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Two specific markers for neural differentiation of embryonal carcinoma cells

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Two multipotential embryonal carcinoma (EC) cell lines, 1003 and 1009, can be induced to form preferentially neural derivatives *in vitro*. Synthesis of specific proteins during neural differentiation was followed by two-dimensional gel electrophoresis. The comparison of protein patterns obtained with neural and non-neural derivatives of these EC cell lines indicates that two changes are specific for the neural pathway: (i) the appearance of a new β-tubulin isoform and (ii) the accumulation of the brain isozyme of creatine phosphokinase already present in small amounts in EC stem cells. These changes were found to take place early in the course of differentiation and to occur even when neurite outgrowth was prevented.

Key words: embryonal carcinoma cells/neural differentiation/β-tubulin isoform/creatine phosphokinase

Introduction

The teratocarcinoma of the mouse (Pierce, 1967; Stevens, 1967) is a unique experimental system for studying some of the determination and differentiation events which occur during embryogenesis (Jacob, 1978; Martin, 1980; Strickland, 1981). Depending on the culture conditions, embryonal carcinoma (EC) cells can either remain undifferentiated or else become committed to various developmental pathways (Kahan and Ephrussi, 1970; Rosenthal *et al.*, 1970; Evans, 1972; Jakob *et al.*, 1973; Martin and Evans, 1974; Nicolas *et al.*, 1975). Recently, it has been possible to induce EC cells to differentiate in particular directions by using either chemical inducers (Strickland and Mahdavi, 1978; Jetten *et al.*, 1979; Jones-Villeneuve *et al.*, 1982) or hormonally defined media (Darmon *et al.*, 1981a).

Three clonal EC cell lines, PCC7-S AzaR1 clone 1009 (1009) (Pfeiffer *et al.*, 1981), C17-S1 clone 1003 (1003) (Darmon *et al.*, 1981a) and P19 (McBurney *et al.*, 1982), although multipotential *in vivo*, can be induced to differentiate preferentially into neural derivatives *in vitro*. Contrary to committed cell lines, such as neuroblastoma or pheochromocytoma lines, these EC cell lines can be used to study the early steps of neural differentiation. Moreover, the ability to induce these cell lines to differentiate into other derivatives (McBurney *et al.*, 1982; Darmon *et al.*, 1982a, 1982b) offers the opportunity of evaluating which changes are specific for neural differentiation.

This differentiation was obtained in the case of 1003 by serum-deprivation, i.e., culture in a serum-free (SF) defined medium and, in the case of 1009 by addition of retinoic acid and dibutyryl cAMP to serum-containing (SC) cultures. The neurons eventually obtained possess veratridine-stimulatable sodium channels, acetylcholine esterase and choline acetyl transferase activities, N6 surface antigen and neurofilament proteins (Darmon *et al.*, 1981a, 1982a, 1982b, 1982c; Pfeiffer *et al.*, 1981; Paulin *et al.*, 1982). In the case of 1003 it is possible to describe several successive steps in the pathway leading to neural differentiation: at day 4–5 of culture in SF medium, the great majority of the cells has differentiated into neuroepithelial cells characterized by their organization into rosettes, by the presence of vimentin intermediate filaments and veratridine-stimulatable sodium channels (Darmon *et al.*, 1981a, 1982b). Neuroepithelial cells are subsequently transformed into pre-neurons (days 6–7), cells with a rounder shape and a small neuritic process, containing both vimentin and the 70 K neurofilament protein. Terminally differentiated neurons (days 9–12) contain both 70 K and 200 K neurofilament proteins. In the case of 1009, terminally differentiated neurons arise more rapidly (day 6); they contain neurofilament proteins. Some glial cells decorated by anti-glial fibrillary acid protein (GFAP) antibodies may be recognized in 1009 but not in 1003 cultures (Paulin *et al.*, 1982).

Here we describe changes in protein patterns occurring during neural differentiation of the 1003 and 1009 EC cell lines. This analysis, as well as a comparative study of non-neural cell lines derived from ECs and mouse embryos, shows that the appearance of a new β-tubulin isoform and accumulation of a 47 K protein, identified as the B isozyme of creatine phosphokinase (BCK), are the most specific changes observed during neural differentiation and occur before terminal differentiation.

Results

Changes in two-dimensional protein patterns occur during neural differentiation of the 1003 and 1009 EC cell lines

Changes in protein patterns occurring during neural differentiation were analyzed by two-dimensional gel electrophoresis. Protein patterns of the supernatant fractions obtained from 1003 cells either in the EC stage or after neural differentiation (10 days in SF defined medium) show several differences. The arrowheads in Figure 1a point to proteins which are preferentially or exclusively expressed by 1003 EC cells while those in Figure 1b point to proteins which appear or increase after neural differentiation. Four significant changes concern relatively abundant proteins which have been identified (Figure 1).

Tubulin isoforms. With the two-dimensional gel system used, purified mouse brain tubulin is resolved into two α- and two β-subunits (not shown). 1003 EC cells synthesize two α-but only one β-isoform. Neuronal differentiation leads to the synthesis of one additional protein which migrates at the same location as the minor brain β-tubulin isoform. This protein can be purified using vinblastine sulfate (inserts in Figure 1a, b) indicating that it has properties similar to tubulin. Identification of this protein as a β-tubulin isoform was by one-

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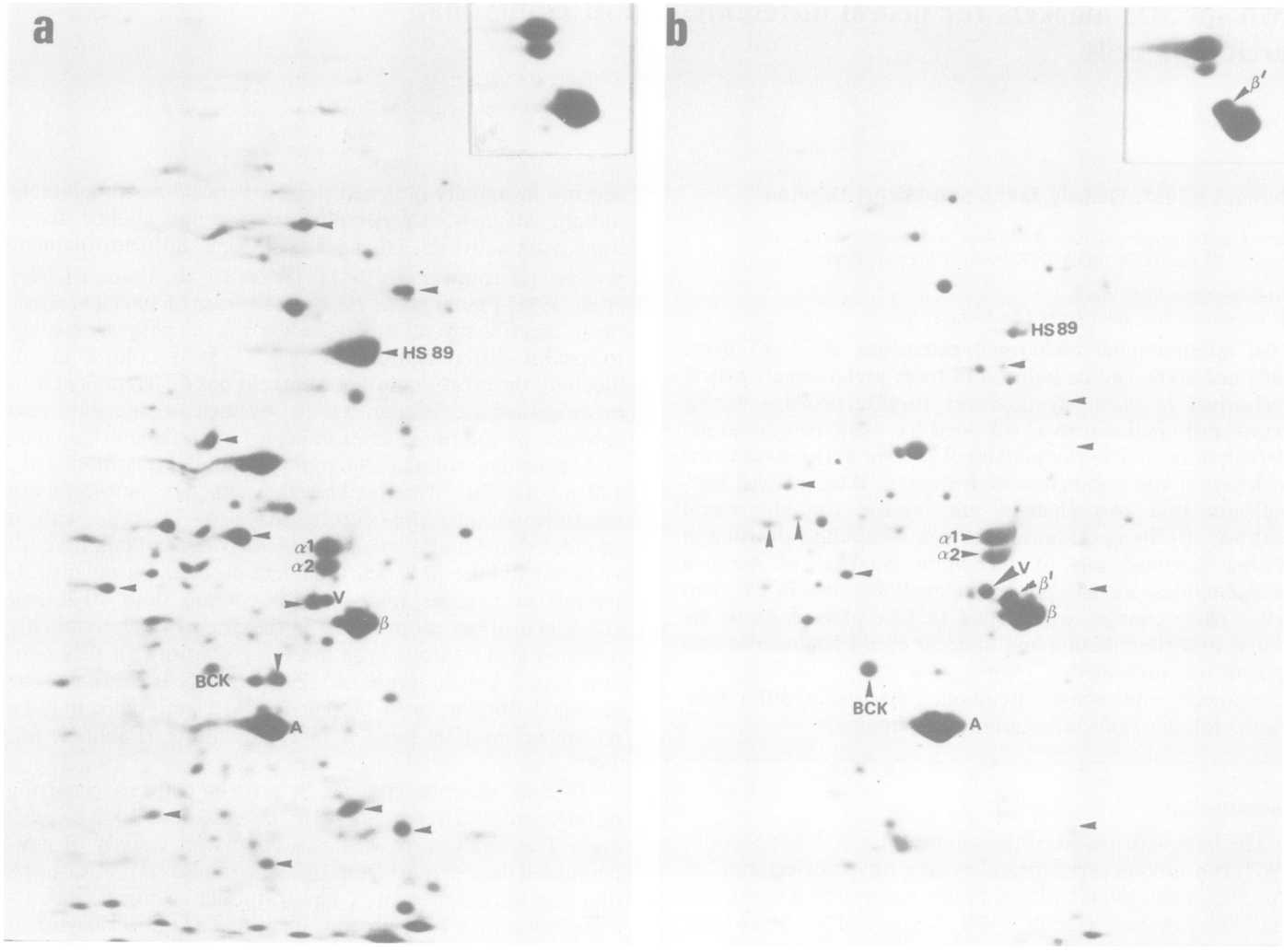

Fig. 1. Two-dimensional autoradiograms of the supernatant fraction of extracts from 1003 cells: **(a)** undifferentiated EC cells cultured in SC medium and harvested during exponentially growth phase; **(b)** neural derivatives obtained by plating EC cells in SF defined medium for 10 days. Labelling with [^{35}S]-methionine was performed overnight before the reported day of harvesting. Inserts represent two-dimensional gels of tubulin partially purified from each extract. Arrowheads point to proteins which are preferentially associated with each stage. A: actin; V: vimentin; α: α-tubulin; β: β-tubulin.

dimensional peptide mapping (Cleveland *et al.*, 1977) (Figure 2a). In this paper this protein will be called β'-tubulin. A stretching in the spots of α1- and α2-isoforms was also seen to occur after neural differentiation. This might correspond to an increase in β-tubulin microheterogeneity similar to that found in mouse brain during development (Denoulet *et al.*, 1982).

Brain isozyme of CPK. The synthesis of a 47 K protein (IEP 6.1) is increased several fold after neural differentiation. Its electrophoretic behavior and its distribution in different cell types and tissues (see below) caused us to compare it with GFAP or BCK. Purified rat BCK was a generous gift of A. Cohen. The peptide map of this 47 K protein shows clear differences when compared with GFAP but strong similarities to rat BCK (Figure 2b and c). The few differences observed in the latter case probably correspond to differences between the rat and mouse proteins.

Vimentin. The synthesis of this intermediate filament protein is low in EC cells but increases at an early stage during the process leading to neural differentiation (Figure 1a and b; see also Figure 4). Similar conclusions were obtained using immunofluorescent detection (Paulin *et al.*, 1982).

Heat-shock proteins. A group of 89 K proteins (HS89) known to be induced by heat-shock in other systems (Bensaude and Morange, 1983) is synthesized at a much lower rate after differentiation.

Two-dimensional electrophoresis of the supernatant fractions of extracts prepared from 1009 cells either in the EC stage or after neural differentiation (6 days with retinoic acid and dibutyryl cAMP) shows very similar changes of the protein patterns. The same four major changes can be observed (Table I). Such a similarity between patterns found during neural differentiation of 1003 and 1009 cells shows that the genotype of the cell line, the conditions used to induce differentiation and the duration of the culture have no influence on the observed changes.

To exclude the possibility that these changes might not be related to alterations of protein metabolism but rather to variations in the solubility of the proteins, the pellet fractions obtained during the preparation of crude extracts were also analyzed by two-dimensional electrophoresis. The solubility of the four proteins seemed to be the same in all culture con-

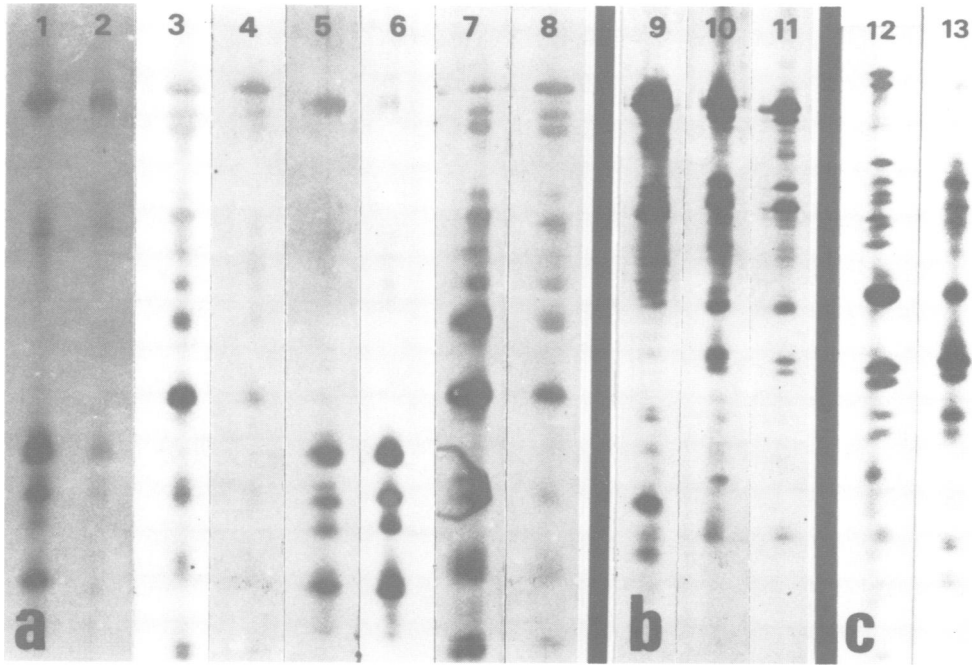

Fig. 2. Peptide mapping. **(a)** Tubulin isoforms: α1 **(1, 5)**, α2 **(2, 6)**, β **(3, 7)**, β′ **(4, 8)**. Spots were exised from two-dimensional gels of purified mouse brain tubulin **(1–4)** and 1003 neural cells **(5–8)**. **(b)** Comparison of GFAP and the 47 K protein: GFAP purified from mouse brain **(9)**, 47 K protein from mouse brain **(10)** and 1003 neural cells **(11)**. **(c)** Comparison of BCK and the 47 K protein: BCK purified from rat brain **(12)** (kindly provided by A. Cohen) and the 47 K protein from mouse brain **(13)**. One or two spots of each protein were excised from the corresponding two-dimensional gels after brief staining and destaining and submitted to V8 *S. aureus* protease (10 ng/slot) according to Cleveland *et al.* (1977). Peptide maps were silver stained.

Table I. The presence of β′-tubulin (β′), BCK, vimentin and HS 89 were checked by two-dimensional electrophoresis in various EC cell lines and their differentiated derivatives. Results are noted as negative (−) or positive (+)

Cell lines	β′	BCK	Vimentin	HS 89
1003, EC	−	+	+	+ + +
1003, neural	+	+ +	+ +	+
10035 C1, mesenchymal	−	−	+ +	+ + +
10031 D4, fibroblastic	−	−	+ +	+ + +
1009, EC	−	+	+	+ + +
1009, neural	+	+ +	+ +	+
1168, myoblastic	−	−	+ +	+
PYS2, parietal yolk sac	−	−	+	+ + +
3 TDM.1, trophoblastoma	−	−	+	+ + +
PCC4 AzaR1, EC	−	+	+	+

Table II. Presence of β′-tubulin and BCK in differentiated cell lines of neural-crest origin and adult mouse tissues. Results are noted as negative (−) or positive (+)

Cell lines or tissues	β′-Tubulin	BCK
B16, melanoma	+	−
PC 12, pheochromocytoma	+	−
NIE115, neuroblastoma	+	−
NIA103, neuroblastoma	+	−
Brain	+	+ + +
Liver	−	−
Spleen	−	−
Kidneys	−	−

ditions: BCK and HS89 were almost exclusively found in the supernatants while tubulin and vimentin were found both in the supernatants and the pellets. Nevertheless, these two latter proteins vary in the same fashion during differentiation (data not shown). Further experiments are needed to determine whether the changes observed are due to variations in the rates of synthesis and/or degradation of these proteins.

Changes in β′-tubulin and BCK are specifically associated with neural differentiation

EC cell lines and their various derivatives provide an opportunity to examine which events are specifically associated with neural differentiation. The results are summarized in Table I. β′-Tubulin is expressed only in neural cell-types. BCK is synthesized at a basal level in EC cell lines and is accumulated during neural differentiation but is not detectable in the non-neural cell types analyzed. On the contrary, changes affecting vimentin and HS89 are observed in various differentiated derivatives and are not, therefore, specific for the neural pathway.

The presence of β′-tubulin and BCK was also investigated in differentiated cell lines of neural-crest origin and in adult mouse tissues (Table II). β′-Tublin, but not BCK, was found in the PC 12 pheochromocytoma line, the B16 melanoma line and the NIE115 and NIA103 neuroblastoma lines. Among adult mouse tissues, both β′-tubulin and BCK were found in the brain but not in the liver, kidneys or spleen (Table II and Figure 3).

Changes in β′-tubulin and BCK occur before terminal differentiation

As reported above, it is possible to divide grossly the dif-

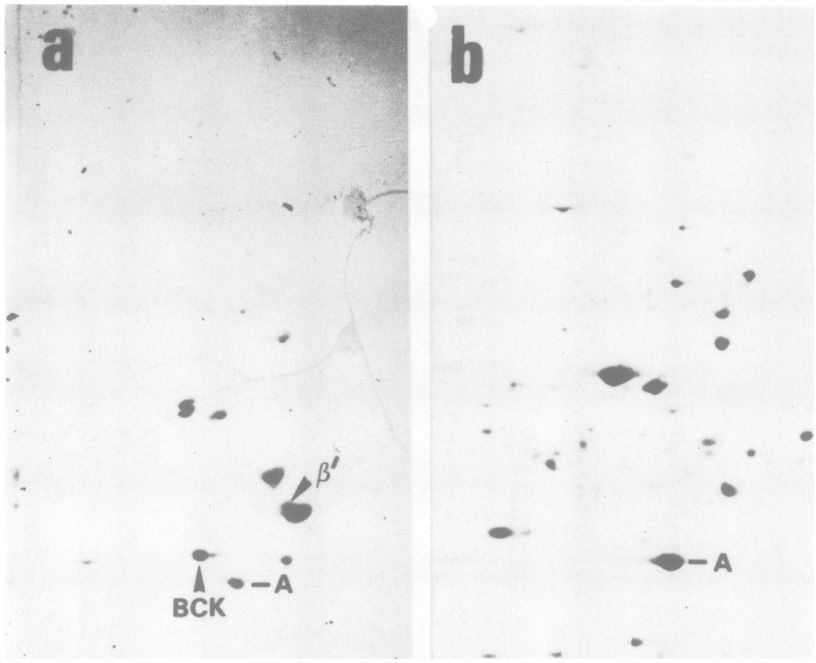

Fig. 3. Two-dimensional electrophoresis of the supernatant fraction of mouse brain (**a**) and spleen (**b**) extracts. Note the high amount of BCK in brain. A, actin.

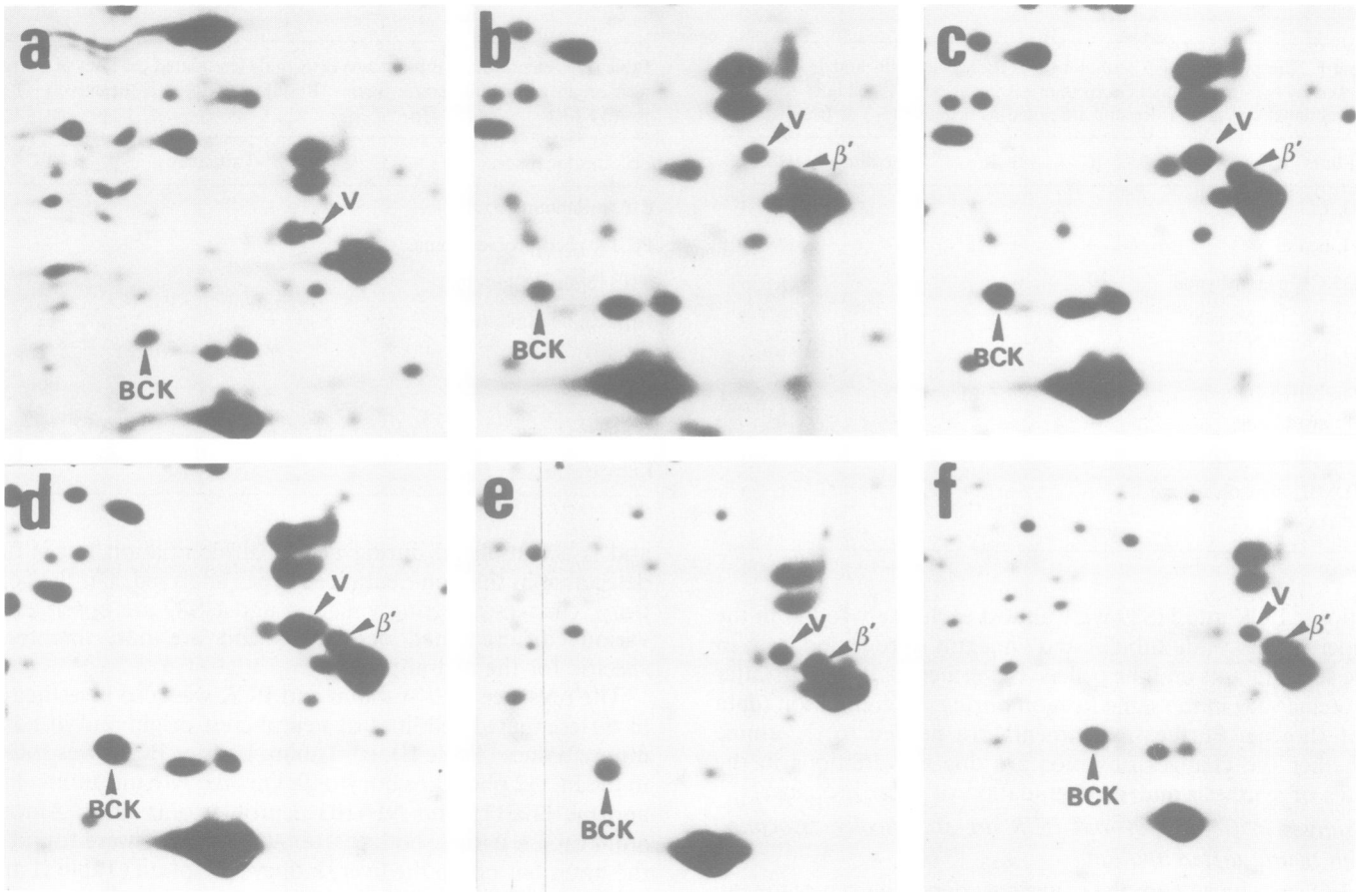

Fig. 4. Two-dimensional autoradiograms of the supernatant fraction of extracts from 1003 EC cells (**a**) or after culture in fibronectin-containing defined medium for 3 (**b**), 5 (**c**), 7 (**d**) and 10 days (**e**); day 5 aggregates in fibronectin-free defined medium (**f**). Labelling with [^{35}S]methionine was performed overnight before the reported day of harvesting. Only the tubulin and BCK region of the autoradiogram are shown. V, vimentin.

ferentiation of 1003 cells into four successive periods. To study them we have analyzed protein patterns after 0, 3, 5, 7 and 10 days of culture in SF defined medium (Figure 4). The appearance of β'-tubulin and the increase in BCK synthesis began to be observed at day 5. Both proteins further accumulated until day 10. Since neurite-bearing cells are extremely rare at day 5, the beginning of these changes seems to be related to the appearance of neural precursors, i.e., neuroepithelial cells and/or pre-neurons.

When 1003 cells are cultured in fibronectin-free defined medium on bacteriological Petri dishes they form aggregates with an epithelial organization similar to that of neuroepithelium (Darmon *et al.*, 1981a). The protein pattern of day 5 aggregates (Figure 4f) shows that β'-tubulin and BCK are synthesized under these conditions at levels similar to those found in terminally differentiated neurons. The changes affecting β'-tubulin and BCK can therefore occur when neurite outgrowth is prevented.

Discussion

During neural differentiation of two EC cell lines, 1003 and 1009, the most important and specific changes affecting protein patterns concern: (i) the appearance of a new β-tubulin isoform (β') and (ii) the accumulation of the brain isozyme of CPK (BCK). A good correlation exists between these *in vitro* changes and events occurring *in vivo*. In the case of tubulin, β'-isoform is expressed in the brain but not in a number of other mouse tissues (Table II). It could provide, therefore, a useful marker for neural differentiation. In the case of CPK, previous studies have revealed an accumulation of the B-isozyme during brain development in the rat (Soreq *et al.*, 1982) and mouse (Vayssière, 1982). However, BCK was also found in the rat uterus, bladder and intestine (Adamson, 1976). This marker must then be used with caution in experimental systems in which other differentiation may occur.

A protein which presents the same electrophoretic parameters as β'-tubulin has been previously described in rat brain (Marotta *et al.*, 1978) and in neuroblastoma cells (Eddé *et al.*, 1982). Our analysis of various cell lines and tissues has revealed a strong association of this protein with neural phenotype. Moreover, Moura-Neto *et al.* (1983) have found β'-tubulin in primary cultures of neuronal, but not of glial cells obtained from embryonic mouse brain. In addition to neuronal cell lines, β'-tubulin is expressed in B16 melanoma cells. This can be related to the fact that melanoma cells bear many similarities to neuronal cells (Fabricant *et al.*, 1977; Bottenstein *et al.*, 1979). Nevertheless, it has to be checked whether β'-tubulin is also present in melanocytes or is only expressed in melanomas.

Our conclusion that β'-tubulin appears early during neural differentiation is strengthened by the finding that this protein is already synthesized in committed but undifferentiated neuronal cell-types, such as pheochromocytoma or neuroblastoma cell lines. In the latter, Spiegelman and co-workers have demonstrated the existence of multiple microtubule organizing centers (MTOCs) which aggregate before neurite outgrowth (Spiegelman *et al.*, 1979). β'-Tubulin could be implicated in such an initiation process of neurite outgrowth.

The results presented here suggest the existence of a complex regulation of BCK during development. BCK, synthesized at a basal level in EC cells, is increased several fold during neural differentiation. In addition, it is absent from non-neural differentiated derivatives. Experiments are in progress to determine if BCK is differently expressed by the inner cell mass and by the trophectoderm of the mouse blastocyst.

BCK and neuronal-specific enolase (NSE) have been identified as being the predominant components of a group of proteins which accumulate at high levels after estrogen treatment in rat uterus (Reiss and Kaye, 1981). The finding of receptors for 17-β estradiol in rat brain and the trophic effects of estrogens demonstrated in nerve cultures (Faivre-Bauman *et al.*, 1981) suggest that these hormones play a role in brain development. It has to be checked whether BCK and NSE are also regulated in the developing brain by estrogen signals.

Materials and methods

Cell cultures

PCC7-S AzaR1 clone 1009 (1009) and C17-S1 clone 1003 (1003) were grown and induced to differentiate into neural derivatives as described previously (Pfeiffer *et al.*, 1981; Darmon *et al.*, 1981a). The protein patterns of these neural derivatives were compared with those obtained from two mesenchymal subclones of 1003, 10035 C1 and 10031 D4, as well as with various EC and differentiated cell lines. The origin, the culture conditions and the phenotype of these cell lines have been described previously: PCC4 (EC) (Jakob *et al.*, 1973), 10035 C1 (mesenchymal) (Darmon *et al.*, 1982a), 10031 D4 (fibroblastic) (Darmon *et al.*, 1983), 1168 (myoblastic) (Darmon *et al.*, 1981b), PYS-2 (parietal yolk sac) (Jakob *et al.*, 1973), 3TDM-1 (trophoblastoma) (Nicolas *et al.*, 1976). B16 (melanoma) (Mather and Sato, 1979), PC12 pheochromocytoma (Green and Tischler, 1976).

Cell and tissue extracts; two-dimensional electrophoresis

Cells were labelled overnight with 30 μCi/ml of [^{35}S]methionine (Amersham, 700 Ci/mmol) before being harvested into buffer A (100 mM MES, pH 6.4; 1 mM EGTA; 1 mM EDTA; 0.5 mM $MgCl_2$; 1 mM GTP) and sonicated (30 W, 30 s three times). Fresh adult mouse tissues were homogenized by 10 strokes in a Teflon/glass potter in buffer A. Supernatant fractions of both extracts were prepared by centrifugation at 12 000 *g* for 15 min at 4°C. Pellet fractions were resuspended in buffer A. Both pellet and supernatant fractions were treated and submitted to isoelectric focusing according to O'Farrell (1975). The pH range of ampholytes was 5–8 and 3.5–10 (4/1). Second dimension electrophoresis (8% acrylamide) was carried out on slab gels (130 x 240 x 1 mm) in the presence of SDS as described previously (Eddé *et al.*, 1982). Two-dimensional gels were autoradiographed for 3–7 days on Kodak industrex A5 films.

Tubulin purification

Partial purification of tubulin from 1003 extracts was performed using vinblastine sulfate as described previously (Eddé *et al.*, 1982). Complete purification of tubulin from mouse brain was performed using the phosphocellulose procedure described by Weingarten *et al.* (1975).

Peptide mapping and silver staining

Limited proteolysis of gel spots performed with *Staphylococcus aureus* V8 protease and peptide analysis were carried out according to Cleveland *et al.* (1977). The gels were silver stained according to Ansorge (1983).

Acknowledgements

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