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POMC gene expression in mouse and hamster melanoma cells

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Expression of mRNA hybridizable to murine POMC cDNA, and immunoreactivity specific for POMC derived peptide, β-endorphin, are reported for the first time in one murine and two hamster melanoma lines The apparent sizes of POMC mRNA transcript were 3 5, 1 5 and 1 kb It is suggested that POMC gene expression may be involved in the autoregulation of the melanoma phenotype at the cellular and tissue levels

Proopiomelanocortin, β-Endorphin, Melanoma

1 INTRODUCTION

Proopiomelanocortin (POMC) is the precursor protein of adrenocorticotropin (ACTH), endorphins, melanotropins (MSH) and lipotropins (LPH) [1,2] Aside from the pituitary gland, the POMC gene is expressed in the central nervous system [1–4], in many peripheral tissues [2–5] and in a variety of neuroendocrine nonpituitary tumors [4–7] The size of mature POMC mRNA transcripts in pituitary is about 1 kb [1,2,4,5], but shorter and longer POMC transcripts have been found in some extrapituitary tissues [2–5] and several tumor lines [5–7]

Melanoma, a cancer of melanocytic origin, is one of the most rapidly increasing malignancies in humans. Its incidence is predicted to rise further due to depletion of the ozone layer [8] POMC-derived peptides are well appreciated for their diverse regulatory functions [1,2]. Of particular interest for melanoma biology are regulation of the differentiated phenotype and proliferation by MSH and ACTH [1], and immunomodulatory functions of β-endorphins, corticotropins and melanotropins [9,10] Although POMC gene expression in melanomas has not been documented, results from two laboratories imply expression in mouse and human melanoma cells [11,12]. To study potential POMC gene expression in malignant mammalian melanocytes, we used variants of Bomirski transplantable hamster melanomas whose proliferative rates are inversely related to the genetically determined degree of cellular differentiation [13], the Bomirski AbCl amelanotic hamster melanoma line [14], and the amelanotic clone #6 of Cloudman S91 mouse melanoma [15] The two latter cell lines express functional MSH receptors [14–16]

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2 MATERIALS AND METHODS

2 1 *Tumor and cell lines*

Randomly bred male Syrian golden hamsters were used for implantation of Bomirski Ab amelanotic, Ml hypomelanotic, and Ma melanotic hamster melanomas Protocols of transplantation and harvesting have been given elsewhere [13] Cloudman S91 (amelanotic clone #6) melanoma cells (gift of Dr J Pawelek [15]) and Bomirski AbCl hamster amelanotic cells were cultured in Ham's F10 medium plus 10% horse serum [14,15] Cultures were supplied with fresh medium 3 times a week The cells were harvested in Ca^{2+} and Mg^{2+}-free Tyrode's balanced salt solution, containing EDTA (1 mM)

2 2 *RNA blotting*

A heat-denatured random prime-labelled POMC cDNA probe was prepared from a 0 923 kb mouse POMC clone (gift of Dr J Roberts [17]), with the aid of a Boehringer-Mannheim kit and [α-^{32}P]dCTP (DuPont, USA) The tumor tissues were pulverized in liquid nitrogen, and the total RNA was isolated by a guanidinium thiocyanate method with subsequent ultracentrifugation of the lysates through a CsCl cushion and phenol/chloroform extraction [18] Total RNA from cultured cells was isolated by a guanidinium hydrochloride method [19], and poly(A)$^+$mRNA was extracted using a 'FAST track' mRNA isolation kit according to the manufacturer's instructions (Invitrogen) Isolations of RNA and Northern blots were performed over the period 1989 and 1990 Forty μg of total RNA and fifteen μg of poly(A)$^+$mRNA was electrophoretically separated through formaldehyde/1% agarose gels by standard procedures [18,20] Ethidium bromide-stained total RNA was photographed under UV light, and the RNA was transferred to Zeta Probe blotting membranes by capillary transfer, overnight The membranes were dried for 2 h at 80°C under vacuum, and then soaked in hybridization buffer (50% deionized formamide, 7% SDS, 5 × Denhardt's solution, 5 × SSC, 20 mM NaH_2PO_4, pH 7 0, and 100 μg/ml denatured salmon sperm DNA) at 42°C for 12 h The heat-denatured random prime-labelled cDNA probe was added to fresh hybridization buffer which included 10% dextran sulphate at 42°C and hybridization was allowed to proceed for 24 h (cf Figs 1 and 2) In some experiments dextran sulfate was not added to the hybridization buffer (cf Fig 3) The membranes were then washed for 5 min with 2 × SSC plus 0 5% SDS, 15 min with 2 × SSC plus 0 1% SDS at room temperature, and then with 0 2 × SSC plus 0 1% SDS at 60–65°C for 2 h After a final 5 min rinse in 0 2×SSC plus 0 1% SDS, the hybridized blots were exposed to Kodak XAR film at −70°C

2 3 *Immunocytochemistry*

The cells were fixed in situ on cover slips in 70% acetone/30%

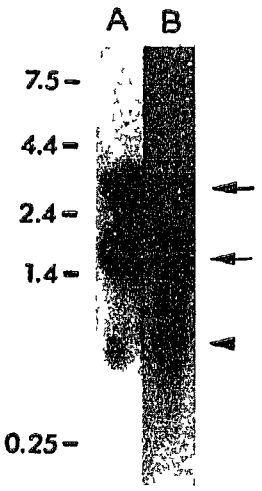

Fig 1 Northern blot analysis of POMC-hybridizable RNA in rodent melanoma lines Northern blot analysis of poly(A)$^+$ mRNA (15 μg) from AbC1 hamster (A) and S91 mouse (B) amelanotic melanoma cells RNA was hybridized to 0 923 kb ^{32}P-labelled mouse POMC cDNA Left, RNA size markers (kb)

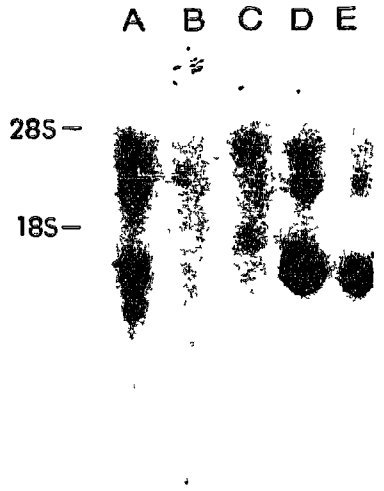

Fig 2 Northern blot analysis of POMC-hybridizable RNA in hamster melanoma lines Northern blot analysis of total RNA (40 μg) from Bomirski Ab amelanotic (A), Ma melanotic (B), Ml hypomelanotic (C) transplantable melanomas, from AbC1 amelanotic melanoma cells (D, cells were cultured in Ham's F10 medium) and from AbC1 melanized cells (E, cells were cultured in Ham's F10 medium plus 200 μM L-tyrosine)

methanol and air dried The cover slips were re-hydrated in PBS and incubated with anti β-endorphin antibody (1 300, 1 500 and 1 1000 dilution, gift of Dr R Allen [21] or rabbit nonimmune serum in PBS plus 0 1% Triton X-100 at room temperature for 1 h After washing with PBS (3 times for 5 min) the cells were incubated with FITC- or biotin-conjugated goat anti-rabbit IgG (1 100 dilution) for 1 h at room temperature After having been washed with PBS, the coverslips were mounted in 50% glycerol in PBS and examined by immunofluorescence microscopy The coverslips incubated with biotin-conjugated antibody were processed further using a biotin–avidin peroxidase kit (Promega) The DAB stained slides were examined by transmission light microscopy

3 RESULTS AND DISCUSSION

Northern blot analysis of poly(A)$^+$mRNA from the AbC1 line and mouse S91 amelanotic clone #6 shows that POMC mouse cDNA hybridizes to mRNA of approximately 3.5 and 1 5 kb (major band) and 1 kb (less abundant species) (Fig 1) Transcripts larger than 1 kb are found in testes, ovary, placenta, adrenal, hypothalamus and in several tumor lines [2,3,5–7] The size heterogeneity of POMC mRNA was explained by alternative splicing, variation in the length of the poly(A)$^+$ tail, or use of alternate upstream transcription initiation sites [2–7]. Since the structure of the POMC gene is similar among all mammalian species [2] and we used a 0 923 kb murine POMC cDNA probe comprising the entire POMC coding sequence (exons 1–3) [17], any of the above mechanisms may be responsible for the presented POMC mRNA size heterogeneity in melanomas

Northern blot analysis of total RNA shows that POMC mouse cDNA hybridized to mRNA of approximate sizes 3 5 and 1 5–1.3 kb in Ab undifferentiated transplantable hamster amelanotic melanoma and the AbC1 hamster melanoma line cultured in vitro (Fig. 2). In 2 other, well differentiated transplantable hamster melanotic melanomas, Ma and Ml, POMC mRNA was not detected (Fig 2). Thus from the point of view of the natural history of Bomirski melanomas [13] the progression from the least malignant melanotic Ma melanoma to the highly malignant amelanotic Ab melanoma coincides (among other phenotypic changes) with an expression of the POMC gene in the established line.

Since L-tyrosine and L-DOPA induce melanogenesis in hamster AbC1 cells through related but distinct mechanisms [14] we tested the effect of those two amino acids on POMC mRNA expression Exposure of AbC1 cells to 200 μM L-tyrosine for 3, 9, 24 and 48 h slightly

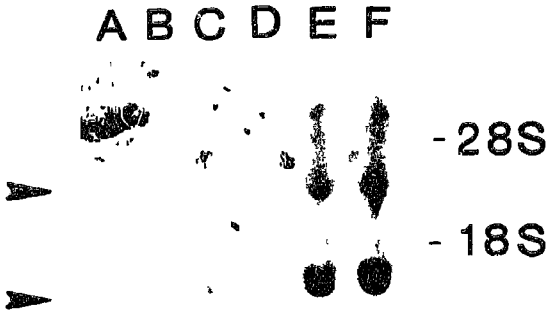

Fig 3 Northern blot analysis of POMC-hybridizable RNA in AbC1 melanoma cells cultured in the presence of L-DOPA The cells were cultured in the absence (A) or in the presence of 50 μM L-DOPA for 0 5 (B), 3 (C), 9 (D), 24 (E) and 48 (F) hours Forty μg of total RNA for each condition tested was used for Northern blot analysis Arrowheads, transcripts hybridized to mouse POMC cDNA

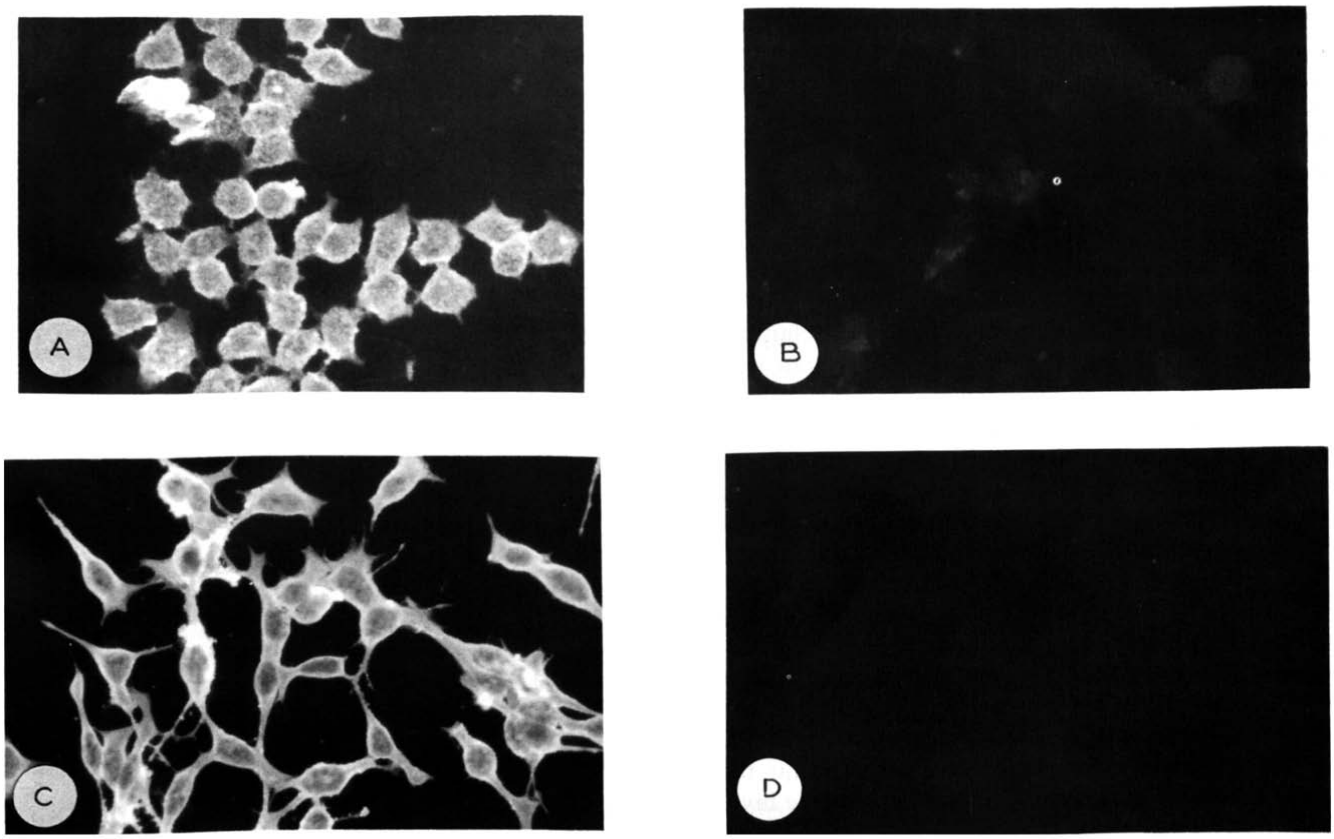

Fig. 4. β-endorphin antigen visualized by indirect immunofluorescence. AbC1 hamster melanoma cells: (A) and (B); S91 mouse melanoma cells: (C) and (D). Cells were incubated with anti β-endorphin antibody (1:300) (A and C) or nonimmune serum (1:300) (B and D). Magnification × 500.

inhibited (cf. Fig. 2) or had no effect on the level of POMC mRNA (not shown). In contrast, culturing of the AbC1 cells in the presence of 50 μM L-DOPA for 24–48 h significantly increased the intracellular concentration of mRNA hybridizable to POMC cDNA (Fig. 3). We believe that this result mandates further detailed studies on a possible link between L-DOPA-induced pathway(s) [20] and POMC mRNA expression.

Immunocytochemical studies on AbC1 hamster and S91 mouse melanoma cells with anti β-endorphin antibodies and indirect immunofluorescent staining demonstrated immunoreactivity against β-endorphin, which

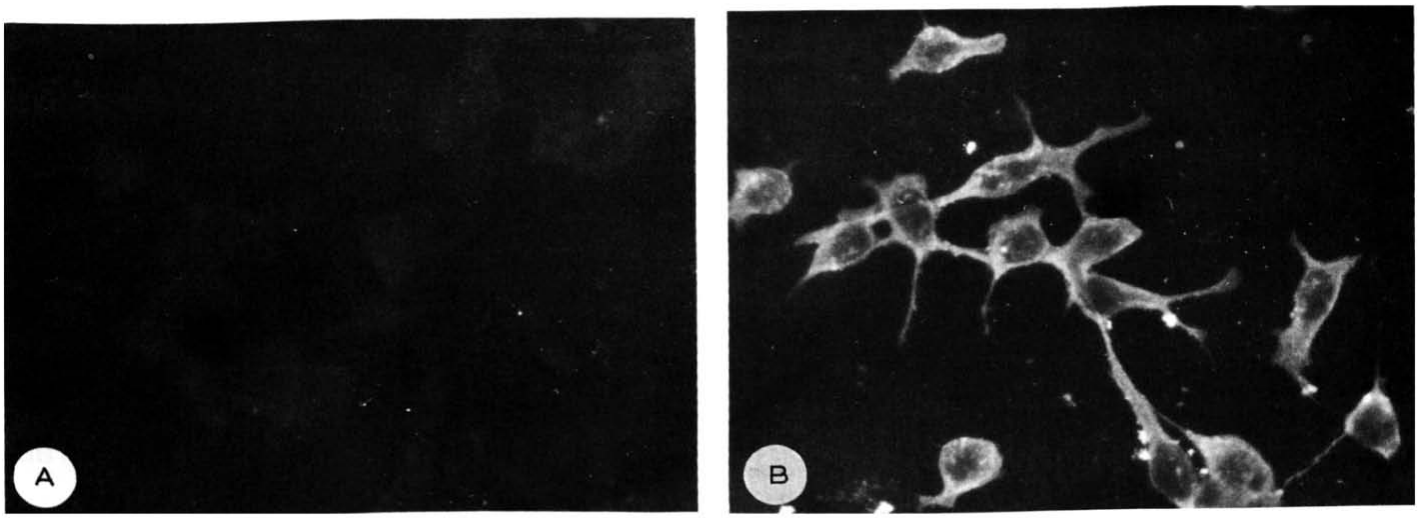

Fig. 5. Specificity control for β-endorphin immunocytochemistry. Acetone/methanol-fixed AbC1 hamster melanoma cells were reacted with (A) Control: anti β-endorphin antibody preabsorbed overnight at 4°C with 3×10^{-4}M β-endorphin prior to incubation with cells; (B) experimental: β-endorphin antibody. Antibody dilution 1:500. Magnification ×650.

was not seen in the case of nonimmune serum (Fig. 4) This immunoreactivity was blocked by preabsorption of anti-β-endorphin antibodies with β-endorphin prior to immunocytochemistry (Fig 5) The same results were obtained using biotin–avidin peroxidase kit for immunocytochemistry (not shown).

The Northern blots and immunocytochemistry presented here provide evidence for the expression of the POMC mRNA and protein in the Ab and AbC1 amelanotic sublines of Bomirski hamster melanoma and the amelanotic subline of Cloudman S91 mouse melanoma. POMC-derived peptides (MSH and ACTH) can regulate melanoma differentiated phenotype [1,10,14,15] and ACTH, MSH and β-endorphin can modulate functions of the immune system [9,10] It is generally accepted that malignant behavior of solid tumors is the result of complex multidirectional interactions between different cell populations including cells of the immune system [22] Therefore the POMC expression in melanomas could imply the existence of autoregulatory loops governing the phenotype and malignancy of melanomas which would involve autocrine and paracrine regulation as well as a crosstalk between melanoma cells and cells of the immune system. According to Orlow et al. [23] Cloudman S91 melanoma cells have intracellular binding sites for MSH This could suggest a possible involvement of a POMC gene in an intracrine regulation of the melanoma phenotype

In summary, we report an expression of POMC mRNA, and immunoreactivity specific for POMC-derived peptide, β-endorphin, in rodent melanomas. This finding forms a basis for further studies into the potential role of POMC gene expression in the autoregulation of the melanoma malignant phenotype, both at the cellular and tissue level

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