REGULATION OF MELANOGENESIS IN CONDITIONALLY IMMORTALISED MOUSE MELANOCYTES EXPRESSING A TEMPERATURE-SENSITIVE SV40 LARGE T ANTIGEN

by

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February, 1999

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ABSTRACT

Regulation of melanogenesis in conditionally immortalised mouse melanocytes expressing a temperature-sensitive SV40 large T antigen

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The transformation of a normal melanocyte to a malignant melanoma involves a series of poorly understood genotypic and phenotypic alterations. *In vitro* models of melanoma formation generated by transforming mouse melanocytes with exogenous oncogenes have revealed that this process is frequently accompanied by a loss of pigmentation. The aim of this study was to establish, and to make use of, unique cell lines to gain further insight into the mechanism(s) by which oncoproteins alter melanocyte differentiation. Primary cultures of mouse epidermal and dermal melanocytes were infected with a retrovirus carrying a temperature-sensitive mutant SV40 large T antigen. Six immortalised cell lines thus generated were analysed by northern and western blots and by enzymatic assays at the permissive temperature of the oncoprotein. Three epidermal and two dermal melanocyte clones remained pigmented and expressed tyrosinase, TRP-1 and -2 genes and the proteins encoded by them. In addition they expressed the *mi* gene and the *c-kit* receptor.

In contrast, one dermal melanocyte clone (DMEL-3) gradually depigmented: this was accompanied by enhanced growth and down-regulation of melanocyte-specific gene expression. At the non-permissive temperature of the oncoprotein, proliferation ceased and DMEL-3 cells repigmented with a time-dependent increase in melanocyte-specific gene expression. Moreover, *mi* gene expression was down-regulated in the DMEL-3 cell line at the permissive temperature and was re-expressed at the non-permissive temperature. These results provided direct evidence for the role of the SV40 large T antigen in melanocyte de-differentiation and emphasized the pivotal role of Mi in this process. Northern blot analysis of DMEL-3 cells cultured at the permissive and non-permissive temperatures revealed that there were no detectable levels of *Pax3* transcripts at either temperature. In addition, *Pax3* expression was absent in the highly pigmented DMEL-2 and melan-a cell lines. These results suggest that Pax3 is not required for *mi* expression and that it is unlikely to be a target of the T antigen-mediated repression of *mi*.

To explore the possibility that other melanocyte markers are also altered as a consequence of alterations in *mi* expression, the DMEL-3 cells were examined for changes in the α-MSH and c-kit receptors. Melanin synthesis and tyrosinase activity assays showed that alterations in *mi* expression did not correlate to responsiveness to α-MSH, suggesting that the MSH receptor gene is not regulated by Mi. Furthermore, northern blot analysis showed that DMEL-3 cells did not express *c-kit* at either the permissive or non-permissive temperature, suggesting that Mi does not regulate *c-kit* expression.

To address the possible role of RB family members in melanocyte differentiation, it was investigated whether melanocyte differentiation is accompanied by an increase in their mRNAs and protein levels. Northern blot analysis strongly suggested that expression of the RB1, p130 and p107 is not altered when DMEL-3 cells were induced to differentiate at the non-permissive temperature. The results from western blot anaysis were inconclusive and require further investigations.

Finally, the pigmented cell lines established in the present study provided a unique opportunity to investigate the stimulatory effect of TPA on melanogenesis because growth curves showed that the cells become TPA-independent. The results showed that stimulation of melanogenesis by TPA in a pigmented melanocyte line, DMEL-2, resulted in an increase in tyrosinase, TRP-1 and TRP-2 proteins and mRNAs. Additionally, TPA increased *mi* gene expression which suggests that Mi is necessary for the TPA-triggered signalling cascade that induces expression of the tyrosinase gene family. These results disclose, for the first time, a mechanistic link between TPA and the transcriptional induction of pigmentation.

CHAPTER 1: INTRODUCTION AND AIMS

1.1 Introduction

Melanoma is a highly aggressive and increasingly common form of cancer. Despite several important advances, the mechanisms underlying the transformation of the melanocyte to a malignant melanoma remains poorly understood. The discovery of oncogenes and an understanding of their ability to convert normal cells (both *in vivo* and in culture) into either an immortalised or a transformed state has paved the way for understanding this process.

Several groups of researchers have transformed murine melanocytes *in vitro* with constitutively expressed oncogenes and have described properties of the resulting cells. The predominant finding has been that melanocytes transformed with a variety of oncogenes including, v-Ha-ras, v-neu, E1A, v-myc, SV40 large T antigen or polyoma middle T antigen acquire growth autonomy from the exogenous mitogen, TPA and show loss of differentiation markers such as, melanin production (melanogenesis) (Donatien et al., 1996; Dooley et al., 1988; Dotto et al., 1989; Halaban et al., 1996; Wilson et al., 1989; Yavuzer et al., 1995). Similar results were reported for melanocytes transformed by either ectopic expression of basic fibroblast growth factor (bFGF) or chemical carcinogens.

The observation that melanocyte de-differentiation frequently accompanies transformation has raised the possibility that these two processes may be regulated by a common mechanism. As suggested by Yavuzer et al. (1995) there may, for example, be a common factor controlling melanocyte differentiation and a subset of genes essential to growth control. Such a factor may therefore act as a common target for the different transforming agents. This possibility has interested both pigment cell biologists and oncologists because it seems likely that studies attempting to elucidate the molecular mechanisms by which transforming agents derail pigment formation will shed light on the molecular basis for the regulation of melanocyte differentiation and transformation. Alternatively, it can be predicted that understanding melanocyte transformation will require a detailed understanding of the differentiation programme of melanocytes. However, despite several advances in these areas of research they remain poorly understood and *in vitro* systems are needed to link melanocyte transformation more directly to differentiation.

Conditional temperature-sensitive mutants of viral oncoproteins provide an appropriate means for establishing *in vitro* systems which can be used to study both melanocyte transformation and the direct effect(s) of the transforming agent on melanocyte differentiation. Temperature-sensitive oncogenes have an advantage in that cells expressing these genes grow well at the

permissive temperature and when they are grown at the non-permissive temperature the transforming agent is inactivated and, in principle, the cells should acquire the morphological and biochemical characteristics of differentiated cells.

The broad aim of this thesis was to establish melanocyte lines expressing a temperature-sensitive mutant SV40 large T antigen and to use these cell lines as *in vitro* models for:

i) obtaining large populations of differentiated melanocytes for studying the regulation of melanogenesis;
ii) addressing the question of whether oncoproteins (transforming agents) play a direct role in altering melanocyte differentiation;
iii) investigating whether any of the cellular proteins known to be inactivated by the SV40 large T antigen play a role in melanocyte differentiation.

The purpose of this review is to provide a general overview of the current understanding of the regulation of melanogenesis (biochemical differentiation) and to introduce key areas of research that have bearing on the aims of this thesis.

1.2 Melanocyte differentiation and melanogenesis

Melanocytes, together with the retinal pigment epithelium (RPE), are responsible for skin, hair and eye colour. Skin and hair follicle melanocytes originate during development as melanoblasts in the neural crest. The melanoblasts migrate along a dorso-lateral pathway to the epidermis before populating the skin and hair follicles, differentiating into dendritic, pigment-producing melanocytes. The most obvious marker of mature melanocytes is the synthesis of melanin within specialised intracellular organelles, the melanosomes. The biochemical process of melanogenesis involves a series of enzymatic steps in which tyrosine is converted into either eumelanin (black and/or brown) or phaeomelanin (red and/or yellow). This is a complex process since it involves numerous genes: genes coding for enzymes involved in catalysing the conversion of tyrosine into melanin as well as genes coding for the structural components of the melanosome. A scheme outlining the current understanding of the enzymatic steps involved in the conversion of tyrosine to melanin is presented in Fig 1.1.

At least three melanocyte-specific enzymes are involved in the production of eumelanin, namely tyrosinase, tyrosinase-related protein 1 and 2 (TRP-1 and TRP-2, or dopachrome tautomerase, DCT). These enzymes constitute the tyrosinase protein family, and while they are structurally similar they have evolved distinct catalytic activities. Tyrosinase (EC 1.14.18.1) is the key enzyme in the melanogenic pathway and its action is rate limiting. Tyrosinase catalyzes the first two steps of melanin biosynthesis, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine

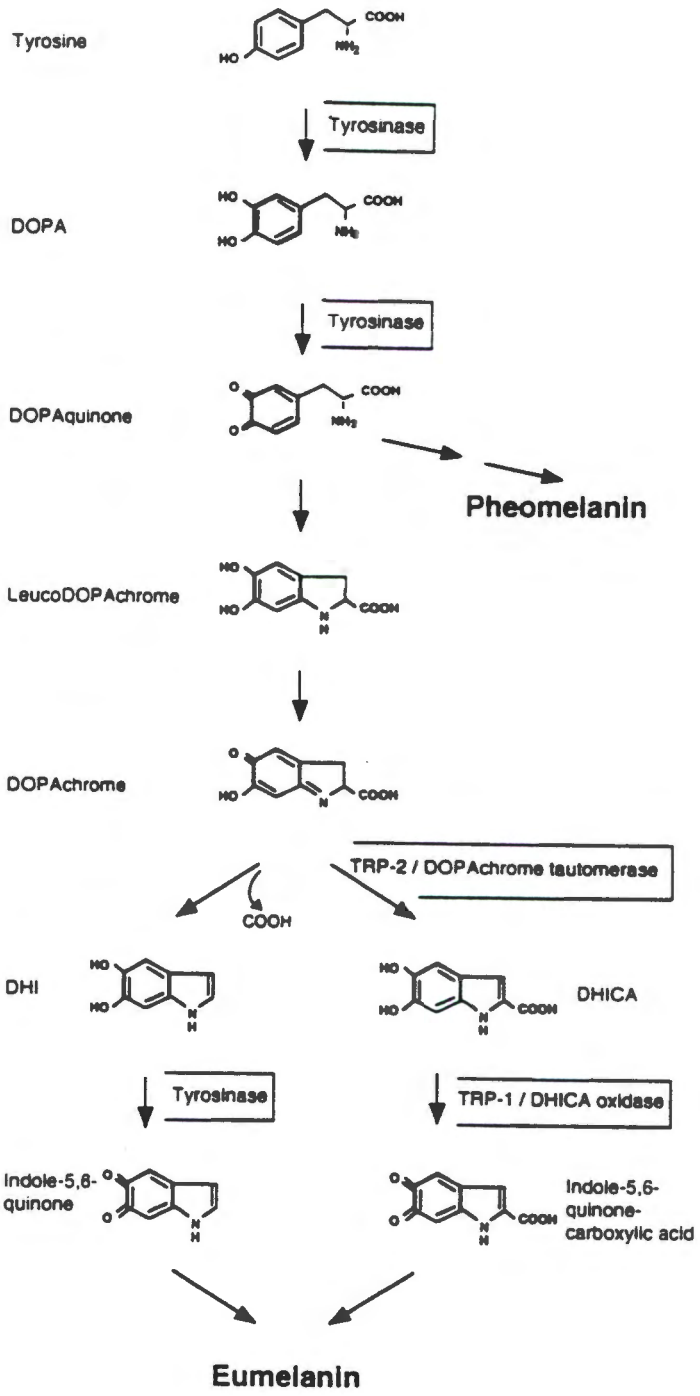


Fig. 1.1. Schematic diagram of the mammalian melanin biosynthetic pathway (from del Marmol and Beerman, 1996)

(DOPA) and its subsequent oxidation to DOPAquinone (Körner and Pawelek, 1982; Tripathi et al., 1992). DOPAquinone undergoes rapid cyclization and rearrangement to sequentially form leucoDOPAchrome and then DOPAchrome. This semi-stable intermediate can either undergo a slow, spontaneous decarboxylation to yield 5,6-dihydroxyindole (DHI), or be enzymatically converted into the stable carboxylated diphenol 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by DCT (EC 5.3.2.3) (Kroumpouzos et al., 1994; Tsukamoto et al., 1992; Yokoyama et al., 1994). Both DHI and DHICA are oxidized again, leading to the formation of reactive quinonic intermediates and, eventually, to the melanin polymer. Tyrosinase catalyses the oxidation of 5,6-dihydroxyindole to indole-5,6 quinone (Körner and Pawalek, 1982). TRP-1 appears to be DHICA oxidase, which acts in the conversion of DHICA into 5,6-quinone 2-carboxylic acid (Jiménez-Cervantes et al., 1994; Kobayashi et al., 1994a, 1994b).

Several other factors are thought to play a role in the biosynthesis of melanin but their specific functions have not yet been clarified. These factors include, for example, the Pmel 17/silver gene family (Kim and Wistow, 1992; Kwon et al., 1991; Kwon et al., 1994; Mochii et al., 1988; Turque et al., 1996; Weterman et al., 1995), the pink-eyed dilution (p) protein (Donatien and Orlow, 1995; Gardner et al., 1992; Rinchick et al., 1993; Rosemblat et al., 1994; Sviderskaya et al., 1997), MART-1/Melan-A protein (Coulie et al., 1994; de Vries et al., 1997; Kawakami et al., 1994), endogenous melanogenic inhibitors (Chakraborty et al., 1989; Hatta et al., 1988; Kameyama et al., 1989), catalases (Halaban and Moellmann, 1990), peroxidases (d'Ischia et al., 1991; Gesualdo et al., 1997) and the proteins encoded by the melanocortin receptor 1 (MC1R) (Chhaijlani and Wikberg, 1992; Mountjoy et al., 1992) and Agouti genes (Bultman et al., 1992; Wilson et al., 1995).

The next section of this review will focus on the tyrosinase gene family because the molecular mechanisms involved in their regulation is crucial for melanogenesis. Over the last decade, the genes encoding the three tyrosinase family members have been isolated from human (Bouchard et al., 1994; Cohen et al., 1990; Kwon et al., 1987), mouse (Jackson et al., 1992; Shibahara et al., 1986; Yamamoto et al., 1987) and chicken (April et al., 1996; 1998a; 1998b) and details of each family member are briefly discussed below.

1.3 The tyrosinase gene family

The existence of a tyrosinase gene family first became apparent when attempts to isolate the mouse gene for tyrosinase led to the identification of three related cDNAs that mapped to different chromosomal loci. Although each family member encodes a distinct enzyme involved in melanin biosynthesis, they share an amino acid homology of 40% and have a number of structural features in common (Jackson et al., 1994).

Tyrosinase is encoded at the *Tyr/TYR* (previously called the *albino* (*c*)) locus and mutations at this locus result in partial or complete loss of melanin production (Jackson and Bennett, 1990; Shibahara et al., 1990; Yokoyama et al., 1990). Northern blot analysis indicates that the processed tyrosinase transcripts are between 2 and 2.5 kb and alternatively spliced forms may be present (Kelsall et al., 1997; Porter and Mintz, 1991). These transcripts cannot be translated into catalytically competent proteins, but nothing is known about their function. Tyrosinase, a multifunctional, copper-containing glycoprotein has three enzyme activities in the melanin biosynthesis pathway (see Fig 1.1). Newly synthesized tyrosinase has a molecular weight of 55 kDa which increases to around 65-75 kDa (or even up to 80 kDa) following its N-glycosylation in the Golgi complex (Chen et al., 1995; Hearing and Tsukamoto, 1991).

The gene encoding tyrosinase-related protein 1 maps to the *Tyrp1 /TYRP1* (previously called the *brown* (*b*)) locus (Chintamaneni et al., 1991; Jackson, 1988). Mutations at the mouse *Tyrp1* locus may result in a brown, rather than a black coat (Jackson et al., 1990), while in humans *TYRP1* mutations have been associated with type 3 oculocutaneous albinism (OCA) (Boissy et al., 1996; Manga et al., 1997; Spritz, 1994). The primary transcript is between 2.1 kb and 3.3 kb (Shibahara et al., 1986; Boissy et al., 1996) and the mature glycosylated TRP-1 protein has a molecular weight of about 75 kDa (Halaban and Moellmann, 1990; Hearing and Tsukamoto, 1991). The enzymatic function of TRP-1 in the melanin biosynthesis pathway has been a subject of much controversy. Several functions have been proposed and disputed, including a low level tyrosinase activity (Jiménez et al., 1989), a catalase/hydrogen peroxidase (Halaban and Moellmann, 1990) as well as a DOPAchrome tautomerase (Winder et al., 1993). It has now been shown that the principal activity of mouse TRP-1 is DHICA oxidase (Jiménez-Cervantes et al., 1994; Kobayashi et al., 1994a, 1994b).

The gene encoding tyrosinase-related protein 2 maps to the *DCT* (previously called the *slaty* (*slf*)) locus (Bouchard et al., 1994; Jackson et al., 1992; Sturm et al., 1994). While mutations at the mouse *DCT* locus may result in a grey (slaty) rather than a black coat (Budd and Jackson, 1995), there is currently no known human disorder associated with the human *DCT* locus. Mammalian transcripts vary from 2.3 to 4.5 kb (Bouchard et al., 1994; Yokoyama et al., 1994) and the molecular weight of the fully processed protein is about 75 to 80 kDa (Tsukamoto et al., 1992).

1.4 Regulation of the tyrosinase gene family

The promoter regions of the tyrosinase, TRP-1 and TRP-2 genes from a number of organisms have been sequenced and compared. Such comparisons have revealed the presence of an 11

bp promoter element, termed the M-box, which is conserved in the promoters of the three tyrosinase family genes in both mouse and human (Budd and Jackson, 1995; Jackson et al., 1991; Lowings et al., 1992; Morrison et al., 1994; Sturm et al., 1995). The M-box contains a core CATGTG E-box motif and functional analyses have shown it to be an important component of the minimum promoter sequences required for melanocyte-specific expression of the tyrosinase gene family members in mammals. The CATGTG motif matches with the CANNTG E-box motif, which is the consensus sequence for binding of the basic/helix-loop-helix/leucine zipper (bHLH-Zip) transcription factor family (Reviewed by López, 1995). This family of transcription factors includes amongst others, TFE3, TFEB, the proto-oncogene, *myc*, the regulators of *myc* function, Mad, Max, and Mxi 1, as well as USF (upstream stimulatory factor), and MyoD-related factors. These proteins bind to DNA as homodimers or heterodimers. DNA binding is mediated via the basic domain, dimerization occurs via the HLH domain and is stabilized by the Zip domain.

The presence of the E-box motif in the tyrosinase, TRP-1 and TRP-2 promoters suggested that this motif might provide a binding site for a melanocyte-specific transcription factor. A potential candidate with a bHLH-Zip domain was cloned and shown to map to the mouse *microphthalmia* locus (*mi*) (Hodgkinson et al., 1993; Hughes et al., 1993). Mice with mutations at the *mi* locus have defects in neural crest-derived melanocytes, including lack of pigmentation in the coat, eye and inner ear (reviewed by Moore, 1995). Most of them also result in microphthalmia, due to defects in the pigmented epithelial layer of the eye. Similarly, the human homologue of the *mi* gene, microphthalmia-associated transcription factor (*MITF*), is mutated in patients with a pigmentation and deafness disorder termed Waardenburg Syndrome type 2 (Hughes et al., 1994; Tassabehji et al., 1994).

Cloning of the cDNA sequences encoding mouse Mi and the human MITF (Hodgkinson et al., 1993; Hughes et al., 1993; Tachibana et al., 1994) has facilitated investigations into the potential of the Mi protein to function as a melanocyte-specific transcription factor. *In situ* hybridisation analyses on sections of mouse embryos show that *mi* gene expression is restricted to several cell types including melanoblasts of the developing eye, skin and inner ear (Hodgkinson et al., 1993). The protein encoded by *mi* is required for melanocyte differentiation (Hodgkinson et al., 1993; Opdecamp et al., 1997) and has been shown to bind the M-box of the tyrosinase (Bentley et al., 1994; Bertolotto et al., 1996; Ganss et al., 1994; Yasumoto et al., 1997), TRP-1 (Bertolotto et al., 1998a; Yavuzer et al., 1995) and TRP-2 (Bertolotto et al., 1998a; Yasumoto et al., 1997) promoters *in vitro*. Co-transfection assays demonstrated that reporter gene expression driven by either a tyrosinase, TRP-1 or an M-box containing the SV40 promoter, is increased in the presence of a Mi/MITF expression plasmid. Mutation of the M-box, or either one of two other elements containing an E-box motif in the tyrosinase promoter, or deletion of the M-box from the

TRP-1 promoter significantly reduced this transactivation (Bentley et al., 1994; Ganss et al., 1994; Hemesath et al., 1994; Yasumoto et al., 1994; Yasumoto et al., 1995; Yavuzer et al., 1995).

The question of whether Mi is involved in the transactivation of the TRP-2 promoter, however, remains unclear. Bertolotto et al. (1998a) have shown that co-transfection of mouse B16 melanoma cells with a TRP-2 reporter construct and an expression plasmid encoding *mi* results in transactivation of the TRP-2 promoter. However similar studies in which human melanoma cells were co-transfected with MITF and either tyrosinase, TRP-1 or TRP-2 promoters linked to a reporter gene suggest that unlike the other two members of the tyrosinase gene family, the TRP-2 promoter is unresponsive to MITF (Yasumoto et al., 1997). Bertolotto et al. (1998a) explained the discrepancy between their results and that of Yasumoto et al. (1997) as resulting from either specific behaviours of human versus mouse microphthalmia or by the different cellular contexts used in their respective experiments.

Ectopic expression of a number of different oncogenes in melanocytes frequently results in de-pigmentation and a co-ordinated down-regulation of tissue-specific gene expression. To explore the potential of Mi to act as a melanocyte-specific transcription factor, Yavuzer et al. (1995) investigated if Mi was the target for E1A-mediated down-regulation of melanocyte-specific gene transcription in E1A-transformed melanocytes. Using quantitative RT-PCR they found that *mi* expression is reduced up to 50-fold in these cells as compared with untransformed controls. This reduction in *mi* expression is likely to be responsible for the concomitant decrease of tyrosinase and TRP-1 gene expression, since these genes are re-expressed in the presence of ectopic *mi* expression. Halaban et al. (1996) provided additional evidence to show that *mi* is downregulated in melanocytes rendered amelanotic by the oncogenes E1A, ras and neu as well as by the aberrant expression of basic fibroblast growth factor (bFGF).

The possibility of Mi functioning as a melanocyte-specific transcription factor was further underscored by studies in which transfection of MITF cDNA converted NIH/3T3 fibroblasts to cells with a melanocyte-like morphology. These cells also exhibit specific induction of tyrosinase and TRP-1 gene expression (Tachibana et al., 1996). The particular line of NIH/3T3 cells used in this experiment already expresses the TRP-2 gene and its expression was not altered by MITF expression.

The above results together with the *mi* phenotype and the fact that *mi* is tissue-restricted makes Mi an attractive candidate as a regulator of pigmentation gene expression. Further progress towards a better understanding of the mechanism by which Mi regulates the pigmentation genes

clearly depends on the identification of potential dimerization partners, Mi-interacting proteins as well as investigation into the mechanism(s) by which Mi is itself regulated.

1.5 Regulation of Mi

Fuse et al. (1996) cloned and characterized the human genomic DNA segment containing a melanocyte-type exon and its 5'-flanking region of the MITF gene. They showed, using transient expression assays, that the 5'-flanking region of 2.3 kb is able to confer preferential expression of a luciferase gene in pigment cells. The promoter region of the MITF gene was found to contain potential cis-acting elements, such as GATA boxes, a cyclic AMP-response element and an interleukin-6-responsive element. However, there were no CATGTG motifs in this promoter region and co-transfection with an MITF cDNA did not lead to the increase in expression of any MITF-luciferase fusion genes. This suggests that the melanocyte-type promoter of the MITF gene is not autoregulated and that the regulatory elements involved in the melanocyte-specific promoter function of the MITF gene may be different from that of the tyrosinase-gene family.

Recently, Watanabe et al. (1998) provided convincing evidence to suggest that PAX3, whose mouse homologue is expressed prior to *mi* in the neural crest of mouse embryos, is a transcriptional activator of MITF expression. PAX3, a transcription factor with a paired domain and homeodomain, is mutated in patients suffering from Waardenburg syndrome type 1 and 3 and these individuals exhibit incomplete pigmentation and hearing impairment. Using co-transfection assays, they show that a reporter gene driven by the MITF promoter region is transactivated in Hela cells in the presence of wild-type PAX3 but not mutant PAX3 cDNAs encoding proteins with mutations in their paired domain and homeodomain. Furthermore, they demonstrate that transactivation of the MITF promoter requires the presence of a 26-bp region that was shown to contain a consensus sequence for binding to the palindromic PAX3 homeodoman and the paired domain.

Brn-2, a POU domain transcription factor, expressed in premelanoblasts, melanocytes and melanoma cells, has also been implicated in transcriptional regulation of *mi* expression. However, the regulation of *mi* by Brn-2 has not been clearly demonstrated and remains controversial. Angus et al. (1995) provide evidence that Brn-2 can function as a transcriptional activator of *mi*. They introduced anti-sense Brn-2 into a melanoma cell line and the resulting cells failed to express Brn-2 and concurrently lost expression of tyrosinase, TRP-1, TRP-2 and *mi*. In contrast, Eisen et al. (1995) reported that in co-transfection assays, the levels of Mi-activated expression of the tyrosinase promoter were repressed in the presence of increasing amounts of a Brn-2 expression vector.

Additional evidence for Brn-2 acting as a negative regulator was provided by studies in which a dominant-positive form of Brn-2 was stably introduced by retroviruses into melanocytes (Goding and Fisher, 1997). The resulting melanocyte cell lines failed to express *mi* and also lost expression of tyrosinase, TRP-1 and TRP-2 which could be reversed by re-expressing *mi* from a second retrovirus. The ability of Brn-2 to repress *mi* expression was confirmed by co-transfection assays in which overexpression of either the wild-type or dominant-positive form of Brn-2 could repress *mi* promoter activity in melanocytes. Finally, when the mouse TRP-1 promoter was used to target ectopic expression of Brn-2 to the retinal pigment epithelium, the resultant transgenic mice developed a microphthalmic phenotype, consistent with loss of *mi* expression.

In addition to Mi being regulated at a transcriptional level, Mi was also reported to undergo post-translational modifications that may link its activity to signal transduction pathways. Germline mutations at loci encoding either Mi, or the cytokine receptor c-Kit, or its ligand Steel factor (Sl) result in similar defects in melanocyte development suggesting a relationship between these factors. Hemesath et al. (1998) have recently described a biochemical link between Kit signalling and the activity of Mi. They showed that when a human melanoma cell line (501 mel) was stimulated with Sl, mitogen-activated protein kinase (MAPK) was activated, which in turn phosphorylated Mi at a consensus target serine. Using a luciferase reporter linked to the tyrosinase promoter, this phosphorylation was shown to upregulate Mi transactivation of the tyrosinase gene promoter. These results, therefore, demonstrate that Mi is phosphorylated by MAPK in response to c-Kit activation and that this leads to upregulation of Mi transcriptional activity.

In a very recent report Price et al. (1998a) examined the mechanism by which Mi is activated by the above pathway. They treated c-kit-expressing melanoma cells (501mel) with Sl factor and demonstrated that the transcriptional coactivator p300/CBP selectively associates with MAPK-phosphorylated Mi and that p300/CBP coactivates Mi transcriptional activity in a manner dependent upon this phosphorylation.

1.6 Regulation of melanogenesis by extracellular factors

Several agents have been found to regulate melanogenesis in mammalian melanocytes and melanoma cells *in vitro* and *in vivo*. These agents include cAMP elevating agents such as forskolin, isobutylmethylxanthine (IBMX) and α melanocyte stimulating hormone (α-MSH), UV irradiation, the endothelins 1, 2 and 3 (Imokawa et al., 1995; Imokawa et al., 1996; Reid et al., 1996; Yada et al., 1991) and phorbol esters such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA). An understanding of the mechanism by which these stimuli regulate melanogenesis has only recently begun to be understood. This section is limited to a review of the effect(s) of α-

MSH and TPA on melanogenesis because they have bearing on areas to which this thesis aims to contribute.

1.6.1 The stimulation of melanogenesis by α-MSH and cAMP

Melanocytes express the MSH receptor which responds to α-MSH to convey signals that are thought to modulate growth and differentiation. Evidence accumulated over many years from several laboratories strongly suggests that in mammals, α-MSH and the α-MSH receptor are involved in enhancing eumelanogenesis by stimulating the enzymatic activity of tyrosinase. Because the regulation of melanin synthesis was thought for a long time to occur at the level of tyrosinase, most studies have focused on how this enhanced enzyme activity may come about and several mechanisms have been proposed. For example, while there is evidence that pre-existing tyrosinase enzyme is activated by α-MSH (Halaban et al., 1983), there is also strong evidence that α-MSH upregulates tyrosinase gene expression (Kuzumaki et al., 1993) as well as tyrosinase protein translation (Aroca et al., 1993).

More recently, TRP-1 and TRP-2 were shown to control distal steps of eumelanin synthesis, suggesting that α-MSH might also regulate TRP-1 and TRP-2 activity and/or expression. However, the regulation of these two tyrosinase family members by α-MSH has not been clearly demonstrated since the hormone appears to elicit different transcriptional responses in different mouse melanoma cell lines. Whereas Aroca et al. (1993), using JB/MS cells, found that α-MSH only upregulated tyrosinase, but had no effect on TRP-1 and TRP-2 transcription, Kuzumaki et al. (1993), using B16-F1 cells reported that α-MSH upregulated both tyrosinase and TRP-1 gene expression.

Recent work by Bertolotto et al. (1996, 1998a) demonstrated that stimulation of melanogenesis by cAMP-elevating agents (forskolin, α-MSH and IBMX) in B16 melanoma cells results in an increase in the amount of tyrosinase, TRP-1 and TRP-2 proteins and mRNAs. In addition, using reporter constructs containing the 2.2 kb, 1.1 kb or 0.6 kb fragments 5' of the transcriptional start sites of the mouse tyrosinase, TRP-1 and TRP-2 genes respectively, they show that cAMP-elevating agents stimulate the transcriptional activity of the promoters of these genes. Deletion and mutation analysis showed that the M-box plays a key role in the regulation of tyrosinase, TRP-1 and TRP-2 promoter activity by cAMP. Finally, they provide data to suggest that by increasing *mi* expression, cAMP increases the binding of Mi to the M-box, resulting in transcriptional activation of the tyrosinase, TRP-1 and TRP-2 gene promoters. The authors therefore hypothesize that microphthalmia is involved in the regulation of the tyrosinase, TRP-1 and TRP-2 promoters by cAMP.

In a subsequent study Bertolotto et al. (1998b) investigated this possibility as well as the mechanism by which cAMP regulates microphthalmia function. They showed that cAMP upregulates the transcription of the microphthalmia gene through a classical cAMP response element that is functional only in melanocytes. Furthermore, using a dominant-negative mutant of microphthalmia, they demonstrate that microphthalmia is required for the cAMP effect on the tyrosinase promoter. In a similar study, Price et al. (1998b) obtained results that confirmed those of Bertolotto et al. (1998b).

1.6.2 The regulation of melanogenesis by 12-O-tetradecanoyl-phorbol-13-acetate (TPA)

TPA is a non-physiological agent that is used routinely for establishing long-term cultures of mammalian melanocytes (Bennett et al., 1987; Eisinger and Marko, 1982; Halaban et al., 1986; Tamura et al., 1987). Withdrawal of TPA from the culture medium of these cells leads to senescence and a loss of pigmentation. TPA is therefore an effective melanocyte mitogen and is capable of stimulating melanogenesis in normal mammalian melanocytes. In contrast, melanocytes transformed with a variety of exogenous oncogenes or chemical carcinogens, lose their pigmented phenotype and their growth is TPA-independent. These transformed melanocytes cannot be stimulated to synthesize melanin upon addition of TPA into their culture medium (Dotto et al., 1989; Melber et al., 1989). Human and some mouse melanoma cells in culture are also to a certain extent growth inhibited in the presence of TPA (Arita et al., 1994; Becker et al., 1990; Halaban et al., 1986; Herlyn et al., 1987; Oka et al., 1996). However, reports in the literature about the melanogenic effects mediated by TPA in melanoma cells are inconsistent and both inhibitory and stimulatory effects have been reported. The reason for these different responses to TPA remains unclear. It is, however, likely that alterations in TPA-responsive signalling occurs during transformation of melanocytes. In order to explore the effects of TPA on melanogenesis in greater depth, a brief summary of signalling by TPA is outlined below.

The major intracellular target for TPA is protein kinase C (PKC) (Nishizuka, 1989). PKC represents a multigene family of serine- and threonine-specific kinases that play crucial roles in signal transduction pathways that are involved in diverse cellular functions, including differentiation, growth control, tumor promotion and cell death. The PKC family is subdivided into three groups: the classical PKC members (α, β, γ) are Ca^{2+} and diacylglycerol (DAG)-dependent, the novel PKCs (δ, ε, η and θ) are Ca^{2+}-independent but DAG-dependent and the atypical PKCs (ζ and λ) are not activated by Ca^{2+} and DAG *in vitro* (reviewed in Livneh and Fishman, 1997). Each member of the PKC family is thought to execute distinct cellular functions since they show differences in their structure, mechanisms of activation and tissue expression (Nishizuka, 1988; Asaoka et al., 1992).

TPA binds to and directly activates both classical and novel members of the PKC family (but not atypical PKCs) in a manner analogous to that of DAG. When activated, PKC phosphorylates specific serine or threonine residues on target proteins that vary depending on the cell type. An important consequence of the activation of PKC, in many cells, is the stimulation of gene transcription which occurs via at least two known pathways (Alberts et al., 1994, see fig. 1.2). In one pathway, PKC activation leads to the phosphorylation of Iκ-B, an inhibitor protein, that is complexed to NF-κB, a transcription factor, in the cytoplasm. Upon phosphorylation of Iκ-B, NF-κB is released so that it can migrate into the nucleus and stimulate the transcription of specific genes, for example the interleukins. In the other pathway, PKC activates a phosphorylation cascade that leads to the phosphorylation of a pivotal protein kinase, MAPK, which in turn phosphorylates and activates nuclear transcription factors. The effects of activated PKC are, therefore, mediated by specific regulatory proteins that interact with distinct cis-acting elements in the 5'-flanking region of the target gene. For example, MAPK phosphorylates and activates TCF-Elk that activates transcription of c-fos. Among other functions, c-fos forms part of the AP-1 (activating protein-1) complex that binds to AP1 sites in gene promoters. Alternatively, MAPK can directly phosphorylate transcription factors which result in stimulation of their transactivation activity.

As mentioned earlier, reports in the literature about the effect of TPA on melanogenesis in melanoma cells are inconsistent, in that both inhibitory and stimulatory effects have been reported. TPA was reported to enhance melanin synthesis in mouse S91 and B16 melanoma cells (Brooks et al., 1980; Friedmann et al., 1990) and human SK23 and HO melanoma cells (Brooks et al., 1980; Huberman et al., 1979), whereas Mufson et al. (1979) reported that TPA delayed, although it did not permanently block, melanin synthesis in B16 melanoma cells. In contrast, other studies have reported that TPA inhibited melanogenesis in mouse S91 (Fuller et al., 1990) and B16 melanoma cells (Ando et al., 1995; Kuzumaki et al., 1993). The molecular mechanisms for the inhibitory effect of TPA in these melanoma cells were shown in part to be mediated by a decrease in tyrosinase (Ando et al., 1995; Fuller et al., 1990; Kuzumaki et al., 1993) and TRP-1 (Kuzumaki et al., 1993) mRNA levels. These results suggest that repression of tyrosinase and TRP-1 gene expression by TPA possibly involves a regulatory site common to their promoters.

While analysis of the 5' flanking sequence in the mouse and human tyrosinase gene revealed putative AP-1 sites, there are no AP-1 binding consensus sequences in the promoter region of the TRP-1 gene (Jackson et al., 1991). It would therefore appear that the AP-1 site may not be involved in mediating the effect of TPA on melanogenesis. Bertolotto et al. (1998c) provided

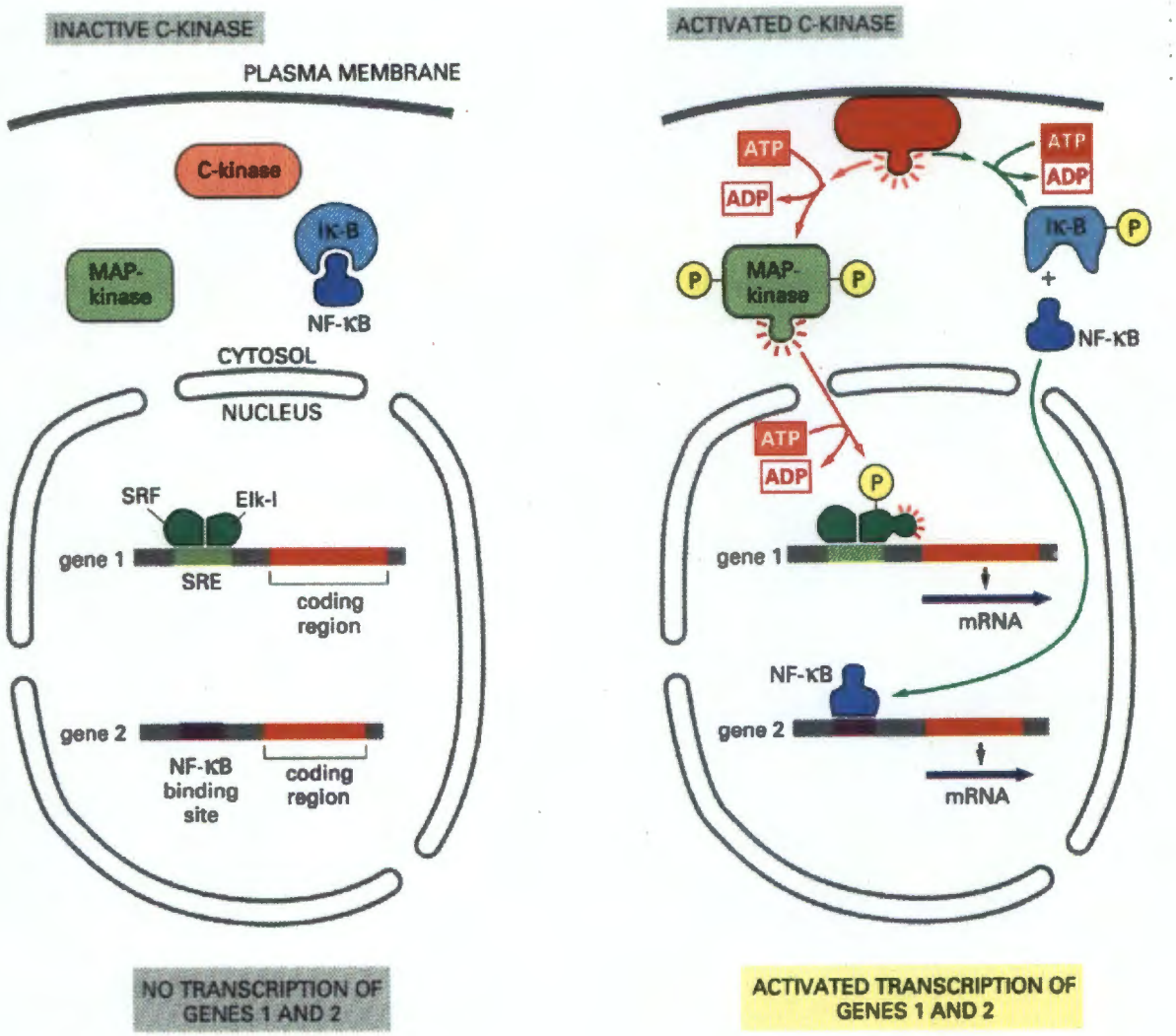


Fig. 1.2. Two intracellular pathways by which activated C-kinase can activate the transcription of specific genes. In one (red arrows) C-kinase activates a phosphorylation cascade that leads to the phosphorylation of a pivotal protein kinase called MAP-kinase, which in turn phosphorylates and activates the gene regulatory protein Elk-1. Elk-1 is bound to a short DNA sequence (called serum response element, SRE) in association with another DNA-binding protein (called serum response factor, SRF). In the other pathway (green arrows) C-kinase activation leads to the phosphorylation of Iκ-B, which releases the gene regulatory NF-κB so that it can migrate into the nucleus and activate the transcription of specific genes (from Alberts et al., 1994).

evidence to show that the M-box, and not the AP-1 sites, in the mouse tyrosinase promoter was the target for TPA-mediated inhibition. In a previous study they found that forskolin, a cAMP-elevating agent, stimulated melanogenesis by increasing the transcriptional activity of the tyrosinase promoter (Bertolotto et al., 1996). They demonstrated that nuclear extracts from forskolin-treated B16 melanoma cells that were exposed to 160 nM TPA for 3 hours, showed decreased binding of Mi to the M-box (Bertolotto et al., 1998c). The authors proposed that TPA decreases Mi binding to the M-box of the tyrosinase promoter, thereby leading to reduced tyrosinase expression and the inhibition of melanogenesis. This can be explained by TPA either regulating Mi expression or by inducing post-translational modifications of Mi that affect its binding to the M-box of the tyrosinase promoter. Indeed, a post-translational mechanism by which TPA regulates Mi activity has been reported by Hemesath et al. (1998). They demonstrated that treatment of a human melanoma cell line (501 mel) with 16 nM (10 ng/ml) TPA for 8 min, results in activation of MAPkinase, which in turn phosphorylates Mi at a consensus target serine. This phosphorylation was shown to upregulate Mi transactivation of the tyrosinase gene promoter. The apparent difference in the effect of TPA on transcriptional regulation in the B16- and 501 mel melanoma cell lines could have resulted from differences in human versus mouse melanoma cell lines or the concentrations of TPA, since Hemesath et al. (1998) used 10-fold less than that used by Bertolotto et al. (1998c).

The reason for the different effects of TPA on melanogenesis in melanoma cells is not known, but the following possibilities are worth noting. Firstly, depending on the concentration of TPA used in the culture medium of cells, it can exert opposite effects on PKC. High concentrations of TPA can initially activate PKC but longer exposures of cells to these concentrations lead to a depletion of certain PKC isoforms (Nishizuka, 1988; Oka et al., 1995). Thus, conflicting results can be obtained depending on the concentration of TPA used and/or the length of time that the cells are exposed to TPA. Alternatively, it is possible that the differential effect of TPA on melanogenesis in different melanoma cell lines results from changes in the expression of certain PKC isoforms during the development of melanoma. For example, while it has not been clearly demonstrated which isoform mediates the effects of TPA on melanogenesis, Park et al. (1993) provided compelling data to suggest that PKC β may be involved. Their data show that the PKC β regulates human melanogenesis through activation of tyrosinase. However, PKC β is not expressed in B16 melanoma cells (Oka et al., 1993) and is frequently absent in human melanoma cells (Oka et al., 1996; Yamanishi and Meyskens, 1994). In the absence of this PKC isozyme, TPA may act on other PKC isozymes which mediate an inhibitory effect on melanogenesis in these cell lines. Given the phenotypic heterogeneity observed among different melanoma cell lines, it would be reasonable to suggest that these cell lines are not particularly suitable for studies aimed at elucidating the effect of TPA on melanogenesis.

The results of studies on the effect of TPA on melanocyte differentiation, including melanogenesis, have been more consistent in normal mammalian melanocytes. Exposure of normal human melanocytes to TPA has been reported to stimulate tyrosinase activity (Abdel-Malek et al., 1992; Chao-Hsing and Hsing-Su, 1991; Halaban et al., 1983) and melanin synthesis (Melber et al., 1989; Friedman et al., 1990). Bennett et al. (1987) also showed that an immortal line of mouse melanocytes are visibly pigmented and dendritic in the presence of TPA. Without TPA, these cells became flat and isometric, without dendrites. Similar results were also reported by Melber et al. (1989) for normal human melanocytes.

In addition, TPA was found to be essential in inducing melanocyte progenitors in mouse neural crest cultures to differentiate (Murphy et al., 1992; Kidson, pers. comm.). Murphy et al. (1992) reported that exposure of mouse neural crest cells to TPA in culture, resulted in pigmented (melanin synthesis) and DOPA-positive (tyrosinase activity) melanocytes. Subsequently, endothelins were found to substitute for TPA in such a system (Reid et al. 1996). Although it is still unclear what physiologic molecule TPA is mimicking, this provided strong evidence that TPA may mimic endothelin signalling and that endothelins/TPA is an essential melanocyte differentiation factor.

Further evidence in support of this possibility comes from studies which have shown that endothelins, like TPA, exert stimulatory effects on melanocyte growth and differentiation. Endothelins are strong mitogens for human melanocytes and endothelin-1 (ET-1) has been shown to increase tyrosinase activity and melanogenesis through binding to a receptor on melanocytes (Yada et al., 1991). Hara et al. (1995) also reported that when added to cultured melanocytes, ET-1 enhances melanocyte dendricity. Swope et al. (1995) also found that ET-1 could replace TPA in the growth medium that they use routinely for culturing normal human melanocytes. Furthermore, binding of endothelins to their G protein-coupled receptor on melanocytes activate the Ca^{2+}-messenger system, which involves both calmodulin and protein kinase C.

It is, therefore, clear that TPA provides a powerful tool for investigating the differentiation process of melanocytes because it substitutes for an endogenous growth/differentiation factor. However, detailed investigations on the stimulatory effects of TPA on normal melanocyte differentiation have, in part, been hindered because TPA itself is a prerequisite for expanding these cells in culture.

1.7 *In vitro* systems for studying melanocyte differentiation

The growing interest in the molecular mechanisms regulating melanocyte differentiation has prompted investigators to develop *in vitro* systems to study this process. Until most recently, investigations were restricted to studies on melanoma cells because they readily give rise to cell lines in culture. However, melanoma cells are often highly heterogeneous and differ from normal cells. Most melanoma cells which grow without growth factors in culture, exhibit a fibroblastic or epithelioid morphology and are often amelanotic, even if the original tumor was highly pigmented (Houghton et al., 1987). They therefore have limited use in the study of 'normal' melanogenesis.

The establishment of *in vitro* methods for isolating and culturing normal mammalian melanocytes marked a major breakthrough in this area. TPA was found to be an effective mitogen and is now used routinely for culturing normal melanocytes. While primary cultures of normal melanocytes have several advantages for the study of melanogenesis, they have three major disadvantages: (1) normal mouse melanocyte cultures senesce within 3 weeks (Bennett et al., 1987) and normal human melanocytes will grow for only a limited period in culture and then senesce (Bennett et al., 1985; Eisinger and Marko, 1982; Herlyn et al., 1987); (2) normal melanocytes have low proliferative potential and it is, therefore, difficult to obtain large numbers of cells, limiting biochemical and molecular analyses; and (3) DNA transfer by transfection or retroviral infection techniques is difficult to carry out in primary cells.

In principle, immortalised melanocyte lines offer a solution to the difficulties experienced with using melanoma cell lines and/or primary melanocytes. Two strategies have been adopted to obtain such cell lines: spontaneous immortalisation and the use of oncogenes.

Rodent cells readily generate spontaneously immortalised cell lines in culture and as a result melanocyte lines from wild-type and black mice have been established (reviewed by Bennett and Sviderskaya, 1998). Similarly, melanocyte lines have also been established from mice that carry germline mutations at loci that encode genes involved in pigmentation (reviewed by Bennett and Sviderskaya, 1998). These cell lines have represented an important resource for genetic and molecular studies of normal and abnormal pigmentation. They are particularly useful in complementation studies which can assist with the characterisation of the genes encoded at the loci for which they carry a mutation. A limitation of spontaneously immortalised melanocyte lines is that their isolation can take a long time and depends largely on chance.

The ability of oncogenes to convert normal cells (both in culture and *in vivo*) either into an immortalised or transformed state could provide an alternative to spontaneously immortalised cell lines. Unlike rodent cells, normal diploid human cells do not readily generate spontaneously

immortalised cell lines in culture. However, expression of viral oncogenes in primary normal human melanocytes have been reported to extend their life span. Albino et al. (1986) showed that when v-Ha- or v-ki-ras oncogenes were introduced and expressed in cultured human melanocytes the resulting cells were initially diploid, pigmented and maintained an obligatory growth requirement for TPA. However, following six months of culture some cells emerged that underwent complete transformation, acquiring phenotypic characteristics of malignant melanoma, chromosomal changes and TPA-independence (Albino et al., 1992).

Zepter et al. (1995) infected cultured normal human melanocytes with a retroviral vector encoding the SV40 large T antigen. The resulting cells were morphologically indistinguishable from their normal counterparts and expressed the tyrosinase gene. Moreover, these T antigen-expressing melanocytes proliferated in the absence of TPA and required reduced levels of exogenous bFGF, indicating growth factor independence. Interestingly, these cells eventually underwent senescence. It should be noted that in another report in which the SV40 large T antigen was introduced into normal human melanocytes, the resulting cells were reported to be unpigmented, growth inhibited by TPA and to have undergone transformation (Melber et al., 1989). None of the above studies were therefore able to obtain stably-pigmented cell lines from human melanocytes.

Exogenous oncogenes have also been expressed constitutively in immortal murine melanocytes. Cells expressing oncogenes v-Ha-ras (Donatien et al., 1996; Dotto et al., 1989; Wilson et al., 1989), v-myc, v-neu (Dotto et al., 1989), adenovirus E1a (Dotto et al., 1989; Halaban et al., 1996; Yavuzer et al., 1995) or polyoma middle T (Dooley et al., 1988) all showed changes in their differentiated properties. The changes include morphological alterations (Donatien et al., 1996; Dotto et al., 1989), loss of expression of the enzymes responsible for melanin synthesis, including tyrosinase, TRP-1 and TRP-2 proteins (Donatien et al., 1996) and inhibition of transcription of the genes coding for tyrosinase and TRP-1 (Halaban et al., 1996; Yavuzer et al., 1995) with concomitant loss of pigmentation. In addition these cell lines loss their growth dependence on TPA.

In general, the above cell lines behaved like transformed cells since they also gained the ability to grow in suspension (in semisolid medium) and some were able to form tumors in mice. While most of the cell lines established above were produced primarily to study melanocyte growth control in relation to melanoma, they have revealed a possible connection between transformation and differentiation. Studies directed at understanding the molecular mechanisms by which oncoproteins can inhibit melanogenesis could, therefore, provide a more indirect but equally effective strategy for identifying the molecular mechanism(s) regulating melanogenesis.

However, the above cell lines are irreversibly transformed and it may be difficult to separate the effect of the oncoprotein on melanogenesis from the transforming process. To date, there have been no reports in the literature of melanocyte cell lines obtained by overexpressing oncoproteins in normal primary mouse melanocytes in culture. It would be of interest to see if oncoproteins exert the same effects on melanocyte differentiation in normal primary melanocytes as described for immortal cells.

In another approach, transgenic (Tyr-SV40E) mice were generated with an integrated fusion gene containing the SV40 early-region sequences, including the large T antigen and small t transforming genes, under the control of the pigment cell-specific mouse tyrosinase promoter (Bradl et al., 1991). Several mouse strains were described which had reduced coat pigmentation that roughly reflected the relative numbers of transgene copies (Bradl et al., 1991). Larue et al. (1993) cultured skin melanocytes from young Tyr-SV40E mice with no apparent skin lesions from hemizygous donors with low, medium and high numbers of transgene copies. The resulting cell lines readily became transformed, with loss of pigment and tyrosinase expression. Importantly, the rate at which these changes occurred was directly related to the numbers of integrated transgene copies in the cells. This suggests that the effect of the SV40 large T antigen on both the growth and pigmentation of melanocytes is dose-dependent. These cell lines and the Tyr-SV40E mouse strains provide potential models of melanoma progression but are probably limited in the study of melanogenesis.

The use of conditional temperature-sensitive (ts) mutants of viral oncogenes provide a novel approach to (a) exploring the molecular mechanisms by which oncoproteins alter pigmentation and (b) obtaining large populations of differentiated melanocytes for studying melanogenesis. Temperature-sensitive oncogenes have an advantage in that the cells grow well at the permissive temperature; however, when the cells are grown at the non-permissive temperature they lose their transformed properties and acquire the morphological and biochemical characteristics of differentiated cells. For example, ts mutants of polyoma and SV40 viruses encode a large T antigen which is transforming at the permissive temperature of 33°C but inactivated at the non-permissive temperature of 39°C. The growth and differentiation of cell lines established by these ts mutant oncogenes can thus be regulated by simply shifting the temperature at which the cells are cultured.

1.8 SV40 large T antigen

Simian virus 40 (SV40) is an oncogenic DNA virus belonging to the papovavirus group. The early region of the SV40 virus encodes two proteins, large (T) and small (t) tumor antigens. Both

these proteins can play a role in the oncogenic potential of this virus. The large T antigen has been shown to interfere with differentiation, as well as to immortalise primary cells in culture. When present at a sufficiently high concentration or when co-expressed with other genes, the large T antigen can also enhance transformation of primary cells (reviewed in Tooze, 1981).

The SV40 large T antigen, a predominantly nuclear phosphoprotein, has multiple biochemical properties and plays a pivotal role in lytic growth of the virus and in transformation of cells. Genetic and biochemical evidence indicates that specific portions of the T antigen molecule are essential for individual T antigen functions (see Fig. 1.3). (For review, see Manfredi and Prives, 1994). A large number of mutants, both deletion and missense, have now been identified and have clearly shown that the replication and transformation functions of SV40 large T antigen can be separated. For example, biochemical analysis has demonstrated that the ability to bind specifically to the SV40 origin of replication, the ability to hydrolyze ATP, and the helicase activity of SV40 large T antigen are not necessary for T antigen-mediated transformation (Auborn et al., 1989; Manos and Gluzman, 1985; Prives et al., 1983).

Extensive studies have demonstrated that specific regions of SV40 large T antigen interacts with a variety of cellular proteins including p53 (Lane and Crawford, 1979; Linzer and Levine, 1979), pRB (DeCaprio et al., 1988; Ewen et al., 1989), p107 (Dyson et al., 1989a), p130 (Hannon et al., 1993), p185 (Kohrman and Imperiale, 1992), p300/CBP (Avantaggiati et al., 1996; Eckner et al., 1996), the key replication enzyme, DNA polymerase α (Dornreiter et al., 1990; Gannon and Lane, 1987; Smale and Tjian, 1986), the transcription factor AP-2 (Mitchell et al., 1987), the heat shock protein, hsp-70 (Sawai and Butel, 1989), and the TATA box binding protein, TBP (Gruda et al., 1993). To date, four regions of SV40 large T antigen have been shown to be important in the ability of the T antigen to transform cells. These regions, identified by letters A-D, are shown schematically in Fig. 1.3. Regions B and D are responsible for binding to the tumor suppressor proteins, pRb and p53, respectively, that normally regulate cell proliferation. T antigen is thought to induce transformation of cells by binding to and inactivating these proteins. The nuclear localization region, C, has been shown to be important for transformation of primary cells and a remaining region, A, at the amino terminus, has been shown to bind to p300, a transcription co-activator. Two other regions, a zinc finger motif and a hydrophobic region embedded within region D, appear to be involved in the general stability of T antigen and therefore mutations in these residues indirectly affect transformation.

Studies on the biological activity of the SV40 large T antigen have provided a wealth of information about the cellular mechanisms involved in normal cell growth and the malfunction of these mechanisms during neoplastic transformation. The emerging picture is that the formation of a complex between the products of viral oncogenes and cellular growth suppressor proteins is

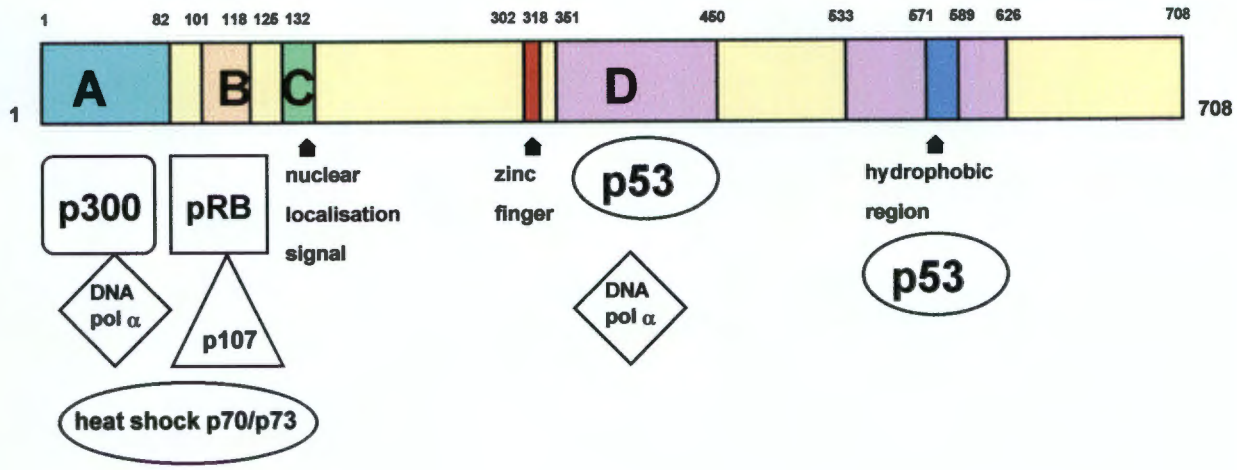


Fig. 1.3. Regions of SV40 large T antigen that are involved in its oncogenic activity. The numbers that are indicated above the schematic diagram represent the amino-acid residues of SV40 large T antigen (out of 708 total amino acids). Four transforming regions are identified by letter. (A) Amino acids 1-82 is the region of binding to DNA polymerase α and to the cellular protein p300. (B) Amino acids 101-118 are the region of binding to pRb and p107. (C) Amino acids 126-132 are the nuclear localization signal. The binding site for the heat shock protein, p73, resides in amino acids 1-178, spanning regions A, B, and C. (D) Amino acids 351-450 and 533-626 are the region of binding to p53 as well as DNA polymerase α. Two other regions involved in the general stability of the protein are also indicated. Amino acids 302-318 is a zinc finger motif and amino acids 571-589 are a hydrophorbic region (adapted from Manfredi and Prives, 1994).

associated with an increased propensity toward neoplastic transformation. Studies of such interactions has led to a clearer understanding of the role of tumor suppressor proteins in cell cycle regulation. The SV40 large T antigen has also been shown to interfere with cellular differentiation but very little is known about the mechanism(s) underlying this effect and whether it is mechanistically related to its transforming ability. A growing body of evidence, however, suggests that the large T antigen exerts its effect on cell proliferation and differentiation by binding, and thus inactivating, a set of regulatory proteins, notably those belonging to the retinoblastoma family of growth suppressors. Since a central interest of the present study is the effect of oncoproteins on melanocyte differentiation, the following section of this review will focus on the role of the retinoblastoma gene product in regulating cell growth and differentiation.

1.9 Dual roles of the retinoblastoma protein in cell cycle regulation and differentiation

Cell differentiation is a coordinated process that includes cell cycle exit and the expression of unique genes to specify tissue identity. While there is much knowledge on each separate step of differentiation, the mechanisms that coordinate cell cycle and tissue-specific events are still not known.

RB1 was the first tumor suppressor gene to be cloned (Dryja et al., 1986; Friend et al., 1986; Lee et al., 1987), and as a consequence has been studied intensively within the context of cell cycle regulation and oncogenesis. However, in addition to its role in regulation of the cell cycle, there is mounting evidence that the retinoblastoma gene product (pRb) also plays a role in cell differentiation. Whether pRb plays a role in differentiation by simply allowing cells to withdraw from the cell cycle or whether it plays a more direct role in tissue-specific gene expression during differentiation has begun to be explored. Because these two processes are almost inextricably linked, the above question has been difficult to resolve. However, recent evidence suggests that in some cell systems, pRb has distinct roles in cell cycle exit and terminal differentiation. For clarity, the next section will deal separately with pRb's role in these two processes.

1.9.1 pRb in cell cycle progression and cell cycle exit

RB1 is a member of a family of genes that encode cell cycle-regulatory proteins. The two other members, p107 (Ewen et al., 1991) and p130 (Hannon et al., 1993; Li et al., 1993), encode proteins that are closely related to pRb in both sequence and function. Each pRb family protein can induce a G1-specific cell cycle arrest when overexpressed in tumor cells (Claudio et al., 1994; Hinds et al., 1992; Zhu et al., 1993). This specific effect is dependent upon two highly conserved domains, termed the A and B boxes or the 'pocket' which are separated by a non-conserved spacer region. pRb, p107 and p130 may act as negative regulators of cell

proliferation through interaction with the E2F transcription factors (Zhu et al., 1993; Claudio et al., 1994; Qin et al., 1995). E2F is a multigene family with at least five members that heterodimerise with another set of transcription factors (DP-1, DP-2 and DP-3) in various combinations to form functionally active DNA-binding complexes (reviewed by LaThangue, 1994; Ormondroyd et al., 1995).

pRb, p107, and p130 are phosphorylated in a cell cycle-dependent manner (Beijersbergen et al., 1995; Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1992; Mayol et al., 1995; Xiao et al., 1996). During early G1 phase, hypophosphorylated pRb binds to E2F and represses its ability to activate transcription. In response to mitogenic stimuli, the cells progress into S phase as pRb becomes increasingly phosphorylated through the action of cyclin-dependent kinases. This causes pRb to dissociate from E2F, which can then regulate transcription of the S phase genes which are its targets (for review see Nevins, 1993). E2F, as mentioned earlier, also forms complexes with p130 and p107 but their times of appearance during the cell cycle differ (for review see Herwig and Strauss, 1997). The predominant E2F complex in quiescent cells is p130:E2F, whereas p107:E2F is detected in cells as they enter the S phase. The specific roles of the p107 and p130 proteins in the cell cycle and growth arrest are still unclear.

Several viral oncoproteins, including simian virus 40 large T antigen (DeCaprio et al., 1988), adenovirus E1A (Whyte et al., 1988) and the human papilloma virus-16 (HPV) protein E7 (Dyson et al., 1989b; Murger et al., 1989) interact with the hypophosphorylated form of pRb and its relatives p107 and p130 by binding to their pocket domains (for review see Herwig and Strauss, 1997). In this way viral oncoproteins promote growth by sequestering the retinoblastoma family proteins (reviewed by Nevins, 1992) and thus disrupting the growth inhibitory E2F:pRb, E2F:p107 and E2F:p130 complexes. This leads to the liberation of transcriptionally active E2F species which triggers S phase entry and uncontrolled proliferation.

1.9.2 pRb and cellular differentiation

The first evidence for a role of pRb in differentiation came from studies of RB1 knockout mice which die *in utero* at a stage when many highly specialized cell types begin to form (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Erythrocytes retain their nuclei and neuronal cells in the developing hind brain and spinal ganglia fail to differentiate further into mature neurons (Jacks et al., 1992; Lee et al., 1992; Lee et al., 1994). pRb is seemingly not required for the differentiation of every cell type however, since certain cell types in RB1-deficient embryos have already begun differentiating by day 14.5 of embryonic development when these mice die. For example the formation of the somites, cardiac and skeletal muscle, bone, and several other tissues have already been initiated in $RB^{-/-}$ mice at this time.

Further evidence in support of a role for pRb in cell differentiation has come from *in vitro* studies. The collective observations suggest that differentiation is accompanied by an increase in transcription of the RB1 gene, changes in the phosphorylation status of pRb and complex formation between pRb and transcription factors that are involved in regulating cellular differentiation. These studies are described in detail below.

To begin to decipher the role of the RB1 gene in differentiation, some investigators have examined whether cell differentiation correlates to changes in the phosphorylation status of the pRb protein. In several systems examined, it was found that an early event in differentiation was the dephosphorylation of pRb. For example, Chen et al. (1989), using western blot analysis, reported that induction of differentiation in several human leukemia cell lines by treatment with the phorbol ester, TPA, or retinoic acid (RA), leads to dephosphorylation of pRb. They further showed that in cells which were unable to differentiate in response to TPA and RA, no change in pRb phosphorylation pattern was observed. Hypophosphorylation of pRb was therefore associated with cell differentiation. Of significance was the observation that dephosphorylation of pRb occurs after the addition of differentiation inducers at a time when the cells are not morphologically differentiated and can still divide. Chen et al. (1989) therefore suggest that pRb dephosphorylation is not merely a consequence of cell cycle arrest. In other systems examined, however, decreased phosphorylation of pRb during differentiation correlated with cell cycle arrest in G0/G1 (Mihara et al., 1989; Resnitzky et al., 1992). In these systems, prevention of pRb phosphorylation and the resulting proliferation block imposed by pRb therefore appear to be essential for subsequent cell differentiation.

As mentioned earlier, increased RB1 mRNA expression has also been associated with differentiation (Coppola et al., 1990). For example, Coppola et al. (1990) studied three mouse cell lineages (erythroid, muscle and B cell) to determine whether there is a correlation between RB1 gene expression and differentiation. They found that when mouse erythroleukemia cells are induced to differentiate with either dimethyl sulfoxide (DMSO) or hexamethylene bisacetimide (HMBA) the process is associated with an increase in expression of RB1 mRNA. Similarly, increased expression of RB1 mRNA was found in myoblasts induced by mitogen depletion to become differentiated myotubes. This increase of RB1 mRNA was shown to correlate to an increase in expression of a muscle specific gene, α-actin. Furthermore, Coppola et al. (1990) show that in a B-cell lineage, RB1 expression is low in pre-B and B cell lines but high in plasmacytomas, which represent late stages of B cell differentiation. Thus, in all three lineages studied, late stages of differentiation were associated with increased amounts of RB1 mRNA.

Other studies have provided more direct evidence for pRb's role in differentiation. Chen et al. (1996a) investigated the possibility that pRb regulates the activity of NF-IL6, a member of the

CAAT/enhancer-binding protein (C/EBP) family of transcription factors, which functions in the generation and maintenance of differentiation in hematopoeitic cells. They demonstrate that wild-type pRb interacts, through its SV40 large T antigen-binding domains, with the nuclear factor NF-IL6. This interaction was shown to occur in monocyte/macrophage precursors precisely when the cells differentiate and continues in terminally specialized cells. Furthermore, they show that pRb directly activates NF-IL6 by enhancing its binding to cognate DNA sequences and by increasing transcription of a gene containing NF-IL6-binding elements in its promoter sequence.

In a different but analogous cell culture system, a correlation was observed between the process of adipocyte differentiation and the positive regulation of the transcription factor C/EBPβ by pRb (Chen et al., 1996b). C/EBPs transactivate the promoters of several adipocyte-specific genes during the differentiation of preadipocytes, including the fatty acid-binding protein 422/aP2 (references within Chen et al., 1996b). Chen et al. (1996b) showed that while embryonic lung fibroblasts (ELFs) prepared from $RB^{+/+}$ mice spontaneously differentiated to a fat-laden phenotype, $RB^{-/-}$ ELFs from an embryo in the same litter did not. To test whether RB1 expression is the key factor in determining the potential of ELFs to differentiate into fat-laden cells, they transfected $RB^{-/-}$ fibroblasts with plasmids containing wild-type or point mutant RB1 cDNA. The fibroblasts transfected with the plasmid expressing wild-type RB1 differentiated into fat-laden cells in response to hormonal treatment. In contrast, they show that untransfected fibroblasts and those transfected with mutant RB1 failed to differentiate along the same lineage. Furthermore, they showed that the process of adipocyte differentiation is regulated by the direct interaction between pRb, through its SV40 large T antigen-binding domains, and the family of transcription factors known as C/EBPs. This interaction activates C/EBPs to regulate the transcription of genes involved in adipocyte differentiation.

Previous studies in *ex vivo* culture systems have also shown that pRb may be important for muscle differentiation (Gu et al., 1993). During differentiation of muscle cells, pRb accumulates in the nucleus and forms complexes with bHLH myogenic transcription factors such as MyoD and myogenin (Gu et al., 1993). Gu et al. (1993) showed that pRb and MyoD directly bind to each other *in vitro* and *in vivo*, through a region that involves the pocket domain of pRb and the basic-helix-loop-helix domain of MyoD. Moreover, this pRb:MyoD interaction is essential for both the ability of MyoD to activate muscle cell-specific transcription as well as for its growth inhibitory activity (Gu et al., 1993). Gu et al. (1993) also demonstrate that the sequences in pRb that bind MyoD overlap with those known to bind to SV40 large T antigen and E1A (DeCaprio et al., 1988; Whyte et al., 1988; Kaelin et al., 1990).

In apparent contradiction to the above results, mice that are genetically deficient in RB1 ($RB^{-/-}$) have a histologically normal skeletal musculature (Clarke et al., 1992). A possible explanation for this is that a different molecule, for example, the structurally related pocket protein p107, may have substituted for the myogenic function of pRb. Indeed, Schneider et al. (1994) have demonstrated, using western blot analysis, that nuclear p107 was induced during myogenic development in $Rb^{-/-}$ mouse skeletal cells, much like pRb in developing wild-type muscle cells. They also show by northern blot analysis, that there is a reciprocal pattern of p107 and RB1 expression in these skeletal muscle cells. The compensatory pattern of p107 expression in $RB^{-/-}$ mouse skeletal cells suggested that, like pRb in wild-type myotubes, this pocket protein might associate with the myogenic bHLH factors present in $RB^{-/-}$ myotubes. Schneider et al. (1994) showed, using different binding analysis, that p107 binds to myogenin which suggests that p107 might function in place of pRb as a partner of myogenic bHLH factors in differentiated $RB^{-/-}$ myotubes. Furthermore, they provide data to show that myogenin and p107 can together mediate the activation of muscle genes in RB1-deficient cells which might therefore explain the myogenic differentiation of $RB^{-/-}$ mouse embryos.

The observation that pRb interacts with the transcription factors, NF-IL6, C/EBPβ and MyoD, through its SV40 large T antigen-binding domains raises the possibility that one way in which the large T antigen and related molecules can interfere with cellular differentiation is through disrupting pRb interactions with other regulatory molecules.

1.10 pRb and melanocyte differentiation

When growth-regulatory molecules such as basic fibroblast growth factor (bFGF) or the dominantly acting oncogenes ras, myc, adenovirus E1A, polyoma middle T antigen, or SV40 large T antigen, are aberrantly expressed in melanocytes they frequently lose their pigmented dendritic phenotype. This would suggest that there may be a common factor controlling melanocyte differentiation and a subset of genes essential to growth control. If one considers the role that pRb plays in controlling growth and differentiation of other cell types, it is possible that it has a similar role in melanocytes. Conclusive proof of such a role for pRb in melanocyte differentiation and growth could serve as a major breakthrough in our understanding of melanocyte differentiation and melanoma formation. Recent investigations have provided preliminary evidence to suggest that pRb might indeed play a role in melanocyte differentiation.

Valente et al. (1996) transfected B16F10 mouse melanoma cells with a human RB1 cDNA under the control of the human β-actin promoter. The resulting cells highly overexpress RB1 mRNA and its product, pRb, show reduced growth rate and increased expression of the differentiation marker melanin (melanogenesis) *in vitro*. While the above data suggest that pRb may be

involved in melanocyte differentiation, one cannot exclude the possibility that induction of differentiation was solely a result of the negative effect of pRb over-expression on cell proliferation.

In a different approach, Yavuzer et al. (1995) used the oncoprotein, E1A, as a probe for factors controlling melanocyte-specific gene expression and differentiation. They showed that expression of the E1A protein in a highly pigmented melanocyte cell line results in a non-pigmented phenotype. Loss of pigmentation was shown to be a consequence of the transcriptional repression of the tyrosinase and TRP-1 genes. The same investigators explored the possibility that the interaction between pRb and E1A was required for repression of the TRP-1 promoter. To this end, wild-type (WT) E1A or E1A mutants that are unable to bind pRb or p300 or both were co-transfected with a TRP-1-CAT reporter gene into B16 mouse melanoma cells. They showed that while the wild-type E1A repressed TRP-1 expression efficiently (32-fold), a E1A protein with a mutation affecting both p300 and pRb binding failed to do so. These results demonstrated that repression of TRP-1 expression by E1A required the sequestration of p300 and/or pRb. Interestingly, a mutant that was able to bind pRb but not p300 repressed TRP-1 expression sixfold less than WT E1A. Repression by the E1A mutant that was able to bind p300 but not pRb, was only twofold lower than WT E1A. While these results suggest that both pRb and p300 are important targets for E1A repression of melanocyte-specific gene expression, it would appear that the p300:E1A interaction is more important.

Moreover, Yavuzer et al. (1995) using reverse transcribed polymerase chain reactions (PCR), showed that *mi* gene expression was reduced around 50-fold in the E1A-expressing melanocytes. They reported that a residual amount of *mi* expression could be detected in E1A-expressing melanocytes if the PCR reactions were allowed to proceed for a further six rounds. Based on their results that showed that tyrosinase and TRP-1 expression was undetectable in E1A-expressing melanocytes, they suggested that the residual amount of Mi present in these cells might be rendered inactive by E1A. Since in muscle cells MyoD requires interaction with pRb for transcription activation, Yavuzer et al. (1995) considered the possibility that a similar interaction between Mi and pRb might be disrupted by E1A. They therefore investigated whether Mi could interact with pRb and showed that *in vitro,* Mi does bind pRb. Yavuzer et al. (1995), therefore, proposed that one mechanism by which E1A can repress transcription of melanocyte-specific gene expression is by disrupting Mi:pRb complexes which may be required for transactivation of these genes. While this speculation seems feasible, there is no evidence that Mi binds pRb *in vivo* nor has it been demonstrated that a Mi:pRb complex can transactivate melanocyte-specific gene expression.

It is also worth noting that in a similar study, Halaban et al. (1996) found that melanocytes expressing an E1A mutant which was unable to bind p300 induced the amelanotic phenotype. This suggested that sequestration of p300 by E1A was not responsible for abrogation of the melanocyte differentiated phenotype and therefore contrasts with the results of Yavuzer et al. (1995). Interestingly, Halaban et al. (1996) showed that melanocytes transfected with an E1A mutant ($p60^-$, pRb^-, $p107^+$, $p300^-$) that bound p107 were morphologically normal and pigmented. These results would suggest that inactivation of p107 by E1A does not result in de-differentiation. However, for some unknown reason these transfectants expressed very low levels of the E1A protein which might explain why this E1A variant was unable to induce the de-differentiated phenotype.

Taken together the results of the above studies suggest, but do not directly and unequivocally show, a role for pRb in melanocyte differentiation. Further investigations are therefore clearly required to confirm a role for pRb in melanocyte differentiation and growth.

1.11 General and specific aims of study

Over the last decade, the development of culture conditions for normal mouse melanocytes has facilitated the establishment of immortal (spontaneously) and transformed cell lines. These cell lines have contributed enormously to our understanding of melanocyte differentiation and transformation. However, despite several important advances, very little is understood about the molecular mechanisms regulating the pigmentation process and the mechanism by which normal melanocytes become malignant *in vivo* remains poorly understood. Moreover, while several studies have provided compelling evidence in support of a connection between the transformation process and the loss of pigmentation, the precise role of transforming agents in the depigmentation process is still unclear. *In vitro* systems are therefore required to establish a more direct link between melanocyte transformation and differentiation.

Because the primary interest of this study was the molecular mechanism(s) regulating melanocyte-specific gene expression and differentiation, the experimental approach adopted was to establish novel conditionally immortalised mouse melanocyte lines with a temperature-sensitive mutant SV40 large T antigen. It was hypothesised that such cell lines will contribute towards the understanding of the molecular mechanisms regulating and de-regulating differentiation in normal and transformed melanocytes respectively by:

i) providing a system in which melanocytes can be induced to differentiate, which can serve as an invaluable resource for studying the molecular mechanisms regulating this process;
ii) providing a system which allows cellular and molecular comparisons between transformed melanocytes (at the permissive temperature) and their equivalent 'normal' cells (at the non-permissive temperature), which could lead to the identification of genes that are differentially

expressed during melanocyte differentiation and transformation and therefore shed light on the mechanism(s) regulating these processes;

iii) allowing identification of a possible factor/mechanism controlling melanocyte differentiation and a subset of genes essential to growth control.

The **specific aims** of this study were therefore:

i) to establish primary cultures of normal melanocytes from newborn mouse skin;

ii) to infect the cultures in i) with a murine recombinant retrovirus containing a temperature-sensitive mutant SV40 large T antigen in order to establish conditionally immortalised melanocyte lines;

iii) to characterise these T antigen-expressing melanocyte lines with regard to features of melanocyte differentiation and growth;

iv) to determine the effect of TPA on melanocyte differentiation;

v) to compare the expression of melanocyte-specific markers in T antigen-expressing melanocytes cultured at the permissive and non-permissive temperatures in order to determine the direct effect of the oncoprotein on melanocyte differentiation;

vi) to investigate whether pRb and/or its relatives, p130 and p107, have a role in melanocyte differentiation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell cultures

Primary cultures of mouse epidermal and dermal melanocytes were obtained from the skins of 0.5-day-old C57BL/6 mice by a procedure described previously for the culture of dermal melanocytes (Tamura et al., 1987). All processing was carried out with sterile instruments under sterile conditions. Briefly, newborn mice were sacrificed by gassing with chloroform. They were then immersed in 70% ethanol and then transferred to petri dishes containing sterile phosphate buffered saline (PBS). The skins were removed from the dorsolateral side of the trunk between the limbs and then cleaned of subcutaneous tissues. The samples were then incubated epidermis side up in a 0.25% solution of trypsin in Ca^{2+}/Mg^{2+}-free PBS (pH 7.2) for 16 hours (hrs) at 4°C.

The epidermis and dermis were mechanically separated with fine forceps and placed separately in 60 mm culture dishes (the dermis of each skin per dish and the epidermis of two skins per dish). Each dish contained 1 ml of melanocyte-specific medium (MSM) which contains Ham's F10 (Highveld Biological) supplemented with 20% heat inactivated (30 minutes 56°C) fetal calf serum (FCS, Highveld Biological), 48 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA, Sigma), 0.1 mM isobutylmethyl xanthine (IBMX, Sigma), 50 μg protein/ml human placental extract (see Appendix to this chapter), 1 mM L-glutamine (Highveld Biological), 200 units/ml penicillin and 100 μg/ml streptomycin (Highveld Biological). In order to obtain a cell suspension the tissues were teased apart with jeweler's forceps and incubated in a total of 3 ml MSM, to allow for optimal attachment, in a 37°C incubator (95.0% air/5% CO_2, 65% humidity) overnight. The following day an additional 2 ml of MSM was added to each dish. Two days after plating, tissue debris was removed and the medium changed and supplemented with 100 μg/ml Geneticin (G418, Sigma) which selectively kills off rapidly dividing cells and has therefore been suggested as a means of eliminating contaminating fibroblasts (Halaban and Alfano, 1984). After 2 days of Geneticin treatment and in all cultures thereafter, cells were grown in MSM without G418.

Mouse B16 melanoma and 3T3 fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 200 units/ml penicillin and 100 μg/ml streptomycin. The mouse melanocyte cell lines, melan-a (black), melan-b (brown) and melan-c (albino) (Bennett et al., 1987, 1989), obtained from Dr. Dorothy Bennett, were grown in Eagle's minimum essential medium supplemented with 5% FCS, 1% sodium pyruvate, 100 μM 2-mercaptoethanol, 200 units/ml penicillin and 100 μg/ml streptomycin and 200 nM TPA. Primary

melanocyte cultures as well as the above cell lines were maintained in a 37°C incubator (95.0% air/5% CO_2, 65% humidity).

2.2 Preparation of retroviral lysate

2.2.1 Maintenance of the ψ2 producer cell line

The ψ2 cell line producing the ecotropic helper-free retrovirus was provided by R.D.G. McKay (National Institute of Neurological Disorders, NIH, Bethesda). This producer cell line, packaging the retrovirus encoding tsA58/U19 T antigen and neomycin resistance, was grown in DMEM (Highveld Biological) supplemented with 5% FCS, 200 units/ml penicillin and 100 μg/ml streptomycin in a 33°C incubator (95.0% air/5% CO_2, 65% humidity).

2.2.2 Collection of retroviral supernatant

The retroviral producer cells were grown to confluency and then incubated overnight in fresh medium containing 10% FCS. The next day the supernatant was collected, centrifuged at 1000 g to remove debris and then filtered through a 0.45 μm filter to remove living producer cells from the retrovirus stock. The retroviral containing supernatant was stored in 10 ml aliquots at -80°C.

2.2.3 Virus titration using NIH 3T3 cells

NIH 3T3 mouse fibroblasts were cultured in DMEM supplemented with 10% FCS, 200 units/ml penicillin and 100 μg/ml streptomycin in a 37°C incubator (95.0% air/5% CO_2, 65% humidity). The day before infection with retroviral-containing supernatant, equal numbers of the cells were plated in 6 X 35 mm dishes. The following day each dish was incubated at 37°C for 2 hrs with 2 mls of one of the following dilutions of retroviral supernatant containing 8 μg/ml polybrene: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} or 10^{-5}. After the 2 hr incubation the medium was replaced with DMEM + 10% FCS. The next day, Geneticin, the neomycin analogue, was included in the medium at 1000 μg/ml to begin neomycin-resistance selection. The selective medium was changed every 3-4 days over a period of 10 days and then the number of resistant colonies was counted visually; this count is presented below.

Table 2.1. Calculation of retrovirus titre

Dilution Factor	No. of colonies
10^0	Completely confluent
10^{-1}	Completely confluent
10^{-2}	Completely confluent
10^{-3}	Clones just merging
10^{-4}	24
10^{-5}	2
10^{-6}	0

The titre was calculated as follows:

$$\frac{\text{Number of colonies X virus dilution}}{\text{virus inoculum volume (ml)}} = \text{cfu/ml}$$

Thus the titre was calculated to be $\frac{2 \times 10^5}{2 \text{ ml}} = 1 \times 10^5$ cfu/ml.

2.3 Retroviral infection of primary mouse epidermal and dermal melanocyte cultures

Seven days after plating, the primary cultures of mouse melanocytes were infected with the retrovirus supernatant in the presence of 8 μg/ml polybrene under the following conditions to ensure successful integration of the retrovirus: at 33°C or 37°C for 2 or 4 hrs. Polybrene was included because it increases the efficiency of infection. After infection, fresh medium was added and 48 hrs later the cultures were subjected to neomycin selection using the neomycin analogue, G418 (Geneticin, Sigma, Gibco) at 200 μg/ml for one month. As opposed to 3T3 cells that are robust and, therefore, require 1000 μg/ml Geneticin for neomycin selection, primary cultures are generally subjected to only 200 μg/ml of the drug. The selection medium was changed every 3-4 days.

After one month, colonies of pigmented cells appeared and they were isolated with the use of cloning rings as follows: The colonies were marked by encircling individual pigmented areas with a pen on the bottom of the culture dish. The medium was aspirated and the cells rinsed with PBS. Sterile cloning rings containing sterile vacuum grease were placed over the marked areas and cells surrounded by the cloning rings were trypsinized and immediately placed into a 96 well tissue culture dish containing MSM. Once confluent the cells were expanded into a 24 well tissue culture dish; then 35 mm dishes; then 60 mm dishes and finally into 10 cm dishes. Clones that grew were cultured at 33°C (unless stated otherwise) in MSM, as described earlier for primary melanocytes, and expanded into cell lines. Cells were frozen in MSM with 10% DMSO before any further analysis.

2.4 Immunodetection of the SV 40 large T antigen

Cells were washed with PBS (pH 7.4), fixed in 2% paraformaldehyde at room temperature (RT) for 15 min and permeabilised in 100% methanol at -20°C for 15 min. T antigen was detected with a mouse monoclonal antibody (PAb 101, ATCC, Gurney et al., 1980) directed against the C-terminus of the SV 40 large T antigen. Fixed cells were incubated with a 1:1 dilution of hybridoma supernatant for 5 hrs at RT and then overnight at 4°C. This was followed by incubation with biotinylated rabbit anti-mouse immunoglobulins (1:200, DAKO) for 1 hr at RT.

The cells were then incubated with a streptavidin biotinylated horseradish peroxidase complex (streptABComplex/HRP, 1:1000, DAKO) and the reaction visualised with the peroxidase substrate, 3,3-diaminobenzidine tetrahydrochloride (DAB). Negative controls were prepared following the same procedure but substituting the primary antibody with 1% FCS in PBS.

2.5 Southern blot hybridisation of genomic DNA

Genomic DNA was isolated from T antigen-expressing melanocytes and from ψ2 and NIH 3T3 cells according to a rapid DNA isolation method (Davis et al., 1986 pp42-43). Samples were subsequently treated with 40 μg/ml of DNAse-free RNase A for 30 min at 37°C to remove contaminating RNA, followed by extraction with phenol:chloroform:isoamyl alcohol. The DNA was precipitated, resuspended in TE (10 mM Tris, pH 7.6; 0.1 mM EDTA, pH 8) and quantified. Twenty micrograms of genomic DNA was digested with a total of 2 units Eco R1 restriction enzyme/μg DNA at 37°C. Steps were taken to ensure even digestion of the high molecular weight DNA, as suggested by Sambrook et al. (1989, pp 9.32). Digested DNA was separated on a 0.8% agarose gel in TBE buffer, run overnight at 6.5 V/cm. The DNA was denatured and transferred onto a nylon membrane (Hybond-N+, Amersham) by capillarity, according to the manufacturer's instructions. Hybridisation was carried out in a hybridisation oven (Hybaid). The blot was prehybridised in 10% dextran sulphate; 6 X SSC; 5 X Denhardt's; 0.5% SDS and 100 μg/ml denatured salmon sperm DNA for 2 hrs at 65°C. An 808 bp Xba 1-Bgl 11 fragment, specific for the sequence conferring resistance to neomycin, was released from a pGK-lacZ plasmid (obtained from R Jaenisch, Massachusetts Institute of Technology, Cambridge) and labelled with [α-^{32}P]-dCTP using a Random-primed DNA labeling kit (Boehringer Mannheim) for use as a probe. Radiolabelled probe was separated from unincorporated label on a Sephadex G-50 column. Denatured probe was added to a final concentration of 1 X 10^6 cpm/ml and hybridisation was carried out at 65°C overnight. Post-hybridisation washes included two low stringency washes in 2 X SSC, 0.1% SDS at RT for 20 min, followed by a higher stringency wash in 0.1 X SSC, 0.1% SDS at 65°C for 10 min. The blot was then exposed to autoradiographic film (AGFA CURIX RP1) at -80°C.

2.6 Melanogenic Assays

Cells were washed three times with cold PBS (pH 7.4), scraped from the dishes into Nonidet P-40/SDS buffer (1% Nonidet P-40, 0.01% SDS, 0.1 M Tris-Cl, pH 7.2, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin), vortexed and incubated at 4°C overnight. Following centrifugation at 14 000 g at 4°C for 15 min, the supernatants were recovered and the protein concentration of each sample was determined by the BCA protein assay system (Pierce

Chemicals). The supernatants were stored at -70°C until use for melanogenic assays. All melanogenic enzyme assays were carried out at pH 6.8, 37°C, for 1 hr.

2.6.1 Tyrosinase is an unusual enzyme in that it has tyrosine hydroxylase, DOPA- and DHI oxidase activity. In this study, tyrosine hydroxylase activity was assayed by a method which measures tritiated water produced during the hydroxylation of L-[3,5-^{3}H]tyrosine to DOPA (Hearing and Ekel, 1976; Hearing, 1987). Briefly, the reaction mixture was set up in round bottom 96 well plates and consisted of 30 µl cell protein, 10 µl of a solution containing antibiotics and the L-DOPA cofactor (1 M KPO_4 buffer, pH 7.2, 1 mg/ml chloramphenicol, 1 mg/ml cycloheximide, 0.1 mg/ml bovine serum albumin, 1000 U/ml penicillin G and 0.25 mM L-DOPA) and 10 µl L-[^{3}H]tyrosine (40 – 50 Ci/mmol, NEN-DuPont, Boston, MA adjusted to 1 Ci/mmol before use). The reaction was incubated for 1 h at 37°C. Forty microliters of each sample was then added to a charcoal slurry (approximately 100 mg charcoal in 1 ml of 0.1 N HCl) and the samples vortexed intermittently over a 1 h period. The charcoal, which is used to adsorb excess [^{3}H]tyrosine, was pelleted by centrifugation (1000 g, 5 min) and a 100 µl aliquot of the supernatant counted for the production of radioactive water.

2.6.2 DOPAchrome tautomerase activity was measured by high performance liquid chromatography (HPLC) as the disappearance of DOPAchrome substrate and the production of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) rather than 5,6-dihydroxyindole (DHI)(Palumbo et al., 1987; Tsukamoto et al., 1992). Calculations of substrate and product concentrations in these reactions are based on comparison of eluting peaks with calibrated curves of known DHI and DHICA standards. DOPAchrome was prepared using the silver oxide method originally described by Körner and Pawelek (1980).

2.6.3 DHICA oxidase activity was measured by HPLC as the disappearance of DHICA above background auto-oxidation. Again, reaction rates are calculated from comparison of eluting peaks with calibrated curves of known DHICA standards. DHICA was a generous gift of Prof. Shosuke Ito (Fujita Health Sciences University).

The results of the above assays are expressed as pmol of product per µg protein per hour at 37°C. Pmol product are calculated from the radioactive measurements or, in the HPLC assays, by comparison with known standards. Extracts of melan-a cells solubilized in extraction buffer were used as positive controls for each assay. All HPLC analysis were performed on a Beckman model 344 instrument with a C18 5 µm-pore column (4.6 mm X 250 mm).

2.7 Melanin Synthesis Assay

The assay that measures melanin synthesis was adapted from that published by Hearing and Ekel (1976) and measures the amount of radioactive melanin formed as [^{14}C]tyrosine is converted to the acid-insoluble melanin biopolymer. This assay therefore reflects the combined melanogenic activities of tyrosinase, TRP-1, TRP-2, and inhibitory factors. Briefly, the cells were harvested with trypsin:EDTA (0.05%:0.02%), solubilised with an equal volume of 0.1% Triton X-100 in 0.1 M phosphate buffer and centrifuged for 5 min at 10000 g. The supernatants were adjusted to equal protein concentrations by the Biuret test (Bradshaw, 1966) or BCA assay (Pierce). A 10 µl aliquot of each supernatant was mixed with 5 µl ^{14}C tyrosine (25 µCi/ml, specific activity 513 mCi/mmol, Amersham), 5 µl L-dopa (0.05 mM in 0.1 M phosphate buffer) and 5 µl of an antibiotic mix containing chloramphenicol (1 mg/ml), cyclohexamide (1 mg/ml), bovine serum albumin (0.1 mg/ml) and penicillin (1000 units/ml). This reaction mix was incubated for 3 hrs at 37°C. Controls were assays in which the supernatants were replaced with phosphate buffer (to determine non-enzymatic incorporation levels). Samples were individually spotted onto Whatman glass-fibre filter discs in 20 µl aliquots and washed in 10% trichloroacetic acid for 15 min and then for 10 min to remove unincorporated ^{14}C-tyrosine. The discs were dehydrated by washing for 5 min in 95% ethanol and then dried overnight in scintillation vials at RT. 200 µl Soluene-350 tissue solubiliser (Packard) was added to lift the reaction products out of the filter and 8 ml toluene scintillation fluid (Packard) was added prior to counting. Samples were counted in a Beckman scintillation counter set for 10 min counts on the ^{14}C channel. Each assay was carried out in triplicate and the results presented as the mean of 3 samples.

2.8 Northern Blot Hybridisation

2.8.1 Northern blot hybridisation of total RNA samples

Expression of *tyrosinase*, *TRP-1*, *TRP-2*, *mi*, *c-kit*, *SV40 large T antigen*, *Pax3* and *β-actin* mRNA was determined by standard northern blot hybridisation procedures (Davis et al., 1986). Probes included:

(i) A 1.9-kb Eco R1 fragment of pmcTyr1, specific for murine *tyrosinase* (Müller et al., 1988).

(ii) A 1.7-kb Hind III fragment of pMT4, which is specific for murine *TRP-1* (Shibahara et al., 1986).

(iii) A 1.8-kb Eco R1 fragment of TRP-2a, which is specific for murine *TRP-2* (Jackson et al., 1992).

(iv) A 1.3-kb Eco R1 fragment of the mouse *mi* cDNA (Yavuzer et al., 1995) or a 1.7-kb Xba 1-Hind 111 fragment of the mouse *mi* cDNA (provided by M. Tachibana, Maryland, USA).

(v) A 1.0-kb Bam H1-Eco R1 fragment of the mouse *c-Kit* cDNA (provided by J-J. Panthier,

Institut National de la Recherche Agronomique, France) was used as probe to c-Kit.

(vi) A 2.0-kb Pvu 11 fragment of the *SV40 large T antigen* cDNA (provided by P. Jat, Ludwig Institute for Cancer Research, London, UK).

(vii) A 0.35-kb Pvu 11 fragment of the mouse *Pax3* cDNA (provided by Y. Furuta, Vanderbilt).

Total RNA was extracted from T antigen-expressing melanocytes, from B16 melanoma, melan-a, and ψ2 fibroblast cells according to Sambrook et al. (1989). Twenty micrograms of RNA from each sample was separated on a 1.1% denaturing, formaldehyde agarose gel at 5 V/cm for 1 hr, stained for 2.5 min with 0.01% ethidium bromide and photographed on a Spectroline transilluminator. The gel was rinsed twice in 10 X SSC for 20 min and then the RNA transferred onto a nitrocellulose membrane (Hybond C, Amersham) by capillarity using 10 X SSC as blotting buffer. The gel was blotted overnight, after which the towelling was replaced and the blotting continued for a further 3 hr. Transfer onto the membrane was checked by transillumination and the position of the rRNA bands marked with pin-pricks. The RNA was then fixed onto the membrane by baking at 80°C for 2 hrs. For northern blot analyses using a 1.7-kb Xba 1-Hind 111 fragment of the mouse *mi* cDNA and a 1.0-kb Bam H1-Eco R1 fragment of the mouse *c-kit* cDNA as probes, a nylon membrane was used and the RNA was ultraviolet crosslinked.

Prehybridisations and hybridisations were carried out according to Davis et al (1986) in a hybridisation oven (Hybaid) overnight at 47°C. After prehybridisation, the blots were hybridised with a probe labelled with [α-^{32}P]-dCTP using a Random-primed DNA labeling kit (Boehringer Mannheim). Radiolabelled probe was separated from unincorporated label on a Sephadex G-50 column. Denatured probe was added to the hybridisation bottle (at a final concentration of 1 x 10^6 cpm/ml) and the blots were hybridised, stripped and then rehybridised with other probes as described later in the results. Blots were washed under high stringency conditions (0.1 X SSC at 68°C for 2 X 10 min) and exposed to autoradiographic film at -80°C. The blots were reprobed with β-actin DNA under the identical conditions to control for comparable loading and transfer of RNA. RNA from B16 melanoma cells was included as a positive experimental control because these cells have been shown to express high levels of the melanocyte-specific genes. RNA from ψ2 fibroblast cells was included as a negative control on all blots.

2.8.2 Northern blot hybridisation of poly(A)$^+$ RNA samples

Expression of pRB, p130 and p107 mRNA was determined by northern blot hybridisation of poly(A)$^+$ enriched RNA from DMEL-3 cells cultured at 33°C and 37°C for 4, 16 and 30 days, mouse B16 melanoma cells, melan-a cells and mouse brain tissue. Four to six 15 cm petri-dishes of subconfluent cells were rinsed in chilled phosphate-buffered saline (PBS) and

trypsinised. The cells were resuspended in cold culture medium with serum to stop the trypsin action and the cell suspensions were collected in 50 ml sterile tubes. The cells were pelleted in a refrigerated centrifuge at 1500 RPM for 5 min, the supernatant was removed and the pellets resuspended in 40 ml of chilled PBS. After the cells were pelleted again in a refrigerated centrifuge at 1500 RPM for 5 min, the supernatant was removed and the cell pellets were immersed in liquid N_2 for 1 min and then transferred onto dry ice and stored at -70°C. Brain tissue was removed from adult mice and immediately placed in 50 ml sterile tubes in liquid N_2. The tissue was minced with the use of a pestle and mortar and then stored at -70°C. Total RNA was extracted from these frozen cell pellets and minced brain tissue according to Sambrook et al (1989, pp 7.12-7.13). To check the integrity of the RNA, 5 μg of each sample was run on a 1% formaldehyde gel. Poly(A)$^+$ RNA was then further purified from total RNA with the 'Fast-track' mRNA isolation kit (Invitrogen) according to the manufacturer's instructions.

Five micrograms of poly(A)$^+$ RNA from each sample was separated on a 1% denaturing formaldehyde agarose gel at 5 V/cm for approximately 4 hours. The gel was rinsed in 1 X MOPS for 30 min followed by a 45 min rinse in 20 X SSC. The poly(A)$^+$ RNA was transferred onto either nitrocellulose (Hybond C, Amersham) or nylon membrane (Hybond N+, Amersham) by capillarity using 20 X SSC as blotting buffer. The gel was blotted overnight after which the towelling was replaced and the blotting continued for a further 3 hours. Following transfer the blots were rinsed briefly in 6 X SSC and then 2 X SSC. The blot was air-dried for 2 hours and then the RNA was fixed onto the membrane by baking at 80°C for 2 hours. After prehybridisation, the blots were hybridised with a probe labelled with [α-^{32}P]-dCTP by random priming (Amersham), stripped and then rehybridised with other probes as described later in the results. Probes included a 848-bp Hind 111 fragment of the mouse p130 cDNA (provided by E. Lam, Ludwig Institute of Cancer Research, St Mary's, London, UK), a 2.88-kb Eco R1-Hind 111 fragment of the mouse pRB cDNA and a Bam H1-Sal 1 fragment of the human p107 cDNA (provided by S. Mittnacht, ICR, London, UK). Prehybridisation, hybridisation and post-hybridisation washes were carried out as described in section 2.8.1.

The membranes containing total RNA or poly(A)$^+$ RNA were autoradiographed at -80°C. After autoradiography, the probe was removed by treating the membranes in 0.1% SDS at 100°C, allowed to cool to room temperature, then reprobed with β-actin DNA.

2.9 Western immunoblotting analysis of tyrosinase, TRP-1, TRP-2, SV40 large T antigen, c-Kit, pRb, p130 and p107

Cells were harvested and solubilized overnight at 4°C in lysis buffer (1% NP-40, 0.01% SDS, 0.1 M Tris-HCl, pH 7.2, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM sodium orthovanadate and 1 mM sodium fluoride), then centrifuged at 12 000 g for 15 min at 4°C, and the supernatants were recovered. Protein concentrations were determined by the BCA assay (Pierce), with bovine serum albumin as the standard. Equal amounts of protein were loaded in each lane and separated on 7.5% SDS gels, and then transferred electrophoretically to Hybond ECL nitrocellulose membranes (Amersham). Equal protein loading was verified by staining the gels with Coomassie brilliant blue after transfer of proteins to membranes. For Western blot analysis of tyrosinase, TRP-1, TRP-2, SV40 large T antigen and c-Kit, blots were blocked for 1 hr at RT in 5% non-fat dry milk powder in TBS/Tween (0.05% Tween-20 in Tris buffered saline), then they were incubated with the following rabbit antisera (all obtained from Dr V. Hearing, National Cancer Institute): αPEP7 (carboxy terminus of tyrosinase, 1:2000), αPEP1 (carboxy terminus of TRP-1, 1:2000) and αPEP8 (carboxy terminus of TRP-2, 1:2000). Mouse monoclonal antibodies to SV40 large T antigen (Pab 101, Santa Cruz Biotechnology) and c-Kit (ACK2, Nishikawa et al., 1991) were employed at dilutions of 0.5 μg/ml and 1 μg/ml respectively. For detection of pRb, p130 and p107, blots were blocked for 1 hour in 5% non-fat dry milk powder in TBS/Tween (0.1% Tween-20 in Tris buffered saline), and then they were incubated with rabbit polyclonal antibodies to pRb, p107 and p130 (C-15, C-18 and C-20 respectively, Santa Cruz) at dilutions 1:2000 (kind gifts from E Lam, Ludwig Institute for Cancer Research, London, UK). The membranes were then incubated with either horseradish peroxidase-conjugated anti-rabbit IgG (1:1500 dilution) or anti-mouse IgG (1:2000 dilution) and the immunoreactive proteins were detected by Enhanced ChemiLuminescence (Amersham) according to the manufacturer's instructions.

2.10 Assessment of the growth characteristics of T antigen-expressing melanocytes

Short-term growth was determined in medium without TPA, or without human placental extract, or with only 2% serum and compared with growth in melanocyte-specific medium which contains TPA, human placental extract and 20% serum. Cells were seeded in triplicate in 12-well plates ($4x10^4$ cells/well), collected by trypsinization and counted on a hemocytometer at intervals of 2-3 days.

2.11 Melanin content measurement

To determine the effect of individual growth-promoting additives on the melanin content of T antigen-expressing melanocytes, cells were grown in experimental and control media for two

weeks. Medium was renewed twice weekly. Cells were harvested separately from each plate by trypsinization and counted by hemocytometer. Melanin contents were determined by heating pellets of 10^6 cells in 1 ml 0.1 M NaOH at 95°C for 90 min (Zepter et al., 1995). Each sample was prepared in duplicate and their optimal density was measured at 475 nm with a Shimadzu spectrophotometer UV-120. Relative values were obtained by comparing absorption values obtained for experimental samples to that of fibroblast samples.

2.12 Hormonal stimulation

To determine the effect of alpha-melanocyte-stimulating hormone (α-MSH) on the DMEL-3 cell line, the following procedures were followed. Cells were seeded at 4000 cells/cm^2 in 10 cm dishes in standard culture medium and left to adhere overnight. The following day the regular medium was replaced with fresh medium with or without α-MSH. Appropriate media were changed daily thereafter for 4 days. MSH used was synthetic bovine α-MSH (Sigma) at 0.2 μM, since this concentration was shown to be the most effective in previous studies (Aroca et al., 1993). Cells were harvested with trypsin-EDTA (0.25% trypsin, 0.05% ethylenediamine tetraacetic acid) and processed for protein and RNA extractions.

2.13 Transfection assays

The transfection experiments to determine if ectopic expression of *mi* can rescue the expression of tyrosinase, TRP-1 and TRP-2 genes were carried out using the FuGENE 6 transfection reagent (Boehringer and Mannheim) according to manufacturer's instructions. DMEL-3 cells grown at the permissive temperature (33°C) were passaged 24 hours before transfection and plated at 1 X 10^5 cells per 35 mm dish. Co-transfections were carried out using a 20:1 molar ratio of an *mi* cDNA under the control of the CMV IE promoter (Yavuzer et al., 1995) to selectable DNA (pBabepuro) in a total of 5.5 μg DNA. Forty-eight hours after transfection, cells were selected in 2.5 μg/ml of puromycin (Sigma). Untransfected DMEL-3 cells and DMEL-3 cells transfected with pBabepuro alone served as negative controls.

To determine the regions of SV40 large T antigen involved in repressing melanocyte-specific gene expression, approximately 2 X 10^6 passage 8 DMEL-3 cells (grown at the permissive temperature, 33°C), were co-transfected with plasmid DNA, using the lipofection reagent DOTAP (Boehringer Mannheim) according to manufacturer's instructions. Cotransfections were carried out using a 20:1 molar ratio of non-selectable DNA to selectable DNA (pBabepuro) in a total of 18 μg DNA. The pBabepuro plasmid which encodes the puromycin resistance gene was used to facilitate the selection of stable transfectants. The three T antigen mutants (non-selectable DNA), *dl*1135, 3213 and 5041, were provided by P.S. Jat (Ludwig Institute for Cancer

Research, London, UK) and are delineated in Table 2.2. Untransfected passage 8 DMEL-3 cells and passage 8 DMEL-3 cells transfected with pBabepuro alone served as negative controls. Forty-eight hours after transfection, cells were selected in 2.5 μg/ml of puromycin (Sigma). Twenty-four hours later, half of the plates were shifted to the non-permissive temperature (37°C). During this time the growth medium was changed every 3-4 days.

Table 2.2. T antigen-encoding plasmids and their protein binding specificities

T antigen derivatives	Specific Protein binding ability		
	p53	pRB, p130, p107	p300
5041	-	+	+
3213	+	-	+
dl1135	+	+	-

Appendix

Preparation of human placental extract

Full-term human placenta was washed extensively with sterile PBS, a portion was minced and homogenized in PBS using a Polytron ultra-homogenizer. The homogenate was centrifuged at 13 000 g for 20 min at 4°C and the supernatant was passed through a 0.45 μm filter. The protein content of the supernatant was determined using the Biorad protein assay and bovine serum albumin as a standard. The supernatant was frozen at -20°C.

CHAPTER 3: RESULTS

3.1 Establishment of primary epidermal and dermal melanocyte cultures

The skins from neonatal (0.5-day-old) C57Bl6 mice were used as a source of both epidermal and dermal melanocyte cultures. After two days in culture, the keratinocytes and fibroblasts dominated the epidermal and dermal cultures, respectively, and melanocytes were distributed sparsely among them. Figure 3.1a,b shows the typical appearance of a two day old epidermal culture. The light-appearing patches that are not in focus, are clumps of epidermal tissue (see arrows). When examined by phase contrast microscopy, single melanocytes (see arrowheads) were randomly distributed among sheets of keratinocytes (Fig. 3.1a). These melanocytes appeared as small dendritic cells with dark cytoplasm and melanin pigment was visible within them by bright field microscopy (Fig. 3.1b).

The keratinocytes and fibroblasts were gradually killed by Geneticin treatment and within 7 days, individual melanocytes could be seen attached to the dishes. In both epidermal and dermal cultures, melanocytes appeared as bipolar or polydendritic cells when examined by phase contrast microscopy (Fig. 3.1c) and melanin was observed both in their cytoplasm and dendrites by bright field microscopy (Fig. 3.1d). Cells continued to proliferate and confluent or subconfluent cultures enriched, for either epidermal or dermal melanocytes, were obtained at about 3 weeks. At this stage, the cultures contained only melanocytes and they were predominantly polygonal or epithelioid in shape (Fig. 3.1e) and pigment granules were clearly seen in them by bright field microscopy (Fig. 3.1f).

3.2 Establishment and isolation of clonal pigmented melanocyte lines expressing the SV40 large T antigen

At the time at which this study was initiated, there was no published procedure for the retroviral infection of primary mouse melanocyte cultures and therefore suitable infection conditions had to be established. A brief account of the approaches attempted is given in chronological order because this clarifies why particular directions were followed at different stages.

The first approach was to infect 3-week old epidermal cultures enriched for melanocytes. After 3 months, no drug-resistant colonies were obtained and only single, senescent-appearing melanocytes were visible. It was concluded that the 3-week old cultures of epidermal melanocytes had probably reached a crisis before infection and were therefore not susceptible to retroviral infection. This experiment was abandoned. To overcome the problem encountered above, the second approach was to infect rapidly-dividing melanoblasts present in early primary

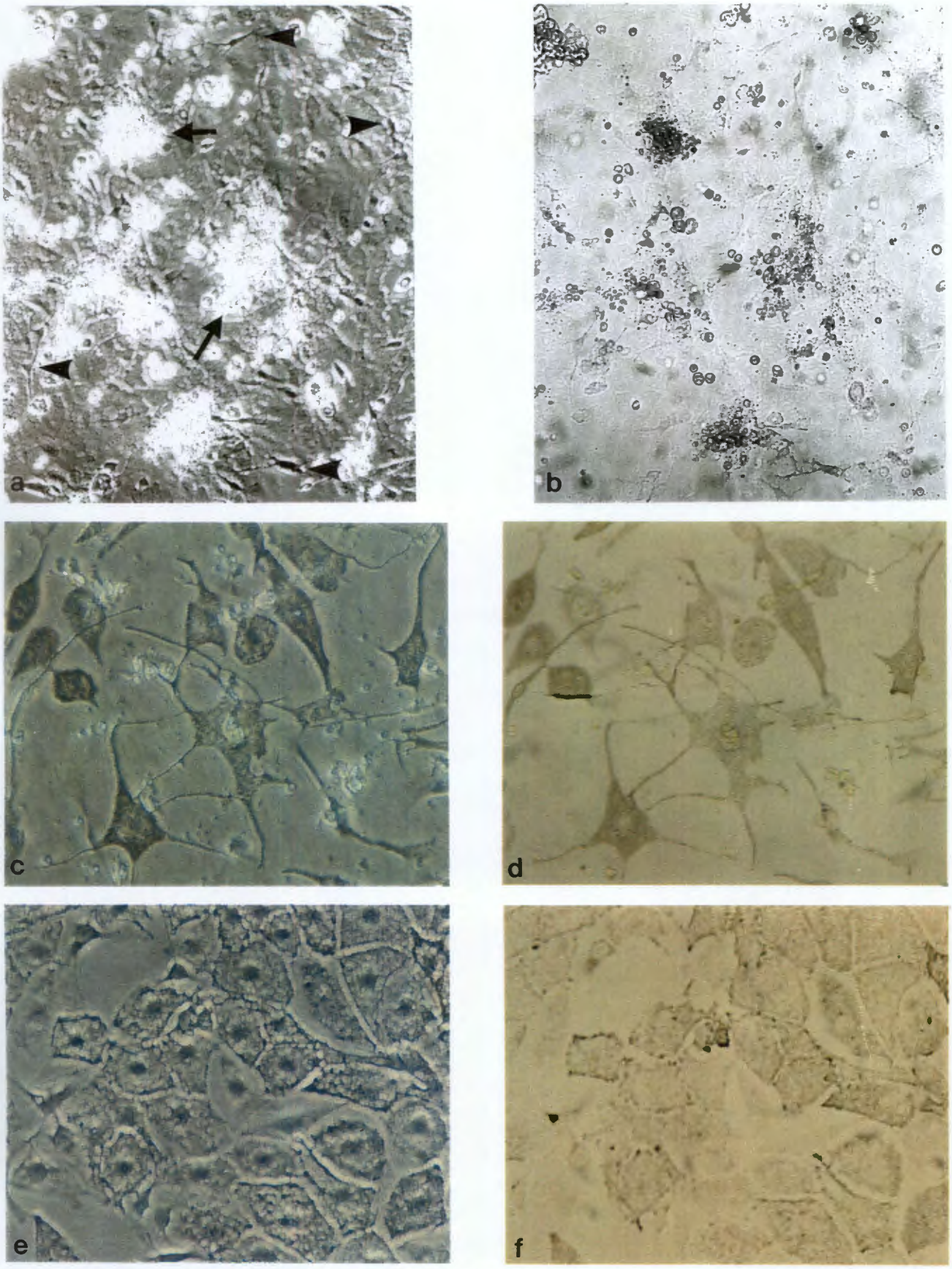


Fig. 3.1. Primary cultures of mouse melanocytes, derived from epidermal cell suspensions, in melanocyte-specific medium. **(a,c,e)** are phase contrast photomicrographs. **(a)** A typical mixture of keratinocytes and epidermal melanocytes after two 2 days in culture. Note sheet of keratinocytes and relative paucity of black, dendritic melanocytes (arrowheads). Refractile masses are clumps of epidermal tissue (arrows). **(c)** After 7 days in culture, melanocytes appear as single bipolar or polydendritic cells. **(e)** After 3 weeks, subconfluent cultures consisted entirely of bipolar to polygonal melanocytes. **(b,d,f)** are bright field photomicrographs of the same fields as **(a,c,e)** respectively, to show colour due to black melanin granules.

dermal cultures. Within 3-4 weeks, 22 unpigmented drug-resistant colonies were obtained and expanded into cell lines. Four cell lines were analysed, at both the permissive (33°C) and non-permissive (37°C) temperatures, for the expression of a set of melanocyte-specific markers, including TRP-2. None of these clones displayed any known features associated with melanocytes. This experiment was therefore also abandoned.

In view of the problems experienced above, the third approach was to infect 7-day old cultures enriched for either proliferating epidermal or dermal melanocytes. Because it was not obvious what the optimal conditions would be for infecting melanocytes, these dishes were incubated with the retroviral supernatant at 33°C or 37°C for either 2 or 4 hrs. Infected cells were grown at the permissive temperature of 33°C in the presence of 200 μg/ml G418 for a minimum of 3 weeks, until drug-resistant colonies were observed. The average number of clones obtained per dish under the different infection conditions is presented in Table 3.1. The dermal cultures gave rise to more clones than the epidermal cultures. The duration of infection at 37°C did not seem to influence the number of clones obtained but infection at 33°C for 4 hrs yielded significantly more clones than the 2 hr infections at this temperature.

Table 3.1. Summary of results obtained under different infection conditions of 7-day old cultures

Source of cultures	Average no. of clones/dish at 37°C for 2 hrs	Average no. of clones/dish at 37°C for 4 hrs	Average no. of clones/dish at 33°C for 2 hrs	Average no. of clones/dish at 33°C for 4 hrs
Epidermis	0.7	1	1.5	3
Dermis	5	6	2.8	5.3

After 4 weeks, 78 pigmented and 4 unpigmented colonies were picked from 18 dishes of primary dermal cultures, and expanded into cell lines. Nine dishes of epidermal cultures gave rise to 6 pigmented and 6 unpigmented clones that were also picked and expanded into cell lines. At the time of picking, these unpigmented clones were thought to be contaminating keratinocytes or fibroblasts. Subsequently, however, several of the pigmented clones became either completely unpigmented (Fig. 3.2a,b), or contained a mixture of pigmented and unpigmented cells (Fig. 3.2c,d), or consisted of cells that contained small amounts of pigment restricted to their perinuclear regions (Fig. 3.2e,f). After expansion, only three epidermal (termed EMEL-1, 2 and 3) and three dermal (termed DMEL-1, 2 and 3) cell lines retained their pigmented phenotype and were selected for further analysis.

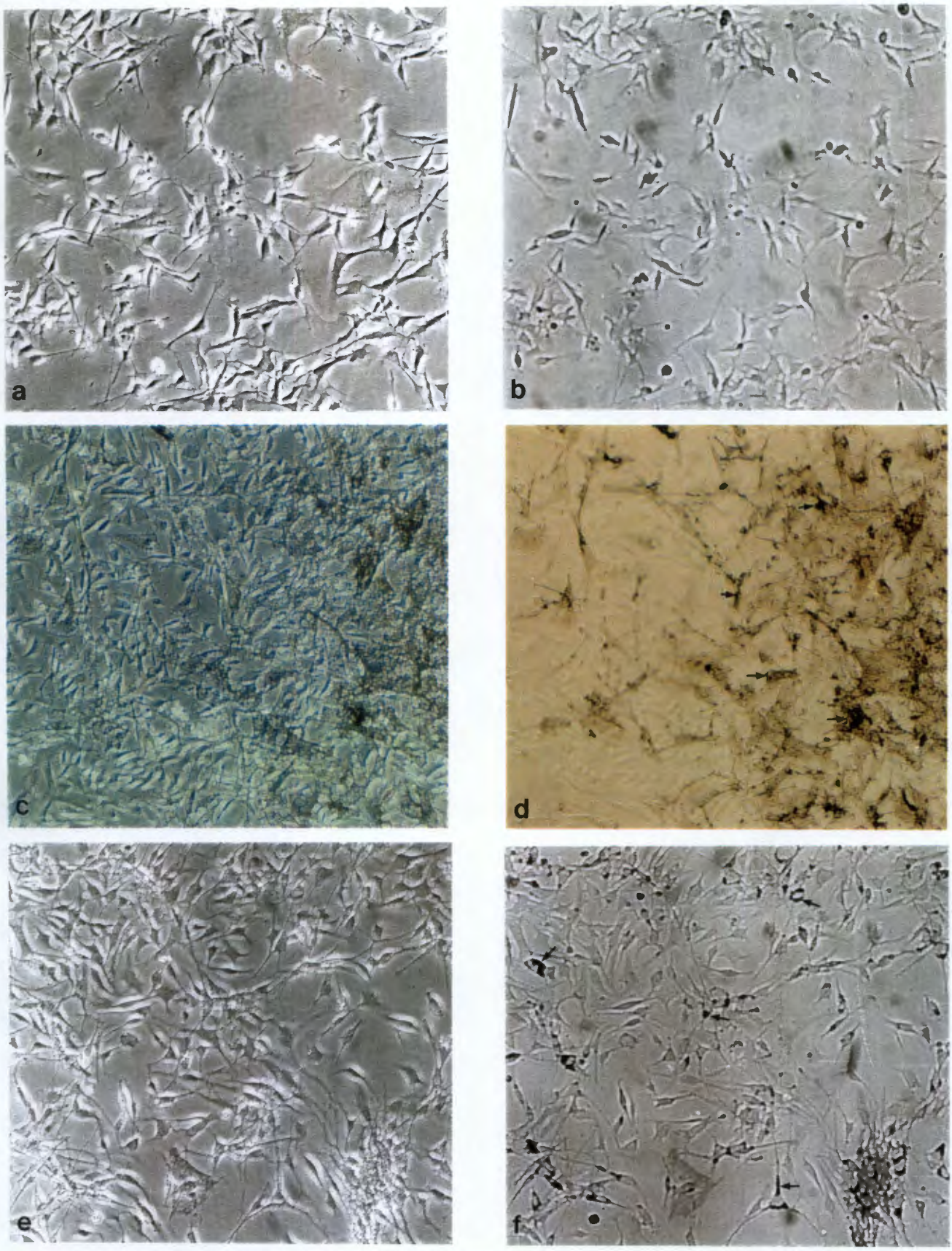


Fig. 3.2. Morphology of clones of melanocytes that have lost their pigmented phenotype during expansion into cell lines. **(a)** A clone of melanocytes that had completely lost their pigmented phenotype (phase contrast). **(b)** Bright field photomicrograph of the same field as (a) showing absence of the melanin pigment. **(c)** A clone consisting of a mixture of pigmented and unpigmented melanocytes (phase contrast). **(d)** Same field as (c) showing only the pigmented cells (bright field). **(e)** Melanocytes of a clone that contain melanin restricted to their perinuclear regions (phase contrast). **(f)** Same field as (e) showing the pigment (bright field).

To confirm that the six selected cell lines expressed the SV40 large T antigen, the cell lines were cultured at 33°C and T antigen expression was detected with a monoclonal antibody directed against the C-terminus of T antigen. The presence of the nuclear T antigen was detected in all six cell lines. An example of the nuclear localization of T antigen in one of the cell lines is shown in Fig. 3.3a. Figure 3.3b shows the absence of nuclear staining in the negative control which was prepared following the same procedure but in which the primary antibody was substituted with 1% FCS in PBS. The presence of T antigen was confirmed by western blot analysis using the same antibody. Figure 3.4 shows that while the dermal lines, DMEL-1, 2 & 3 appeared to have comparable levels of T antigen, these levels were higher than that found in the epidermal lines, EMEL-1, 2 & 3. Interestingly, compared to the EMEL-1 & 3 cell lines, the EMEL-2 cell line had much lower levels of the T antigen.

Southern blotting was used to determine whether the six selected cell lines were clonal. Genomic DNA prepared from the cells was digested with Eco R1 and blots hybridised to a cDNA sequence of the neomycin resistance gene. Since Eco R1 cuts once in the provirus and yields a fragment containing an intact neomycin resistance gene (Fig. 3.5a), analysis of the Eco R1 digest with a probe specific for the neomycin resistance gene should indicate the number of proviral inserts. A single hybridisation band in each cell line indicated that all six cell lines contained a single integrated retroviral genome (Fig. 3.5b) and confirmed that they were clonal. As expected, DNA from the ψ2 cells had a single hybridisation band while DNA from the NIH 3T3 cells (negative control) showed no hybridisation. These results confirmed that the EMEL-1, 2 & 3 and DMEL-1, 2 & 3 cell lines each arose from a single pigmented melanocyte and not a mixture of melanocytes and other cell types.

3.3 T antigen-expressing melanocytes retain the characteristics of fully differentiated melanocytes

To determine the effect of the SV40 large T antigen on melanocyte differentiation, EMEL-1, 2 & 3 and DMEL-1, 2 & 3 cells were cultured at the permissive temperature (33°C) and assessed for their ability to express melanocyte-specific markers.

3.3.1 Epidermal and dermal-derived melanocytes are morphologically normal and actively synthesize melanin

To ascertain the effect of the T antigen on melanocyte morphology, all six cell lines were examined by phase contrast and bright field microscopy. The epidermal melanocyte lines exhibited a morphology typical of cultured epidermal melanocytes: the cells were bipolar, tripolar, or dendritic with dark cytoplasm when examined by phase contrast microscopy (Fig. 3.6a,c,e). Under bright field microscopy, pigment granules were visible within the cytoplasm and dendrites

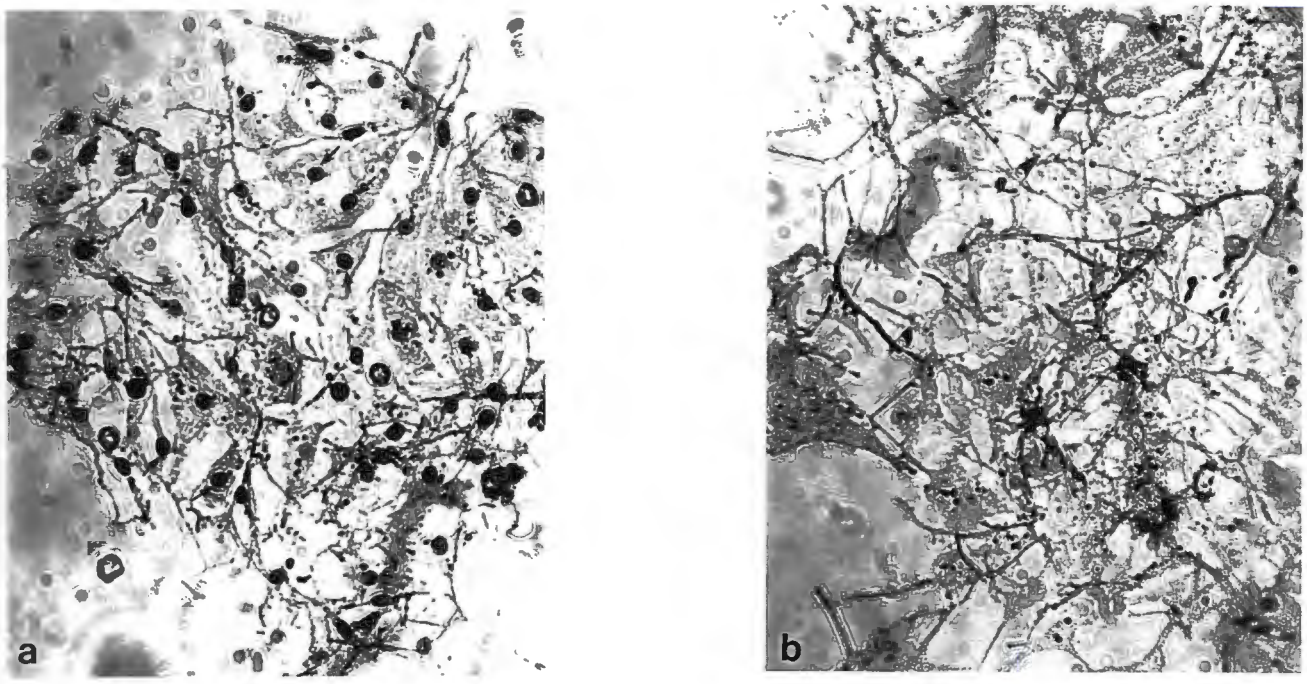


Fig. 3.3. Immunocytochemical detection of the SV40 large T antigen. **(a)** Shows a bright field photomicrograph of DMEL-2 cells immunostained with the monoclonal antibody Pab 101, specific for the large T antigen. Staining was observed in the nuclei of all the cells. **(b)** Staining was not observed in the nuclei of cells treated as in (a) but in which the primary antibody was substituted with 1% serum in PBS.

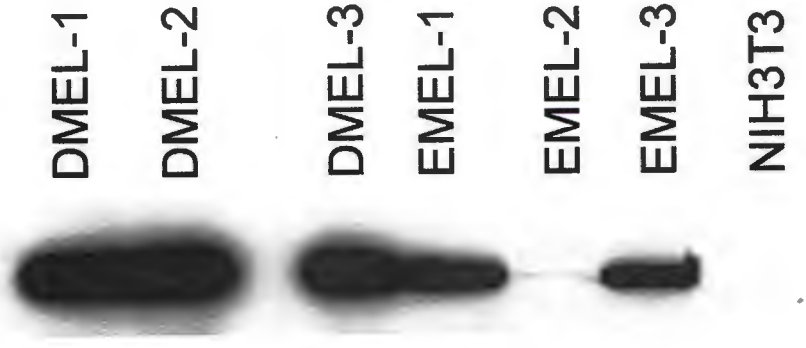


Fig. 3.4. Comparison of levels of T antigen protein in T antigen-expressing melanocytes. T antigen-expressing melanocyte cell lines cultured at 33°C at no more than 10 passages were solubilised in lysis buffer and 12.45 μg of protein from each extract was separated on a 7.5% polyacrylamide gel. After electrotransfer to a nitrocellulose sheet the proteins were stained with the monoclonal anti-SV40 large T antigen antibody (0.5 μg/ml) and visualised by enhanced chemiluminescence (Amersham). NIH 3T3 fibroblasts were used as a negative control.

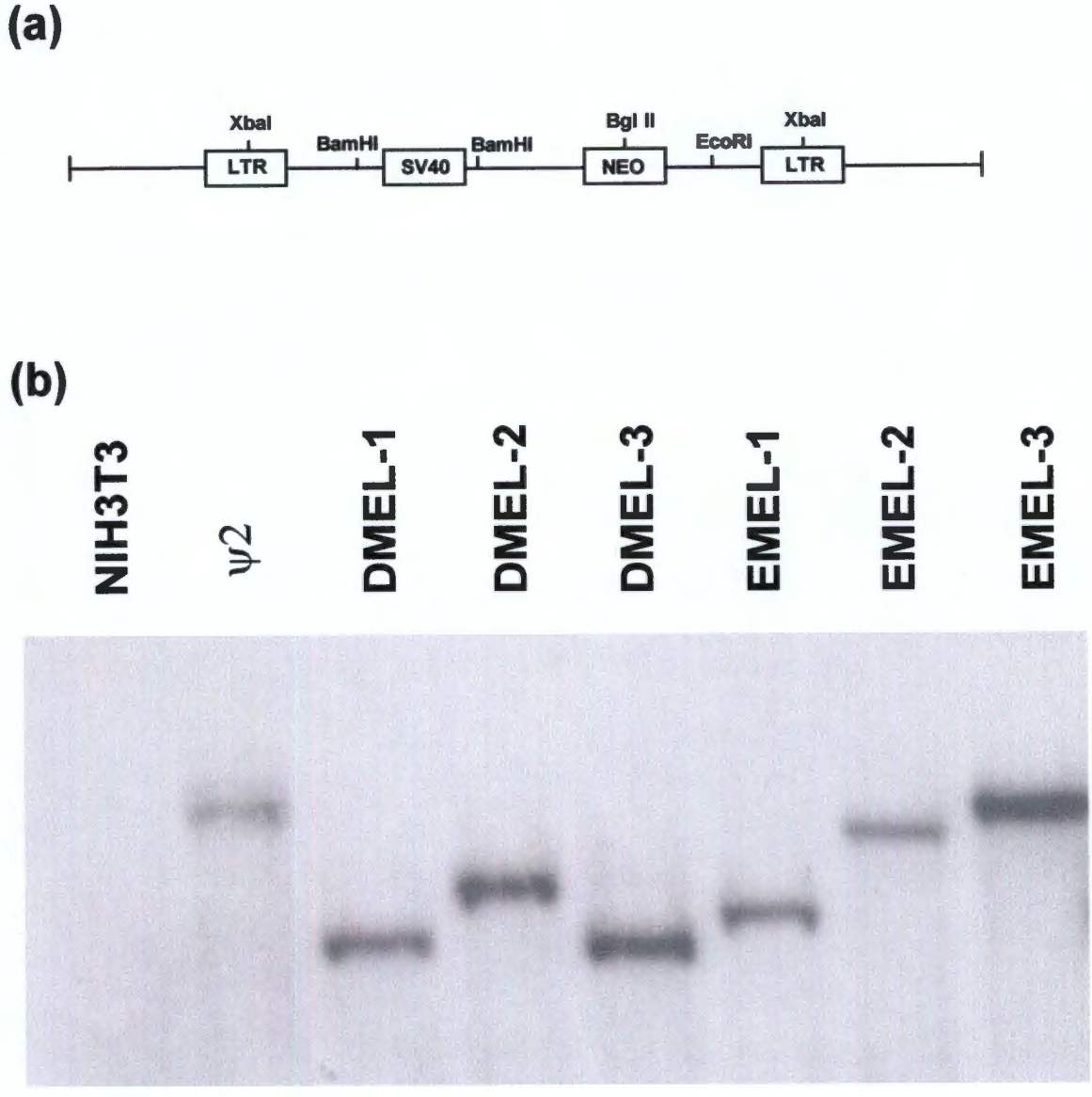


Fig. 3.5. Detection of proviral insertion by Southern blot hybridisation to a radiolabelled neomycin-resistance cDNA probe. **(a)** A schematic representation of the integrated proviral DNA. Since Eco R1 cuts once in the provirus, hybridisation of the Eco R1 digest with the neomycin-resistance (neo) probe should reveal a single band. **(b)** Southern blot analysis shows that all cell lines contain a single retroviral insert. Twenty micrograms of genomic DNA was digested with Eco R1, transferred onto a Nylon+ membrane and hybridised to a ^{32}P-labelled probe covering the neo sequence.

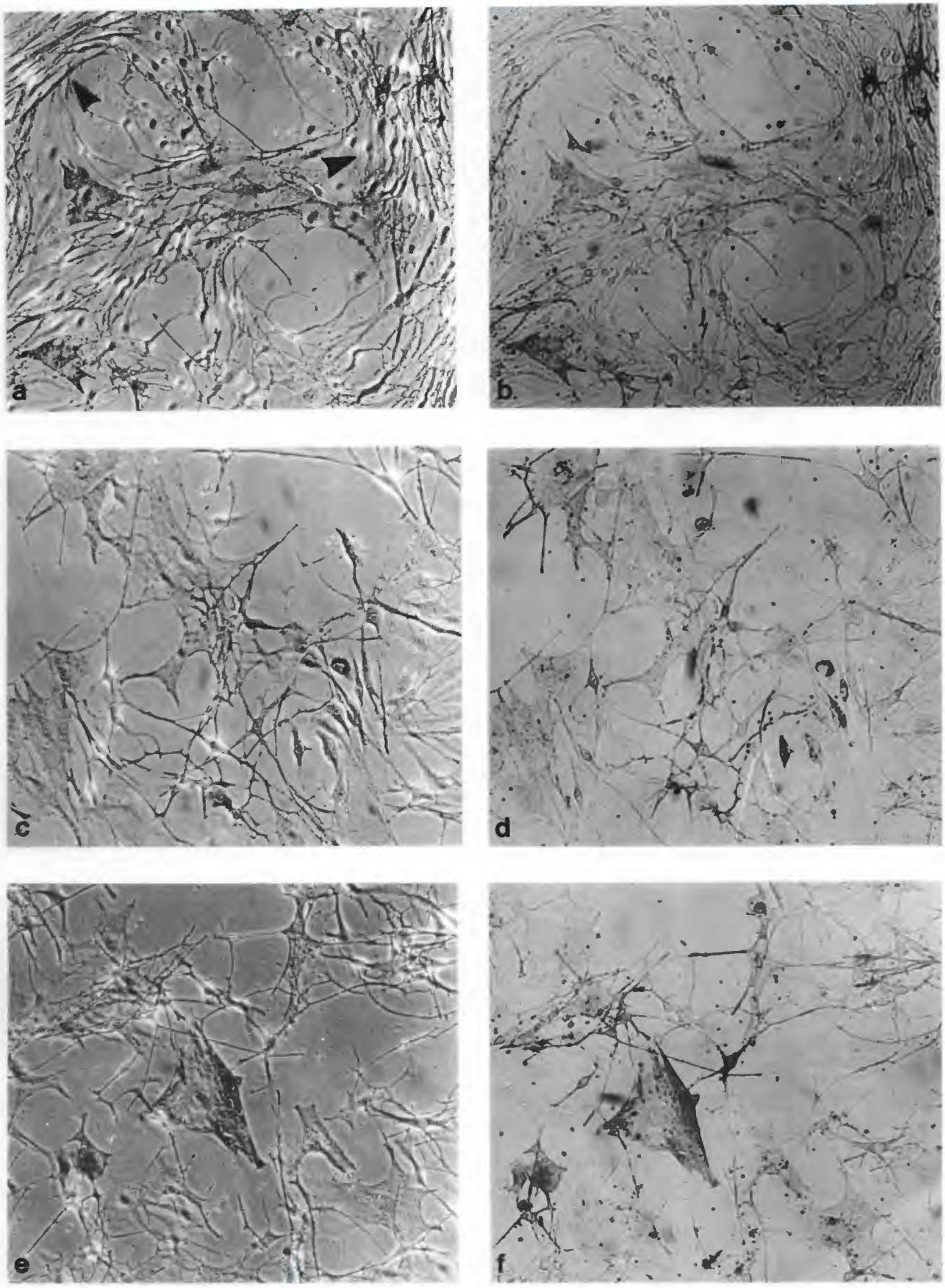


Fig. 3.6. Morphology of semiconfluent cultures of T antigen-expressing epidermal melanocytes. Phase contrast **(a,c,e)** and bright field **(b,d,f)** photomicrographs depict the typical appearance of the epidermal melanocyte lines. **(a)** EMEL-1 at passage 12, **(c)** EMEL-2 at passage 11 and **(e)** EMEL-3 at passage 13. **(b,d,f)** are the same fields as (a,c,e) showing pigment granules in the cytoplasm and dendrites of the cells. Arrowheads depict the elongated appearance of cells at confluence.

of all the cells (Fig. 3.6b,d,f). At confluence, the cells were homogeneous in shape with an elongated appearance (see arrowheads in Fig. 3.6a,b) and although they were closely juxtaposed, they never formed multiple layers.

The dermal melanocyte lines had a morphology different from the epidermal cell lines: the cells were mainly bipolar, typical of dividing melanocytes (Fig. 3.7a,c,e) and visibly pigmented (Fig. 3.7b,d,f). Cell bodies of the dermal cell lines were much smaller than those of the epidermal cell lines (compare Fig. 3.6a & 3.7a). At confluence, they became tripolar and epithelioid (Fig. 3.7g,h) but never formed foci. After six months in culture (approximately passage 10), the EMEL-1, 2 & 3 and DMEL-1, 2 & 3 retained the highly melanotic phenotype of their original counterparts. The above results indicate that at this stage, T antigen expression had no effect on the pigmented phenotype of these cell lines. This was confirmed by assays for melanin synthesis (Table 3.2).

Table 3.2. Melanin synthesis in T antigen-expressing melanocytes at no later than passage 10

Cell lines	^{14}C-Tyrosine incorporation/100 µg protein
ψ2	400.00 ± 52.0
B16	2577.00 ± 16.7
EMEL-1	1580.00 ± 20.3
EMEL-2	2060.00 ± 12.0
EMEL-3	2639.00 ± 16.4
DMEL-1	1825.00 ± 42.0
DMEL-2	1672.00 ± 0.6
DMEL-3	1326.00 ± 39.3

Epidermal cell lines (EMEL-1, 2 & 3) appeared, on average, to be synthesizing melanin at a higher rate than the dermal cell lines (DMEL-1, 2 & 3). Compared to the EMEL-1 & 2 and DMEL-1, 2 & 3 cell lines, the B16 melanoma cells had a higher rate of melanin formation. This result was interesting because the visual examination of the pellets of all the pigmented cell lines used in this assay indicated that B16 melanoma cells were the least pigmented. This suggests that there was no direct correlation between visible pigmentation and melanin synthesis.

3.3.2 Epidermal and dermal melanocytes express tyrosinase, TRP-1, TRP-2 and *mi* genes

The next aim was to examine the effect of the T antigen on the expression of melanocyte-specific genes known to be involved in melanin biosynthesis. Northern blots were prepared and hybridised with specific cDNA probes for mouse tyrosinase, TRP-1 and TRP-2 genes. The same

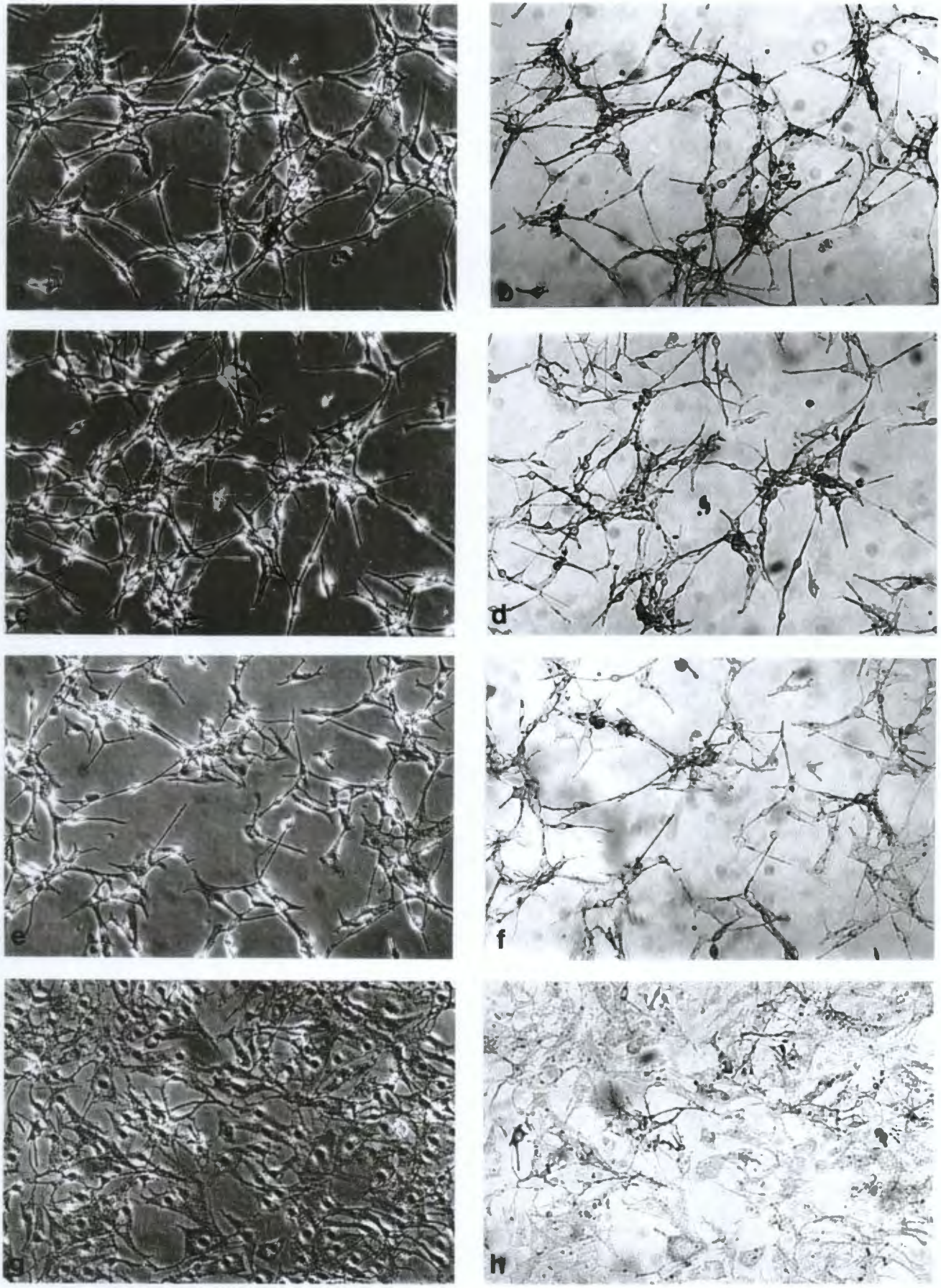


Fig. 3.7. Morphology of cultures of T antigen-expressing dermal melanocytes. Phase contrast **(a,c,e,g)** and bright field **(b,d,f,h)** photomicrographs depict the typical appearance of the dermal melanocyte lines at passage 5. **(a)** DMEL-1, **(c)** DMEL-2 and **(e)** DMEL-3. **(g)** Typical appearance of dermal melanocytes at confluence. **(b,d,f,h)** are the same fields as **(a,c,e,g)** showing pigment granules in the cytoplasm and dendrites of the cells.

filters were stripped and reprobed for all the genes. RNA from B16 melanoma cells and RNA from the ψ2 fibroblast cell line were included on all blots as positive and negative controls and hybridisation with a β-actin cDNA served as a loading control. The results from these experiments are presented in Fig. 3.8.

When hybridised with a mouse tyrosinase cDNA, two transcripts of approximately 2.5 kb and 8.0 kb were detected in all six T antigen-expressing melanocyte cell lines (see arrowheads) and both signals were present even after very high stringency washing. The 2.5 kb and 8.0 kb transcripts were first detected after 9 and 14 days, respectively, of autoradiographic exposure. This suggested that the 8.0 kb transcript was expressed at lower levels than the 2.5 kb transcript. The detection of more than one transcript is consistent with a previous report showing that multiple transcripts of the mouse tyrosinase gene are generated by alternative splicing (Ruppert et al., 1988). As can be seen in Fig. 3.8, the expression of the tyrosinase gene appeared to be highest in the epidermal cell lines and the B16 melanoma cells expressed levels similar to the dermal cell lines. The expression of tyrosinase was melanocyte-specific since no bands were observed in the ψ2 fibroblast RNA.

For TRP-1, a single transcript of approximately 3.7 kb was observed in all six cell lines as well as in the B16 melanoma cells. The TRP-1 transcript could be detected as early as 1 day after autoradiographic exposure indicating that TRP-1 is significantly more abundant than tyrosinase. Similarly, the autoradiographs for TRP-2 were exposed for 1 day and clearly showed a very intense 2.4 kb transcript in all six cell lines indicating a higher prevalence of TRP-2 mRNAs. TRP-2 expression was higher in epidermal than in the dermal cell lines and the B16 melanoma cells expressed TRP-2 mRNAs at levels similar to the dermal cell lines.

To determine whether there was a correlation between the expression of the tyrosinase gene family and *mi* in T antigen-expressing melanocytes, the above northern blots were hybridised with a mouse *mi* cDNA. After 11 days of exposure, a single band of approximately 7.0 kb was obtained in all six cell lines, as well as in B16 melanoma cells (See Fig. 3.8). The long exposure time for the *mi* result could indicate low prevalence of the *mi* mRNA in the above cell lines. Alternatively, it could indicate that stripping the blots repeatedly resulted in some RNA being lost.

The expression of the tyrosinase, TRP-2 and *mi* genes in Fig. 3.8 appeared to be highest in the epidermal melanocyte cell lines. To determine whether this was indeed the case, the levels reflected by these autoradiographs needed to be corrected for (1) RNA loading and (2) the use of separate blots for the epidermal and dermal cell lines. This was estimated as follows: (1) Less

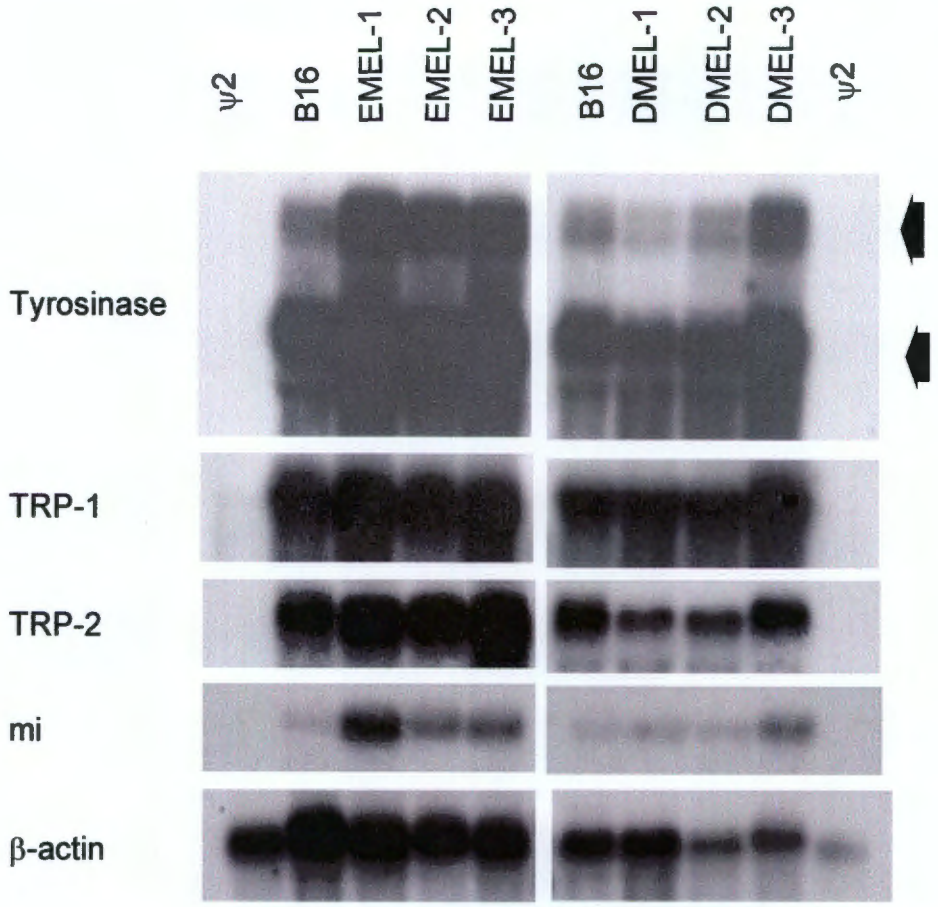


Fig. 3.8. Northern blot analysis of melanocyte-specific gene expression in pigmented T antigen-expressing melanocytes. Total RNA (20 μg) from each sample was separated on a formaldehyde gel followed by electrophoretic transfer onto nitrocellulose membranes. The same blots were repeatedly hybridised to probes specific for tyrosinase, TRP-1, TRP-2, *mi* or for β-actin as a loading control. Arrowheads indicate two tyrosinase transcripts.

exposed autoradiographs were scanned densitometrically and the levels of tyrosinase, TRP-1, TRP-2 and *mi* were calculated relative to β-actin. (2) Each blot contained RNA that was prepared from the same B16 melanoma cells so that values were calculated relative to the respective level of B16melanoma cells in each blot. Bar graphs of these values for EMEL-1, 2 & 3 and DMEL-1, 2 & 3 are shown in Fig. 3.9. The results suggested that, on average, the epidermal cell lines express higher levels of the tyrosinase, TRP-1, TRP-2 and *mi* genes.

3.3.3 Melanocyte-specific proteins are present in T antigen-expressing melanocytes

To determine whether the levels of message correlated to levels of tyrosinase, TRP-1 and TRP-2 proteins in EMEL-1, 2 & 3 and DMEL-1, 2 & 3 cell lines, western immunoblotting was carried out. Extracts from all six cell lines were analyzed using the anitibodies αPEP7, αPEP1 and αPEP8 which recognise tyrosinase, TRP-1 and TRP-2 proteins, respectively (Tsukamoto et al., 1992). NIH 3T3 fibroblasts were included as non-melanocytic negative control cells. The results from these experiments are presented in Fig. 3.10.

Immunoblotting using antibodies specific for the tyrosinase protein revealed an immunoreactive band of approximately 75 kDa in the EMEL-1, 2 & 3 and DMEL-1 & 2 cell lines which was not detected in the DMEL-3 cell line. This band represents the glycosylated form of tyrosinase (Chen et al., 1995; Hearing and Tsukamoto, 1991; Halaban and Moellmann, 1990). The 75 kDa protein was not detected in the NIH 3T3 fibroblasts.

As for the tyrosinase result, antibodies specific to the TRP-1 protein recognised the glycosylated form of TRP-1, which was detected as a major band of approximately 80 kDa band, in the EMEL-1, 2 & 3 and DMEL-1 & 2cell lines but not in the DMEL-3 cell line. It is worth noting that a minor background band of 69 kDa was also detected in the NIH 3T3 fibroblasts. This result is occasionally seen (in NIH 3T3 cells and other non-melanocytes) in other laboratories and it has been suggested that it represents a nonspecific reaction (personal communication, V. Hearing).

Antibodies to TRP-2 identified a major band of approximately 64 kDa in all 6 melanocyte lines. A faint band of approximately 75 kDa was present in the EMEL-1, 2 & 3 and DMEL-1 cell lines but was undetectable in the DMEL-2 and 3 cell lines. Since the 64 kDa protein probably represents the de novo form of TRP-2, these results suggest that all 6 cell lines contain high levels of the TRP-2 protein. Shorter exposures of the autoradiograms revealed that this protein was absent in NIH 3T3 fibroblasts. However, longer exposures of the autoradiograms indicated that this protein was present at low levels in the NIH 3T3 fibroblasts.

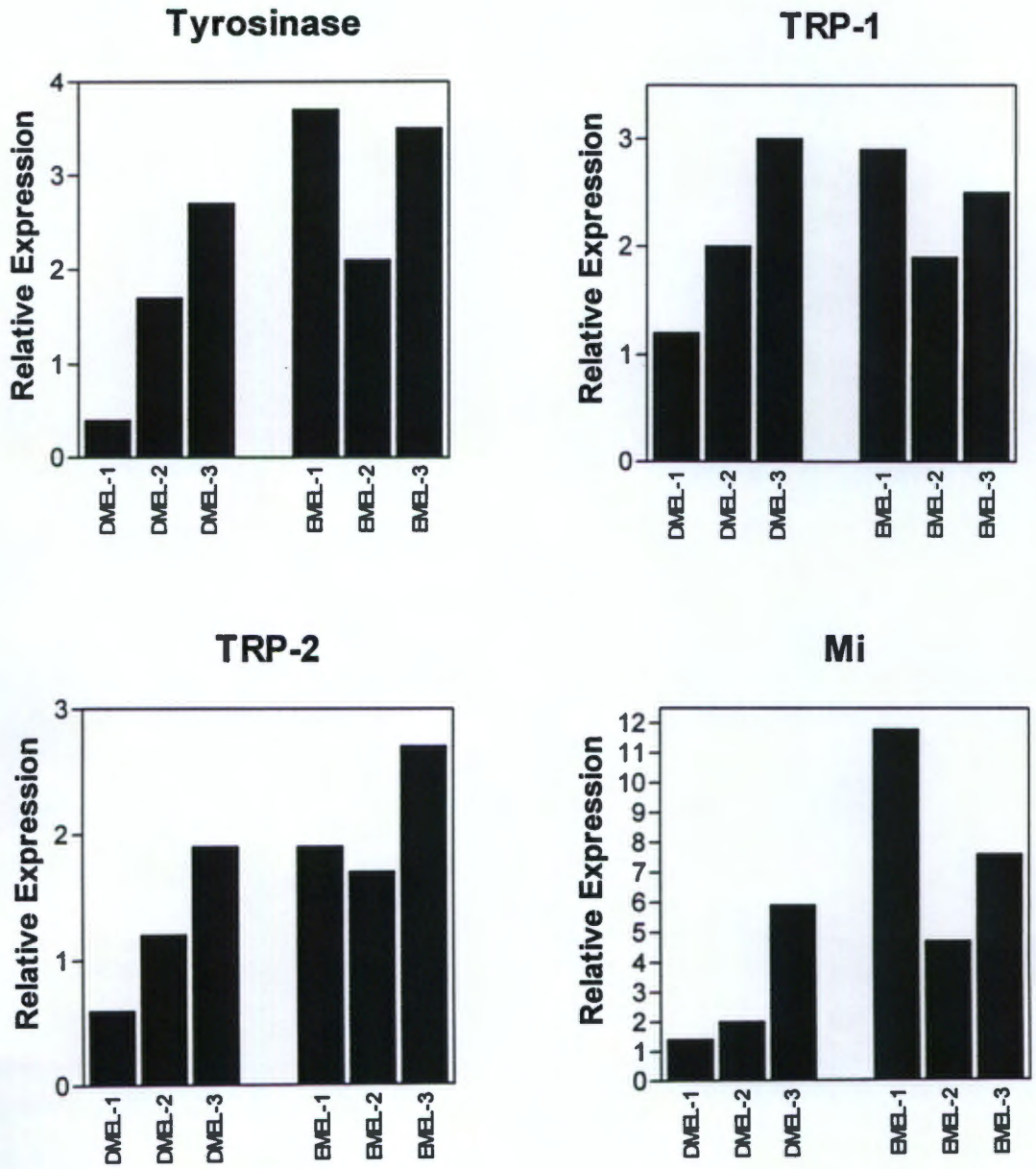


Fig. 3.9. Expression of tyrosinase, TRP-1, TRP-2 and *mi* in epidermal (EMEL-1, EMEL-2 and EMEL-3) and dermal (DMEL-1, DMEL-2 and DMEL-3) cell lines. Densitometric scans of the autoradiographs in Fig. 3.8 were standardised to β-actin and are indicated as a ratio of expression relative to B16 melanoma cells.

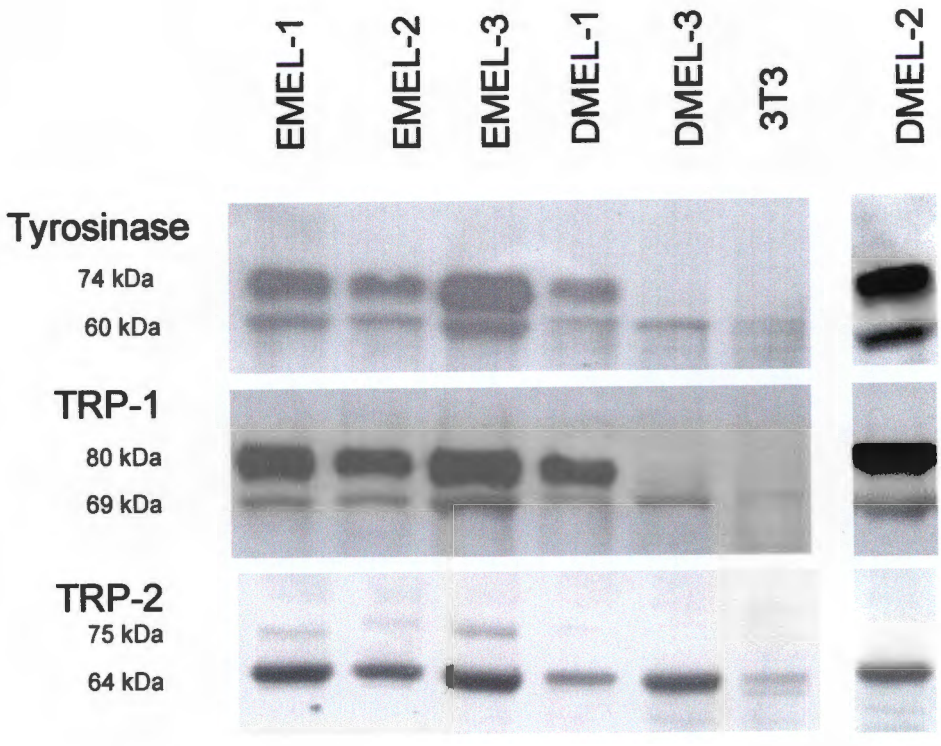


Fig. 3.10. Western immunoblot analysis of levels of melanocyte-specific proteins. T antigen-expressing melanocyte cell lines cultured at 33°C were solubilised in lysis buffer and 20 μg protein from each extract was separated on SDS gels. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to tyrosinase (αPEP7), TRP-1 (αPEP1) or TRP- 2 (αPEP8) and the bands were visualised by chemiluminescence. Molecular weight sizes (in kilodaltons) of the proteins are indicated on the left. NIH 3T3 cells were used as a negative control.

3.4 Proliferation of T antigen-expressing melanocytes is stimulated by, but not dependent on TPA

Previous studies have shown that in general, normal mouse melanocytes in culture have obligatory growth requirements for TPA and a natural growth factor provided by serum. In contrast, transformed melanocytes proliferate independently of TPA and growth factors required by normal melanocytes. When the EMEL-1, 2 & 3 and DMEL-1, 2 & 3 cell lines were originally established from primary melanocyte cultures, they were grown in melanocyte-specific medium (MSM) which contained TPA, human placental extract and 20% fetal calf serum. To evaluate the effect of these individual growth-promoting additives on T antigen-expressing melanocytes, the growth of DMEL-2 cells was assessed in the following media:

i) MSM = basic medium (Ham's F10 and IBMX) + 48 nM TPA + 50 μg protein/ml human placental extract + 20% FCS
ii) MSM - HPE = MSM without human placental extract
iii) MSM - TPA = MSM without TPA
iv) MSM2% = MSM with 2% instead of 20% FCS

DMEL-2 cells were selected for detailed analysis since all six cell lines were shown to exhibit similar melanocyte-specific characteristics. The results of this experiment are illustrated in Fig. 3.11. When the growth of the cells was compared in MSM and MSM - HPE, there was no difference in the proliferation rate of the cells. HPE was, however, retained in the culture medium to ensue consistency in experimental conditions. Cells grown in MSM - TPA revealed that the absence of TPA did not lead to quiescence of the cells but it did result in decreased proliferation as compared to cells grown in MSM. This result suggested that while TPA was not an obligatory mitogen for T antigen-expressing melanocytes, it was able to stimulate the growth of these cells. To determine the relative importance of serum in the culture medium, the growth of DMEL-2 cells was compared in MSM and MSM2%. Lowering the serum concentration in the medium from 20% to 2%, reduced cell numbers by almost 50%. Since MSM2% contains TPA, it can only be concluded that 2% serum was sufficient for growth (albeit slower growth) when TPA was present in the culture medium.

3.5 TPA stimulates melanogenesis

3.5.1 TPA enhances the dendritic pigmented phenotype of melanocytes

While investigating the growth of the DMEL-2 cells in different media (as described above), differences in the morphology of the cells were noticed: Cells grown in medium without TPA

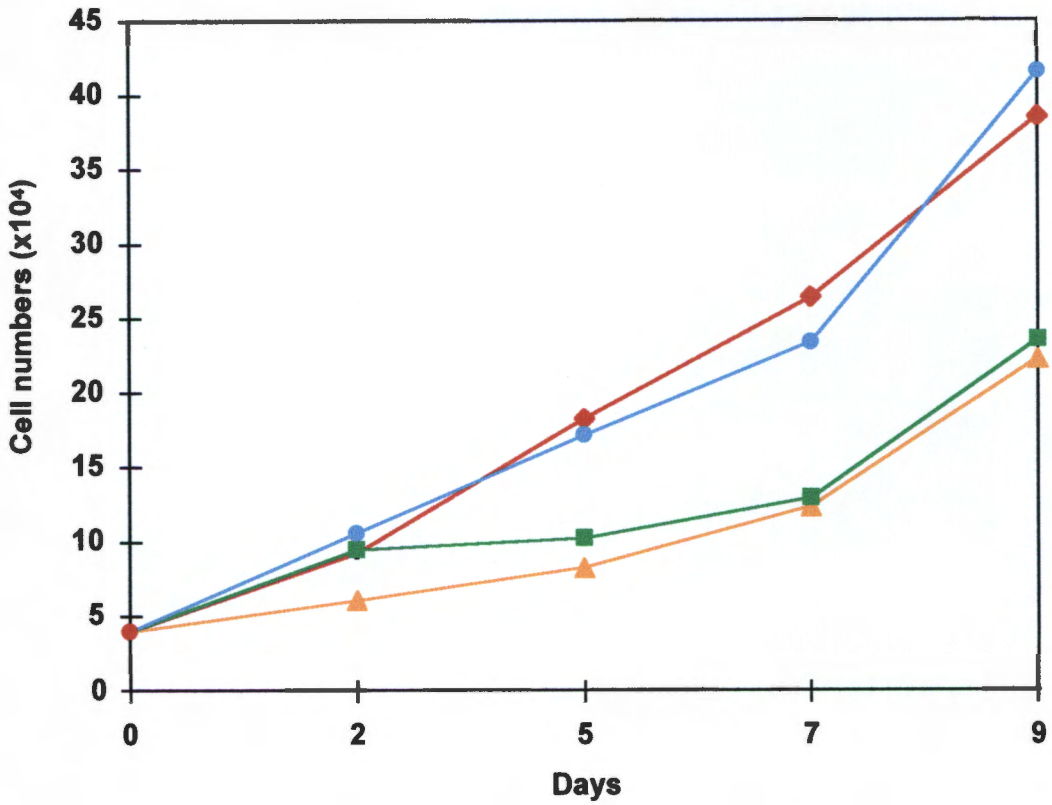


Fig 3.11. Growth kinetics of DMEL-2 cells cultured at 33°C in MSM (blue line), MSM-HPE (red line), MSM-TPA (orange line) and MSM2% (green line). Each value is the average of duplicate wells. The standard error between duplicate wells ranged between 5 and 15%.

became polygonal, lost their dendritic morphology (Fig. 3.12a) and exhibited minimal pigmentation when examined by bright field microscopy (Fig. 3.12b). In contrast, cells grown in medium with TPA were dendritic and pigmented (Fig. 3.12c,d). Furthermore, the removal of TPA from the medium resulted in a 5.3-fold decrease in melanin content of these cells (Table 3.3). There was only a slight decrease of 1.7- and 1.4-fold in melanin content when either human placental extract was excluded from the medium or, when the serum concentration was dropped from 20% to 2%, respectively.

Table 3.3. Effect of TPA, HPE and FCS on melanin content in DMEL-2 cells

Samples	OD_{475}	Fold increase above 3T3 fibroblasts
3T3 fibroblasts	0.028 ± 0.001	1.0
MSM	0.403 ± 0.005	14.4
MSM - HPE	0.296 ± 0.003	10.6
MSM - TPA	0.076 ± 0.003	2.7
MSM2%	0.278 ± 0.005	9.9

3.5.2 TPA treatment results in increased levels of tyrosinase,TRP-1 and TRP-2 protein levels

To further explore the effect of TPA on pigmentation, the levels of the melanogenic proteins were measured in TPA-treated and untreated DMEL-2 cells. Western immunoblotting of extracts of DMEL-2 cells, grown at 33°C in either the presence or absence of 48 nM TPA for 24 days, was carried out using the antibodies, αPEP7, αPEP1 and αPEP8. Protein extracts from melan-a cells were included on all blots as positive experimental controls. The results are presented in Fig. 3.13.

Immunoblotting, using antibodies specific for tyrosinase, revealed the presence of two immunoreactive proteins of approximately 60 kDa and 75 kDa in TPA-treated DMEL-2 cells (lane 3). These two proteins, which were also detected in melan-a cells (lane 1) represent the *de novo* and mature glycosylated forms of the protein, respectively. When TPA was removed from the medium, the 60 kD protein was detected at similar levels to those found in TPA-treated DMEL-2 cells but the 75 kDa protein was completely absent (lane 4). These results suggest that TPA stimulated the synthesis of tyrosinase (because the total amount of tyrosinase protein was higher in TPA-treated cells).

Similarly, using antibodies specific to the TRP-1 protein, two immunoreactive proteins (69 kDa and 80 kDa) were detected in TPA-treated DMEL-2 cells (lane 3) and in melan-a cells (lane 1).

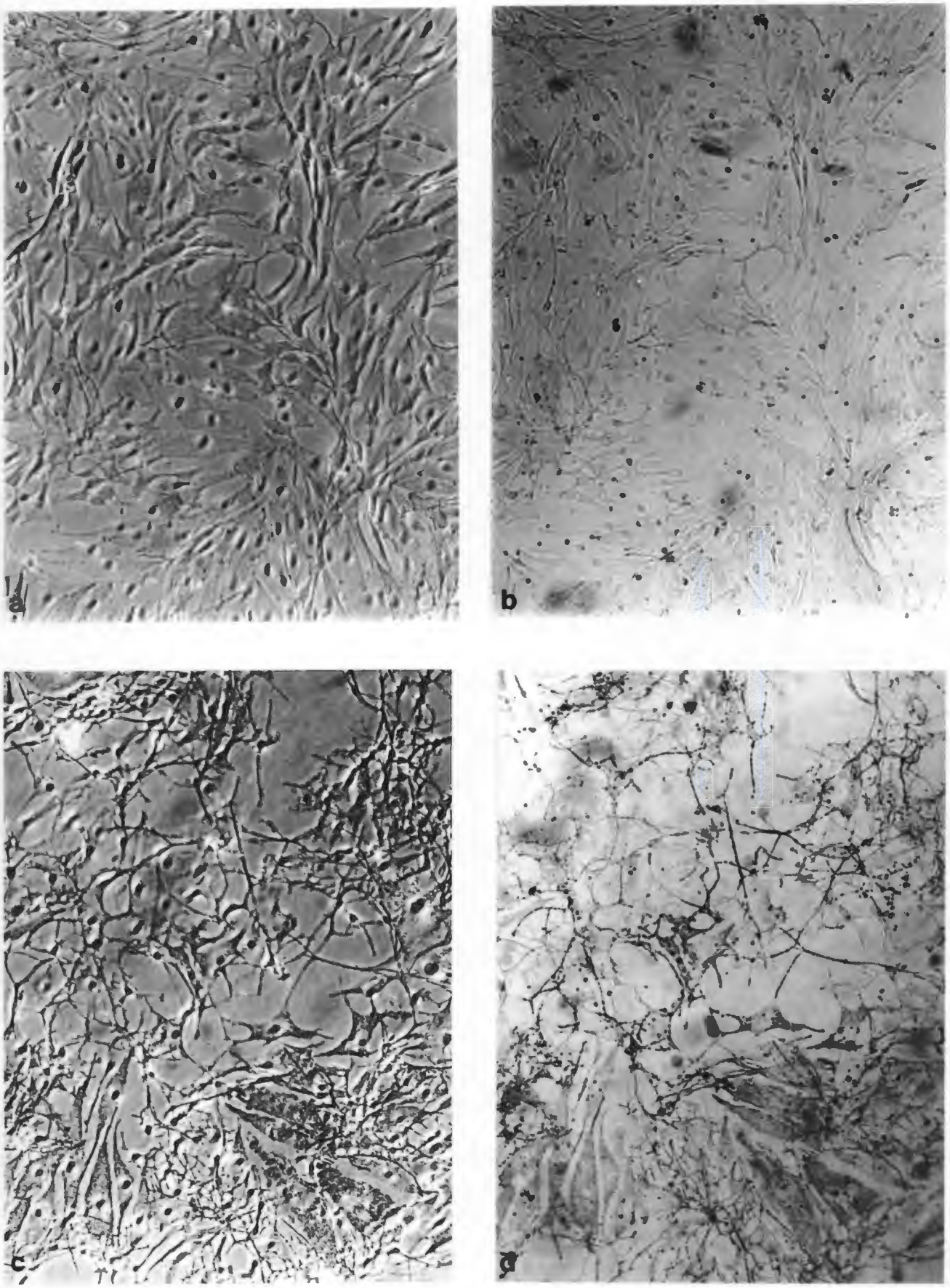


Fig. 3.12. Morphology of DMEL-2 cells cultured in the absence **(a,b)** or presence **(c,d)** of TPA. Phase contrast micrographs of DMEL-2 cells grown in medium in which TPA was withdrawn for 14 days **(a)** and in medium containing 48 nM TPA **(c)**. **(b,d)** are bright field micrographs of the same fields as (a,c) showing the absence or presence of pigment granules, respectively.

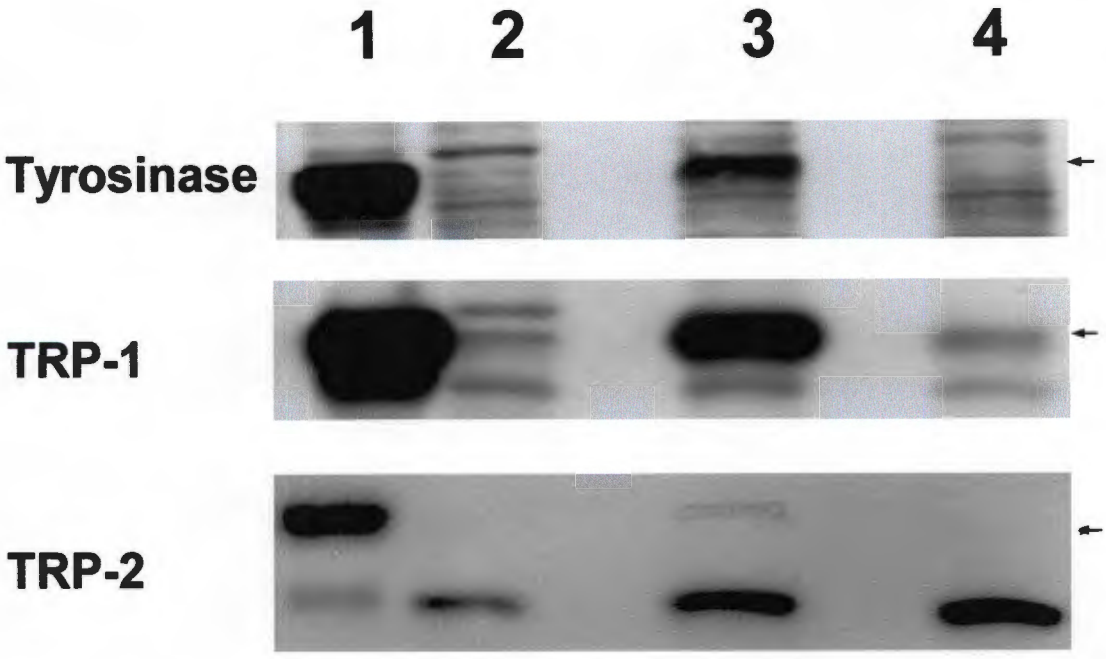


Fig. 3.13. Western immunoblot analysis of levels of melanocyte-specific proteins in TPA treated and untreated DMEL-2 cells. The cells were solubilized in lysis buffer and 30 μg protein from each extract was separated on 7.5% SDS gels. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to tyrosinase (αPEP7), TRP-1 (αPEP1) or TRP-2 (αPEP8) and the bands visualised by chemiluminescence. Lane 1, melan-a cells (positive control); lane 2, NIH 3T3 fibroblasts (negative control); lanes 3 and 4, DMEL-2 cells cultured at 33°C with or without 48 nM TPA, respectively.

The glycosylated form of TRP-1 was dramatically lower in cells cultured in TPA-free medium (lane 4). Likewise, TPA-treated and untreated cells expressed similar levels of a 69 kDa protein that reacted with antibodies specific for TRP-2 (compare lanes 3 and 4). The glycosylated form of the TRP-2 protein was detected very faintly in TPA-treated cells and was not detectable in untreated cells. These results suggest that TPA was able to stimulate the synthesis of the TRP-1 and TRP-2 proteins. Interestingly, unlike the T antigen-expressing DMEL-2 cells, a very intense band corresponding to the glycosylated form of TRP-2 was detected in melan-a cells (lane 1).

3.5.3 The effect of TPA on tyrosinase, TRP-1 and TRP-2 protein levels is not dose-dependent

Previously, it was reported that TPA caused a dose-related stimulation of tyrosinase activity in normal human melanocytes (Chao-Hsing, 1991; Abdel-Malek et al., 1992). Western blot analysis was therefore used to compare the levels of tyrosinase, TRP-1 and TRP-2 proteins in DMEL-2 cells grown at 33°C in either 48 nM TPA or 200 nM TPA. These two concentrations were selected because they are the most frequently used in procedures published for the culture of primary and immortalised mouse melanocytes. Figure 3.14 shows that there was no appreciable difference in the amount of tyrosinase, TRP-1 and TRP-2 proteins present in DMEL-2 cells grown in either 48 nM TPA (lane 1) or 200 nM TPA (lane 4). These results suggest that 48 nM TPA is possibly a saturating dose.

3.5.4 T antigen does not modulate the effect of TPA on tyrosinase, TRP-1 and TRP-2 protein levels

Because oncoproteins are known to alter the differentiation programme of melanocytes, the next question asked was whether the T antigen could modulate the effect of TPA on levels of tyrosinase and TRP-1 proteins. To answer this question DMEL-2 cells were grown at the permissive (33°C) and non-permissive (37°C) temperatures for 3 and 5 days in either 48 nM TPA or 200 nM TPA. As can be seen in Fig. 3.14, DMEL-2 cells grown in 48 nM TPA at either 33°C (lane 1) or 37°C for 3 and 5 days (lanes 2 & 3 respectively) produced similar levels of tyrosinase, TRP-1 and TRP-2 proteins. Similarly, a comparison of the levels of tyrosinase, TRP-1 and TRP-2 protein in cells grown in 200 nM TPA at 33°C (lane 4) or 37°C for 3 and 5 days (lanes 5 & 6 respectively) revealed that there were no appreciable differences in the levels of these proteins. Therefore, the T antigen did not appear to alter the responsiveness of DMEL-2 cells to TPA.

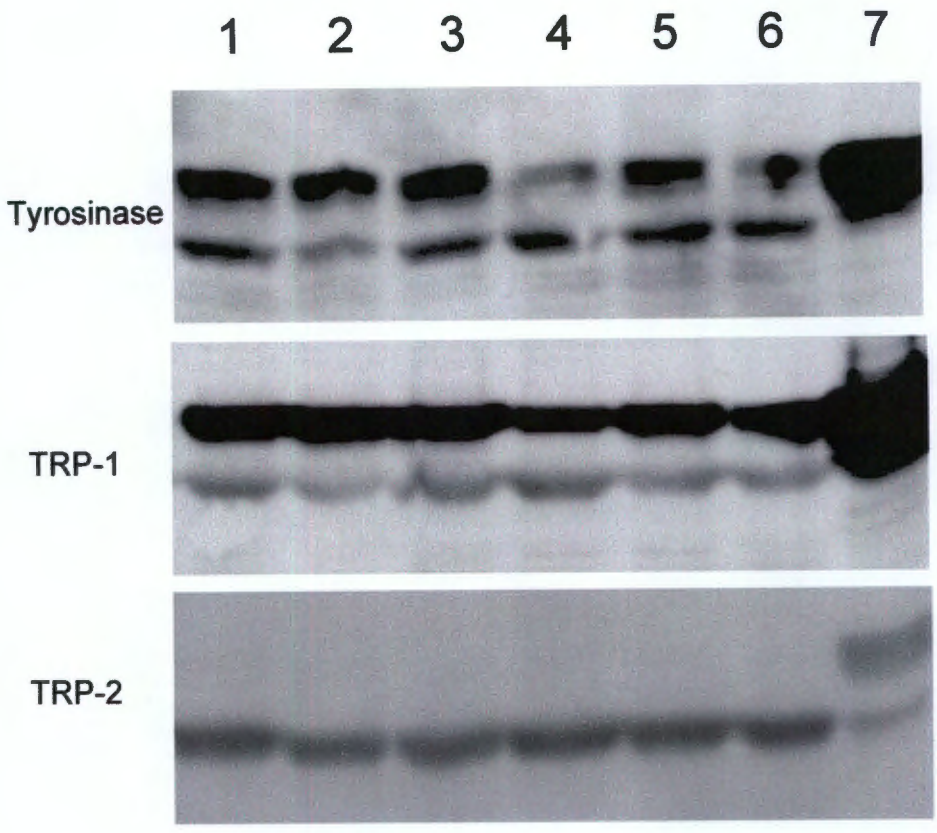


Fig. 3.14. Western immunoblot analysis of levels of melanocyte-specific proteins in DMEL-2 cells cultured at 33°C and 37°C in either 48 nM TPA or 200 nM TPA. The cells were solubilized in lysis buffer and 38 μg protein from each sample was separated on 7.5% SDS gels. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to tyrosinase (αPEP7), TRP-1 (αPEP1) or TRP-2 (αPEP8) and the bands were visualised by chemiluminescence. Lanes 1, 2 and 3, DMEL-2 cells grown in 48 nM TPA at either 33°C or, 37°C for 3 and 5 days, respectively; lanes 4, 5 & 6, DMEL-2 cells grown in 200 nM TPA at either 33°C or, 37°C for 3 and 5 days, respectively; lane 7, melan-a cells (positive control).

3.5.5 Expression of tyrosinase, TRP-1 and TRP-2 genes are co-ordinately up-regulated by TPA

Northern blot analysis was performed to determine whether the increase in the levels of tyrosinase, TRP-1 and TRP-2 proteins in response to TPA treatment resulted from an increase in the levels of the encoding mRNA. A northern blot was prepared using total RNA from DMEL-2 cells cultured in the presence or absence of 48 nM TPA at 33°C for 10 and 17 days. Figure 3.15 (lanes 2 and 4) shows that DMEL-2 cells, grown in the presence of TPA, expressed both tyrosinase transcripts of approximately 2.5 kb and 8.0 kb, whereas no detectable tyrosinase mRNA was found in cells grown in the absence of TPA for 10 (lane 3) and 17 (lane 5) days.

Similarly, a 3.7 kb TRP-1 transcript was observed in DMEL-2 cells grown with TPA (lane 2 and lane 4) and this signal decreased the longer the cells were grown without TPA (lane 3 (10 days) and lane 5 (17 days)). Likewise, an intense 2.4 kb TRP-2 transcript was detected in lanes containing RNA from TPA-treated DMEL-2 cells (lanes 2 and 4). When the cells were cultured without TPA, the amount of TRP-2 mRNA decreased after 10 days (lane 3) and was further lowered to undetectable levels after 17 days (lane 5).

3.5.6 TPA increases *mi* expression

To investigate the mechanism by which TPA increased tyrosinase, TRP-1 and TRP-2 mRNA levels, the level of expression of the *mi* gene was determined. As can be seen in Fig. 3.15, DMEL-2 cells treated with TPA expressed high levels of *mi* (lanes 2 and 4) and there was no detectable expression in untreated cells (lanes 3 and 5). These results indicate that TPA could be stimulating melanin synthesis by stimulating *mi* expression and consequently increasing the expression of the tyrosinase, TRP-1 and TRP-2 genes. To explore the possibility that TPA is acting via Pax3 to increase *mi* expression, northern blot analysis was performed. A northern blot containing the same samples as described in section 3.5.5 was probed with a mouse *Pax3* cDNA and no detectable *Pax3* transcripts were found in cells cultured in either the presence or absence of TPA (results not shown).

3.6 The SV40 large T antigen induces loss of melanocyte-specific markers in DMEL-3 cells which are re-expressed at the non-permissive temperature

3.6.1 DMEL-3 cells gradually acquire morphological features typical of transformed melanocytes

Following six months of continual passage (approximately passage 10) at 33°C, the DMEL-3 cells, unlike the DMEL-1 and 2 cell lines, gradually lost their differentiated phenotype. At

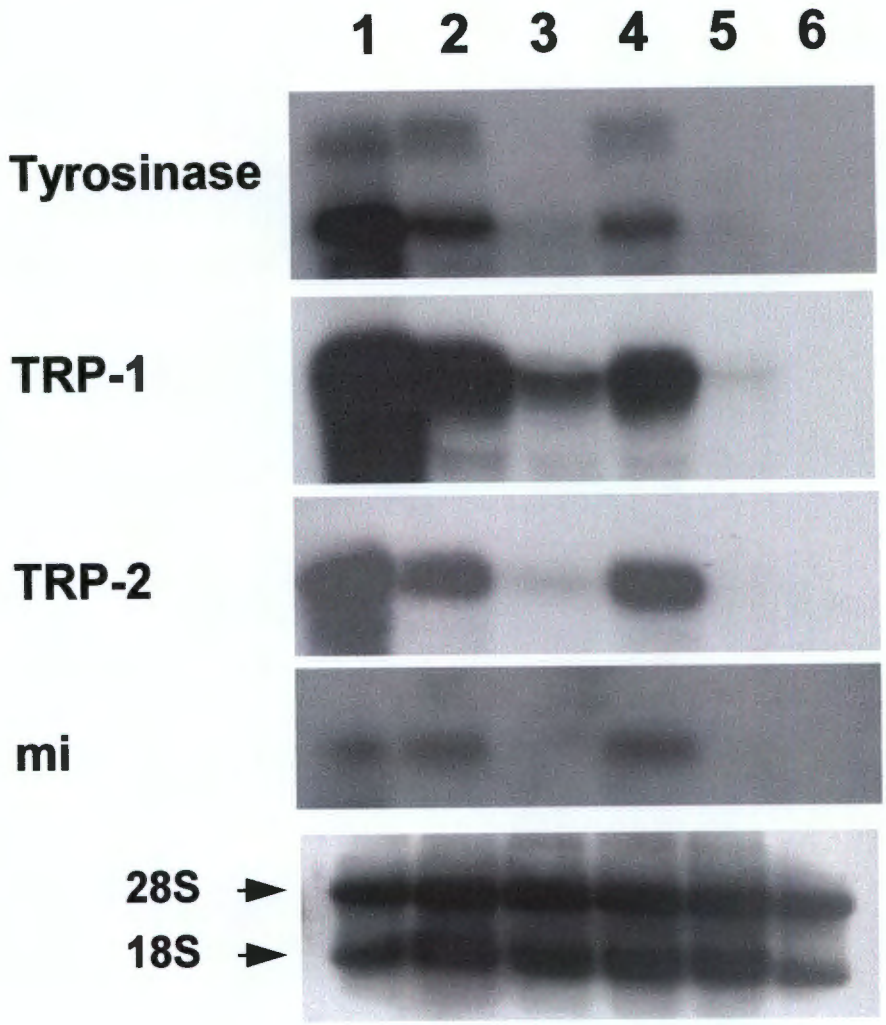


Fig. 3.15. Northern blot analysis of melanocyte-specific gene expression in DMEL-2 cells cultured at 33°C with or without 48 nM TPA. Total RNA (20 μg) from each sample was separated on a formaldehyde gel followed by electrophoretic transfer onto nitrocellulose membranes. The same blot was repeatedly hybridised to probes specific for tyrosinase, TRP-1, TRP-2 or *mi*. Ethidium bromide staining of the 28S and 18S rRNAs shows the quality and quantity of the RNAs loaded. Lane 1, B16 melanoma cells (positive control); lanes 2 and 4, TPA-treated DMEL-2 cells; lanes 3 and 5, DMEL-2 cells cultured without TPA for 10 and 17 days, respectively; lane 6, ψ2 fibroblasts (negative control).

passage 10, the DMEL-3 cells were much less pigmented and dendritic (Fig. 3.16a,b) than cells of the same cell line at earlier passages (see Fig. 3.7e,f). Eventually, at passage 17, the cells displayed the characteristic morphology of a transformed cell line: that is, they were mostly unpigmented and larger with fewer dendrites (Fig. 3.16c,d). However, they did not form colonies in soft agar assays nor did they form foci in culture.

To ascertain whether the T antigen was directly involved in the de-differentiation process, it was investigated whether reversion to the normal melanocyte morphology could be achieved by inactivating the oncoprotein at the non-permissive temperature. The approach was thus to culture passage 10 and 17 DMEL-3 cells at both the permissive (33°C) and non-permissive (37°C) temperatures and to compare the morphology of the cells using light microscopy. After 3 days of incubation at 37°C, the morphology of passage 10 cells had visibly changed. The cells reverted to a more dendritic and visibly pigmented phenotype and some of the cell bodies became smaller in size (Fig. 3.16e,f). When passage 17 cells were transferred to 37°C, the cells became more dendritic but, in contrast to passage 10 cells, they did not visibly repigment (even after 21 days at this temperature). It is possible, however, that partial reversion had occurred but that the changes were not detectable microscopically. To explore this question further, a more detailed biochemical and molecular analysis was carried out on DMEL-3 cells, and by way of comparison, on DMEL-2 cells. (The DMEL-2 cell line was selected as a representative of the five cell lines which did not depigment).

3.6.2 Depigmentation in DMEL-3 cells results from the co-ordinate down-regulation of tyrosinase, TRP-1 and TRP-2 gene expression

Passage 13 or 14 DMEL-2 cells grown at 33°C and at 37°C for 2 and 4 days, and passage 15 or 17 DMEL-3 cells grown at 33°C and at 37°C for 2, 4 and 21 days, were assayed for their ability to synthesize melanin. The assay was carried out on three separate occasions in triplicate with different passages and are therefore presented separately as cpm/100 μg protein and as a percentage of B16 melanoma cell activity (Table 3.4).

Results showed that, compared to B16 melanoma cells, DMEL-3 cells cultured at 33°C had low levels of melanin synthesis activity. At 37°C, however, melanin synthesis was stimulated in a time-dependent manner. Compared to DMEL-3 cells, the DMEL-2 cells showed much higher melanin synthesis activity at 33°C which also increased at 37°C. The above results indicate that although DMEL-3 cells did not visibly repigment within 21 days at 37°C, they did regain the ability to synthesize melanin at this temperature. A similar pattern of results was obtained when each of the melanogenic enzymes was assayed separately. For these assays, passage 17

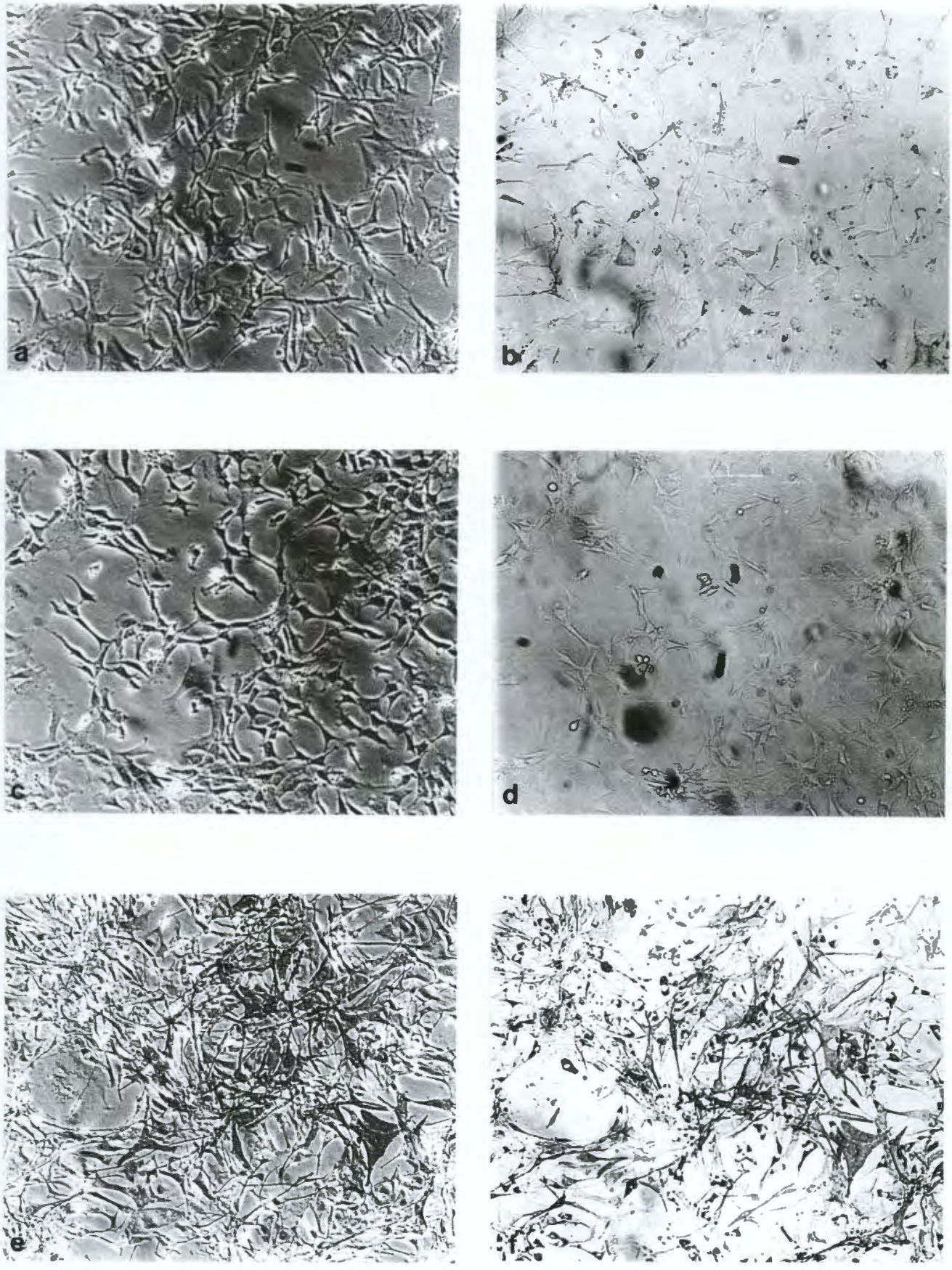


Fig. 3.16. Morphology of DMEL-3 cells cultured at 33°C and 37°C. Figures **a,c,e** are phase contrast photomicrographs and **b,d,f** are the equivalent bright field photomicrographs. They show DMEL-3 cells cultured at 33°C at passage 10 **(a,b)** and passage 17 **(c,d)** and at 37°C at passage 10 **(e,f)**.

Table 3.4. Melanin formation in DMEL-2 and 3 cells cultured at 33°C and 37°C

Cell lines	Assay 1		Assay 2		Assay 3	
	cpm/100 μg protein	% activity of B16 cells	cpm/100 μg protein	% activity of B16 cells	cpm/100 μg protein	% activity of B16 cells
ψ2 Fibroblasts	1066 ± 171	31	893 ± 252	63	1146	31
B16 melanoma cells	3428 ± 324	100	1421± 62	100	3700	100
DMEL-2 (33°C)	ND	ND	1043 ± 27	74	2238	61
DMEL-2 (37°C for 2 days)	ND	ND	1310 ± 5	92	2406	65
DMEL-2 (37°C for 4 days)	ND	ND	859 ± 17	61	2422	66
DMEL-3 (33°C)	1333 ± 219	39	624 ± 41	44	1242	34
DMEL-3 (37°C for 2 days)	1550 ± 72	45	721 ± 3	51	1341	36
DMEL-3 (37°C for 4 days)	2246 ± 324	66	1092 ± 44	77	1752	47
DMEL-3 (37°C for 21 days)	1998 ± 181	58	1213 ± 68	85	2049	55
ND Not determined						

DMEL-3 cells were cultured at 33°C and 37°C for 4 and 21 days and then assayed for tyrosinase (tyrosine hydroxylase), TRP-1 (DHICA oxidase) and TRP-2 (DOPAchrome tautomerase) activity. The results of the tyrosine hydroxylase and DOPAchrome tautomerase assays are shown in Table 3.5 and are expressed in pmol/μg protein/hour. The results for DHICA oxidase activity was too low to be detected by this assay and they were therefore considered unreliable for analysis.

Table 3.5. Melanogenic activities of DMEL-3 cells at 33°C and 37°C

CELLS	TYROSINE HYDROXYLASE pmol/μg protein/hr	DOPACHROME TAUTOMERASE pmol/μg protein/hr
DMEL-3 (33°C)	1.0	1.6
DMEL-3 (37°C for 4 days)	1.7	4.9
DMEL-3 (37°c for 21 days)	2.0	30.2
melan-a (37°C)	6.1	14.7

Taken together, the above results suggest that the inhibition of melanin synthesis in passage 17 DMEL-3 cells cultured at 33°C results from low levels of tyrosinase, TRP-1 and TRP-2 activity. To determine whether these decreases were due to down-regulation of gene activity, northern blot analyses were carried out to compare tyrosinase, TRP-1 and TRP-2 expression in passage 17 DMEL-3 cells cultured at the permissive (33°C) and non-permissive (37°C) temperatures.

DMEL-2 cells grown at 33°C and 37°C were also included for comparative analyses. Fig. 3.17A (lane 2) shows that the DMEL-2 cells grown at 33°C expressed two tyrosinase transcripts of approximately 2.5 kb and 8.0 kb. The level of both tyrosinase transcripts increased slightly when DMEL-2 cells were grown at 37°C for 2 days (lane 3) and 4 days (lane 4). In contrast, Fig. 3.17B, lane 2, shows that no detectable tyrosinase mRNA was found in DMEL-3 cells grown at 33°C but a faint signal corresponding to a 2.5 kb transcript was detected in DMEL-3 cells cultured at 37°C for 2 days (lane 3). The intensity of the signal increased the longer the cells were cultured at 37°C (lane 4 (4 days) and lane 5 (21 days)). A comparison of the TRP-2 gene expression in DMEL-2 and 3 cells at 33°C and 37°C is shown in Fig. 3.17A and B, respectively. A very intense 2.4 kb transcript was obtained in DMEL-2 cells at 33°C (lane 2) and the intensity of this transcript increased slightly when DMEL-2 cells were cultured at 37°C for 2 days (lane 3) and 4 days (lane 4). In contrast, DMEL-3 cells showed a weak signal at 33°C (lane 2) and the intensity of the signal increased with time when the cells were cultured at 37°C for 2 days (lane 3), 4 days (lane 4) and 21 days (lane 5). An analysis of TRP-1 gene expression in DMEL-2 cells at 33°C and 37°C is shown in Fig. 3.17A. A single transcript of 3.7 kb was observed in DMEL-2 cells grown at 33°C (lane 2) and this signal increased slightly when the cells were grown at 37°C for 2 days (lane 3) and 4 days (lane 4). While TRP-1 expression was not determined for DMEL-3 cells at 33°C, Fig. 3.17C. (lane 3) shows a faint signal corresponding to a 3.7 kb transcript in DMEL-3 cells cultured at 37°C for 2 days. The intensity of this signal increased, the longer the cells were cultured at 37°C (lanes 4 (4 days) and 5 (21 days)).

Tyrosinase, TRP-1 and TRP-2 transcripts were detected in B16 melanoma cells but were completely absent in the ψ2 fibroblast cell line.

3.6.3 *mi* expression is down-regulated in depigmented DMEL-3 cells

To shed light on the mechanism by which the downregulation of melanocyte-specific genes might occur at the permissive temperature (33°C), the level of expression of the microphthalmia gene was determined. Given the possibility that stripping blots repeatedly could be resulting in loss of RNA (as mentioned on page 50) and thus inaccurately reflecting low levels of *mi* expression, new northern blots were prepared using the same batch of RNA that was probed for the expression of the tyrosinase, TRP-1 and TRP-2 genes. As can be seen in Fig. 3.18A, the DMEL-2 cells expressed high levels of *mi* at 33°C and, as with tyrosinase, TRP-1 and TRP-2, there was only a slight increase at 37°C. A comparison of the level of *mi* expression obtained in this experiment (for DMEL-2 and B16 melanoma cells) to that obtained in Fig 3.8 would indeed suggest that large amounts of RNA are lost during stripping of northern blots. In contrast, Fig. 3.18B shows that in DMEL-3 cells, low levels of *mi* message were detected in cells cultured at

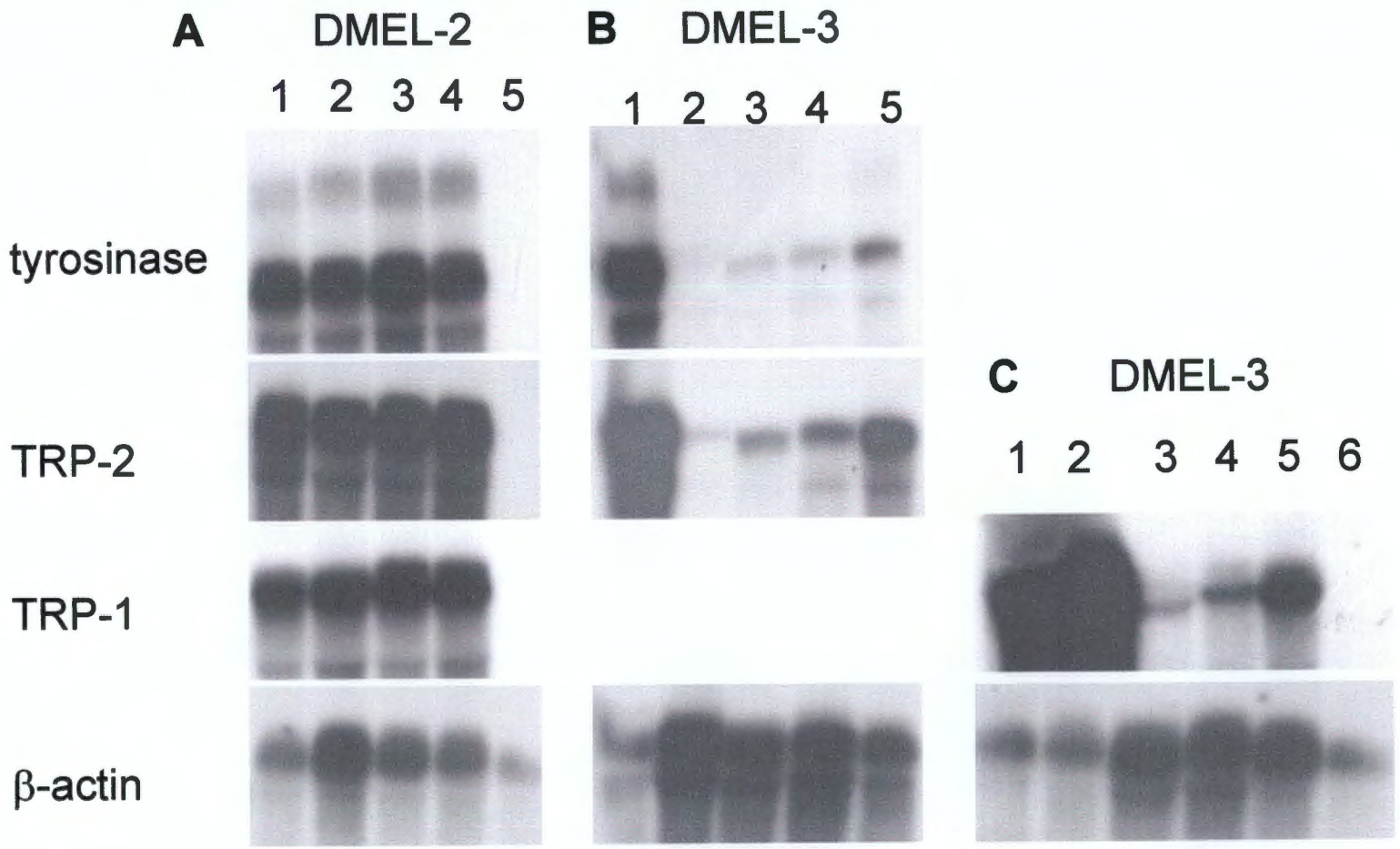


Fig. 3.17. Northern blot analysis of melanocyte-specific gene expression in DMEL-2 and 3 cells cultured at 33°C and 37°C. The same blots were repeatedly hybridised to probes specific for tyrosinase, TRP-1, TRP-2 or β-actin as a loading control. An exception is the blot containing RNA from DMEL-3 cells probed with TRP-1. Total RNA (20 μg) of each sample was separated on a formaldehyde gel followed by electrophoretic transfer onto nitrocellulose membranes. In **A**: lane 1, B16 melanoma cells; lane 2, 3 & 4, passage 13 DMEL-2 cells cultured at 33°C and, 37°C for 2 and 4 days, respectively; lane 5, ψ2 fibroblasts. In **B**: lane 1, B16 melanoma cells; lanes 2, 3, 4 & 5, passage 17 DMEL-3 cells cultured at 33°C and, 37°C for 2, 4 & 21 days respectively. In C: lane 1, B16 melanoma cells; lane 2, passage 8 DMEL-3 cells; lanes 3, 4 & 5, passage 17 DMEL-3 cells cultured at 37°C for 2, 4 and 21 days, respectively; lane 6, ψ2 fibroblasts.

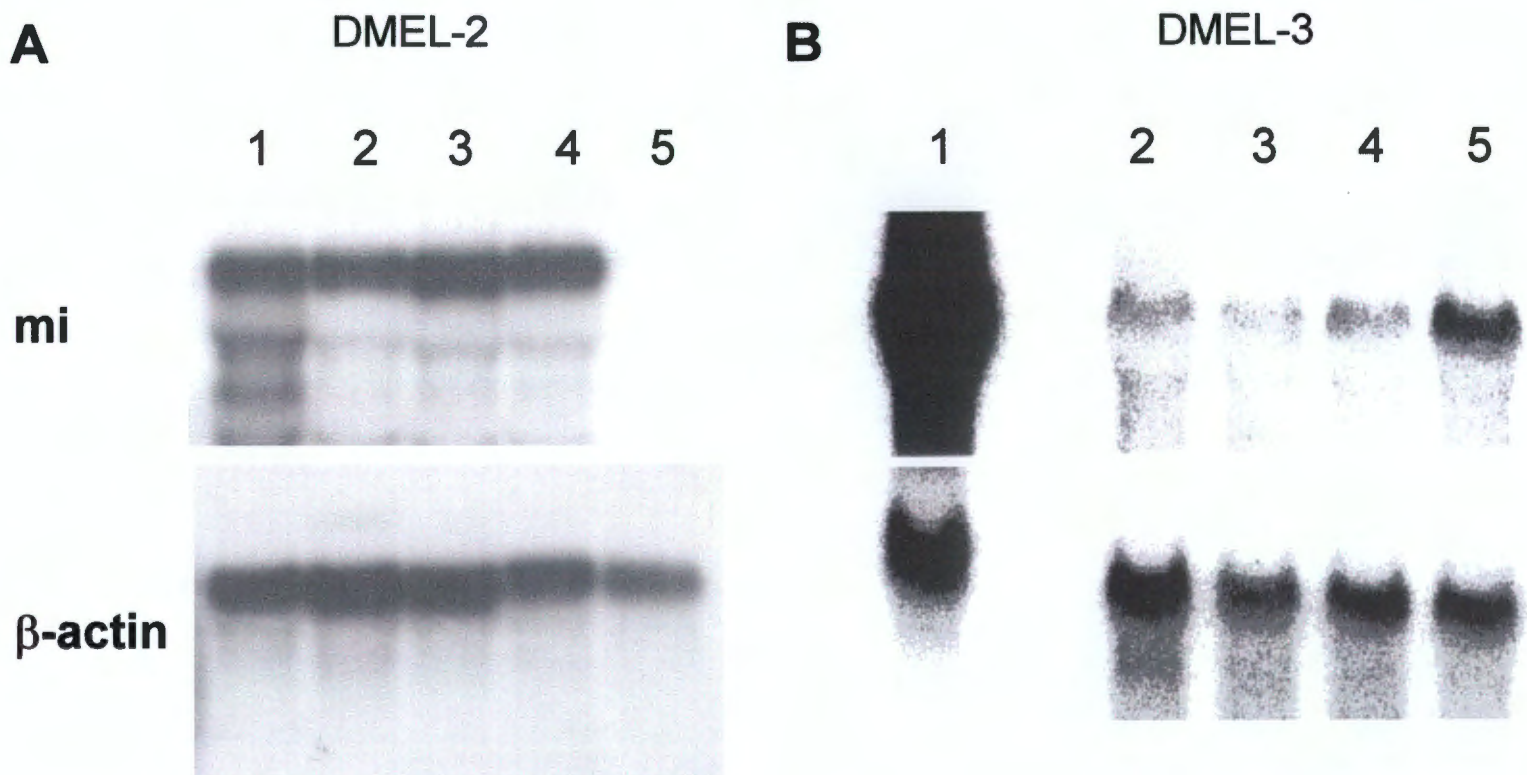


Fig. 3.18. Northern blot analysis of microphthalmia expression in DMEL-2 and 3 cells cultured at 33°C and 37°C. Total RNA (20 μg) of each sample was separated on a formaldehyde gel followed by electrophoretic transfer onto nitrocellulose membranes. In **A**: lane 1, B16 melanoma cells; lane 2, 3 & 4, passage 13 DMEL-2 cells cultured at 33°C and, 37°C for 2 and 4 days, respectively; lane 5, ψ2 fibroblasts. In **B**: lane 1, melan-a cells; lanes 2, 3, 4 & 5, passage 17 DMEL-3 cells cultured at 33°C and, 37°C for 2, 4 & 21 days, respectively.

33°C. After only 4 days of culture at 37°C, mi message began to increase. The ratio of *mi* expression relative to β-actin expression was calculated from the phospho-analyzer readings and the following ratios were obtained: at 33°C, 1:1; at 37°C for 2, 4 and 21 days, 1:1.1; 1:1.2 and 1:2.4, respectively. These results show that *mi* gene expression is down-regulated in DMEL-3 cells cultured at 33°C and that the level of its expression begins to increase after 4 days at 37°C. There was therefore a direct correlation between the re-expression of the *mi* gene at 37°C and that of the tyrosinase, TRP-1 and TRP-2 genes.

The above results raised the possibility that one mechanism whereby the T antigen might downregulate expression of the tyrosinase gene family is by repressing *mi* expression. If this was the case, then ectopic expression of *mi* in DMEL-3 cells cultured at 33°C should rescue the expression of endogenous tyrosinase, TRP-1 and TRP-2 genes. DMEL-3 cells were therefore transfected with an expression vector encoding a *mi* cDNA. To facilitate the selection of stable transfectants, a plasmid encoding the puromycin resistance gene (pBabepuro) was co-transfected into the cells. DMEL-3 cells transfected with only the pBabepuro plasmid and untransfected DMEL-3 cells, served as controls. A week after the cells were grown in medium supplemented with 2.5 μg/ml puromycin, a significant percentage of cells detached from the culture surfaces of all dishes and died. This mortality was initially most noticeable in untransfected dishes. However, puromycin-resistant colonies of cells soon appeared in both transfected and untransfected dishes. A similar pattern of results was obtained for concentrations of puromycin ranging from 2.5 μg/ml to 7.5 μg/ml. Since the survival of untransfected and "transfected" DMEL-3 cells was similar in medium supplemented with various concentrations of puromycin, this experiment was abandoned.

It should be pointed out that before the above transfection experiments were performed, a dose-curve was constructed to determine what concentration of puromycin was required to kill untransfected DMEL-3 cells (results not shown). The cells were plated in 35 mm dishes at a density of 6×10^4 cells/dish and duplicate dishes were treated with the following concentrations of puromycin over a period of 10 days (media containing the appropriate concentration of puromycin were renewed every 3 days): 0 μg/ml; 0.625 μg/ml; 1.25 μg/ml; 2.5 μg/ml; 5.0 μg/ml and 10 μg/ml. Results from the dose curve suggested that the cells were sensitive to puromycin at concentrations above and including 2.5 μg/ml. It would therefore appear that DMEL-3 cells, cultured for prolonged periods in the presence of puromycin, developed drug resistance.

3.6.4 *Pax3* expression is not detected in DMEL-2 and 3 cells

To investigate the mechanism by which *mi* expression is down-regulated at the permissive temperature (33°C), northern blot analysis was performed to determine the level of *Pax3* gene expression. Northern blots were prepared using the same batch of RNA that was probed for the expression of the *microphthalmia* gene. *Pax3* expression could not be detected in DMEL-3 cells cultured at either the permissive or non-permissive temperatures (results not shown). Similarly, *Pax3* transcripts were completely absent in pigmented DMEL-2 and melan-a cells (results not shown). These results suggested that *Pax3* was not expressed in "normal" and T antigen-expressing melanocytes and that there was therefore no direct correlation between the expression of the *mi* gene and that of the *Pax3* gene.

3.7 Melanogenesis is not favoured at 37°C

At this stage of the study it was considered important to confirm that DMEL-3 cells were re-expressing melanocyte-specific markers at the non-permissive temperature (37°C) due to the inactivation of the T antigen and not because melanogenesis is favoured at this temperature. If the latter was the case, one would predict that the levels of the melanogenic proteins and their enzymatic activities would be higher at 37°C than at 33°C. To test this hypothesis, a study was done using melan-a cells, an immortalised mouse melanocyte cell line in which properties of normal melanocytes are retained. The cells were grown at 37°C and 33°C for 18 days, solubilized, and the proteins used for both western blot analysis and enzymatic assays for tyrosinase and TRP-2 activity.

Western blots showed that the levels of tyrosinase and TRP-1 proteins were higher in cells grown at 33°C (Fig. 3.19). In contrast, the levels of TRP-2 protein were not influenced by the temperature at which the cells were cultured. Furthermore, the cells grown at 33°C exhibited almost twice the tyrosine hydroxylase activity of cells grown at 37°C (Table 3.6). Similarly, DOPAchrome tautomerase activity was stimulated 1.5 fold in melan-a cells grown at 33°C compared to 37°C. These results demonstrate that, if anything, melanogenic activity was higher at 33°C than at 37°C.

Table 3.6. Melanogenic activities of melan-a cells at 33°C and 37°C

CELLS	TYROSINE HYDROXYLASE pmol/μg protein/hr	DOPACHROME TAUTOMERASE pmol/μg protein/hr
melan-a cells (33°C)	11.9	22.0
melan-a cells (37°C)	6.1	14.7

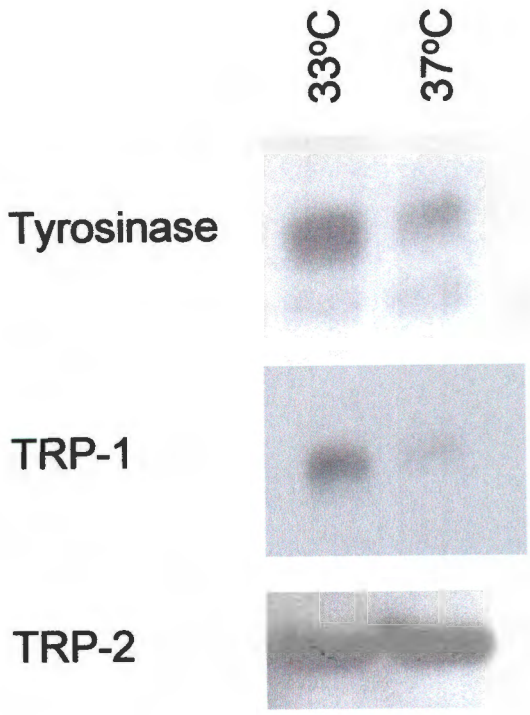


Fig. 3.19. Western blot analysis of levels of melanocyte-specific proteins in melan-a cells cultured for 18 days at 33°C and 37°C. The cells were solubilized in lysis buffer and 25 μg of protein from each sample was separated on 7.5% SDS gels. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to tyrosinase (αPEP7), TRP-1 (αPEP1) or TRP-2 (αPEP8) and the bands visualised by chemiluminescence.

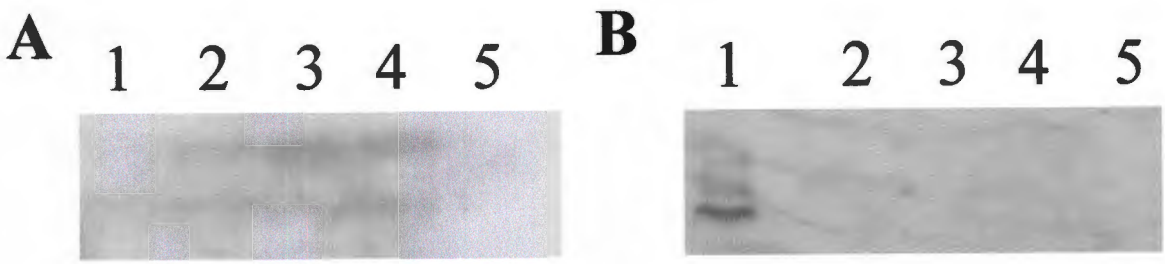


Fig. 3.20. Northern blot analysis of c-kit expression in DMEL-2 and 3 cells cultured at 33°C and 37°C. In **A**: lane 1, B16 melanoma cells; lanes 2, 3 & 4, passage 13 DMEL-2 cells cultured at 33°C, and 37°C for 2 and 4 days, respectively; lane 5, ψ2 fibroblasts. In **B**: lane 1, melan-a cells; lanes 2, 3, 4 & 5, passage 17 DMEL-3 cells cultured at 33°C and, 37°C for 2, 4 and 21 days, respectively.

3.8 Does T antigen expression affect signalling pathways known to regulate melanogenesis?

3.8.1 *c-kit* is expressed in pigmented DMEL-2 cells but not in unpigmented DMEL-3 cells

To establish whether the T antigen affects *c-kit* expression in DMEL-2 and DMEL-3 cells, blots previously probed with *mi* were rehybridised to a *c-kit* cDNA. Fig. 3.20A shows the results of a blot containing total RNA from DMEL-2 cells grown at 33°C and 37°C, probed for *c-kit* expression. Two transcripts of approximately 8.2 kb and 5.8 kb were detected in DMEL-2 cells grown at 33°C (lane 2) and the levels of both transcripts increased slightly when these cells were grown at 37°C for 2 days (lane 3) and 4 days (lane 4). A single transcript of approximately 6 kb was detected in melan-a cells only (lane 1, Fig. 3.20B) and no signal was obtained for DMEL-3 cells grown at either 33°C or 37°C.

In a repeat experiment, a northern blot of poly(A)$^+$ RNA from DMEL-3 cells grown at 33°C and at 37°C for 4, 16 and 30 days revealed once again an absence of *c-kit* transcripts in DMEL-3 cells in all cases (even after 30 days at 37°C when the cells had visibly repigmented) (not shown). Consistent with this result, c-Kit protein was not detected by western blot analysis with the monoclonal antibody, ACK2, which recognises c-Kit (not shown). These results suggest that *c-kit* expression is repressed in DMEL-3 cells grown at the permissive temperature (33°C) and that it is not re-expressed when the T antigen is inactivated at 37°C.

3.8.2 α-MSH stimulates melanogenic activities in DMEL-3 cells grown at 33°C and 37°C

To determine whether DMEL-3 cells were able to respond to α-MSH and whether such responsiveness was altered by T antigen expression, passage 14 DMEL-3 cells were grown at 33°C and 37°C in melanocyte-specific medium in the presence and absence of 0.2 μM α-MSH for 4 days, with daily changes of the medium. The cells were then harvested and their melanogenic activities measured.

Following MSH treatment of DMEL-3 cells at 33°C and 37°C, it was noticed that morphological changes had occurred. At 33°C, both untreated control cells and treated cells were visibly unpigmented. However, unlike the untreated cells, some MSH-treated cells became dendritic (compare Figs 3.21a & b). This observation was interesting because it suggested that even at the permissive temperature (33°C), MSH was able to induce dendrite formation, a key feature of melanocyte differentiation. As expected, both treated and untreated dishes cultured at 37°C contained pigmented cells. There was, however, a significant increase in the number of pigmented cells after MSH treatment (compare Figs 3.21c & d). This observation suggested that

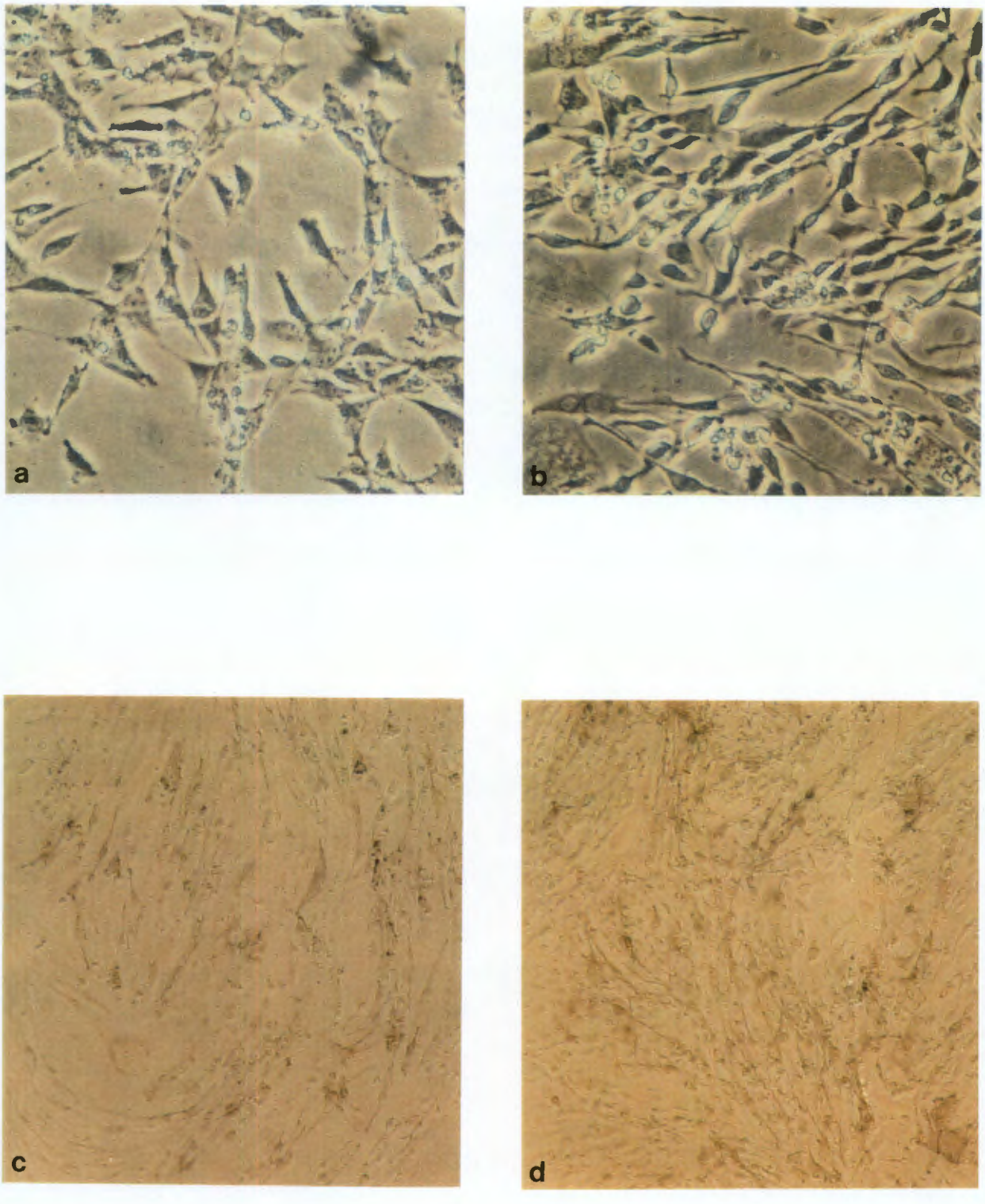


Fig. 3.21. Phenotype of DMEL-3 cells cultured at 33°C and 37°C in either the absence or presence of α-MSH for 4 days. Phase contrast photomicrographs show the morphology of DMEL-3 cells cultured at 33°C in the absence **(a)** or presence **(b)** of α-MSH. Bright field photomicrographs show colour due to black melanin granules in DMEL-3 cells cultured at 37°C in the absence **(c)** or presence **(d)** of α-MSH.

MSH was able to stimulate pigment synthesis in DMEL-3 cells grown at the non-permissive temperature (37°C).

To assess more accurately the effect of MSH on melanin synthesis in DMEL-3 cells, the melanin formation assay was carried out on two separate occasions and each sample was assayed in triplicate. The results, expressed as cpm/100 μg protein are presented in Table 3.7.

Table 3.7. Effect of MSH on melanin synthesis in DMEL-3 cells at 33°C and 37°C

Samples	Experiment 1 cpm/100 μg protein			Experiment 2 cpm/100 μg protein		
	Control	+ MSH	MSH/ Control	Control	+ MSH	MSH/ Control
DMEL-3 at 33°C	1053 ± 86	1389 ± 36	1.3	382 ± 78	473 ± 10	1.2
DMEL-3 at 37°C	1104 ± 102	1407 ± 36	1.3	1581 ± 50	1912 ± 59	1.2

In experiment 1, when DMEL-3 cells were exposed to 0.2 μM α-MSH for 4 days, there was only a 1.3-fold stimulation of melanin synthesis at both the permissive (33°C) and non-permissive (37°C) temperatures. This suggests that the T antigen did not effect the responsiveness of DMEL-3 cells to MSH. However, in this experiment, MSH treatment was initiated only one day after the cells had been transferred to 37°C and it has been reported that the ts mutant large T antigen is inactivated at the non-permissive temperature after 2-4 days (Almazan and McKay, 1992). In experiment 2, therefore, DMEL-3 cells were cultured at 37°C for 21 days before MSH treatment was initiated. A comparison of melanin synthesis in untreated DMEL-3 cells at 33°C and 37°C confirmed that inactivation of the T antigen at 37°C resulted in increased melanin synthesis. However, both at 33°C and 37°C, MSH induced a proportionately equal increase in melanin synthesis. These results were therefore similar to those obtained in experiment 1.

Results of the above experiments indicated that the DMEL-3 cells were only slightly responsive to the melanogenic stimulatory effect of MSH and that T antigen expression did not affect this responsiveness. To confirm these results, and to investigate the effect of MSH on the activity of individual melanogenic enzymes, assays measuring tyrosine hydroxylase and DOPAchrome tautomerase (DCT) activities of tyrosinase and TRP-2, respectively, were carried out. The results of these assays are presented in Table 3.8 and are expressed in pmol/μg protein/hour. Tyrosine hydroxylase activity was stimulated around 1.6- and 1.4-fold after MSH treatment at 33°C and 37°C, respectively. As in the case of the results obtained for the melanin formation assay, there was no significant difference in stimulation between cells that were treated at 33°C and 37°C. MSH did not stimulate DCT activity in DMEL-3 cells at either 33°C or 37°C, although a slight

stimulation was observed in melan-a cells. DCT activity was, however, 10-fold more in DMEL-3 cells cultured at 37°C than at 33°C.

Table 3.8. Effect of MSH on melanogenic activities of DMEL-3 cells at 33°C and 37°C

Melanogenic activities	Samples	Control pmol/μg protein/hr	+MSH pmol/μg protein/hr	Stimulation MSH/Control
Tyrosine hydroxylase	DMEL-3 at 33°C	0.55	0.88	1.6
	DMEL-3 at 37°C	1.51	2.04	1.4
	melan-a	4.32	5.05	1.2
DOPAchrome tautomerase	DMEL-3 at 33°C	6	6	1.0
	DMEL-3 at 37°C	60	60	1.0
	melan-a	12	18	1.5

3.9 The growth of DMEL-2 and DMEL-3 cells is temperature-sensitive but not TPA-dependent

Since DMEL-3 cells gradually lost several features of differentiated melanocytes, whereas DMEL-2 cells did not, the effect of TPA on the growth of the DMEL-2 and DMEL-3 cell lines was compared. This is important because transformed melanocytes become TPA-independent. Short-term growth of the cells was compared at 33°C and 37°C in melanocyte-specific medium (MSM) with and without 48 nM TPA (Fig. 3.22a,b).

It was found that over a period of nine days, DMEL-3 cells grew about nine times faster than the DMEL-2 cells when cultured at 33°C in medium with TPA. As expected, at 37°C in MSM with TPA, both cell lines grew much more slowly than their counterparts at 33°C. Removal of TPA resulted in a 40-50% decrease in proliferation of both DMEL-2 and 3 cells cultured at 33°C. Similarly, at 37°C the removal of TPA resulted in a 67% decrease in proliferation of DMEL-3 cells. It was not possible to determine the effect of TPA on the growth of DMEL-2 cells at 37°C because the cells showed very little potential to proliferate at this temperature. This analysis indicated that at the permissive temperature (33°C), melanocyte cell lines in this study did not senesce after removal of TPA, confirming the results from a previous experiment (Fig. 3.11) that expression of the T antigen in these cells induced growth autonomy from TPA. Furthermore, the decline in proliferation when the cells were grown at the non-permissive temperature (37°C) demonstrated that the cells were conditionally immortalised by the ts mutant T antigen.

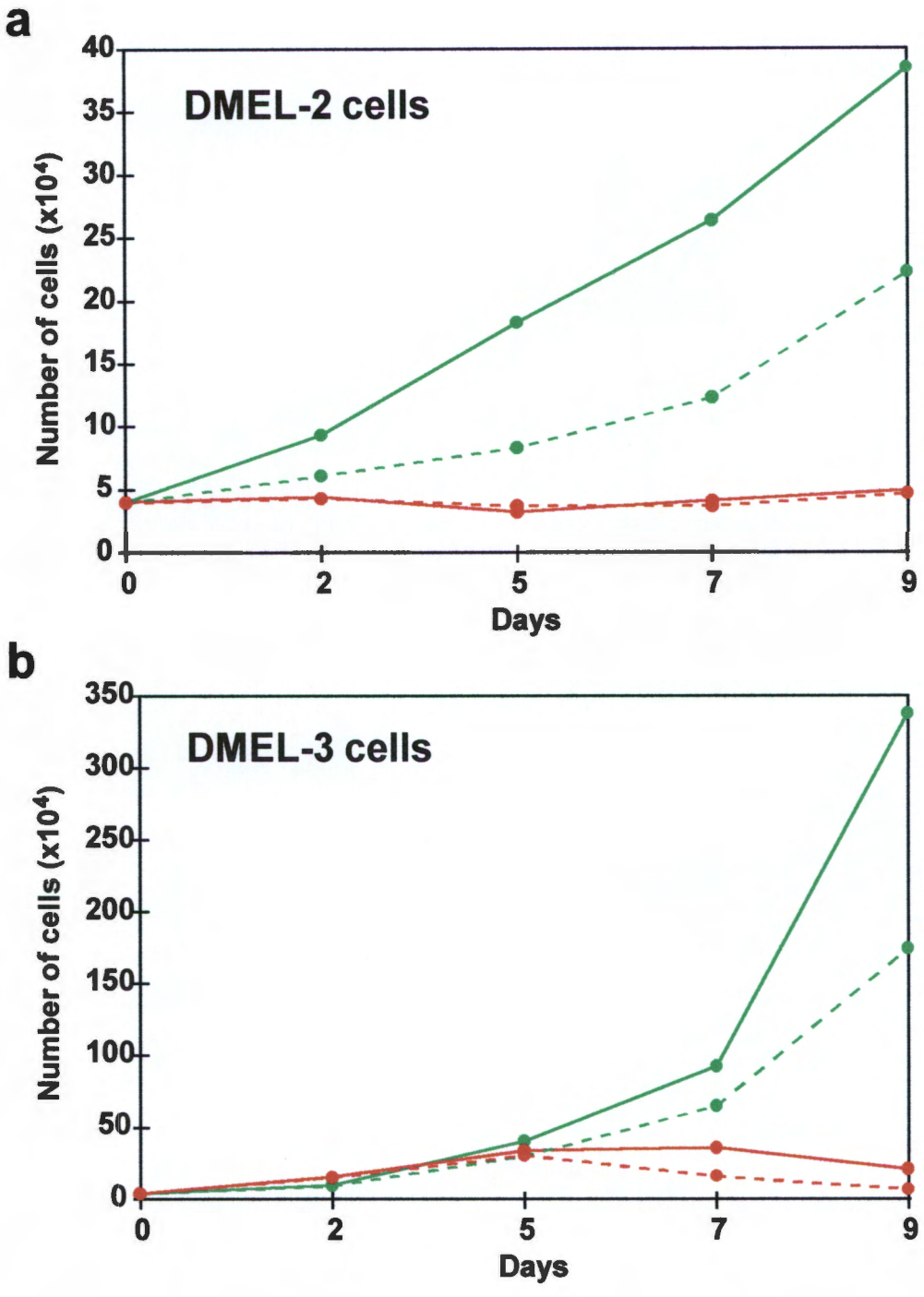


Fig 3.22. T antigen-expressing melanocytes show reduced dependence on TPA for growth. Growth kinetics of **(a)** DMEL-2 cells and **(b)** DMEL-3 cells cultured at 33°C (green) and 37°C (red) in MSM with (solid line) or without (broken line) TPA. Each value is the average of duplicate wells. The standard error between duplicate wells ranged between 5 and 15%.

3.10 Loss of pigmentation and an increase in proliferation rate in DMEL-3 cells is not associated with increased levels of T antigen

The results of the experiments described above raise two interesting questions. Firstly, why did the differentiation and growth characteristics of late passage DMEL-3 cells cultured at 33°C differ from earlier passages and from the pigmented cell lines established in this study? Secondly, when cultured at the non-permissive temperature (37°C), why were passage 17 DMEL-3 cells not able to revert to the fully differentiated state of earlier passages?

It was hypothesized that late passage DMEL-3 cells become de-differentiated and proliferated faster because they contain higher levels of the T antigen. To test this hypothesis, western blot analysis was performed to compare the levels of T antigen in early passage pigmented DMEL-3 cells and late passage unpigmented DMEL-3 cells, as well as to assess how these levels compared to those in DMEL-2 cells. This analysis was carried out on three separate occasions, using protein from DMEL-3 cells at passages 9 and 16 and from DMEL-2 cells at passages 11 and 18. Similar results were obtained on each occasion and an example of one of these experiments is shown in Fig. 3.23A. A 94 kDa band representing the large T antigen was seen in DMEL-3 cells (lanes 2 and 3) and DMEL-2 cells (lanes 4 and 5) but not in melan-a cells (lane 1). A visual comparison of the intensity of the bands in passage 9 (lane 2) and 16 (lane 3) DMEL-3 cells revealed that they contained similar levels of the T antigen indicating that late passage DMEL-3 cells do not contain higher levels of T antigen than earlier passages. Similarly, passage 11 DMEL-2 cells (lane 4) and passage 18 DMEL-2 cells (lane 5) contain similar levels of the T antigen. Furthermore, DMEL-2 and 3 cells contained equal amounts of T antigen. As expected, the levels of message for the T antigen (Fig. 3.23B) were similar in passage 9 (lane 2) and passage 16 (lane 3) DMEL-3 cells (see arrowhead).

The present study used 37°C as the non-permissive temperature instead of the recommended non-permissive temperature of 39°C. It was hypothesized that the inability of DMEL-3 cells to revert to their fully differentiated state at 37°C was due to incomplete inactivation of the T antigen at this temperature. To test this hypothesis, western blot analysis was used to compare the levels of T antigen in passage 8 and 15 DMEL-3 cells cultured at 33°C, 37°C and 39°C for 4 days. The 4-day period was selected because previous studies have reported that the T antigen is not detected in cells expressing the temperature-sensitive mutant large T antigen after 2 to 4 days at 39°C (Almazan and McKay, 1992). The results from this experiment are shown in Fig. 3.24.

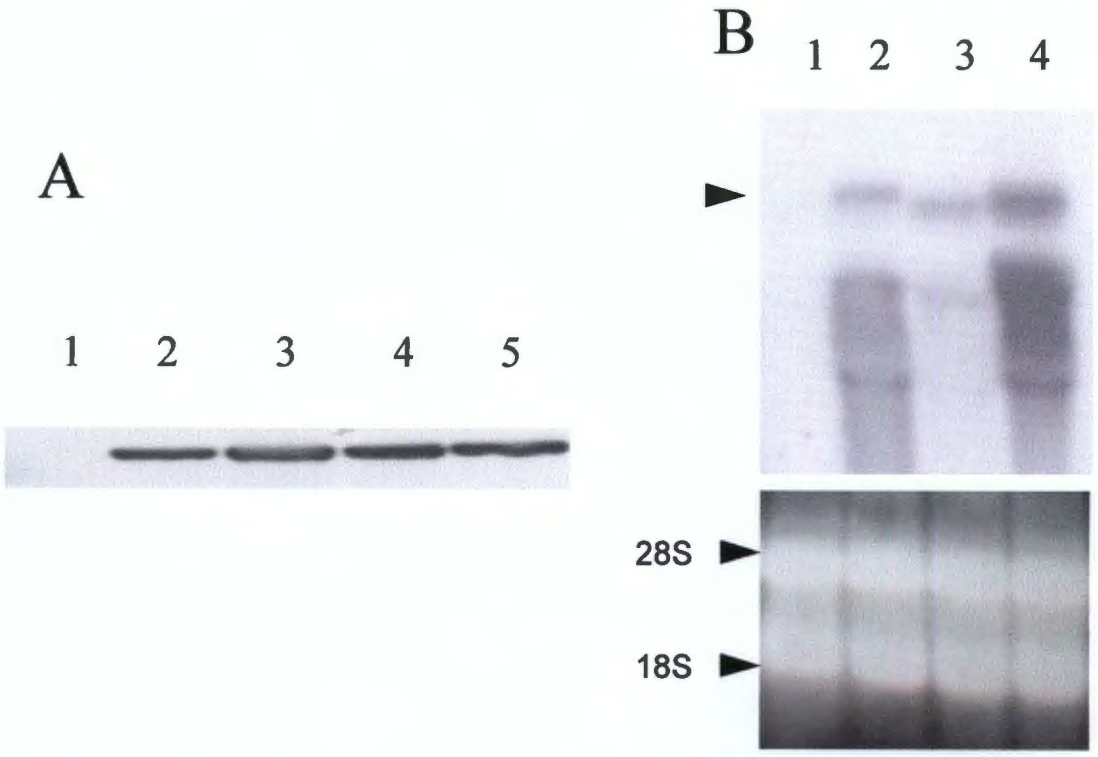


Fig. 3.23. A, Levels of T antigen protein in DMEL-2 and 3 cells at 33°C. Each lane of a 7.5% polyacrylamide gel was loaded with 30 μg of soluble intracellular protein obtained by detergent lysis. After electrotransfer to nitrocellulose sheets the proteins were stained with the monoclonal anti SV40 large T antigen antibody (0.5 μg/ml) and visualised by enhanced Chemiluminescense (Amersham). Lane 1, melan-a cells; lanes 2 and 3, passage 9 and 16 DMEL-3 cells, respectively; lanes 4 and 5, passage 11 and 18 DMEL-2 cells respectively. **B**, Northern blot analysis of T antigen expression (see arrowhead) in passage 9 (lane 2) and passage 16 (lane 3) DMEL-3 cells. Ethidium bromide staining of the 28S and 18S rRNAs show the quantity and quality of the RNA loaded. Lane 1, B16 melanoma cells (negative control); lane 4, ψ2 fibroblasts (positive control).

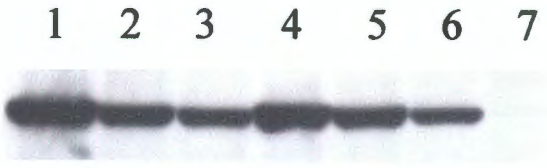


Fig. 3.24. Levels of T antigen in passage 8 and 15 DMEL-3 cells cultured at 33°C, 37°C and 39°C. Each lane of a 7.5% polyacrylamide gel was loaded with 30 μg of soluble intracellular protein obtained by detergent lysis. After electrotransfer to nitrocellulose sheets the proteins were stained with the monoclonal anti SV40 large T antibody (0.5 μg/ml) and visualised by enhanced Chemiluminescense. Lanes 1, 2 & 3, passage 8 DMEL-3 cells cultured at 33°C, 37°C and 39°C, respectively; lanes 4, 5 & 6, passage 15 DMEL-3 cells cultured at 33°C, 37°C and 39°C respectively; lane 7, NIH 3T3 fibroblasts (negative control).

Immunoblotting using the Pab101 antibody specific for the T antigen protein revealed an immunoreactive band of approximately 94 kDa in all lanes containing protein from DMEL-3 cells (lanes 1 to 6). The intensity of the band obtained in passage 8 DMEL-3 cells cultured at 33°C (lane 1), 37°C (lane 2) and 39°C (lane 3) decreased as the temperature at which the cells were cultured increased. Similar results were obtained for passage 15 DMEL-3 cells cultured at 33°C (lane 4), 37°C (lane 5) and 39°C (lane 6). No signal was obtained in the NIH 3T3 fibroblasts (lane 7). These results show that: (1) although the levels of T antigen were lower at the recommended temperature of 39°C, T antigen was still detectable and (2) the levels of T antigen present in the two passages of DMEL-3 cells were similar at the different temperatures.

3.11 Mechanism by which SV40 large T antigen inhibits differentiation in DMEL-3 cells

3.11.1 T antigen does not affect the expression of the RB gene family

To determine whether any of the RB family members is involved in melanocyte differentiation, this study investigated whether there were any correlative changes in the expression pattern of RB1, p130 and p107 mRNA when DMEL-3 cells were induced to differentiate at the non-permissive temperature of 37°C. Passage 15 DMEL-3 cells were cultured at 33°C and 37°C for 4, 16 and 30 days. As reported earlier, DMEL-3 cells cultured at 33°C and 37°C for 4 and 16 days were unpigmented, whereas after 30 days at 37°C, melanin was clearly visible (Fig. 3.25a,b), confirming that the cells had re-differentiated. To determine whether there was any correlation between this re-differentiation and expression of the RB gene family, a northern blot of poly(A)$^+$ RNA from DMEL-3 cells cultured at 33°C and at 37°C for 4, 16 and 30 days as well as poly(A)$^+$ RNA from melan-a cells, B16 melanoma cells and adult mouse brain tissue was probed with cDNAs for RB1, p130 and p107.

When probed with a mouse RB1 cDNA, a 4.7 kb band of equal intensity was obtained in all lanes (Fig. 3.26A). When this blot was stripped and reprobed with a mouse p130 cDNA a 4.8 kb band was detected in all lanes (Fig. 3.26A). These results indicate that there were no significant changes in RB1 and p130 expression in DMEL-3 cells induced to differentiate by inactivation of the T antigen. Interestingly, the signal was strongest in melan-a and B16 melanoma cells.

Finally, the above blot was probed with a human p107 cDNA. Two bands of 4.9 kb and 2.4 kb were detected in DMEL-3 cells cultured at 33°C and 37°C but not in melan-a cells, B16 melanoma cells or brain tissue (Fig. 3.26A). There was no change in the intensity of these bands when DMEL-3 cells were induced to re-differentiate. The absence of p107 in melan-a cells, B16 melanoma cells and brain tissue suggests that p107 is up-regulated in T antigen-expressing melanocytes. To test the validity of this conclusion, a northern blot containing total RNA from

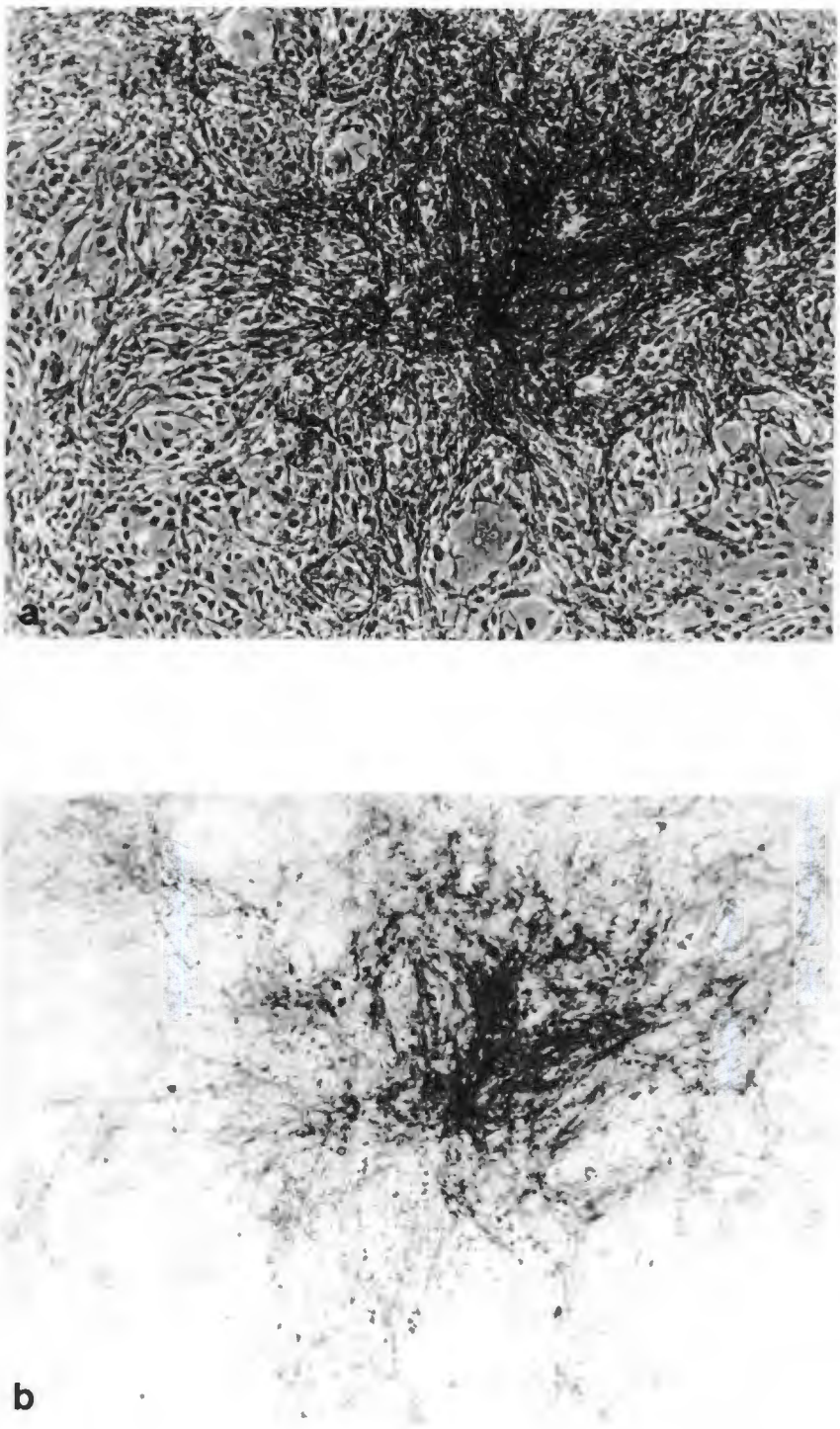


Fig. 3.25. Morphology of passage 15 DMEL-3 cells induced to differentiate at 37°C. **(a)** Phase contrast photomicrograph of DMEL-3 cells at 37°C for 30 days. **(b)** Bright field photomicrograph of the same field as (a) showing pigmentation typical of differentiated melanocytes.

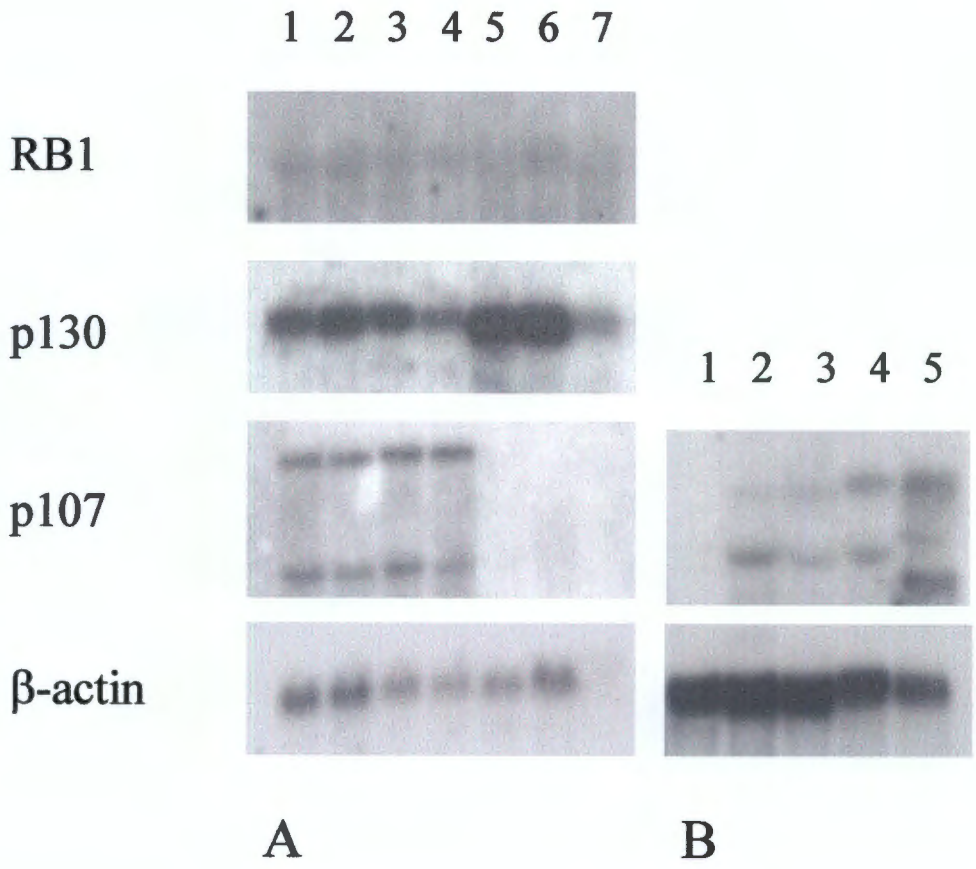


Fig. 3.26. Expression of the RB gene family members. Northern blot hybridisation analysis was performed on DMEL-3 cells cultured at 33°C and 37°C. Poly(A)$^+$ RNA (5 μg) from each sample was separated on a formaldehyde gel followed by electrophoretic transfer onto nylon membranes. **A**, The same blot was repeatedly hybridised to a mouse RB1 cDNA, a mouse p130 cDNA and a human p107 cDNA. In **A**: lanes 1, 2, 3 & 4, passage 15 DMEL-3 cells cultured at 33°C and, 37°C for 4, 16 and 30 days, respectively; lane 5, melan-a cells; lane 6; B16 melanoma cells and lane 7, adult mouse brain tissue. **B**, Northern blot containing total RNA (20 μg) from DMEL-2 cells cultured at 33°C and 37°C, probed with a human p107 cDNA. Lane 1, B16 melanoma cells; lanes 2, 3 & 4, DMEL-2 cells cultured at 33°C and, 37°C for 2 and 4 days, respectively; lane 5, ψ2 fibroblasts.

pigmented DMEL-2 cells cultured at 33°C and 37°C as well as B16 melanoma and ψ2 fibroblast cells was also probed with p107. Figure 3.26B shows that while DMEL-2 and ψ2 cells express the p107 gene, no detectable expression was observed in B16 melanoma cells. The signals obtained in DMEL-2 cells grown at 33°C and 37°C was of equal intensity.

3.11.2 Changes in the levels and phosphorylation status of the pRb protein family in DMEL-3 cells induced to differentiate

To investigate whether phosphorylation of any of the pRb family proteins is modulated during melanocyte differentiation, the phosphorylation status of these proteins was examined in unpigmented DMEL-3 cells at 33°C and in DMEL-3 cells, induced to re-differentiate, at 37°C. Since the more highly phosphorylated forms of the pRb family proteins have slower mobilities upon SDS-PAGE than the least phosphorylated forms, immunoblotting could be employed to gauge the phosphorylation status of these proteins. Cell extracts were prepared from passage 16 DMEL-3 cells cultured at 33°C and 37°C for 19 days, as well as from melan-a, melan-b, melan-c and B16 melanoma cells. Figure 3.27 shows immunoblot analyses with antibodies specific to pRb, p130 and p107.

Both the hypo- and hyper-phosphorylated forms of the pRb protein can be seen in DMEL-3 cells cultured at 33°C (lane 1) and 37°C (lane 2), as well as in melan-a (lane 4), melan-b (lane 5) and melan-c (lane 6) cells. No detectable pRb protein was found in B16 melanoma cells (lane 7). A visual comparison of the levels and phosphorylation status of the pRb protein in DMEL-3 cells cultured at 33°C and 37°C, revealed that there was a moderate increase in both forms of the protein at 37°C. Melan-a, melan-b and melan-c cells contained comparable levels of both forms of the pRb protein.

When blots were probed with antibodies specific to the p130 protein, two bands were detected in all lanes. The lower band corresponded to approximately 130 kDa suggesting that these bands probably represent the hypo- and hyper-phosphorylated forms of the p130 protein. The hyperphosphorylated band was equally intense in all lanes suggesting that all the samples contained equal levels of this form of p130. Interestingly, judging from the intensity of the lower band in all the lanes, the hypophosphorylated form of the protein was present at much lower levels in DMEL-3 cells cultured at 33°C (lane 1) and 37°C (lane 2) compared to melan-a (lane 4), melan-b (lane 5) and melan-c (lane 6) cells.

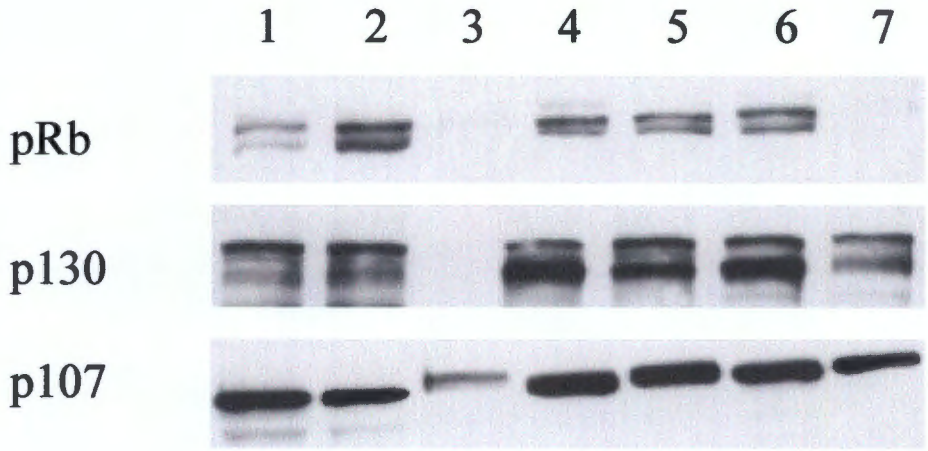


Fig. 3.27. Levels and phosphorylation status of the pRb protein family in DMEL-3 cells at 33°C and 37°C. Passage 16 DMEL-3 cells were induced to differentiate at 37°C for 19 days and differentiation was assessed by visible pigmentation. The cells were solubilized in lysis buffer and approximately 60 μg of protein from each sample was separated on 7.5% SDS gels. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to pRb, p130 and p107 and the bands visualised by chemiluminescence. Lane1, DMEL-3 cells at 33°C; lane 2, DMEL-3 cells at 37°C; lane 3 (for pRb and p107), senescent melanocytes; lanes 4, 5 & 6, melan-a, melan-b & melan-c cells, respectively.

By using antibodies specific to the p107 protein, a broad band of approximately 107 kDa, which probably represents a mixture of different phosphorylated forms of the protein, was detected in all samples. The intensity of the band was slightly stronger in DMEL-3 cells cultured at 33°C (lane 1) compared to those at 37°C (lane 2). Interestingly, melan-a (lane 4), melan-b (lane 5), melan-c (lane 6) and B16 melanoma cells (lane 7) contained significant levels of the p107 protein. These results therefore did not correlate with the results obtained from northern blot analysis.

3.11.3 Identification of the regions of T antigen required for inhibiting melanocyte-specific gene expression and differentiation

To determine which regions of T antigen were required for inhibiting melanogenesis in DMEL-3 cells at 33°C, the cells were transfected with the T antigen mutants, dl1135, 5041 and 3213, described in Table 2.2. These T antigen mutants have previously been used to determine the region(s) of the T antigen required for transformation of cells. The advantage of using the DMEL-3 cell line to assay T antigen mutants is that it should allow the identification of regions required to maintain the de-differentiated state at the non-permissive temperature. Cells from each transfection were incubated at both the permissive and non-permissive temperatures. Since passage 10 DMEL-3 cells were previously shown to visibly re-pigment at the non-permissive temperature, these cells were used in this experiment. To detect which mutants were able to inhibit re-pigmentation at the non-permissive temperature, paired dishes of transfected cells that were cultured at the permissive and non-permissive temperatures, were examined by light microscopy. Mutants that were able to bind the cellular proteins that are required for pigmentation, were expected to yield colonies of cells that were unpigmented at the non-permissive temperature. Over a period of several weeks, re-pigmentation was not detected in any of the transfected dishes at the non-permissive temperature (results not shown). However, the untransfected control cells also did not visibly re-pigment (results not shown). Since analysis of these results was dependent on the repigmentation of the control cells at 37°C, it was not possible to make any conclusive deductions from the above experiments. The following observations are, however, worth noting: (1) Cells from all dishes were unpigmented and fibroblast-like at the permissive temperature (33°C). At the non-permissive temperature (37°C), however, cells transfected with the T antigen mutant 5041 which encodes a protein that is unable to bind p53, were dendritic. The morphology of these cells was indistinguishable from cells transfected with only the control pBabepuro plasmid, and from untransfected DMEL-3 cells at the non-permissive temperature. In contrast, cells transfected with the two other mutants, dl1135 and 3213 that encode proteins that bind p53 but are defective in binding p300 and the RB family members, respectively, resembled their counterparts at the permissive temperature. (2) At the non-permissive temperature, untransfected cells, cells transfected with pBabepuro

alone and cells transfected with the mutant 5041 grew much slower than cells transfected with either the 3213 or dl1135 mutants. This would suggest that the two mutants, 3213 and dl1135, were able to complement the growth defect of DMEL-3 cells at 37°C. Due to time limitations on the present study, this transfection experiment could not be further analysed nor could it be repeated.

CHAPTER 4: DISCUSSION

The stepwise progression from a normal melanocyte to malignant melanoma involves a series of poorly understood genotypic and phenotypic alterations. One of the reasons for this poor understanding, particularly about the molecular events and controls that are altered during this progression, is that there are very few models in which all the steps in this progression are represented. Several groups have attempted to establish *in vitro* models of melanoma formation by transforming mouse melanocytes with exogenous oncogenes. These studies have resulted in a growing understanding of the alterations in the growth characteristics of transformed melanocytes. In addition, although this was not the prime aim of most of these studies, they also reported on concurrent changes in differentiation. A consistent finding in all these studies was that when melanocytes became transformed, there was always a change from TPA dependence to independence and at the same time, the cells became unpigmented. At face value these results would suggest that the exogenously introduced transforming oncogenes are directly involved in the changes observed in both proliferation and differentiation. Interestingly, in most instances these transformation-related changes were reported to occur immediately following oncogene expression, suggesting that *in vitro* at least, the transformation process occurs in a single step. However, clinical and pathological observations have defined cutaneous lesions which represent steps in melanoma progression, clearly indicating that the transformation process is not a single step process. One can therefore conclude that the cell lines established in the above studies do not mirror the sequential manner in which melanoma occurs *in vivo*. Why is it then that melanoma progression *in vivo* is clearly a multistep process involving a number of different changes, whereas *in vitro* it can be brought about by the introduction of a single oncogene? The most likely reason for this is that in all of the above *in vitro* studies, the investigators have introduced oncogenes into already immortal melanocyte lines. Thus the cells had already passed through the first steps of transformation and undergone some of the earliest changes characteristic of tumor cells. It would therefore appear that these proposed *in vitro* models are not entirely suitable for delineating the key events in early melanoma progression.

The hypothesis that an oncogene alone is unable to bring about complete transformation in a single step could be tested by introducing oncogenes into primary (non-immortal) melanocytes. This approach was adopted by Albino et al. (1992), who infected normal human melanocytes with a retrovirus containing the viral Ha-ras oncogene. They reported that the resulting cells acquired transformation-related changes in a minimum of two phases. In the early phase, the oncogene induced limited alterations, including changes in morphology and anchorage-dependent growth. At this stage, the cells remained pigmented and TPA-responsive for approximately six months. Subsequently, in what appeared to be a second phase of

transformation (associated with spontaneous chromosomal changes), the cells became TPA independent, depigmented and acquired tumorigenic potential. Transformation therefore occurred in a sequential manner. In a similar type of experiment, Zepter et al. (1995) introduced the SV40 large T antigen into normal human melanocytes but obtained somewhat different results. The growth rate of these T antigen-positive cells increased and the cells proliferated in the absence of TPA. Similar to the results obtained in the early phase by Albino et al. (1992), however, their cells were pigmented and tyrosinase-positive. The cells of Zepter et al. (1995) were unable to initiate tumors in nude mice and eventually underwent senescence.

Although Albino et al. (1992) were able to establish an *in vitro* model of time-dependent tumorigenesis, the transforming oncoprotein could not be inactivated and therefore their model made it difficult, if not impossible, to study the following questions: Is de-differentiation a direct effect of oncogene expression and, if so, is it mechanistically related to the transforming activity of the oncoprotein? Secondly, what is the role of the oncogene in maintaining the transformed phenotype over time? Because the cell lines produced in the present study were derived from primary melanocytes, and because the oncoprotein could be inactivated by a temperature alteration, it was possible to address the above questions.

The first issue addressed in the present study was to determine whether the temperature-sensitive (ts) mutant SV40 large T antigen, that was expressed in primary mouse melanocytes, was directly and immediately able to bring about changes both in proliferation and differentiation. The six clonal cell lines established in the present study were therefore characterised fully with regard to features of melanocyte differentiation and growth dependence on TPA. It was established that during continued passage over at least six months at the permissive temperature, these cell lines expressed the T antigen and retained several features of normal differentiated melanocytes. They remained pigmented, dendritic, actively synthesizing melanin and expressed the tyrosinase, TRP-1 and TRP-2 genes and the proteins encoded by them. In addition, they expressed the *mi* gene and the *c-kit* receptor. These results demonstrate that alterations in the differentiation programme of melanocytes is not necessarily an early event induced by the T antigen. This is similar to studies that show that differentiated cell types expressing the SV40 large T antigen can maintain a proliferative state while the differentiated phenotype is retained. For example, Greenwood et al. (1996) infected primary cultures of rat brain and retinal endothelial cells with a recombinant retrovirus encoding the SV40 large T antigen. The cell lines derived expressed the T antigen and retained the morphology and markers characteristic of the primary culture cells. Similarly, transfection of primary testicular cells with a plasmid containing the SV40 large T antigen gave rise to immortalised peritubular, Leydig and Sertoli cell lines that retained their morphology and differentiated properties

(Hoffman et al., 1992). It is also worth noting that melanocytes derived from skin explants of transgenic mice carrying a recombinant gene composed of DNA coding for a ts mutant SV40 large T antigen under the control of the human vimentin promoter were also pigmented at the permissive temperature and their growth was TPA-dependent (Vicart et al., 1994)

To determine whether the growth of the pigmented T antigen-expressing melanocytes in the present study showed alterations in TPA-dependence, a representative cell line, the DMEL-2, was assessed for its ability to proliferate with and without TPA. Over the 9-day growth period tested, cells grown in TPA proliferated almost 50% more than those deprived of TPA. Importantly, the cells grown without TPA continued to proliferate and did not senesce. The growth of DMEL-2 cells was therefore stimulated by, but not dependent on, TPA. The cell lines established in the present study thus differ from all other melanocyte cell lines that have been established to date because the cells remained pigmented but were TPA-independent. These results suggest that an early event induced by the SV40 large T antigen in primary cultures of normal mouse melanocytes is release from TPA dependence, without loss of TPA-responsiveness and without alterations in the differentiation programme of the cells.

During the course of the above investigations, one of the six cell lines, the dermal melanocyte cell line DMEL-3, acquired a more fibroblastic morphology, grew very rapidly and became depigmented at the permissive temperature. Even though they were unpigmented, low levels of *mi* and TRP-2, though not tyrosinase, transcripts were detected by northern blot hybridisation (levels of TRP-1 were not examined). Because the oncoprotein could be inactivated, this provided an ideal tool for investigating the question of whether the T antigen plays a direct role in the de-differentiation process. Proliferation ceased on inactivation of the large T antigen and the DMEL-3 cells re-expressed melanocyte-specific markers, including the tyrosinase gene family, as well as *mi* message, in a time-dependent manner. These results convincingly demonstrate that (1) the T antigen initially had a role in suppressing the pigmented phenotype; (2) this suppression is directly linked to alterations in *mi* expression and function; and (3) this process is reversible at this stage. If Mi is acting as a melanocyte master gene one might predict that other melanocyte-specific markers are also altered as a consequence of alterations in *mi* expression.

To explore this hypothesis, this study also examined the question of whether the expression of the α-MSH and c-kit receptors is altered by the changes in *mi* expression. When paired cultures of DMEL-3 cells were treated with α-MSH at the permissive and non-permissive temperatures, melanin synthesis and tyrosinase activity increased by the same magnitude at both temperatures. This suggested that DMEL-3 cells have α-MSH receptors which are responsive to

α-MSH. Because levels of *mi* expression did not correlate to responsiveness to α-MSH at permissive and non-permissive temperatures, it suggested that the MSH receptor gene is not regulated by Mi.

However, this data should be interpreted with caution because responsiveness of DMEL-3 cells to α-MSH was very low at both temperatures. This low response of DMEL-3 cells may be related to the culture conditions used in this study. For example, the experimental medium used in the present study contained TPA which has been shown to block or reduce pigmentation by the POMC peptides (Hunt et al., 1994; McLeod et al., 1995). In addition, in several earlier reports where melanocytes failed to show a pigmentary response to α-MSH (De Luca et al., 1993; Friedmann et al., 1990; Halaban et al., 1983; Hedley et al., 1998; Ranson et al., 1988), TPA was present as a mitogen in the culture media. It is, therefore, quite possible that the inclusion of TPA masks potential melanogenic responses to POMC peptides. An alternative explanation for the poor response of DMEL-3 cells to MSH may be related to early changes at the MSH receptor. It is possible, for example, that the T antigen could alter expression of the MSH receptor and that re-expression of this receptor was not obtained at the non-permissive temperature. The most plausible cause for the poor response of DMEL-3 cells to α-MSH, however, may relate to the presence of IBMX in the culture medium. It is very possible that high levels of cAMP induced by IBMX maximises the melanogenic response of the cells and that further addition of α-MSH was not able to increase melanogenesis. The above speculations remain to be tested.

When DMEL-3 cells were examined for *c-kit* expression, no message could be detected at either the permissive or non-permissive temperatures. *c-Kit* transcripts were, however, present in DMEL-2 and melan-a cells. These results were confirmed by western blot analysis and suggest that, at the very least, Mi homodimers do not regulate *c-kit* expression. This is in agreement with the results of Askan and Goding (1998) who demonstrated that Mi homodimers were unable to bind the atypical E-box element in the *c-kit* promoter either *in vitro* or in the yeast one-hybrid assay. However, as pointed out by the authors, their experiments used Mi homodimers and therefore the possibility that Mi heterodimers might be involved in regulating *c-kit* expression cannot be ruled out. Similarly, the results of the present study do not exclude the possibility that the SV40 large T antigen could be disrupting formation of such heterodimers.

The inability of DMEL-3 cells to re-express *c-kit* at the non-permissive temperature at a stage when they were able to re-express *mi*, suggested that the irreversible loss of *c-kit* expression is an early irreversible change induced by the SV40 large T antigen. This is in line with evidence that *c-kit* expression is lost in tumorigenic primary melanomas and metastases. While the

functional relevance of this modulation remains to be evaluated, it is possible that signalling pathways that normally regulate growth are bypassed during transformation of melanocytes and that more efficient pathways are mobilized. This might lead to eventual loss of the molecules that convey signals that normally regulate cell growth.

With increased passage of DMEL-3 cells at the permissive temperature, loss of differentiation and enhanced growth (results not shown) became more pronounced and more difficult to reverse at the non-permissive temperature. Eventually, it was not possible to induce the cells to re-express *mi* and the tyrosinase gene family at all. There is a number of possible explanations for these findings. The first possibility is that over a period of time, the levels of T antigen increased, resulting in increased suppression of differentiation as was shown by Larue et al. (1993). Western blot analyses in the present study, however, showed that early passage (pigmented) and late passage (unpigmented) DMEL-3 cells contained similar levels of the T antigen. This correlated to the results of northern blot analysis which showed that equal levels of large T mRNA were present in early and late passage DMEL-3 cells. Furthermore, it might be argued that DMEL-3 cells originally contained higher levels of T antigen than the other pigmented cell lines. However, a comparison of levels of T antigen in DMEL-2 and 3 cells showed that they express similar levels of the T antigen protein. Therefore the depigmented phenotype of DMEL-3 cells at the permissive temperature was not associated with increased levels of the T antigen. In addition, a comparison of T antigen levels in DMEL-3 cells at the permissive and non-permissive temperature revealed that the difference in the ability of early and late passage DMEL-3 cells to re-differentiate at the non-permissive temperature was not due to higher levels of the T antigen in the late passage cells at this temperature.

A second possible explanation for ultimate irreversibility of the de-differentiated state of DMEL-3 cells is that the large T antigen acts as a primary transforming stimulus which, over time, triggers a cascade of changes that prohibit reversal of oncogene-induced phenotypic alterations in the DMEL-3 cells. Inactivation of the oncoprotein in late passage DMEL-3 cells cultured at the non-permissive temperature would therefore not be sufficient to reverse this process. This theory is supported by experiments with transgenic mice in which the SV40 large T antigen could be conditionally expressed in the salivary gland (Ewald et al., 1996). In this system, the oncogene is transcribed in the absence of tetracycline and is silent in the presence of tetracycline. The temporal progression of T antigen-induced morphological changes in the submandibular gland, and their potential reversibility were investigated over a 7-month period after birth. Expression of T antigen in the submandibular gland of transgenic mice from the time of birth induced cellular transformation and extensive ductal hyperplasia by 4 months of age. The hyperplasia was reversed when T antigen expression was silenced by exposing the mice to tetracycline for 3

weeks. When T antigen expression was silenced after 7 months, however, the hyperplasia persisted even though the T antigen was absent. Interestingly, epithelial cells from the submandibular tissue of the transgenic mice were polyploid and this polyploidy could be reversed at 4 months of age but not at 7 months in the absence of T antigen. These results support a model of time-dependent, multistep tumorigenesis, in which virally transformed cells eventually lose their dependence on the viral oncoprotein for maintenance of the transformed state.

An interesting question that remains to be answered is why did the DMEL-3 cells, but not the other five cell lines analysed in the present study, depigment? It should be pointed out that while all six cell lines analysed in the present study contain a single retroviral insert, Southern blot analysis show that the insert has been incorporated into a different part of the host genome. This might have important implications on how these melanocytes behave because the site of insertion could differentially affect the expression of genes that regulate melanocyte proliferation or melanogenesis.

Mechanism by which T antigen causes depigmentation in DMEL-3 cells

Having established that there is a window period in which the T antigen directly but reversibly affects melanocyte differentiation, the next question was to explore the mechanism whereby the T antigen inhibits the melanogenic process. The first indication that the T antigen could be altering pigmentation in the DMEL-3 cells was the failure to detect the glycosylated forms of the tyrosinase, TRP-1 and TRP-2 proteins in this cell line. A possible explanation is that T antigen was interfering with either their post-translational processing or synthesis. It is interesting to note that the cytosolic heat shock protein, hsp 70, known to influence protein folding in the endoplasmic reticulum (ER) is bound by the T antigen (Sawai and Butel, 1989). It is therefore possible that incorrect folding of the tyrosinase, TRP-1 and TRP-2 proteins as a result of inactivated hsp 70 prevents them from exiting the ER and this results in their degradation and/or the retardation of their maturation. The latter possibility is supported particularly by the observation that the pigmented cell lines, established in the present study, express high levels of the TRP-2 gene and the *de novo* TRP-2 protein, but frequently had undetectable or low levels of the glycosylated TRP-2 protein. This alteration was clearly not due to an experimental artefact because under the same conditions, the melan-a cells were shown to contain low levels of *de novo* TRP-2 protein and high levels of glycosylated TRP-2 protein.

Some investigators have used the oncoprotein E1A as a tool for understanding the control of melanocyte-specific gene expression and differentiation (Halaban et al., 1996; Yavuzer et al., 1995). Yavuzer et al. (1995) demonstrated that E1A induced loss of pigmentation in immortal

murine melanocytes and this correlated to loss of expression of the TRP-1 and tyrosinase genes. They also showed that repression of TRP-1 by E1A correlated to E1A binding to pRb and p300. The demonstration that Mi binds pRb *in vitro* suggested that the pRb:Mi interaction may be essential for the ability of Mi to activate transcription of the melanocyte-specific genes. The authors proposed that since E1A binds pRb, repression of melanocyte-specific gene expression by this viral oncogene may result, in part, from the disruption of Mi:pRb complexes. Since the SV40 large T antigen also binds pRb, the above mechanism may, in part, provide an explanation for the depigmentation in the DMEL-3 cells. To address the possible role of pRb in melanocyte differentiation, the present study investigated whether melanocyte differentiation is accompanied by an increase in transcription of the RB1 gene and increased levels of the pRb protein (as has been shown by Copolla et al. (1990) and Chen et al. (1989) respectively). Because p107 and p130 are structurally similar to pRb and may also possibly play a role in differentiation, an analysis of these two proteins was also included.

Northern blot analysis showed that unpigmented DMEL-3 cells, cultured at the permissive temperature (33°C), express similar levels of RB1 mRNA as DMEL-3 cells induced to pigment at the non-permissive temperature (37°C). Western blot analyses, however, revealed that the hypophosphorylated form of pRb increased when the DMEL-3 cells were induced to differentiate at 37°C and that there was also a slight increase in the hyperphosphorylated form of pRb at this temperature. Similar results were obtained for p130. This would suggest that either the levels of pRb and p130 increased during differentiation or that these proteins are less stable in the presence of the SV40 large T antigen. Since the mRNA levels of pRb and p130 did not increase during differentiation, the former possibility seems unlikely. With regard to the latter possibility, it is of interest to note that the N terminus of the SV40 large T antigen shares sequence homology with the J domain of the DnaJ (heat shock protein 40) family of molecular chaperones (Kelley and Landry, 1994). This J domain of T antigen is required for cellular transformation and has been reported to mediate a perturbation of the phosphorylation status of p130 and p107 and to promote the degradation of the p130 protein (Stubdal et al., 1997). Such a mechanism could possibly account for lower levels of pRb and p130 proteins at the permissive temperature.

As with RB1 and p130, there was no alteration in p107 gene expression. Overall, the results therefore show that there are no alterations in the expression of the pRb gene family in the presence or absence of the large T antigen. However, unlike the pRb and p130 proteins, differentiation was not associated with an increase in levels of the p107 protein. These western blot analyses are, however, difficult to interpret because the Rb-related proteins are bound and presumably inactivated by the SV40 large T antigen at the permissive temperature. At the non-permissive temperature, the SV40 large T antigen is degraded, thereby liberating the active form

of the proteins. Release of this form of the proteins at the non-permissive temperature alone might therefore be sufficient for their activity. Thus a comparison of the levels of the Rb-related proteins at the permissive and non-permissive temperatures by western blot analyses does not necessarily reflect the functioning of these proteins at the two temperatures.

Additional information on the role of the Rb family in melanocytes can be deduced from the comparisons of their expression in melanocytes with and without T antigen. The present study shows that although melan-a and B16 melanoma cells express the RB1 gene at levels similar to those found in DMEL-3 cells, they expressed higher levels of the p130 gene and no detectable p107 message. These results possibly suggest that the T antigen down-regulates p130 and up-regulates the p107 genes. To test the hypothesis that the T antigen up-regulates p107 expression, a northern blot containing RNA from DMEL-2 melanocytes, B16 melanoma cells and ψ2 fibroblasts (cell line producing T antigen) was probed for p107 expression. The results revealed that the DMEL-2 and ψ2 fibroblast cell lines, both of which express T antigen, express p107, whereas B16 cells, which do not contain T antigen, do not express p107. If the differences between the T antigen-expressing melanocytes established in the present study, and the melan-a and B16 melanoma cells can be believed, then they might provide important clues about the changes brought about during oncogene-mediated transformation of melanocytes. For example, it is possible that in response to constitutive expression of the SV40 large T antigen, melanocytes up-regulate the tumor-suppressor p107 in an attempt to control growth. A possible explanation for the observation that melan-a cells do not express any detectable levels of p107 mRNA transcripts but contained high levels of p107 protein might be that p107 mRNA is unstable in spontaneously immortalised melan-a cells. Further studies are clearly necessary to determine whether pRb and its relatives play a role in melanocyte-specific gene expression and differentiation.

Down-regulation of *mi* expression

Depigmentation in the DMEL-3 cell line at the permissive temperature was shown to be a consequence of the down-regulation of the tyrosinase, TRP-1 and TRP-2 genes and this correlated to reduced levels of *mi* mRNA transcripts. At the non-permissive temperature, *mi* expression increased in a time-dependent manner in a pattern similar to that observed for the tyrosinase gene family. These results are consistent with the possibility that Mi is the central regulator of melanocyte-specific gene expression and suggests that it is the potential target for T antigen-mediated de-differentiation in melanocytes. These results are similar to studies which show that *mi* expression is also reduced in E1a-transformed melanocytes (Halaban et al., 1996; Yavuzer et al., 1995) and that ectopic expression of Mi was able to prevent repression of the tyrosinase and TRP-1 promoters in the presence of E1A (Yavuzer et al., 1995).

To date, there have been no investigations directed at understanding how *mi* is down-regulated in E1A or SV40 transformed melanocytes. This is a challenging question because, so far, only the human *microphthalmia* promoter sequence has been cloned and there is limited information on the regulation of *mi* expression (see section 1.5). One possibility is that *mi* is autoregulated and that oncoproteins interfere with this autoregulation either by directly binding to and inactivating Mi or by preventing complexing with a necessary transcription partner. This possibility however appears unlikely since there are no CATGTG motifs in the published sequence of the MITF promoter and co-transfection of human melanoma cells with an MITF cDNA did not lead to an increase in expression of MITF-luciferase fusion genes (Fuse et al., 1996). However, it is possible that the cloned promoter fragment does not represent the full regulatory sequence or that Mi may bind either upstream or intronic enhancer sequences. Thus the possibility of autoregulation cannot be excluded.

Another possible mechanism for *mi* downregulation could be via the inactivation/repression of a positive transcriptional regulator of the *mi* gene. A potential candidate is, the paired-box homeodomain transcription factor, Pax3. Mutations in *Pax3* cause hypopigmentation and hearing impairment in individuals suffering from Waardenburg Syndrome 1 and 3 which possibly results from a fault in melanocyte differentiation. Interestingly, very recent evidence provided by Watanabe et al. (1998) suggest that Pax3 may be an upstream regulator of the *mi* gene. The present study therefore explored the possibility that Pax3 may be a potential target for T antigen-mediated repression of *mi* gene expression. Northern blot analysis of DMEL-3 cells, cultured at the permissive and non-permissive temperatures, revealed that there was no detectable levels of *Pax3* transcripts at either temperature. In addition, *Pax3* expression was also absent in the highly pigmented DMEL-2 and melan-a cell lines.

The inability to detect *Pax3* expression in pigmented melanocyte cell lines that express high levels of *mi* would suggest that Pax3 is not expressed in fully differentiated melanocytes and that it is unlikely to be a target for T antigen-mediated repression of *mi* expression in DMEL-3 cells. It is, however, possible that the onset of *mi* expression during melanocyte development may be dependent on Pax3 but that once the initial expression of *mi* is established, its subsequent expression becomes independent of Pax3. This possibility is similar to the role of Pax3 in muscle development. *Pax3* expression has been reported to be required for myogenic cell specification but appears to be repressed as precursors cells activate expression of the myogenic gene cascade (Williams and Ordahl, 1994). Given the results of Watanabe et al. (1998) and the fact that mutations in *Pax3* give rise to hypopigmentation, it is clear that Pax3 must play a role in the melanocyte lineage. However, reports on the expression of *Pax3* during

melanocyte development are inconclusive. For example, Goulding et al. (1991) examined mouse embryos at various stages of development for *Pax3* expression and they reported, although the results were not shown, that "at no time was *Pax3* expression detected in melanocytes". Furthermore, they concluded that *Pax3* is only expressed during embryogenesis because no adult tissues contained detectable levels of *Pax3* mRNA. In contrast, it appears that in keeping with their origins in the neural crest, premelanoblast cell lines, as well as melanocytes at later stages of development, do express *Pax3* (D.C. Bennett, unpublished, personal communication). Similarly, Watanabe et al. (1998) reported on the presence of Pax3 protein in a melanoma cell line, although, again, the data was not shown. It is worth noting that unlike the present study in which total RNA was used to detect *Pax3* expression, Bennett reportedly used poly(A)$^+$ RNA. It is therefore possible that differentiated melanocytes do express *Pax3* but at levels too low to be detected on blots containing total RNA. More sensitive assays, such as quantitative RT-PCR, are currently underway and it is hoped that the results from these experiments would clarify this issue.

Another possibility that should be explored is whether the SV40 large T antigen activates a negative transcriptional regulator of the *mi* gene. However, only a single candidate negative regulator, the POU domain transcription factor Brn-2, has been identified and it is still not clear whether it indeed acts as a repressor or activator of the *mi* gene. Further work is clearly necessary before the precise role of oncoproteins in melanocyte de-differentiation is established.

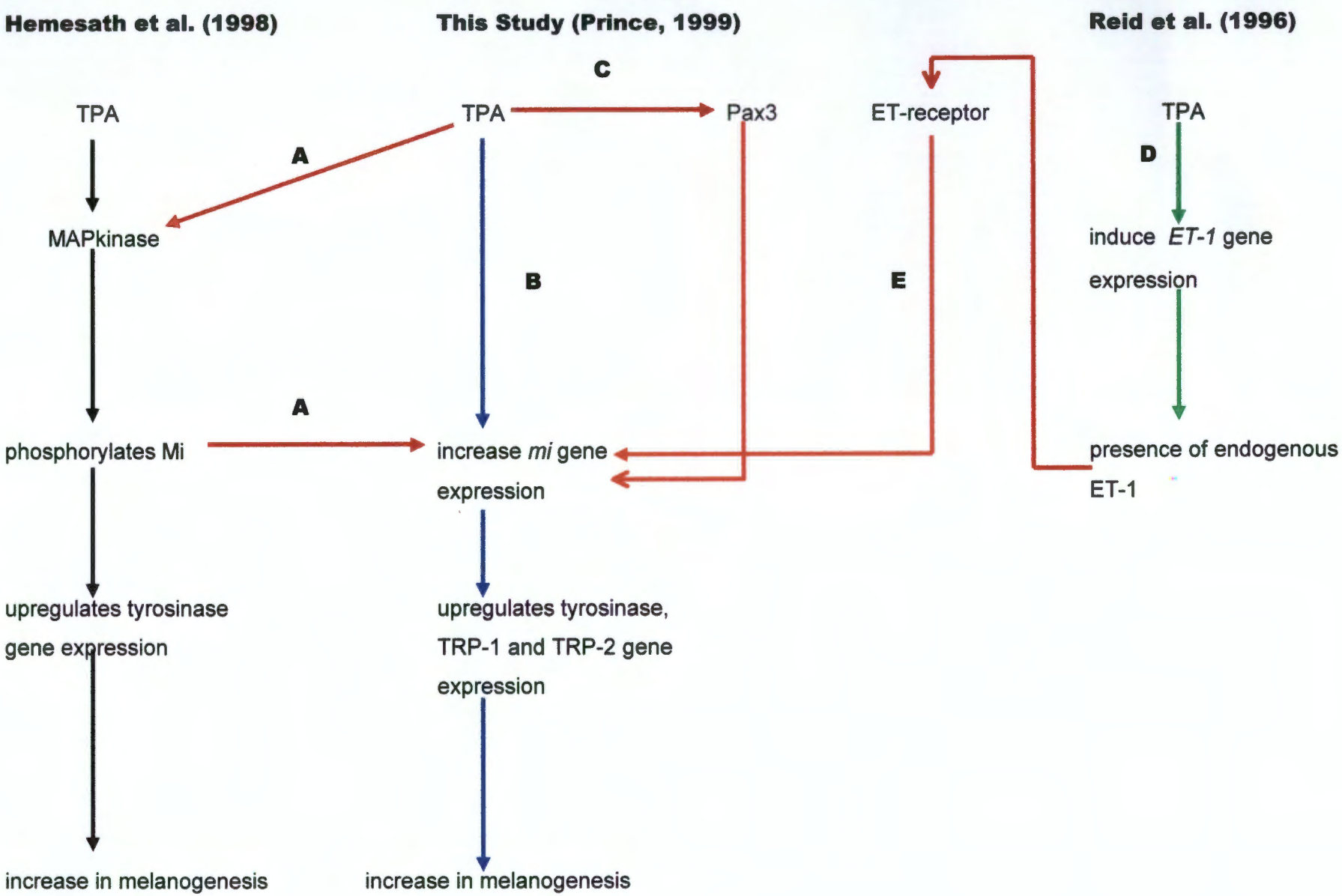
While there is substantial evidence that Mi activates transcription of the tyrosinase and TRP-1 genes through specific E-box elements, most notably the highly conserved M-box, the role of Mi in the regulation of the TRP-2 gene remains in dispute. Halaban et al. (1996) report that, unlike the tyrosinase and TRP-1 genes, TRP-2 gene expression is not altered in E1A-transformed melanocytes. However, examination of their published Figures shows that, compared with the pigmented clones transfected with the pSVneo control plasmid, there does appear to be a reduced level of TRP-2 protein and mRNA in the amelanotic melanocytes. Their observation that TRP-2 expression is down-regulated but not shut down in the absence of *mi* expression would suggest that Mi, even if not essential, may have a role in TRP-2 gene expression. It is possible that in the present study, either the large T antigen itself is somehow directly responsible for down-regulating TRP-2 expression, or that the large T antigen is inactivating another, as yet unidentified, factor involved in TRP-2 transcription. These last two alternatives might explain the differences between the results in this study and those of Halaban et al. (1996). It is possible, at the very least, that E1A and large T antigen do not exert the same effect on TRP-2 expression.

TPA stimulates melanogenesis by regulating *microphthalmia* expression

In vitro studies have shown that TPA not only acts to regulate melanocyte progenitor cell number but is also essential for inducing pigment in pluripotent mouse neural crest cells (Murphy et al., 1992). These findings suggest that TPA stimulates differentiation of the melanocyte progenitors into fully mature melanocytes. Since TPA is not a physiological agent, its action is clearly mimicking some *in vivo* pathway involved in the melanogenic process. Subsequent studies in the same mouse neural crest system have shown that endothelins can substitute for this role of TPA (Reid et al., 1996). Further evidence for the role of TPA in melanogenesis have also come from studies that have shown an increase in tyrosinase activity (Abdel-Malek et al., 1992; Chao-Hsing and Hsin-Su, 1991; Halaban et al., 1983) and melanin synthesis (Melber et al., 1989) in normal melanocytes treated with TPA. However, the study of the stimulatory effect of TPA on melanogenesis has, in part, been hindered because normal melanocytes in culture frequently require TPA as an obligatory growth factor. To date, there have been no reports on the effect of TPA on the expression of the pigmentation genes in normal melanocytes and the mechanism by which TPA stimulates melanogenesis has not been elucidated. The pigmented cell lines established in the present study provided a unique opportunity to address this issue because, as described earlier, the cells become TPA-independent but continue to be TPA-responsive.

In this investigation it was found that the levels of tyrosinase, TRP-1 and TRP-2 proteins and mRNAs were increased during TPA-induced melanogenesis in DMEL-2 cells. This study also shows, for the first time, that *mi* gene expression is up-regulated by TPA and that this directly correlates with the increase in tyrosinase, TRP-1 and TRP-2 gene expression. This suggests that the TPA signalling pathway is stimulating melanogenesis by increasing *mi* expression. Interestingly, Hemesath et al. (1998) obtained a somewhat different result when they treated human melanoma cells with TPA. They demonstrated that TPA stimulation results in activation of MAP kinase which in turn phosphorylates Mi. This phosphorylated form of Mi was shown to increase transactivation of a tyrosinase gene promoter reporter construct. Taken together, the results of the present study and that of Hemesath et al. (1998) would suggest that TPA regulates the expression of the tyrosinase-gene family by increasing Mi activity through both transcriptional and post-translational mechanisms (see blue and black arrows in Fig 4.1). The following possible models, also depicted in Fig 4.1, could explain this dual effect.

Firstly, in theory, it is possible that the *mi* gene is autoregulated and that the increase in *mi* expression seen in the present study may be due to TPA increasing the affinity of Mi for its target sequences in the *mi* promoter (as demonstrated by Hemesath et al. (1998) for tyrosinase (see A in Fig 4.1)). Cloning and expression studies of *mi* promoter sequences from other species are clearly necessary to explore this further.


Hemesath et al. (1998)
This Study (Prince, 1999)
Reid et al. (1996)
TPA
MAPkinase
phosphorylates Mi
upregulates tyrosinase gene expression
increase in melanogenesis
A
A
TPA
B
increase mi gene expression
upregulates tyrosinase, TRP-1 and TRP-2 gene expression
increase in melanogenesis
C
Pax3
E
ET-receptor
TPA
D
induce ET-1 gene expression
presence of endogenous ET-1

Secondly, the presence of TPA-responsive DNA elements, AP1 and AP2, in the *mi* promoter would provide a mechanistic link between TPA signalling and stimulation of tyrosinase, TRP-1 and TRP-2 via regulation of *mi* expression (see B in Fig 4.1). However, analysis of the cloned fragment from the human MITF promoter reveals that there are no AP1 and AP2 binding consensus sequences. Another alternative is that TPA may be altering the functioning of a transcriptional regulator of the *mi* gene, such as Bm-2 or Pax3 (see C in Fig 4.1).

Thirdly, the endothelin 1 (ET-1) gene has been shown to contain a TPA-responsive element and, as has been suggested by Reid et al. (1996), TPA may be stimulating melanogenesis by inducing endogenous endothelin production (see D in Fig 4.1). This endothelin (ET) would act on its own endothelin receptor, thus creating an autocrine and/or paracrine loop which mediates the effects observed in the present study (see E in Fig 4.1). Interestingly, several of the effects observed for TPA in the present study are similar to the reported effects for endothelins on normal melanocytes (Hara et al., 1995; Imokawa et al., 1995, 1996, 1997; Lahav et al., 1996; Nataf et al., 1996; Yada et al., 1991); for example, stimulation of melanocyte proliferation, melanin synthesis, dendrite formation and increased tyrosinase and TRP-1 mRNA gene expression. In the light of the results in the present study it would be interesting to establish whether endothelins also have an effect on *mi* expression. Finally, to further explore the mechanism by which TPA stimulates melanogenesis will require a more detailed understanding of its effects on the various PKC isoforms. This should lead to a clearer understanding of the role of PKC in pigmentation.

Future considerations

A number of questions has arisen from the investigations carried out in the present study. A few of these questions, and possible directions for future studies, are briefly discussed below.

Pax3 has recently emerged as a potential regulator of *mi* expression. It is therefore of interest that *Pax3* expression could not be detected in any of the melanocytes tested in the present study, data which conflicts with unpublished reports that *Pax3* is expressed in premelanoblasts and is indeed up-regulated during melanocyte differentiation. In view of the key role that Mi plays

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Fig. 4.1. Possible models for mechanism(s) by which TPA stimulates melangenesis. **A, B, C, D & E** (red arrows) represent possible mechanisms by which TPA stimulates melanogenesis. **Black arrows**, data provided by Hemesath et al., (1998); **blue arrows**, data provided by present study; **green arrows**, data proposed by Reid et al., (1996).

in melanocyte development, the question of what regulates *mi* expression is of paramount importance. One possible approach to confirming the results of the present study would be to carry out a more sensitive assay, such as quantitative RT-PCR, for investigating *Pax3* expression. In addition, detailed investigations into the expression pattern of the *Pax3* and *mi* genes in melanocytes at various stages of development would be most valuable.

One theory put forward in the present study is that the T antigen derails pigmentation by inactivating cellular proteins that are required for regulating melanogenesis. To test this theory, an initial approach could be to repeat the experiment which was designed to establish the domain(s) of the T antigen that are involved in inducing the depigmented phenotype. This would involve stably transfecting DMEL-3 cells with T antigen mutants that are unable to bind either the pRb-related proteins, or p53, or p300, and to assess whether any of these mutants is able to maintain the de-differentiated phenotype at the non-permissive temperature. To overcome the problem experienced in the present study, where the untransfected control cells did not visibly re-pigment at the non-permissive temperature, much earlier passages of the DMEL-3 cells should be used. It is important to note that in the present study the results were analysed by assessing the visible "re-pigmentation" of the cells. However, as has been shown earlier in this study, certain passages of DMEL-3 cells do re-express melanocyte-specific genes at the non-permissive temperature even though visible pigmentation was not evident. An alternative approach may, therefore, be to carry out molecular analyses of the stable transfectants obtained in the present experiment.

One of the most interesting findings of the present study was that TPA regulates expression of the *mi* gene. An obvious line of investigation would be to carry out transient transfection assays in which *mi* promoter reporter constructs are introduced into DMEL-2 cells treated with and without TPA. This would lead to the identification of key promoter elements required for mediating the effect of TPA on *mi* expression which could reveal potential upstream regulators of *mi* gene regulation. Such studies await the cloning of the mouse *mi* regulatory sequences.

In conclusion, the unique feature of the DMEL-3 cells generated in this study is that the differentiation process can be reversibly controlled by regulating the activity of the temperature-sensitive oncoprotein. These cells therefore provide an ideal tool for carrying out differential display analyses or microchip searches for identifying and cloning genes specifically involved in melanocyte transformation and differentiation. It should, however, be emphasised that given the irreversible changes that occur in the DMEL-3 cells, the power of this cell line in the use of melanocyte differentiation is limited to early passages.

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