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Heat shock proteins are methylated in avian and mammalian cells

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

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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 $500 \times g$) 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 $500 \times g$, 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-

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

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[*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

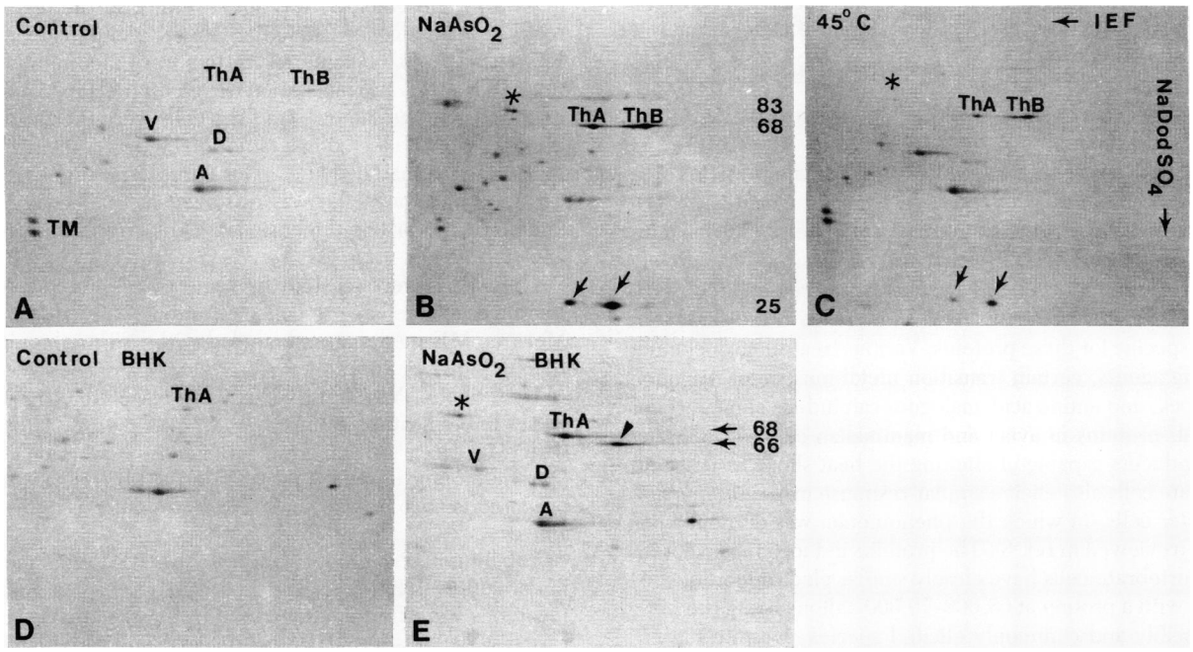

FIG. 1. Two-dimensional gel electrophoresis of avian and mammalian proteins induced by sodium arsenite and heat shock in cells grown in tissue culture showing thermins (Th A, Th B), HSP 25 (arrow), HSP 66 (▼), HSP 83 (*), actin (A), desmin (D), vimentin (V), and tropomyosin (TM). (*A*) Proteins extracted 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

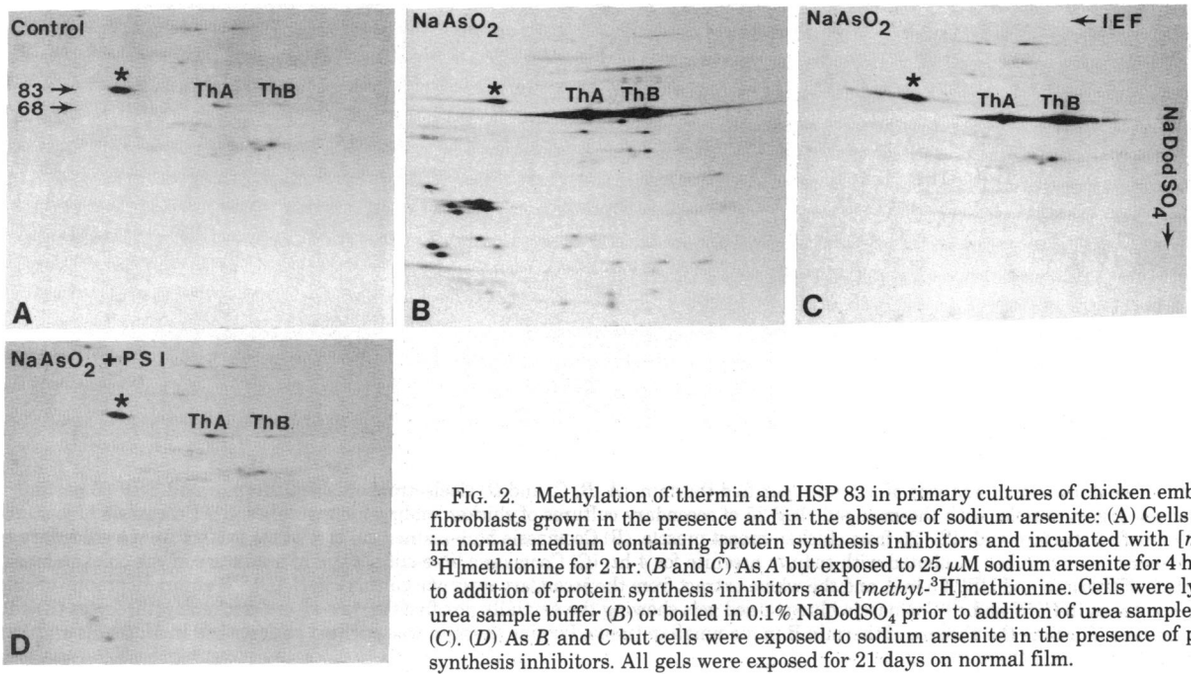

FIG. 2. Methylation of thermin and HSP 83 in primary cultures of chicken embryonic fibroblasts grown in the presence and in the absence of sodium arsenite: (A) Cells grown in normal medium containing protein synthesis inhibitors and incubated with [*methyl*-^3H]methionine for 2 hr. (*B* and *C*) As *A* 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-

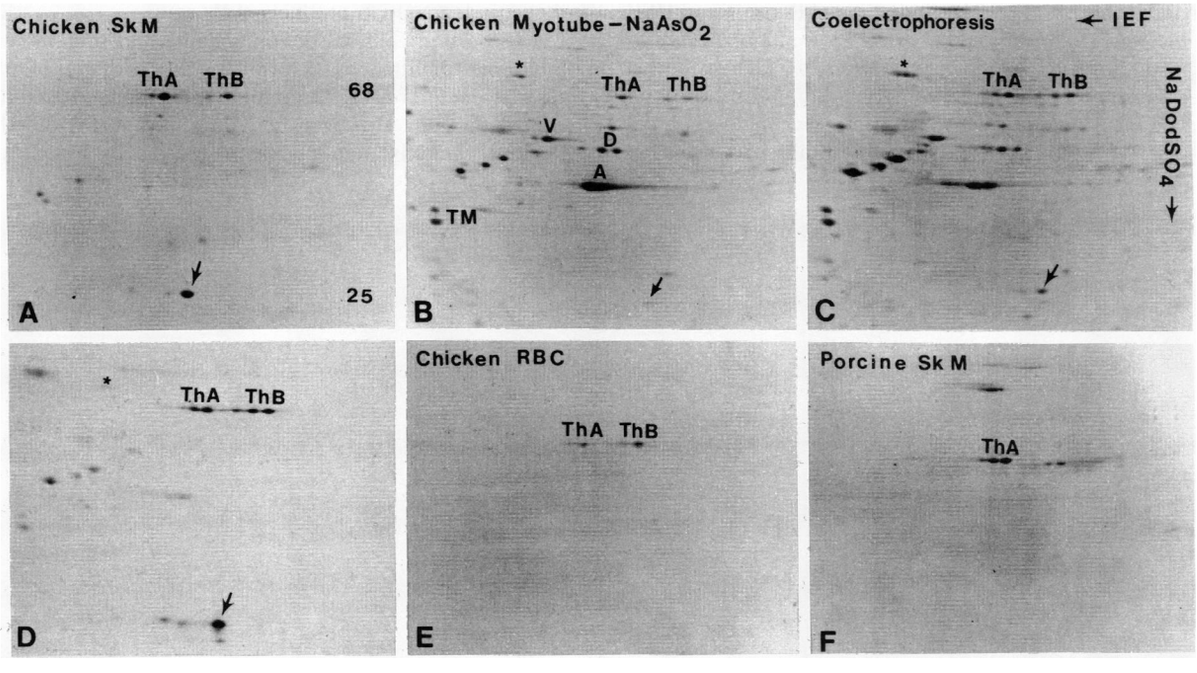

FIG. 3. Two-dimensional analysis of partially purified thermin. (*A*, *B*, *C*, and *D*) Coelectrophoresis of thermin and HSP 25 partially purified from chicken breast muscle, with thermin and HSP 25 of secondary cultures of chicken embryonic myotubes. (*A*) Coomassie blue-stained gel of partially purified 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.

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