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Spontaneous high expression of heat-shock proteins in mouse embryonal carcinoma cells and ectoderm from day 8 mouse embryo

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When submitted to a heat-shock, mouse embryonal carcinoma (EC) and fibroblast cells show very different behavior. All the EC cells so far analyzed express very high levels of several heat-shock proteins (HSP) in the absence of stress and independent of their origin and culture conditions. In such cells, the 89-kd, 70-kd and 59-kd HSP are the most prominent proteins after actin. In addition, the 89-kd and 59-kd HSP are not stimulated by an arsenite shock in contrast to what is observed with fibroblasts or cells of the parietal yolk sac type. Arsenite induces the synthesis of a 105-kd polypeptide in fibroblasts but not in EC cells. *In vitro* differentiation of F9 cells induced by retinoic acid and dibutyryl cAMP is accompanied by a decrease in the spontaneous relative abundance of HSP and restores the arsenite-induced synthesis of the 105-kd polypeptide. EC cells are usually believed to be similar to inner cell mass cells of mouse blastocyst. Furthermore, data in the literature together with our own results suggest that the same three HSP are also spontaneously expressed in high amounts in the early mouse embryo.

Key words: differentiation/mouse embryo/heat shock/retinoic acid/teratocarcinoma

Introduction

When subjected to a 'stressing' change of culture conditions, eukaryotic cells decrease the synthesis of most of their proteins, except for a dozen polypeptides which are then produced in higher amounts (Ashburner and Bonner, 1979; Schlesinger *et al.*, 1982). Irrespective of the stress (temperature rise, starvation, anoxia, addition of transition metal ions or drugs to the culture medium) the same group of very conserved polypeptides, called the heat-shock proteins (HSP), are synthesized. Apart from one or two low mol. wt. HSP (Atkinson, 1981), the general pattern of HSP synthesis before or after a stress is not thought to be related to cell type. Interestingly, recent reports have demonstrated a connection between HSP and viral transformation: the synthesis of a 70-kd HSP is specifically enhanced by an early gene product of adenovirus (Nevins, 1982), by papovavirus infection (Khandjian and Türler, 1983); in addition a 89-kd HSP binds to the tyrosyl-protein kinases of avian sarcoma viruses (Oppermann *et al.*, 1981; Brugge *et al.*, 1981; Lipsich *et al.*, 1982). A link between HSP expression and differentiation was suggested when Ireland and Berger (1982) established that ecdysterone increased the synthesis of low mol. wt. HSP (from 23 to 27 kd) in embryonic *Drosophila* cells. Therefore, we felt it was of interest to investigate early mouse embryonic cells for modifications of the HSP synthetic pattern using mouse embryonal carcinoma (EC) cells as a model system. EC cells, which are derived from teratocarcinomas, are very similar to the pluripotent cells of the early mouse embryo. They can be maintained in culture in an undifferentiated state; they may differentiate either spontaneously (Nicolas *et al.*, 1976) or following exposure to appropriate inducers (Strickland, 1981) or due to changes in culture conditions (Darmon *et al.*, 1981).

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Results

Arsenite shock of mouse fibroblasts

After 1 h exposure to arsenite-containing media, protein synthesis decreases dramatically. Three to four hours later the synthetic activity is almost restored to its original level. However, analytical gel electrophoresis reveals a new protein synthetic pattern (Figures 1, 2 and 4). Several polypeptides – identical to the HSP (Ashburner and Bonner, 1979; Johnston *et al.*, 1980) – are produced in higher amounts than prior to the stress. The mol. wt. of the major polypeptides are: 105 kd, 89 kd, 70 kd, 68 kd, 59 kd and 24 kd (arrows in Figure 2b). The 89-kd, 70-kd and 59-kd polypeptides are clearly present in non-stressed cells. The 89-kd species appears as a doublet in mono-dimensional polyacrylamide gel electrophoresis whereas two-dimensional gels reveal a multiplet structure (data not shown) with an isoelectric point between that of α and β tubulins.

EC cells

As with fibroblasts, the pattern of protein synthesis in EC

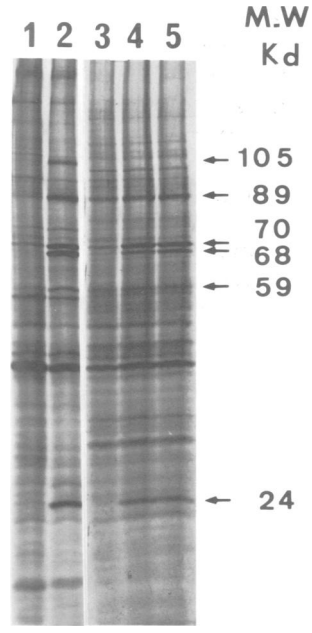

Fig. 1. One-dimensional polyacrylamide gel electrophoresis (Laemmli, 1970) of total extracts from [^{35}S]methionine-labelled cells: secondary mouse embryo fibroblasts untreated **(lane 1)** or exposed to medium containing 150 μM sodium arsenite before labelling **(lane 2)**; F9 EC cells untreated **(lane 3)** or exposed to media containing 50 μM **(lane 4)** or 150 μM **(lane 5)** sodium arsenite.

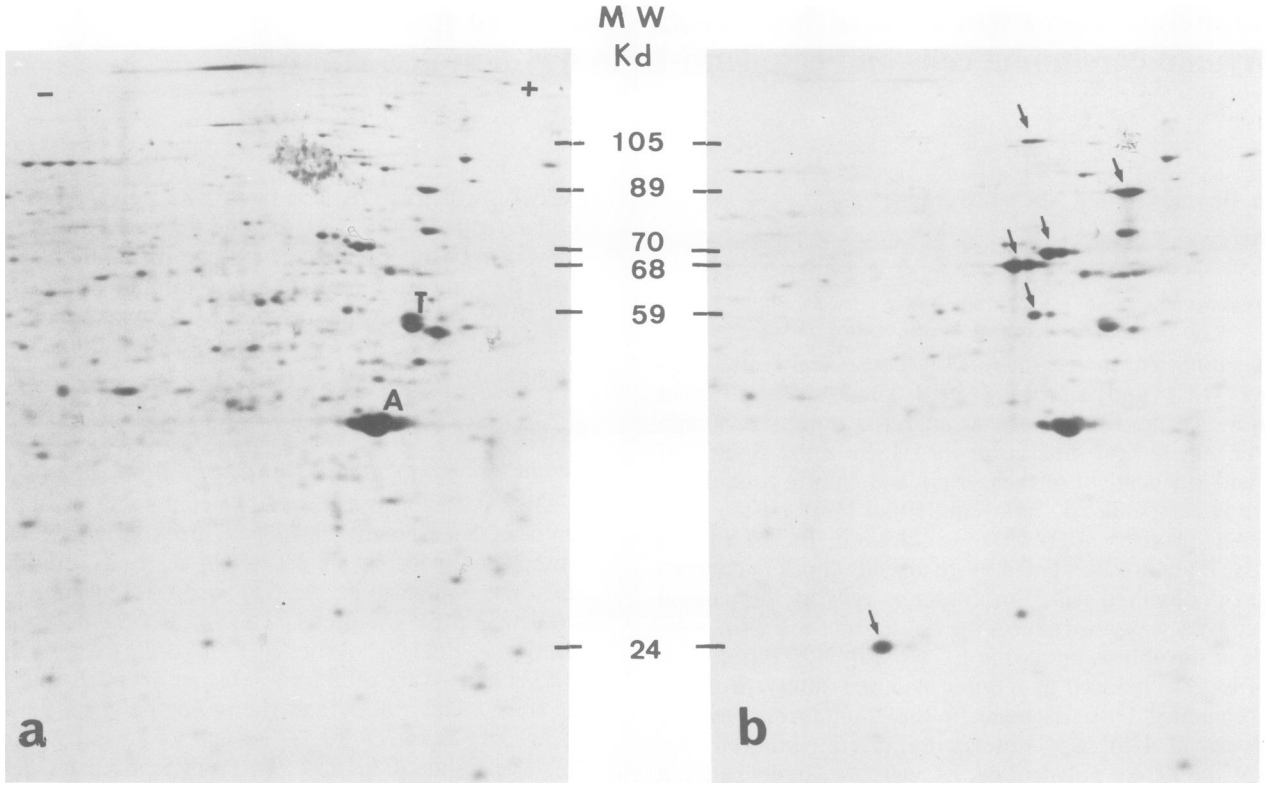

Fig. 2. Two-dimensional polyacrylamide gels of total extracts from [^{35}S]methionine-labelled fibroblasts: **(a)** untreated or **(b)** exposed to medium containing 150 μM sodium arsenite. Arrows indicate the most prominent arsenite-stimulated proteins quoted in the text. A: actin, T: tubulins.

cells of the F9 line (Bernstine *et al.*, 1973) is altered 3–4 h after a 1 h exposure to arsenite-containing medium (Figures 1, 3 and 4). The same 24-kd, 68-kd and 70-kd HSP (arrows in Figure 3b) are expressed at increased levels. However, little or no change in 105-kd, 89-kd and 59-kd HSP (when compared to actin) is detectable. In the absence of stress, F9 cells already express as much 89-kd and 59-kd HSP (arrows in Figure 3a) as arsenite-shocked fibroblasts. The 70-kd HSP is also spontaneously highly expressed but remains inducible by arsenite. Such a high spontaneous HSP expression might be due to the culture conditions. However, this is unlikely since F9 cells analyzed either at various times after plating or after growth at different densities or after being propagated and labelled at 32°C (Figure 3c) instead of 37°C, always displayed a very similar pattern of protein synthesis. The high spontaneous expression of HSP observed with F9 cells is not dependent on the method used for detecting the proteins. Staining the gels with Coomassie brilliant blue also clearly reveals the 89-kd, 70-kd and 59-kd HSP as the major cellular proteins after actin.

Three other EC cell lines of various origins were analyzed: PCC4 Aza_R1 (Lehman *et al.*, 1974), C17-S_1-1003 (Darmon *et al.*, 1981) and PCC7-S-1009 (Pfeiffer *et al.*, 1981) and in every case, as with the F9 cells, the 89-kd, 70-kd and 59-kd HSP were found to be the major cellular proteins after actin (Figure 5a).

Effect of differentiation

When treated with retinoic acid and dibutyryl cAMP (DBcAMP) during 7 days, F9 cells differentiate into parietal endoderm cell type if plated on gelatin-coated tissue culture dishes (Strickland, 1981). In such cells, in the absence of stress, the 89-kd HSP is expressed at levels similar to those found in fibroblasts of the 3T6 cell line, or in secondary fibroblasts, or in the parietal yolk type cells of the PYS-2 cell line (Figure 5b). A similar decrease in the expression of 89-kd HSP was observed following the neuronal-like differentiation of PCC7-S-1009 cells induced by retinoic acid and in C17-S_1-1003 cells induced by serum deprivation (B.Eddé, personal communication). Furthermore, the pattern of protein synthesis after an arsenite-shock is the same for fibroblasts (Figure 2b), or for PYS-2 cells and for F9 cells treated for 7 days with retinoic acid (Figure 6). Treatment of the F9 cells with DBcAMP alone has no detectable effect on the pattern of protein synthesis.

Discussion

In mouse fibroblasts, arsenite enhances the synthesis of six major HSP. However, in EC cells of the F9 line, it increases the synthesis of only three of them. In this respect, the behaviour of the F9 cells is unusual. Differences in HSP stimulation in different cell types have already been reported (Atkinson, 1981; Buzin and Petersen, 1982), but concern only the low mol. wt. HSP. Our most striking result is the elevated proportions of 59-kd, 70-kd and more specifically 89-kd HSP found in mouse EC cells of different origins in the absence of stress. This is not due to the fast growing characteristics of the EC cells, since when propagated at 32°C the protein pattern is the same as at 37°C. Furthermore, fast growing cells such as B16 melanoma cells (unpublished data) or 3T6 mouse fibroblasts do not express such high levels of HSP.

EC cells are usually believed to be very similar to the pluripotent cells of the early mouse embryo. Therefore, the protein pattern of embryonal ectoderms from day 8 mouse embryo was analyzed by silver staining in order to exclude that

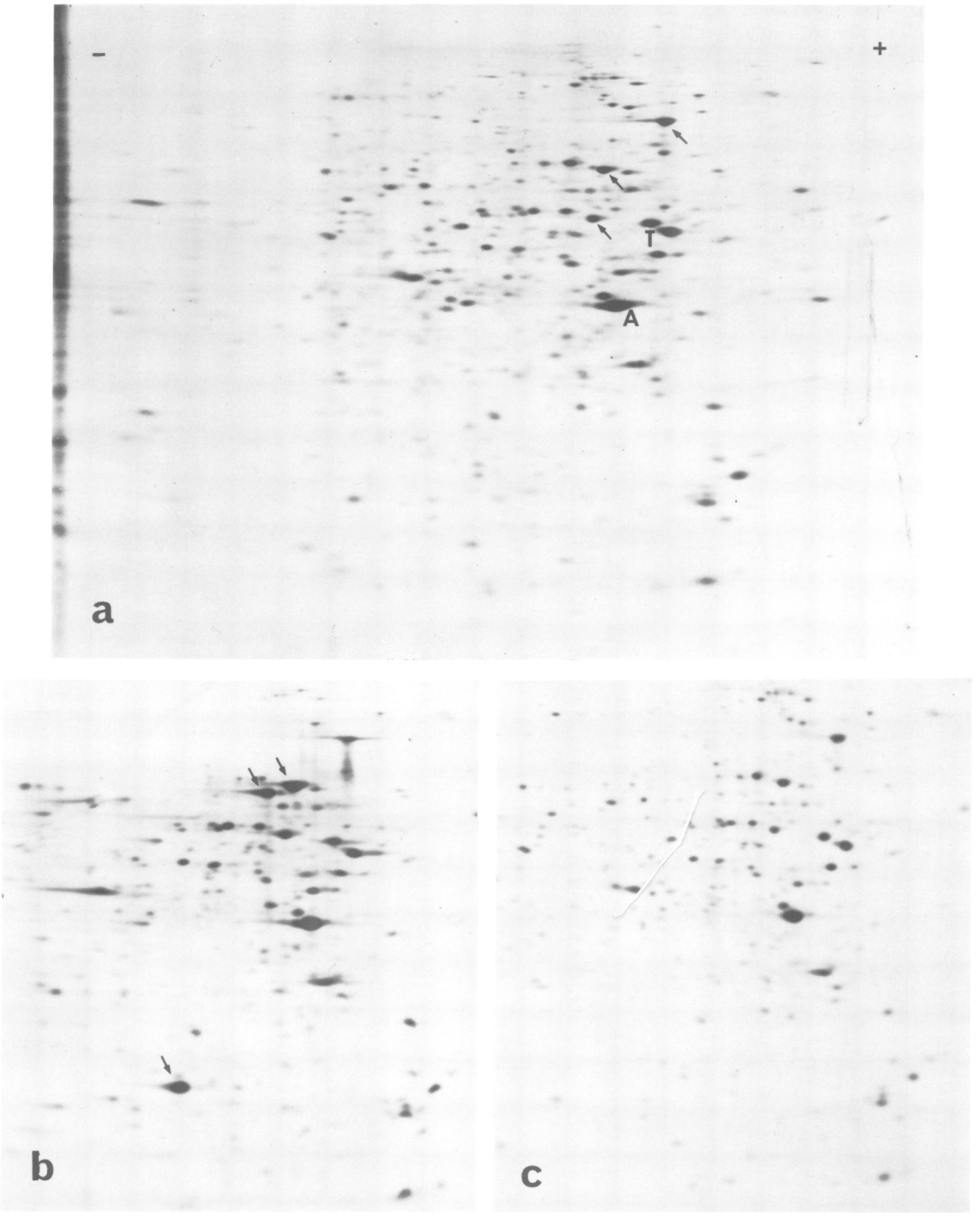

Fig. 3. Two-dimensional polyacrylamide gels of total extracts from [^{35}S]methionine-labelled F9 EC cells untreated and propagated at 37°C. **(a)** untreated; **(b)** exposed to media containing 150 μM sodium arsenite or **(c)** propagated at 32°C. Arrows point to the most prominent HSP. A: actin, T: tubulins.

dissection and labelling conditions could be responsible for HSP enhanced synthesis. Indeed, the two-dimensional pattern shown in Figure 7 is very similar to that obtained with EC cells which were silver stained (data not shown) or [^{35}S]-methionine-labelled (Figure 3a). Reexamination of previously published two-dimensional protein patterns from isotopic labelled early mouse embryos (Abreu and Brinster, 1978; Braude, 1979; Dewey *et al.*, 1978) confirms that at least the 89-kd HSP is also spontaneously expressed in high amounts in the pre-implantation embryo. This polypeptide has apparently intrigued Dewey *et al.* (1978) because of its failure to focus in the pH gradient. These authors referred to it as 'the comet'. In early *Drosophila* embryos (Graziosi *et al.*, 1980; Loyd *et al.*, 1981) the HSP equivalent to the mouse 89-kd and

70-kd have also been found to be spontaneously very abundant. In addition, the heat induction of HSP synthesis is reported to depend heavily upon the embryonic stage in

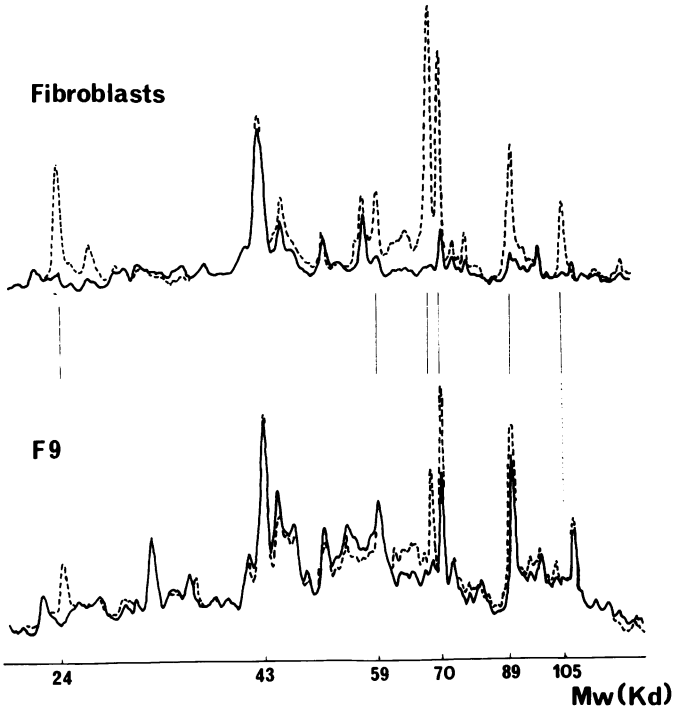

Fig. 4. Scanning the one-dimensional polyacrylamide gels of [^{35}S]methionine-labelled fibroblasts or F9 EC cells; controls (solid lines) and arsenite shocked (dashed lines).

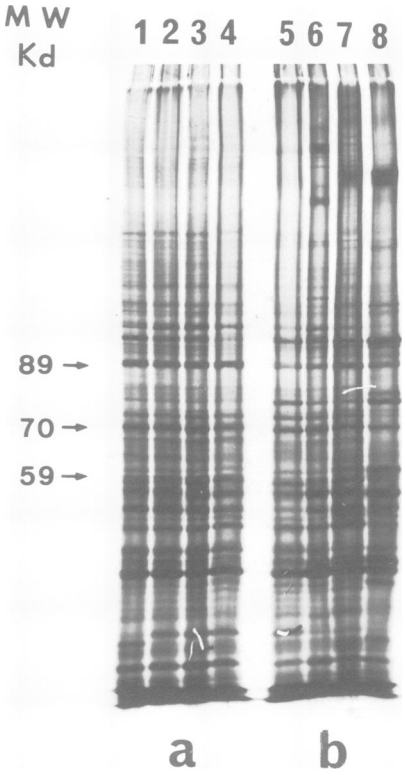

Fig. 5. One-dimensional polyacrylamide gel electrophoresis of total extracts from [^{35}S]methionine-labelled cells; **(a)** of EC type C17-S_1-1003 **(lane 1)**, PCC7-S-1009 **(lane 2)**, PCC4-aza^{R}1 **(lane 3)** and F9 **(lane 4)**; **(b)** differentiated 3T6 mouse fibroblasts **(lane 5)**, secondary mouse embryo fibroblasts **(lane 6)**, PYS-2, parietal yolk sac type cells **(lane 7)** and F9 cells which have been induced to differentiate by 7 days of retinoic acid and DBcAMP treatment **(lane 8)**.

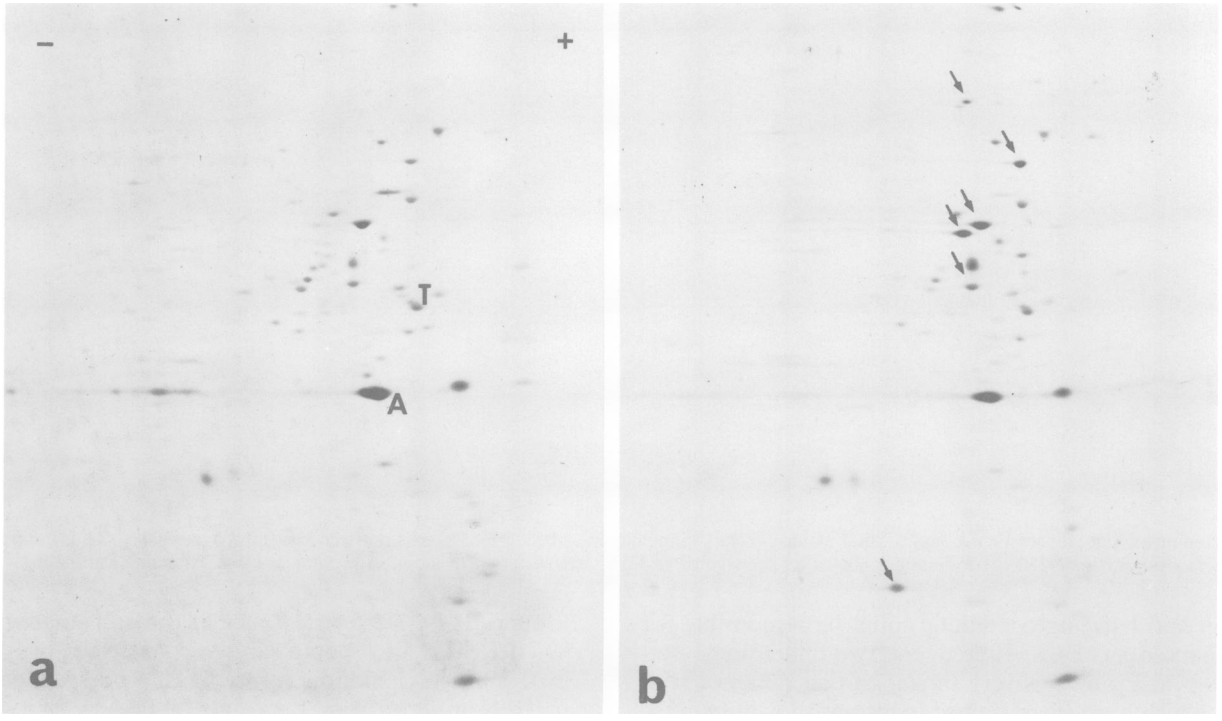

Fig. 6. Two-dimensional polyacrylamide gels of total extracts from [^{35}S]methionine-labelled differentiated F9 cells after 7 days of exposure to retinoic acid and DBcAMP. **(a)** Untreated; **(b)** exposed to medium containing 150 μM sodium arsenite. Arrows point to most prominent arsenite-induced proteins. A: actin, T: tubulin.

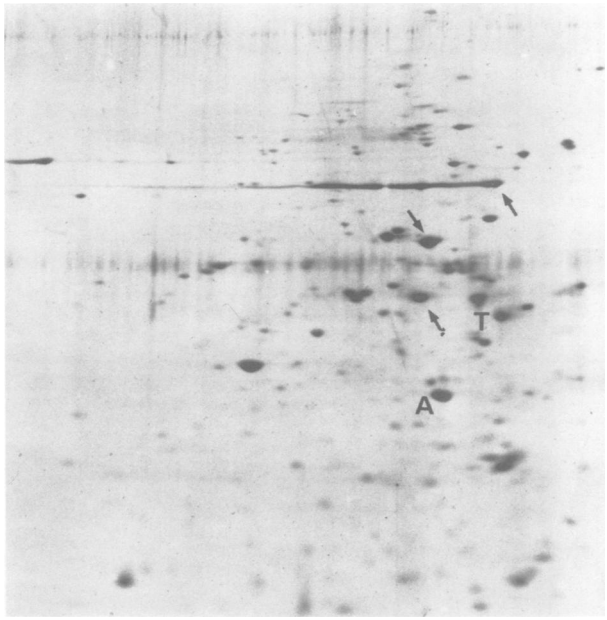

Fig. 7. Two-dimensional gel of silver stained mouse embryonal ectoderms (three embryos). Arrows point to the most prominent HSP. A: actin, T: tubulin.

Drosophila (Graziosi *et al.*, 1980) and sea urchin (Roccheri *et al.*, 1981).

Little is known about the physiological significance and function of the HSP. A mild heat-shock, stimulating HSP synthesis, has been shown to enhance survival and recovery of protein synthesis after a subsequent higher temperature heat-shock in Chinese hamster ovary cells (Li and Werb, 1982) and *Drosophila* cells (Petersen and Mitchell, 1981). According to the latter, it also prevents heat-induced developmental defects in *Drosophila* pupae. It is tempting, therefore, to consider the HSP synthetic pattern in mouse embryonic cells as a special adaptation of these cells to the stress conditions to which they are exposed after fertilization.

Materials and methods

Cell culture

Unless otherwise stated in the text, cells were grown at 37°C in tissue culture dishes with Dulbecco modified Eagle's media supplemented with foetal calf serum (FCS) in a 15% CO_2 atmosphere. 15% FCS was used for EC cells, 10% FCS for fibroblasts and 5% FCS for PYS-2, a cell line analogous to parietal endoderm (Lehman *et al.*, 1974). F9 EC cells were plated on gelatin-coated tissue culture dishes. For *in vitro* differentiation experiments, retinoic acid (Sigma) was added at 2×10^{-7} M final concentration, and the next day DBcAMP (Sigma) was added at 10^{-3} M final concentration and cells were cultured in the presence of retinoic acid and DBcAMP with a medium change every 48 h. Primary mouse fibroblasts were prepared by C.Marle from 12-day embryos.

Cell labelling

Cells were usually labelled for 3 h with 150 μCi of [^{35}S]methionine (Amersham, 1350 Ci/mmol) in 1.5 ml of methionine-free media in each 50 mm dish. After labelling, cells were washed twice with serum-free media, then flushed off or scraped with a rubber policeman, centrifuged and resuspended in a small volume of serum-free medium, which was then saturated with urea (Schwartz and Mann) and brought to 2% Nonidet P40, 5% mercaptoethanol (Aldrich), 0.1% SDS (Aldrich). Samples were frozen at −70°C until use for electrophoresis.

Arsenite treatment

Cells were exposed for 1 h to 150 μM sodium arsenite (Prolabo). Three hours later they were labelled as described.

Gel electrophoresis

Isoelectrofocusing (O'Farrell, 1975) was performed with 15 cm polyacrylamide gels containing 1.6% 3.5/10 and 0.4% 4/6 ampholines (LKB): 550 V were applied to the gel tubes for 16 h and then 1000 V for 1 h. Gels were extruded and kept at −20°C until used for the second dimension. They were then incubated for 15 min in Laemmli sample buffer (Laemmli, 1970).

After electrophoresis, the gels were either stained by Coomassie brilliant blue R-250 (Bio-Rad) or fixed in 30% ethanol, 10% acetic acid and dried. Autoradiographs were performed with Ilford Rapid R Type S films. One-dimensional gels were scanned with a VERNON photometer.

Visualisation of embryo protein pattern

Embryonal ectoderms from day 8 mouse embryos (129 strain) were dissected manually by M.T.Schnebelen, rinsed in phosphate buffered saline before freezing and lyophilisation. Samples were dissolved in O'Farrell sample buffer for electrophoresis as already described. The two-dimensional polyacrylamide gel was then silver stained according to the procedure of Ansorge (1982).

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