IEF/NaDodSO$_4$/polyacrylamide gel electrophoresis. A, Rat serum albumin; B, chicken serum albumin; C, rat spinal cord 68,000-dalton polypeptide (Fig. 2*A*); D, BHK-21 68,000-dalton polypeptide (Fig. 3*A*); E, CEF 68,000-dalton polypeptide (Fig. 3*B*); F, pig brain high-speed pellet 68,000-dalton polypeptide (Fig. 2*D*); G, chicken skeletal myofibril 68,000-dalton polypeptide (Fig. 3*D*); H, cycled brain microtubule-associated 68,000-dalton polypeptide (Fig. 2*E*). The peptide maps in lanes G and H were prepared at a different time than those depicted in lanes A–F and the gel was run longer to increase the resolution of the peptides. In both cases the 68,000-dalton polypeptide from all these sources exhibited indistinguishable peptides.

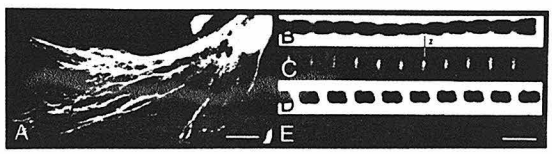


FIG. 5. Immunofluorescent localization of the 68,000-dalton polypeptide. (*A*) CEF; bar = 10 μm. (*B* and *C*) Chicken skeletal myofibril. Z, Z line. (*D* and *E*) Chicken skeletal myofibril that had been allowed to react with preimmune serum. *A*, *C*, and *E* are fluorescence images; *B* and *D* are phase-contrast images. Bar = 3 μm for *B–E*.

mediate filaments (2, 26). In isolated myofibrils, immunofluorescence indicated that the 68,000-dalton polypeptide is concentrated in the Z-line region of the myofibril (Fig. 5 *B* and *C*). The antigen also remains associated with the Z lines after solubilization of the actin and myosin filaments with 0.6 M KI (not shown). Preimmune sera were uniformly negative both with myofibrils (Fig. 5 *D* and *E*) and with fibroblasts (not shown).

DISCUSSION

The 68,000-Dalton Polypeptide as a Component of 10-nm Neurofilaments. It has been well documented in the literature that mammalian 10-nm neurofilaments contain a 68,000-dalton polypeptide as their major constituent (8, 12, 15, 16). This polypeptide remains associated with the filaments under a variety of chemical treatments (8, 12, 15) and thus appears to be an integral component of the filaments. However, it is not clear whether this polypeptide serves as a subunit for these filaments, because it has not yet been purified to homogeneity and shown to assemble into intermediate filaments *in vitro*. On the basis of its purification with 10-nm filaments from brain, Berkowitz *et al.* (14) and Runge *et al.* (15) have concluded that the 68,000-dalton polypeptide is not a component of microtubules but rather of the 10-nm filaments that copurify with them during the purification of the microtubule protein by cycles of polymerization–depolymerization. Our results confirm the conclusions of these authors and show clearly that the 68,000-dalton polypeptides isolated from preparations of 10-nm filaments from rat spinal cord or pig brain, as well as from microtubule protein purified by cycles of polymerization–depolymerization, are highly homologous if not identical proteins. The presence of tubulin as the major component of isolated brain 10-nm filaments remains unexplained but is in accordance with the observations of Runge *et al.* (15).

Presence of the 68,000-Dalton Polypeptide in Nonneuronal Cells and in Skeletal Myofibrils. The most interesting conclusion to be reached from the results presented here is that the 68,000-dalton neurofilament-associated polypeptide is a major component of many different nonneuronal cell types of both avian and mammalian origin. In particular, this protein is present in cytoskeletal preparations, which are composed predominantly of intermediate (10-nm) filaments and do not contain any detectable microtubules or tubulin. Thus it seems likely that the 68,000-dalton polypeptide is also associated with intermediate filaments in nonneuronal cells. Indeed, immunofluorescence microscopy with antibodies raised against this protein confirms this contention.

A 68,000-dalton polypeptide designated tubulin assembly protein (TAP) has been purified from microtubule protein isolated from brain by cycles of polymerization–depolymerization as a protein that stimulates microtubule assembly *in vitro* (27). Antibodies to this protein were shown by immunofluorescence to decorate cytoplasmic filamentous structures that resemble microtubules. It was therefore concluded that this polypeptide was associated with cytoplasmic microtubules even though the fluorescent images would also be consistent with intermediate filament staining (27). From our results it appears that at least a portion of this polypeptide is associated with intermediate filaments in a number of cell types grown in tissue culture as judged by immunofluorescence and its presence in the microtubule-free, intermediate-filament-rich, cytoskeletons prepared from these cells.

The comparative peptide map analysis of the 68,000-dalton polypeptide from a variety of avian and mammalian cell sources indicates that it is a conserved protein and a major cytoplasmic constituent. We have previously reported that the cell types studied here, namely BHK-21 cells, CEF, and embryonic chicken skeletal myotubes contain and synthesize simultaneously two distinct major intermediate-filament components, desmin and vimentin (4, 6). The presence of the 68,000-dalton polypeptide in these cells indicates that a third major intermediate-filament component can also coexist in the cytoplasm of these cells. This observation raises interesting and intriguing questions about the functional specificity and regulation of assembly of intermediate filaments in higher eukaryotic cells.

We have previously demonstrated that desmin and vimentin associate with the peripheries of myofibril Z discs and that they remain associated with the Z disc after extraction of actomyosin with 0.6 M KI. Furthermore, the two proteins are deposited initially as cytoplasmic filaments early during muscle differentiation and myofibril assembly, and later during myogenesis they transit onto the Z disc (5, 6). From the results presented here, the 68,000-dalton polypeptide is also a component of the myofibril Z disc and remains associated with this structure after short extraction of the actomyosin with 0.6 M KI. The function of this polypeptide during myogenesis and myofibril assembly or as a component of cytoplasmic intermediate filaments remains to be elucidated.

We thank Dr. C. O'Connor and B. L. Granger for their helpful comments, J. Sauer for her help in the preparation of the manuscript, and I. Lielausis for her technical assistance. This work was supported by a grant from the National Institutes of Health (PHS GM 06965) and by a grant from the Muscular Dystrophy Association of America.

1. Lazarides, E. (1980) *Nature (London)* **283,** 249–256.
2. Franke, W. W., Schmid, E., Osborn, M. & Weber, K. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5034–5038.
3. Franke, W. W., Schmid, E., Weber, K. & Osborn, M. (1979) *Exp. Cell Res.* **118,** 95–109.
4. Gard, D. L., Bell, P. B. & Lazarides, E. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3894–3898.
5. Granger, B. L. & Lazarides, E. (1979) *Cell* **18,** 1053–1063.
6. Gard, D. L. & Lazarides, E. (1980) *Cell* **19,** 263–275.
7. Tuszynski, G. P., Frank, E. D., Damsky, C., Buck, C. A. & Warren, L. (1979) *J. Biol. Chem.* **254,** 6138–6143.
8. Schlaepfer, W. W. & Freeman, L. A. (1978) *J. Cell Biol.* **78,** 653–662.
9. Schlaepfer, W. W. (1977) *J. Ultrastruct. Res.* **61,** 149–157.
10. Schlaepfer, W. W. (1977) *J. Cell Biol.* **74,** 226–240.
11. Schlaepfer, W. W. (1978) *J. Cell Biol.* **78,** 369–378.
12. Liem, R. K. H., Yen, S.-H., Salomon, G. D. & Shelanski, M. L. (1978) *J. Cell Biol.* **79,** 637–645.
13. Hoffman, P. N. & Lasek, R. J. (1975) *J. Cell Biol.* **66,** 351–366.
14. Berkowitz, S. A., Katagiri, J., Binder, H. K. & Williams, R. C., Jr. (1977) *Biochemistry* **16,** 5610–5617.
15. Runge, M. S., Detrich, W. H., III & Williams, R. C., Jr. (1979) *Biochemistry* **18,** 1689–1698.
16. Thorpe, R., Delacourte, A., Ayers, M., Bullock, C. & Anderton, B. H. (1979) *Biochem. J.* **181,** 275–284.
17. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
18. Hubbard, B. D. & Lazarides, E. (1979) *J. Cell Biol.* **80,** 166–182.
19. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
20. Schlaepfer, W. W. (1978) *J. Cell Biol.* **76,** 50–56.
21. Shelanski, M. L., Gaskin, F. & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 765–768.
22. Borisy, G. G. & Olmsted, J. B. (1972) *Science* **177,** 1196–1197.
23. Brown, S., Levinson, W. & Spudich, J. A. (1976) *J. Supramol. Struct.* **5,** 119–130.
24. Starger, J. M., Brown, W. E., Goldman, A. E. & Goldman, R. D. (1978) *J. Cell Biol.* **78,** 93–109.
25. Lazarides, E. & Balzer, D. R., Jr. (1978) *Cell* **14,** 429–438.
26. Hynes, R. O. & Destree, A. T. (1978) *Cell* **13,** 151–163.
27. Lockwood, A. H. (1978) *Cell* **13,** 613–627.

(2) Sodium Arsenite Facilitates the Turnover of Phosphate in Cultured Chicken Myotube Tropomyosin

Summary

Both the α and β subunits of tropomyosin are phosphorylated in embryonic and adult skeletal muscle. Here we demonstrate that the phosphate groups on α- and β-tropomyosin in cultured chicken myogenic cells have half-lives of 7- and 21-hr, respectively under normal growth conditions. After sodium arsenite treatment, the half-lives of α- and β-tropomyosin phosphates are reduced to 1.5- and 5-hr, respectively. This facilitated phosphate turnover is reversible and may be specific for tropomyosin since it is not observed with other major phosphorylated proteins, such as the intermediate filament proteins desmin and vimentin. The results suggest that vicinal dithiols may be involved in the enzymatic activities which are responsible for the removal of phosphate from tropomyosin.

Introduction

Tropomyosin, a two chain coiled-coil molecule (1), in association with actin and troponin in skeletal muscle, is a key regulatory protein for muscle contraction. It is composed of two different but homologous forms, α- and β-tropomyosin, with molecular weights of 33,000 and 36,000, respectively. The ratio of α- and β-tropomyosin varies depending on the source of muscle (2,3,4) and also changes during development (5,6); the majority of the tropomyosin exists as a heterodimer (αβ) rather than a homodimer (α_2 or β_2) (4). It has been shown that tropomyosin is phosphorylated in skeletal and cardiac muscle of several different species (7,8,9). In addition, the phosphorylation of tropomyosin appears to change, both during embryonic development and during cultured myotube differentiation (6). Nevertheless, the functional significance of the two tropomyosin forms and their phosphorylation are currently obscure.

Here we report that sodium arsenite, which interacts with vicinal dithiols to form a non-dissociable complex (10), specifically reduces phosphate incorporation into cultured chicken myotube tropomyosin during a short [^{32}P]-phosphate pulse

labeling period. We also present evidence which indicates that arsenite facilitates the removal of phosphate from tropomyosin, presumably by activating a tropomyosin specific phosphatase.

Materials and Methods

Chicken embryonic myotubes were grown in tissue culture as described previously (11). The cells were incubated with or without sodium arsenite (25 μM) for 4 hr, after which they were washed twice with phosphate-free minimal essential medium, then [^{32}P]-phosphoric acid (New England Nuclear) was added to 100 μCi/ml, and incubation was continued for 2 hr. After rinsing three times with phosphate buffered saline, the cells were scraped off the plates and collected by centrifugation. The cell pellets were lysed in 1% SDS and subjected to two dimensional gel electrophoresis (12,13). The gels were routinely stained, destained and dried for autoradiography.

The protein spots corresponding to α- and β-tropomyosin as well as the intermediate filament protein, desmin (8,13), were excised from the dried gels and placed into scintillation vials. The polypeptides were subsequently eluted with 1 ml of 0.1% SDS in 100 mM NH_4HCO_3 overnight; 10 ml of Aquasol-2 scintillation fluid (New England Nuclear) was added to each vial and then counted. The ratios of the [^{32}P]-radioactivity of α-and β- tropomyosin to desmin were then calculated and compared between experimental and control cells to quantitate the arsenite effect on [^{32}P]-phosphate incorporation into tropomyosin.

To study the reversibility of the arsenite effect, cells were incubated with 25 μM sodium arsenite for 4-hr, the chemical was then removed by rinsing three times with growth medium, and the incubation was allowed to continue. At various time points, the cells were pulse labeled with [^{32}P]-phosphate and the [^{32}P]-radioactivity in α- and β-tropomyosin as well as in desmin was determined by the procedure described above. The ratio of [^{32}P]-radioactivity of tropomyosin to that of desmin was compared to evaluate the recovery.

To determine the half-life of phosphate on tropomyosin, the cultures were first double-labeled with 50 μCi/ml of [^{32}P]-phosphate and 10 μCi/ml of [^{3}H]-leucine (New England Nuclear, specific activity 122 Ci/mmole) for 16 hr in phosphate- and leucine-free minimal essential medium supplemented with 10% calf serum. The cells were rinsed three times with growth medium and then incubated with or without sodium arsenite for between 2 and 7 hr. Subsequently, the cells were harvested at several different time points and the [^{32}P]- and [^{3}H]-radioactivity in tropomyosin were determined. The ratio of [^{32}P]/[^{3}H]-radioactivity was used to estimate the half-life of phosphate in tropomyosin.

Results

1. Arsenite reduces [^{32}P]-phosphate incorporation into tropomyosin.

Tropomyosin and the two intermediate filament proteins, desmin and vimentin, are among the major [^{32}P]-phosphate-accepting polypeptides in cultured chicken myotubes during a short pulse labeling period (8,13). After comparing the autoradiograms of arsenite-treated cells with those of control cells, we found that the [^{32}P]-phosphate incorporation into tropomyosin is reduced with arsenite. Quantitative changes have been analyzed in terms of relative incorporation of [^{32}P]-phosphate into α- and β-tropomyosin to that of desmin. Desmin was chosen primarily because it is specifically expressed in myogenic cells, since vimentin is also expressed in fibroblasts (11). Table I shows the [^{32}P]-phosphate incorporation of tropomyosin relative to that of desmin at several different stages of development. The results indicate that the relative [^{32}P]-phosphate incorporation is reduced to 10-30% and 20-50% of the control levels for α- and β-tropomyosin, respectively, after arsenite treatment. Qualitatively, the reduction by arsenite is independent of the developmental stage of the culture.

Table 1. Reduction of [^{32}P]-phosphate incorporation into tropomyosin relative to desmin at different stages of in vitro chicken muscle development

Days after cell plating	$\left(\frac{\alpha\text{-tropomyosin}}{\text{desmin}}\right)^{(a)}$		$\frac{\left(\frac{\alpha\text{-tropomyosin}}{\text{desmin}}\right)_{(+)}}{\left(\frac{\alpha\text{-tropomyosin}}{\text{desmin}}\right)_{(-)}}$	$\left(\frac{\beta\text{-tropomyosin}}{\text{desmin}}\right)^{(a)}$		$\frac{\left(\frac{\beta\text{-tropomyosin}}{\text{desmin}}\right)_{(+)}}{\left(\frac{\beta\text{-tropomyosin}}{\text{desmin}}\right)_{(-)}}$
	(+)[b]	(-)[b]		(+)[b]	(-)[b]	
1 day[c]	0.23	1.9	0.12	0.15	0.57	0.26
4 days[d]	0.4±0.1	4.2±0.2	0.10±0.01	0.3±0.1	1.7±0.1	0.2±0.1
9 days[d]	0.69±0.04	2.2±0.1	0.31±0.03	0.45±0.05	0.97±0.03	0.46±0.06
12 days[e]	0.6±0.1	2.5±0.3	0.24±0.08	0.38±0.06	1.1±0.1	0.35±0.04

(a) the ratio of [^{32}P]-radioactivity.

(b) (+): sodium arsenite treated cells, (-): control cells.

(c) single determination.

(d) duplicate determinations.

(e) six determinations.

The interpretation that arsenite reduces the phosphate incorporation into tropomyosin is correct only if the [^{32}P]-phosphate incorporation into desmin and the specific activity of [^{32}P]-ATP do not change significantly. Indeed, we found that the ratio of [^{32}P]-phosphate incorporation into desmin to trichloroacetic acid precipitable material does not change significantly. Alternatively, the [^{32}P]-phosphate incorporation into desmin relative to total amount of desmin remains unchanged (data not shown). The low concentration of arsenite (25 μM) used in this study has no effect on oxidative phosphorylation (14,15) and the intracellular level of [^{32}P]-ATP should remain unchanged. We therefore conclude that arsenite specifically reduces the [^{32}P]-phosphate incorporation into tropomyosin during a short pulse labeling period.

2. Reversibility of the arsenite effect on tropomyosin phosphorylation.

Since the [^{32}P]-phosphate incorporation into desmin remains unchanged upon sodium arsenite treatment, we compared the ratio of [^{32}P]-phosphate incorporation into tropomyosin to desmin between arsenite treated and control cells to quantitate the recovery from the arsenite effect. The results of such experiments (Figure 1) indicate that the [^{32}P]-phosphate incorporation into both α- and β-tropomyosin eventually can recover after the removal of arsenite. However, it appears that 24-48 hr of recovery is necessary for tropomyosin [^{32}P]-phosphate incorporation to return to normal levels. The recovery process appears similar at two different developmental stages of the cultured cells.

3. Turn-over of phosphate on tropomyosin.

Myogenic cells were double-labeled with [^{3}H]-leucine and [^{32}P]-phosphate and the radioactivity incorporated into tropomyosin was then quantitated after various chase periods to determine the half-life of phosphate on tropomyosin. Figure 2 shows the results of such studies for both α- and β-tropomyosin. Under normal growth conditions, the phosphates on α- and β-tropomyosin have half lives of 7 and 21 hr, respectively. These are reduced to 1.5 and 5 hr after sodium arsenite treatment.

Figure 1. Recovery of Arsenite Effect on Tropomyosin. The results of five day old (0) and eight day old myogenic cultures (●) are present here. (A) α-tropomyosin, (B) β-tropomyosin.

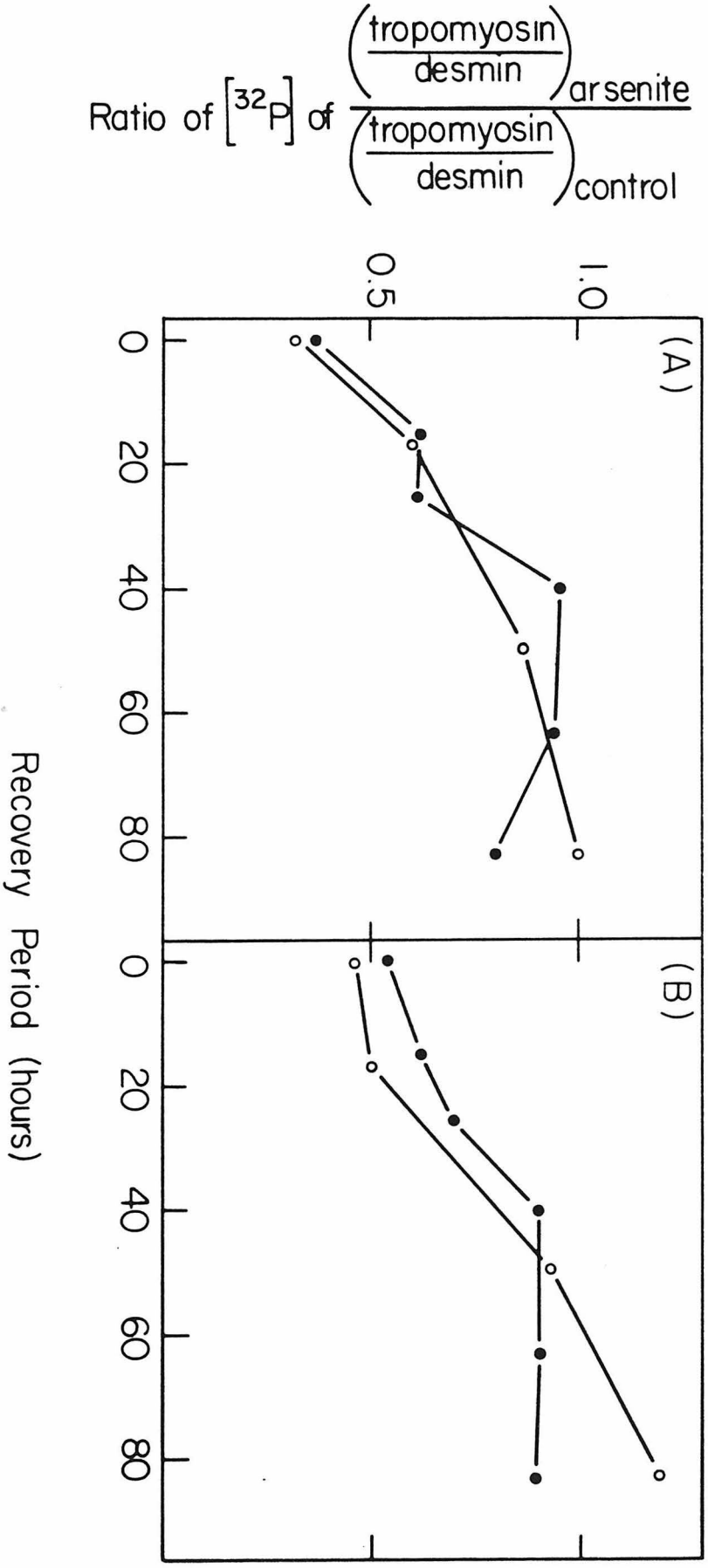

Ratio of [32P] of (tropomyosin/desmin)arsenite / (tropomyosin/desmin)control
0.5
1.0
(A)
(B)
0
20
40
60
80
Recovery Period (hours)

Figure 1

Figure 2. Phosphate Turn-over of Tropomyosin. The results from 10 day old myogenic cells are presented here. Open circles (0) and triangles (Δ) represent the results of α- and β-tropomyosin for the control, respectively; closed ones (●, ▲) represent arsenite treated cells. Sodium arsenite was added to the cultures at 2 hrs and removed at 7 hrs. (A) The ratio of [^{32}P]/[^{3}H]-radioactivity is plotted directly versus the chasing period. (B) The natural logarithm of the ratio relative to those of 2 hr time points is plotted versus the chasing period minus 2 hr. A straight line was drawn for the first three time points. Assuming the decay follows the first order kinetics dR/dt = -kR, then k can be determined by the straight line (B), and half-life ($t_{1/2}$) = 0.693/k.

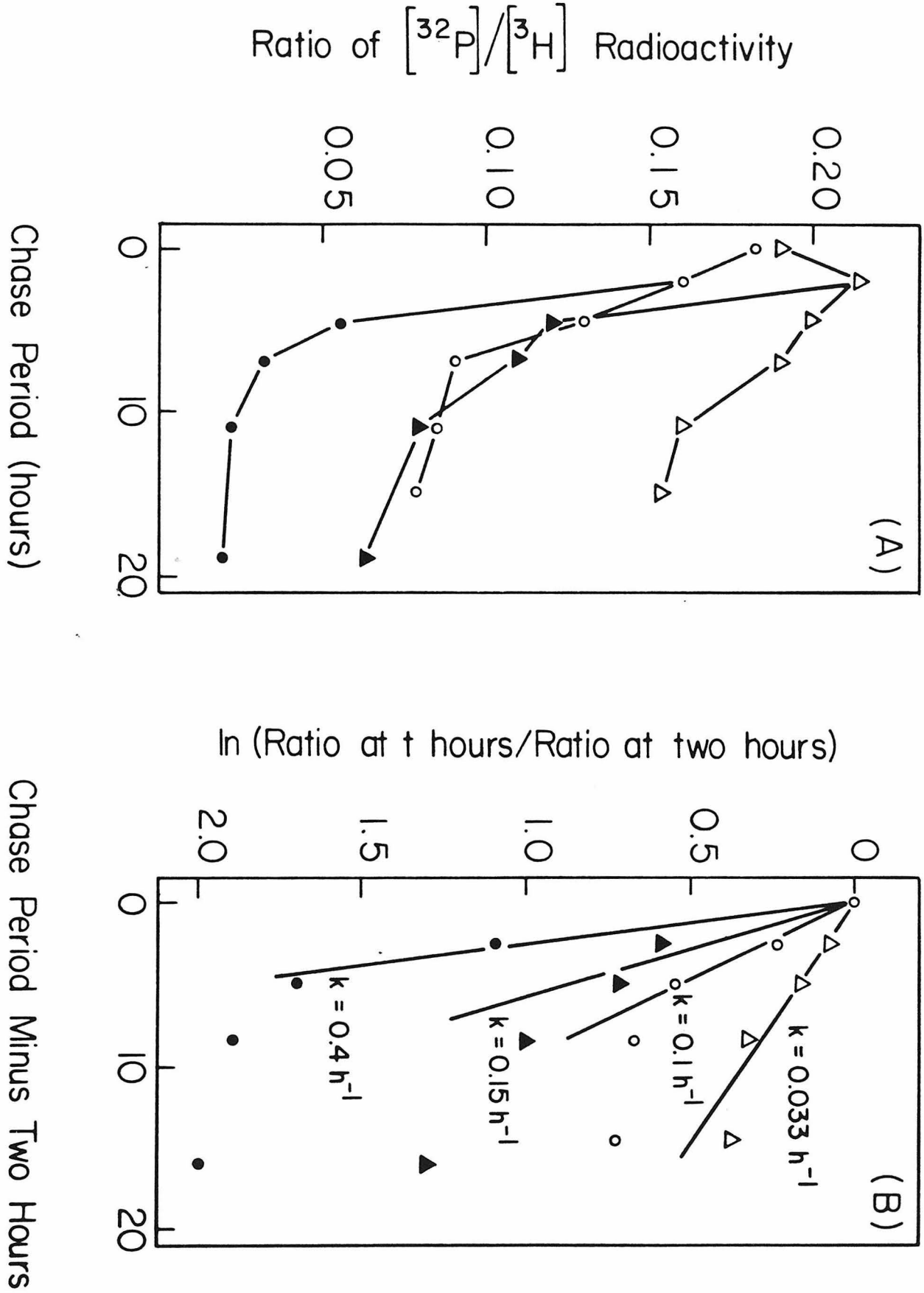

Ratio of [32P]/[3H] Radioactivity
0.05
0.10
0.15
0.20
0
10
20
(A)
Chase Period (hours)
ln (Ratio at t hours/Ratio at two hours)
2.0
1.5
1.0
0.5
0
0
10
20
k = 0.4 h-1
k = 0.15 h-1
k = 0.1 h-1
k = 0.033 h-1
(B)
Chase Period Minus Two Hours

Figure 2

In addition, incubation with sodium arsenite for less than 2.5 hr is sufficient to potentiate the phosphate turn-over of tropomyosin. These results suggest that arsenite may induce a tropomyosin-specific enzyme system which is responsible for the removal of phosphate groups from tropomyosin.

Discussion

Based on published results, arsenite has a strong affinity for vicinal dithiols (10). The inhibition of dithiol enzymes, such as aldehyde dehydrogenase, by arsenite occurs even in the presence of a large excess of 2-mercaptoethanol (16). However, a similar amount of 2,3-dimercaptopropanol can reduce the arsenite inhibitory effect on the enzyme (16). Since the enzyme(s) involved in tropomyosin phosphorylation has not been studied, it is difficult to explain exactly by what mechanism arsenite affects tropomyosin phosphorylation. Nevertheless, we believe that it is reasonable to assume that vicinal dithiols are involved. This could be related to phosphatase catalytic activities, or alternatively, vicinal dithiols may be involved in some regulatory mechanism. In addition, the fact that 24 to 48 hr are necessary for the recovery from arsenite treatment (Figure 1) is consistent with the assumption that a non-dissociable complex is formed after arsenite treatment, necessitating de novo protein synthesis for recovery.

From the evidence presented here it is clear that arsenite facilitates the turn-over of phosphate on tropomyosin. However, at present we cannot correlate this greater turn-over rate with the three-fold reduction of [^{32}P]-phosphate incorporation into tropomyosin duirng a short pulse-labeling period. Because of faster phosphate turn-over, more substrates become available for kinase after arsenite treatment. Consequently, one would expect more [^{32}P]-phosphate incorporation into tropomyosin instead of the observed decrease in incorporation. One could speculate that aresenite may affect the kinase as well.

It has been documented that α- and β-tropomyosin are homologous to each other and that the amino acid sequence around the site of phosphorylation is highly conserved (4,17). Since arsenite has a similar effect on both α- and β-tropomyosin phosphorylation, it is likely that a similar set of enzymes is responsible for the phosphorylation of both α- and β-tropomyosin. From the data present here, it is possible that sodium arsenite treatment may help elucidate the enzyme system responsible for the phosphorylation of this protein and the functional significance of tropomyosin phosphorylation as well.

Acknowledgements

We thank John Ngai for his comments on the manuscript. This work was supported by grants from the National Institutes of Health (GM-06965), the National Science Foundation and the Muscular Dystrophy Association of America. E.L. is also a recipient of a Research Career Development Award from the National Institutes of Health.

References

1. Phillips, G. M., Lattman, E. E., Jr., Cummins, P., Lee, K. Y., and Cohen, C. (1979) Nature 278, 413-417.
2. Cummins, P., and Perry, S. V. (1974) Biochem. J. 141, 43-49.
3. Dhoot, G. K., and Perry, S. V. (1979) Nature 278, 714-718.
4. Bronson, D. D., and Schachat, F. H. (1982) J. Biol. Chem. 257, 3937-3944.
5. Roy, R. K., Sreter, F. A., and Sarkar, S. (1979) Develop. Biol. 69, 15-30.
6. Montarras, D., Fiszman, M. Y., and Gros, F. (1982) J. Biol. Chem. 257, 545-548.
7. Mak, A., Smillie, L. B., and Barany, M. (1978) Proc. Nat. Acad. Sci. U.S.A. 75, 3588-3592.
8. O'Connor, C. M., Balzer, D. R., and Lazarides, E. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 819-823.
9. Montarras, D., Fiszman, M. Y., and Gros, F. (1981) J. Biol. Chem. 256, 4081-4086.
10. Stocken, L. A., and Thompson, H. S. (1946) Biochem. J. 40, 529-535.
11. Gard, D. L., Bell, P. B., and Lazarides, E. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 3894-3898.
12. O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4007-4012.
13. Gard, D. L., and Lazarides, E. (1982) Mol. Cell. Biol. 2, 1104-1114.
14. Crane, R. K., and Lipmann, F. (1953) J. Biol. Chem. 201, 235-243.
15. Fluharty, A., and Sanadi, D. R. (1970) Proc. Nat. Acad. Sci. U.S.A. 46, 608-616.
16. Jakoby, W. B. (1958) J. Biol. Chem. 232, 89-97.
17. Mak, A. S., Smillie, L. B., and Stewart, G. R. (1980) J. Biol. Chem. 255, 3647-3665.