SYNTHESIS AND ACTIVITY OF TYROSINASE IN MOUSE SKIN MELANOCYTES

by

Nkosana Cyril Nkabinde

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March, 1990

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LIST OF ABBREVIATIONS

cAMP	cyclic-adenosine-3',5'-monophosphate
dbc-AMP	dibutyrylcyclic-AMP
DCF	dopachrome conversion factor
DCOR	dopachrome oxidoreductase
DDA	2',5'-dideoxyadenosine
DEAE	diethylaminoethyl
DEP	diethylpyrocarbonate
DMEM	Dulbecco's minimal essential medium
DOPA	dihydroxyphenylalanine
EDTA	ethylenediaminotetraacetate
FCS	foetal calf serum
GGA	glucose albumin agar
HPE	human placental extract
IBMX	isobutylmethylxanthine
MSH	melanocyte stimutating hormone
PAGE	polyacyrlamide gel electrophoresis
PBS	phosphate buffer saline
PEG	polyethylene glycol
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TPA	12-O-tetradecanoyl-phorbol-13-acetate

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ABSTRACT

Tyrosinase (E.C. 1.14.18.1) is a key enzyme in the biosynthesis of melanin. The control of melanin sythesis was explored in skin melanocytes of the following strains; wild type (C57BL/6J-C/C) (which maximally synthesize melanin at normal mammalian body temperature, Himalayan (C57BL/6J-c^h/c^h) (which maximally synthesize melanin at temperatures below 37°C) and albino (Balb C-c/c) (a mutant which does not synthesize melanin)

The effect of α-MSH on tyrosinase activity was initially investigated. A skin culture tyrosinase assay that made it possible to measure the effect of α-MSH on the activity of this enzyme in vitro was first developed. It was found that α-MSH activated the wild type and Himalayan tyrosinase in a dose-dependent manner and that this activation did not require the de novo synthesis of new enzyme.

The role of glycosylation on the wild type and particularly the Himalayan tyrosinase activity was next investigated. The results do not support, but are not in conflict with the theory that the Himalayan tyrosinase is inherently underglycosylated.

Translation and transcription as additional control mechanisms of tyrosinase activity was finally investigated. The correlation between the levels of tyrosinase activity, abundance of the enzyme and the synthesis of tyrosinase mRNA in wild type, Himalayan and albino mice was determined. It was shown that the levels of newly synthesized tyrosinase and tyrosinase mRNA transcripts were higher in the wild type than in the Himalayan skin. This could account for the reduced tyrosinase activity in the Himalayan mutant at normal body temperature. Low levels of tyrosinase mRNA were found in the albino skin though there was no immunodetectable enzyme in this tissue.

CHAPTER I

GENERAL INTRODUCTION

1.1 INTRODUCTION

The development of a fertilized egg into a mature organism does not simply produce a multicellular mass of identical cells. Instead, intracellular events leading to unique structural and functional products take place. At the molecular level, differential gene activity in different cells results in the synthesis of the specific proteins which are characteristic of the various cell types.

While differentiated cells produce unique proteins, the mechanisms that control the synthesis of these end products are not so obvious. The mystery of the differentiative pathway has considerably deepened following the realization that different cells within the same organism have an identical and complete sets of genes. How tissue specific master genes direct the expression of mRNA in these different cell types remains to be elucidated. Since differentiated cells change the expression of their final product in response to various agents, it is still not clear how this process occurs and whether such changes are regulated at the level of transcription or post-transcription.

To answer these questions, the present study has made use of the melanocyte, a pigment cell whose differentiated condition is characterized by the formation of melanin. A pigment cell

provides an ideal model system to study differentiation events, since melanin synthesis is catalyzed by a single key enzyme, tyrosinase, which can easily be assayed *in vitro*. Although melanin formation is primarily regulated by tyrosinase, how the synthesis and activity of this enzyme in particular are controlled by various endogenous and exogenous factors is poorly understood. In order to explore in depth the control mechanisms of tyrosinase activity, normal, mutant and transformed mouse melanocytes were used in this investigation

1.2 ORIGIN AND DIFFERENTIATION OF MELANOCYTES

The neural crest is a transient embryonic structure which gives rise to numerous and diverse derivatives including peripheral neurons, glial and Schwann cells, endocrine and pigment cells (Le Douarin, 1982). These cells migrate along various routes through the extra-cellular matrix to reach their destinations. Pigment cells migrate as large round amoeboid cells called melanoblasts via the sub-epidermal route to their final epidermal sites, where they become fully differentiated melanocytes (Mayer, 1973; Le Douarin, 1982; Nakayama *et al*., 1988) (Fig. 1.1). Melanocyte differentiation is characterized by dendrification (Prestin *et al*., 1987), detectable tyrosinase activity (Pomerantz,

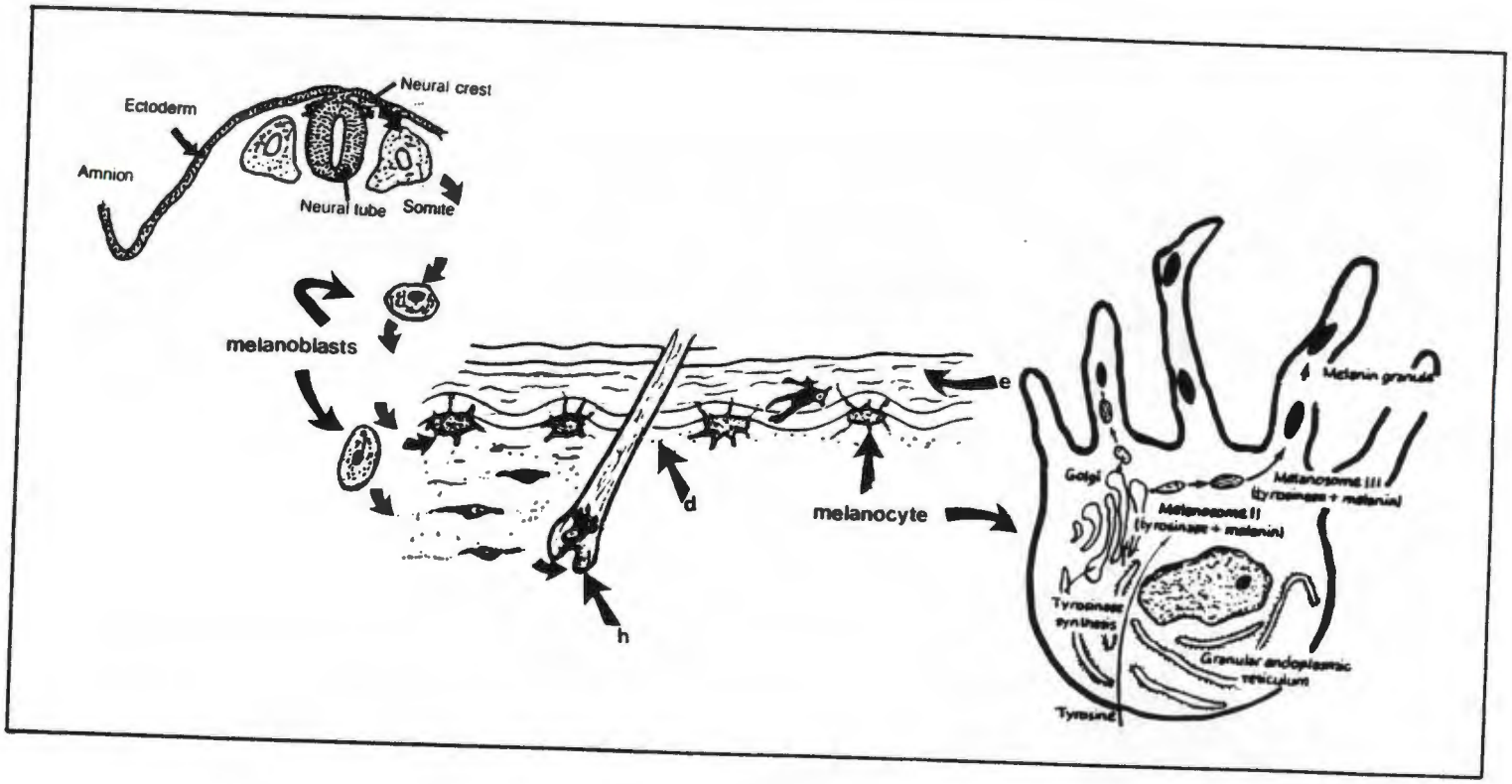

Fig. 1.1: Scheme showing the origin, migration and differentiation of melanocytes. e, epidermis; d, dermis; h, hair bulb.

1966; Pawelek et al., 1973; Halaban et al., 1983; Bennett et al., 1986) and melanin deposition within melanosomes (Steinberg, 1976; Mufson, 1979; Hu et al., 1982).

1.3 BIOCHEMISTRY OF MELANIN BIOSYNTHESIS

Melanin is synthesized, within the melanosome, from the amino acid, tyrosine. The key enzyme in this biosynthetic pathway is tyrosinase (monophenol monooxygenase, E.C. 1.14.18.1 (Hearing et al., 1980; Garcia-Cánovas et al., 1982; Hearing & Jiménez, 1987). As shown in Figure 1.2., mammalian tyrosinase first catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) which is also a cofactor of this initial reaction (Nagatsu et al., 1964; Pomerantz, 1964, 1966; Oikawa et al., 1972). The second catalytic step of tyrosinase is the oxidation of DOPA to DOPAquinone and subsequent steps resulting in melanin formation are thought to occur autocatalytically. Tyrosinase must therefore have at least three binding sites, one each for binding of tyrosine and DOPA as substrates and a third for the binding of DOPA as a cofactor (Pomerantz & Warner, 1967; Hearing & Ekel, 1976). In addition, recent evidence indicates that tyrosinase may also catalyze the oxidation of 5,6-dihydroxyindole to indole-5,6-quinone (Korner & Pawelek, 1982), suggesting that there might be a fourth binding site.

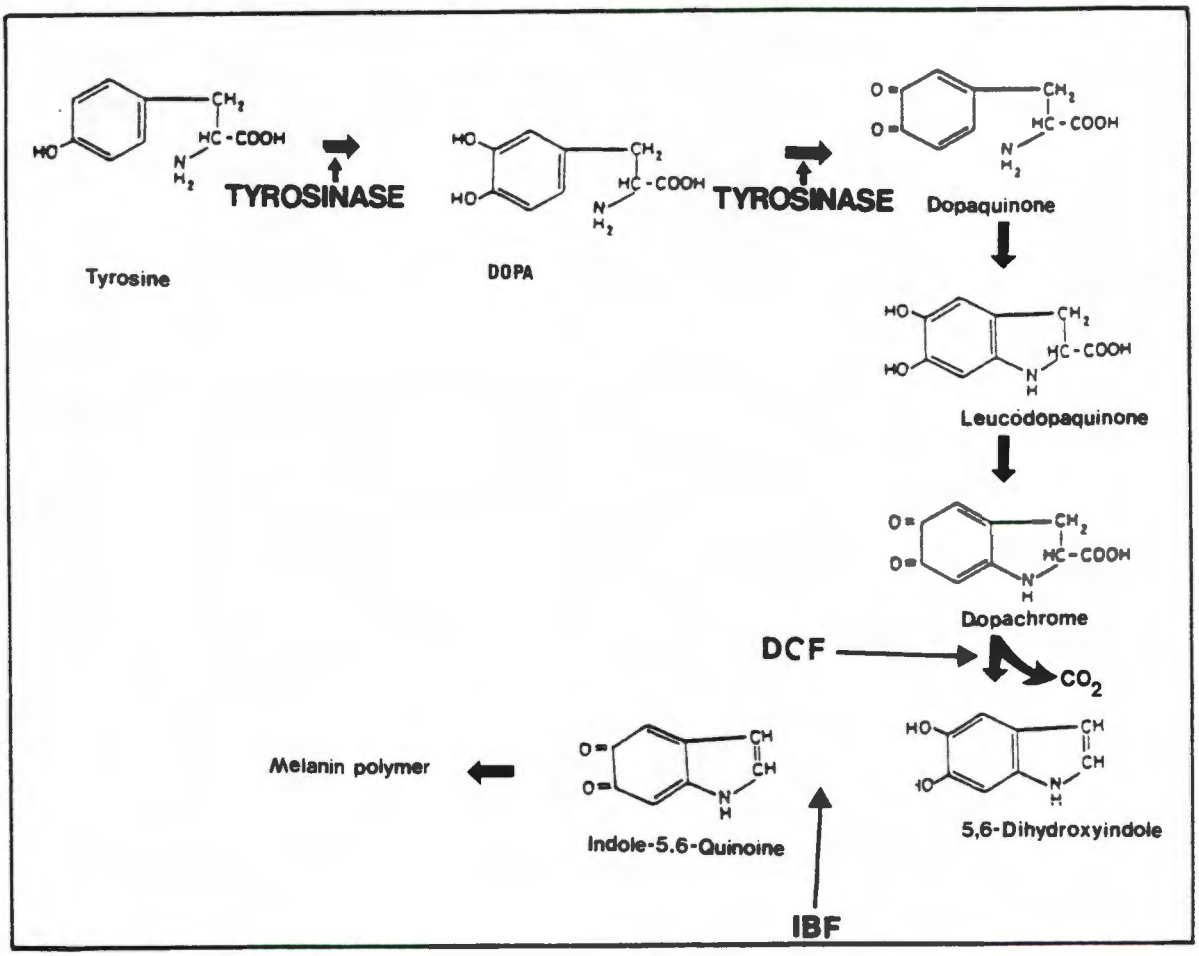

Fig. 1.2: Scheme showing the melanin biosynthetic pathway and control steps distal to tyrosinase. (Based on studies as detailed in the text). Note the third reaction catalyzed by tyrosinase (i.e. 5,6-dihydroxyindole to indole-5,6-quinone) and the sites of action of IBF (indole blocking factor) and DCF (dopachrome conversion factor).

Even though the conversion of DOPAquinone to melanin is thought to be autocatalytic, another enzyme, dopachrome oxidoreductase (DCOR), a 34 kd protein (Barber *et al*., 1984), has been shown to catalyze the conversion of dopachrome to 5,6-dihydroxyindole. This enzyme (DCOR), which is referred to by Hearing *et al*. (1981) as the dopachrome conversion factor (DCF), is closely associated with the catalytic activity of tyrosinase *in vivo* inside the melanosome.

Most importantly, yet another factor termed the indole blocking factor (IBF), has been shown to affect melanin formation negatively by inhibiting the conversion of 5,6-dihydroxyindole into indole-5,6-quinone. The indole blocking factor is thought to be an adaptation which prevents the premature formation of toxic melanin precursors in the cytoplasm. Thus, inside the melanosome, the site of melanin formation *in vivo*, the indole blocking factor is replaced by dopachrome conversion factor (Hearing *et al*., 1982).

1.4 FACTORS THAT REGULATE TYROSINASE ACTIVITY

Although tyrosinase is known to be the key enzyme involved in the biosynthesis of melanin in pigment cells, the question of how its activity is regulated still remains to be fully elucidated. Besides genetic factors, a number of parameters which regulate melanin synthesis by modulating the activity

of tyrosinase have been identified. These include exogenous factors such as:

(1) Ultra-violet radiation in humans (Quevedo et al., 1965; Rosdal & Szabo, 1978; Rosen, 1987; and Lester et al., 1988).

(2) Melanocyte stimulating hormone in mice (Burchill et al., 1986; Seerchurn et al., 1988) and other mammals (Weatherhead & Logans, 1981).

and endogenous factors like:

(3) Inhibitors and activators of tyrosinase (Hearing, 1973; Chen, 1975; Korner & Pawelek, 1977; Korner & Pawelek, 1980; Barber et al., 1984; Garcia-Boron et al., 1985; and Pawelek et al., 1980 Hearing et al., 1982).

(4) Availability of substrates (Slominski et al., 1988) and cofactors (Hearing et al., 1978; Martinéz et al., 1987)

(5) Intra-melanosomal pH (Devi et al., 1987; Garcia-Cánovas et al., 1988)

(6) Post-translational glycosylation (Hearing et al., 1981; Imokawa & Mishima, 1982; Imokawa & Mishima, 1983; Imokawa & Mishima, 1986; and Halaban et al., 1988).

(8) Environmental temperature (Coleman, 1962; Chen & Chavin, 1975; Kidson & Fabian, 1979; 1981; Halaban et al., 1988; Fernandez & Bagnara, 1989).

The relevant literature on some of the factors covered in this thesis will be discussed below and possible mechanisms action of these factors described.

1.5 GENETIC CONTROL OF MELANIN SYNTHESIS

The inheritance of coat color in mice was one of the first mammalian traits to be studied (Cuenot, 1905; Castle & Little, 1910). Since then, geneticists have attempted to identify genes that influence pigmentation. To date, significant progress has been made in this direction as more than 50 genetic loci that affect melanin formation have already been described (Bennett et al., 1989; Brilliant et al., 1989). This discussion will be restricted to the role of the A, B, C and E loci and their interactions during melanogenesis.

The C or albino locus, situated on chromosome 7, controls the availability of active tyrosinase and determines whether or not the melanin pigment is formed. One of the well described mutation at this locus is that of albinism, which is caused by undetectable or reduced levels of tyrosinase activity, resulting in the inability to synthesize melanin. The albino phenotype is therefore characterized by white coat color due to the absence of melanin in pigment cells (i.e. amelanotic melanocytes). Other known alleles of this locus include; the

Himalayan (c^h), chinchilla (c^{ch}) and extreme dilution (c^e) mutants mice.

Previously, it was shown that the C/c heterozygotes of the F_1 generation displayed an intermediate coat color and tyrosinase activity when compared with their C/C and c/c homozygous parents (Coleman, 1962). This observation led to the assumption that the C locus encodes the structural gene for the pigment synthesizing-enzyme tyrosinase (Silvers, 1979; Kwon et al., 1987; Halaban et al., 1988; Ruppert et al., 1988). In contrast to this view, the finding that some albino mice and hamster species displayed significant levels of tyrosinase activity gave rise to the hypothesis that the C locus does not code for the structural tyrosinase gene, but rather a regulatory protein involved in melanin formation (Hearing, 1973; Pomerantz and Li, 1974). In the light of this controversy, a lot of effort has been focused on generating DNA probes so as to perform a detailed analysis of the C locus at the molecular level (Shibahara et al., 1986; Kwon et al., 1987, 1988; Ruppert et al., 1988).

The first cDNA thought to code for tyrosinase, called pMT4, was isolated by Shibahara et al. (1986), but was later shown by Müller et al. (1988) to hybridize to genomic DNA of mice carrying large C locus deletions. Furthermore, expression studies showed that the cDNA insert of pMT4 produced a

protein with no tyrosinase activity, suggesting that this is not the authentic tyrosinase gene. These findings suggested that the pMT4 cDNA, which was shown by Jackson (1988) to map to the B locus, does not encode for tyrosinase. Recently, a number of genes which were mapped to the C locus have been sequenced and cloned. These include cDNA's such as the pme134 (Kwon et al., 1987), MTY811 (Kwon et al., 1988), pmTyr1 (Ruppert et al., 1988). These cDNA's are thought to code for tyrosinase because their corresponding genomic DNA has been shown to be absent in C-locus deletion mice, but present in wild type mice. The transfection of these cDNA's into cells that do not normally synthesize melanin (e.g. breast cancer cell line, albino melanocytes, etc.) has been shown to confer tyrosinase activity on these cells (Müller et al., 1988; Yamamoto et al., 1989). These findings clearly show that the C locus is the structural gene for tyrosinase. However, this conclusion has been challenged by Shibahara (1988) who by the Southern blot analysis showed that the genomic DNA from the C-locus-deleted mice gives the same signal as that of wild type mice, suggesting that the C locus does not code for tyrosinase. Thus, the question of whether the C locus is the structural or regulatory tyrosinase gene still remains to be elucidated and may perhaps in future be resolved by the use of transgenic mice.

The A or agouti locus situated on chromosome 2, determines whether eumelanin (black melanin) or phaeomelanin (yellow melanin) is produced by hair follicular melanocytes. The melanocytes of wild type mice at this locus first produce eumelanin, followed by phaeomelanin and then again eumelanin. Due to this sequential production of eumelanin and phaeomelanin (Galbraith, 1964; Sakurai et al. 1975), the hair of these animals is characterized by a yellow and black band pattern. The melanocytes of non-agouti animals produce only eumelanin and as a result, their hair is uniformly black. Although it is known that sulphydryl compounds such as cysteine are elevated during phaeomelanin synthesis in agouti mice, the precise switch-mechanism which determines whether phaeomelanin or eumelanin is produced remains to be elucidated.

Understanding how these two types of melanin are produced is further complicated by the fact that, in addition to the A locus, it is also dependent on the E locus (situated on chromosome 8). Previous studies have shown that while alleles at the A locus exert their effects by modifying the local tissue environment outside the melanocyte (Silvers & Russell, 1955; Silver, 1979), those at E locus act within melanocytes (Lamoreux & Mayer, 1975).

The brown or B locus (situated on chromosome 4) also affects the production of melanin. The pMT4 cDNA (Shibahara et al., 1986) mentioned earlier in this review, maps to this locus. Evidence to date (as revealed by electron microscopy) indicates that this locus is involved in determining the shape of melanosomes. In homozygous black mice (non agouti), eumelanin is synthesized on ellipsoidal melanosomes, whereas in homozygous brown mice, eumelanin is formed in smaller rounded melanosomes.

It is clear from this discussion that various genetic loci interact to direct the synthesis and type of melanin produced in the melanocytes of wild type and mutant mice.

1.6 TYROSINASE GLYCOSYLATION AND ITS ACTIVITY

Tyrosinase is a glycoprotein with at least four to six potential glycosylation sites (Okhura et al., 1984; Hearing & Jiménez, 1987; Kwon et al., 1988; Hoganson et al., 1989; Kwon et al., 1989). Its ultimate structure is affected by the nature and extent of glycosylation. Glycosyltransferases (enzymes which add specific sugar residues to the protein core) add neutral sugar residues such as mannose, N-acetylglucosamine, galactose and fucose and terminal charged sialic sugar residues to the non-glycosylated tyrosinase isozyme, T_3, to form T_1 and T_2 isozymes (Hearing

et al., 1978, 1981; Imokawa & Mishima, 1982; Laskin & Piccini, 1986; Hearing & Jiménez, 1987). Previous studies have shown that the digestion of T_1 tyrosinase isozyme with neuraminidase (which cleaves off terminal sialic acid residues) results in T_3, the de novo form of tyrosinase Imokawa & Mishima, 1986). In contrast, Hearing et al. (1978, 1981) demonstrated that the treatment of T_1 with neuraminidase does not result in the T_3 native form of tyrosinase. The results of the latter studies seem reasonable since the sugar composition of the T_1 isozyme includes residues such as mannose, galactose and sialic acid. Therefore, the conversion of T_1 to T_3 should result from the combined activity of α-mannosidase, β-galactosidase and neuraminidase. The fully glycosylated form of tyrosinase, T_1, is packaged into coated vesicles and finally delivered to melanosomes, where it complexes with membrane components to form a high molecular weight complex T_4 (> 250 000 Da) (Hearing, 1987).

Glycosylation has been shown to affect the half-life of various glycoproteins by rendering them resistant to proteolysis (Older et al., 1978) and by facilitation of proper folding (Paulson, 1989). The use of drugs such as tunicamycin or glucosamine (inhibitors of the synthesis of N-linked core glycosylation) has been shown to result in the aggregation of most glycoproteins in the endoplasmic

reticulum (Kornfeld & Kornfeld, 1985; Rademacher et al., 1988). These results demonstrate the role of N-linked core glycosylation in intra-cellular translocation of glycoproteins. The inhibition of the synthesis of tyrosinase N-linked sugars by these drugs has already been tested in cultured B16 mouse melanoma cells. As expected, the treatment of these cells with either glucosamine (1 mg/ml) or tunicamycin (0.4 μg/ml) resulted in marked loss of pigment inside the melanosome as visualized by electron microscopy (Imokawa & Mishima, 1982). The depigmentation in these melanoma cells caused a 80-86% reduction in the incorporation of [^{3}H]-mannose after 5 to 10 hours of glucosamine treatment. Electrophoresis of tyrosinase under these conditions showed an increase in the T_3 isozyme accompanied by a substantial decrease in the T_1 form of the enzyme. Tyrosinase activity in the melanosomal fraction of glucosamine-treated melanoma cells was found to be significantly lower than that of the control. These results demonstrate that the loss of pigment in the melanosomes is due to the lack tyrosinase activity caused by the inhibition of the translocation of the enzyme.

The inhibition of tyrosinase transportation to the melanosomes (similar to that induced by glucosamine or tunicamycin in vitro) has recently been identified by Imokawa et al. (1988) in the chinchilla mouse (genotype a/a, c^{ch}/c^{ch}, stain PW). In this study, it was shown using organ culture

and electron microscopy that the level of tyrosinase activity in Golgi-associated endoplasmic reticulum of lysosome (GERL) and coated vesicles was within normal range, but no activity was detected in melanosomes. The electrophoretic pattern of tyrosinase determined by SDS-PAGE showed the levels of the T_1 isozyme in chinchilla melanocytes to be much lower than that of the wild type black mouse (C57BL/6J strain). However, the supplementation of the culture medium with either 2 mM theophylline or dbc-AMP, resulted in markedly increased melanin deposition in the melanosomes as revealed by electron microscopy, suggesting the promotion of tyrosinase translocation by these agents. In contrast, it was found that the presence of α-MSH (1 mg/ml) in the culture medium did not result in increased melanin deposition in the melanosomes of this mutant. Although theophylline and dbc-AMP were effective promoters of melanogenesis in chinchilla melanosomes, their mechanism of action is not known. One cannot assume that these agents act through their conventional c-AMP mechanism of action since in RPMI melanoma cells the stimulation of melanin formation by theophylline does not involve this pathway (Steinberg & Whittaker, 1976). In addition, α-MSH, which is known to act via this pathway, did not enhance melanosomal melanin formation in this mutant. The important conclusion from this study is that <u>in vitro</u> agents such as theophylline and dbc-AMP can override and correct for the genetic defect in the chinchilla mouse.

Following the description of the functional role of core glycosylation on tyrosinase, the discussion below will be restricted to the significance of terminal sugars on the activity of this enzyme. In general, terminal sugars are known to significantly alter the charge of glycoproteins, thereby affecting their solubility and conformational properties (Berman & Lansky, 1985). In a recent study, a 50- to 100-fold variation in sialyltransferase mRNA levels were measured in different rat tissues, which were shown to correlate with the levels of enzyme activity (Paulson et al., 1989). The role of sialic acid residues on tyrosinase activity has been demonstrated by using neuraminidase, which hydrolyzes the bonds joining the terminal sialic acid with internal sugar residues. The treatment of T_1 tyrosinase isozyme purified from B16 melanoma cells resulted in a change in the pI from 3.3 to 4.2 as well as a 21% reduction in the activity of this enzyme (Hearing et al., 1981).

Retinoic acid (RA), a vitamin A derivative, is an agent with diverse biological functions including cell differentiation (Yaar et al., 1981), cancer prevention in experimental animals (Moon et al., 1983) and pattern formation in developing limbs (Summerbell, 1983). In pigment cells, retinoic acid has been shown to promote melanogenesis in Hs 939 human and S91 mouse melanoma cells (Lotan et al., 1978,

1981; Lotan, 1979; Lotan & Lotan, 1980). A dose-dependent increase in melanization caused by retinoic acid (10^{-10} M to 10^{-6} M) was shown to result from a corresponding increase in tyrosinase activity in these cells. What needs to be elucidated is the mechanism by which retinoic acid promotes melanogenesis. Available evidence so far indicates that retinoic acid-stimulated tyrosinase activity in melanoma cells is accompanied by increased activity of sialyltransferases (Deutsch & Lotan, 1983; De Luca, 1977; Lotan et al., 1984). These results suggested that retinoic acid activates tyrosinase by facilitating the attachment of terminal sialic acid sugar residues. Recently, tyrosinase and cell surface glycoproteins have been shown to share the same intra-cellular glycosylation machinery (Imokawa & Mishima, 1988) (see Fig. 1.3). It is therefore likely that retinoic acid-stimulated activity of sialyltransferases may not only affect tyrosinase, but other cell surface glycoproteins. Since these cell surface glycoproteins are known to be mainly involved in metastasis of malignant cells (Hart et al. (1989), the elucidation of the role of glycosylation on tyrosinase activity may lead to a better understanding of the metastatic properties of melanoma cells in vivo.

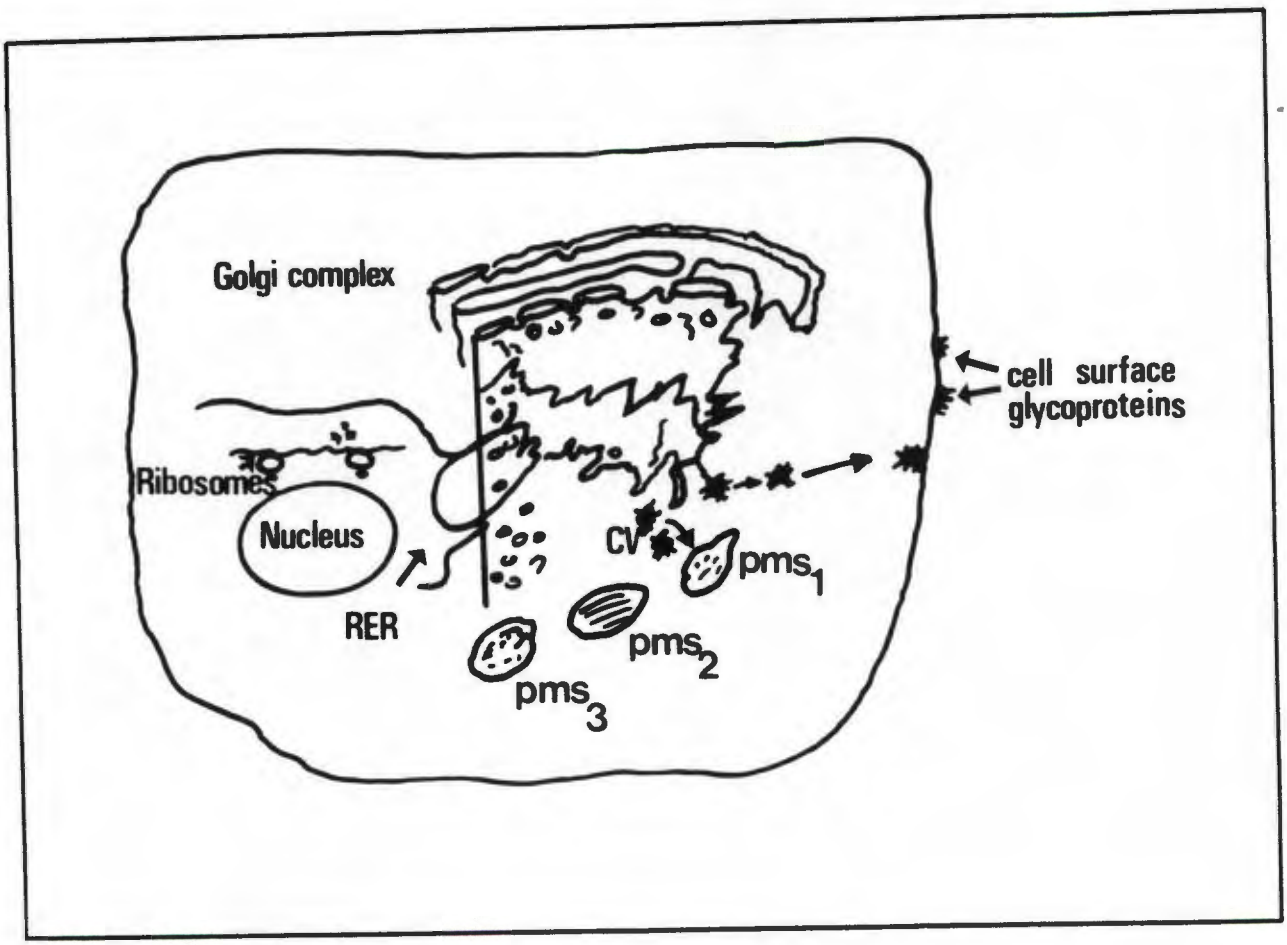

Fig. 1.3: Scheme showing the glycosylation pathway shared by tyrosinase and cell surface glycoproteins (After Imokawa & Mishima, 1988). pms, pre-melanosomes.

1.7 REGULATION OF MELANOGENESIS BY MSH

1.7.1 Effect of MSH on tyrosinase activity and its signal transduction pathway.

Melanocyte stimulating hormone (α-MSH), Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH2, which is synthesized in the pituitary gland, modulates integumental pigmentation of many vertebrate species. The two forms of this peptide hormone (α- and β-MSH) are derived from a 31 kd precursor hormone called pro-opiocortin (Fig. 1.4). Within this large pro-opiocortin fragment are the sequences of the 39 and 91 amino acid residues of adrenotropic hormone (ACTH) and γ-lipotropin respectively, which result from the specific proteolysis of this prohormone. ACTH is further cleaved after the 13^{th} amino acid residue to yield α-MSH and a CLIP peptide consisting of amino acid residues 18-39 of the original ACTH molecule. Soon after the discovery of α-MSH, another form of this peptide, β-MSH, was isolated from the pituitaries of various mammalian species including humans. β-MSH, which is cleaved from β-lipotropin, comprises amino acid residues 41-58 of this fragment. Human pituitaries have been shown to lack α-MSH but to contain β-MSH which is longer (22 amino acids) than the β-MSH's (18 amino acids) from other mammalian pituitaries. It is interesting to note that α- and β-MSH, which are derived from different fragments, have

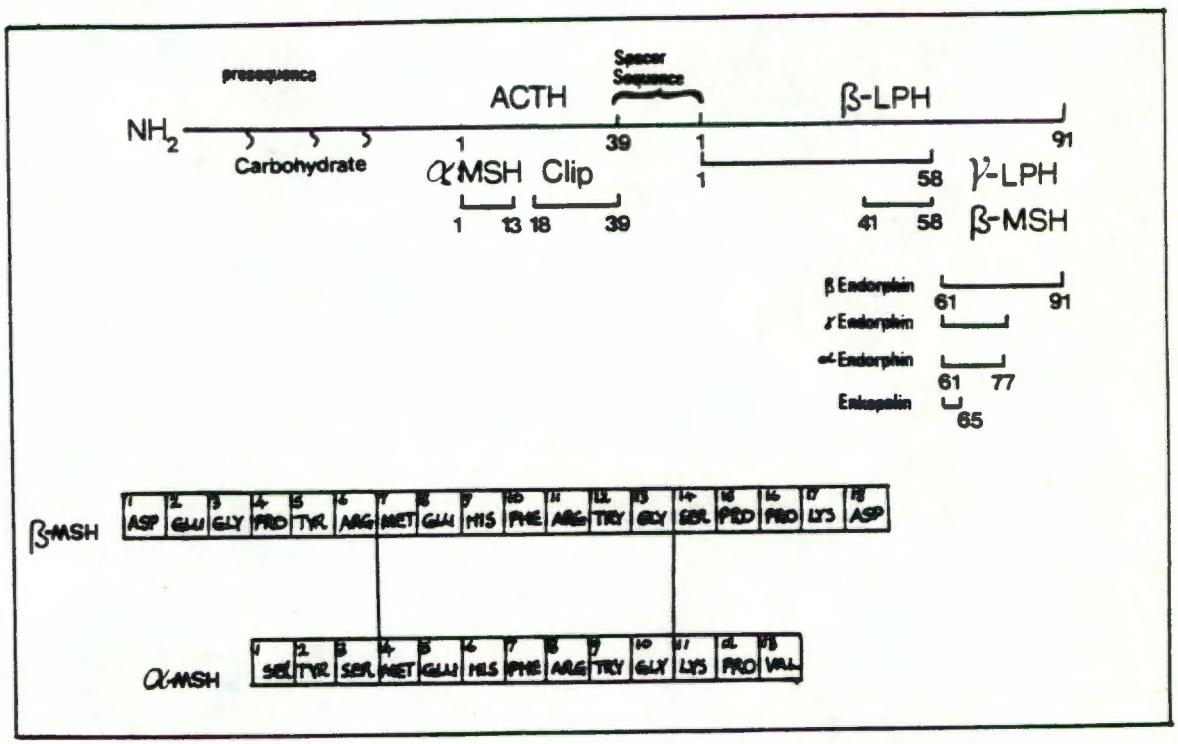

Fig. 1.4: Schematic representation of the known biologically active peptides derived from pro-opiomelanocortin which is found in the vertebrate pituitary (modified from Mac E. Hadley, 1989). Note the sequence homology between β-MSH (amino acids 7-13) and α-MSH (amino acids 4-10). ACTH, adrenocorticotropic hormone, LPH, lipotropin, MSH, melanocyte stimulating hormone.

identical effects on skin pigmentation (Pomerantz & Chiang, 1970; Seerchurn et al., 1988). Although the evolutionary significance of these two forms of MSH is not clear, their identical effects may be due the fact that they both contain the sequence, Met-Glu-His-Phe-Arg-Try-Gly, which has been shown to be the minimal amino acid sequence necessary for the unique biological activity of MSH (Abdel-Malek et al., 1985) (see also Fig 1.4).

In general, MSH is known to be involved in the control of central body temperature (Samson et al., 1981; Murphy & Lipton, 1982), facilitation of selective learning functions (Handelman et al., 1983), camouflage of lower vertebrates such as amphibians (Kaestin et al. 1976; Fukuzuwa & Bagnara, 1989) and mammalian seasonal color change (Rust, 1965). With respect to tyrosinase, α-MSH has been shown to increase the activity of this enzyme in various mammalian melanocytes including humans (Lerner & Mc Guire, 1964; Kitano, 1976) mice (Geschwind, 1966; Pomerantz & Chuang, 1970; Burchill et al., 1986; Seerchurn et al., 1988), hamsters (Weatherhead & Logan, 1981), and guinea pigs (Snell, 1964). What many pigment biologists have attempted to elucidate is the mechanism by which MSH increases tyrosinase activity in pigment cells. To date, several conflicting views on this subject have been reported.

A number of peptide hormones such as thyroid stimulating hormone (TSH), leutinising hormone (LH) and follicle stimulating hormone (FSH) are known to bind to specific receptor sites of the target cells and stimulate the activity of adenylate cyclase (a transmembrane enzyme), thereby instigating the formation of cyclic-3',5'-adenosine monophosphate (c-AMP) (De Robertis & De Robertis, 1987) (see Fig. 1.5). Like these hormones, α-MSH has been shown to promote melanogenesis in melanoma cells by increasing the levels of intra-cellular c-AMP (Bitensky et al., 1972; Wong & Pawelek, 1973; Varga et al., 1974; Wong & Pawelek, 1977; Fuller & Viskochil, 1979; Abdel-Malek et al., 1985; Satya et al., 1987; Abdel-Malek et al., 1987). The view that MSH acts through a c-AMP dependent mechanism, has been reinforced by the fact that known stimulators of the c-AMP pathway such as dibutyryl c-AMP, theophylline and isobutylmethylxanthine (IBMX) mimic the melanogenic effect of this peptide hormone (Hirobe & Takeuchi, 1977; Kwon et al., 1988).

However, the role of c-AMP as an intra-cellular messenger which mediates melanogenesis has been challenged by a few recent studies (Hill et al. 1989; Abdel-Malek et al., 1989). In one these studies, Hill et al. (1989) used F10, B16 F1 and B16 1° melanoma cell lines to study the mechanism of MSH action. These melanoma cells were cultured for 5 days in the presence of MSH (10^{-6} M to 10^{-9} M) which increased the intra-

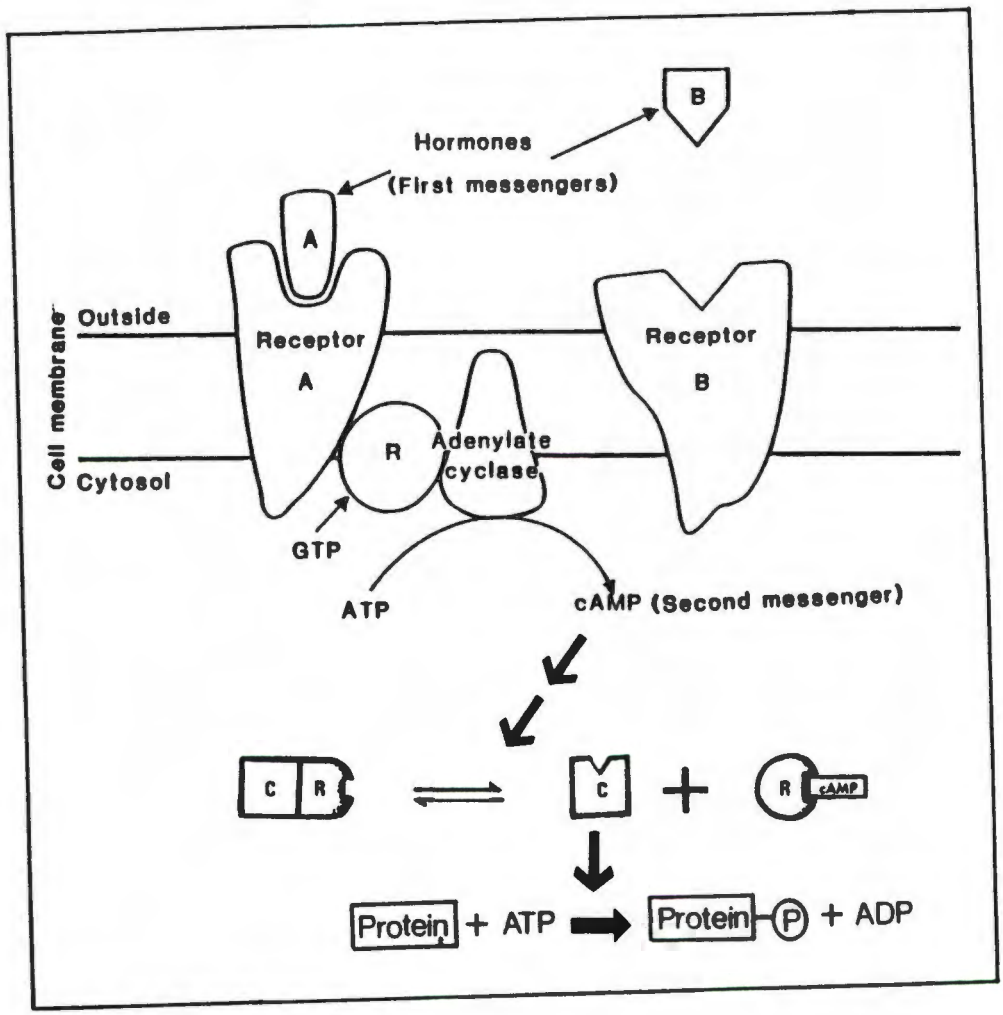

Fig. 1.5: Scheme showing the activation of adenylate cyclase by hormones (first messengers) and the production of intracellular c-AMP (second messenger) (After De Robertis and De Robertis, 1987). R, regulatory protein which binds GTP (Guanosine triphosphate). Note the activation of protein kinase by c-AMP. This enzyme has a catalytic (C) and a regulatory (R) subunit which forms an inactive complex. The free subunit is an active form, which phosphorylates proteins in the reaction.

cellular levels of c-AMP in all the three cell lines. Despite this increase in the levels of c-AMP, tyrosinase activity and melanin content were only elevated in B16 F1 and F10 cell lines, but not in the B16 1° cells. The mutation in these B 16 1° melanoma cells did not result in the absence of MSH receptors or a defective adenylate cyclase system, since c-AMP levels increased. Because of these results, they next explored the possibility that endogenous inhibitors caused the failure of MSH to increase tyrosinase activity in these cells. Tyrosinase activity measured in the mixture of B16 F1 and B16 1° cell lysates showed that the addition of lysate prepared from the latter cell line did not reduce tyrosinase activity in B16 F1 cells, suggesting the absence of inhibitors. Subsequently, the effect of calmodulin antagonists, N-[6-amino hexyl]-5-chloro-naphthalene-2-sulphonamide and 5-iodo-C_8-(N-[6-aminoacyl]-5-iodo-naphthalene-1-sulphonamide), on B16 1° melanoma tyrosinase activity was investigated . These antagonists of calmodulin (a calcium-binding protein which modulates the activity of many enzymes by transmitting the message carried through increased intracellular levels of calcium) caused an increase of tyrosinase activity in these mutant B16 1° melanoma cells. The precise role of the calmodulin pathway on melanogenesis is not known, however, these results showed that the inhibition of this pathway promoted melanogenesis. Calmodulin and calcium may act to inhibit tyrosinase activity

and the stimulatory effects of these calmodulin antagonists could be due to the release of this inhibition. These results are important because they show that in addition to c-AMP, calcium could also be involved in regulating melanogenesis.

In a separate study, further evidence that MSH may not act via c-AMP has been put forward (Abdel-Malek et al., 1989). In this work, 2',5'-Dideoxyadenosine (DDA), a potent inhibitor of adenylate cyclase, was used to investigate the role of the c-AMP-dependent mechanism in MSH-mediated activation of tyrosinase. Cloudman S91 melanoma cells were cultured for 48 hours in media with or without DDA and supplemented with either α-MSH (10^{-7} M) or 10 μg/ml Postglandins E_1 and E_2 (PGE). DDA effectively inhibited α-MSH- and PGE-stimulated increase of c-AMP formation, but resulted in the synergistic increase in tyrosinase activity. Subsequently, it was demonstrated that the treatment of these melanoma cells with either α-MSH or PGE in the presence of DDA, resulted in the recruitment of more cells to the G_2 phase of the cell cycle than in control culture media. Electron microscopy revealed more fully melanized melanosomes in those cells recruited to the G_2 phase by α-MSH when compared to untreated cells. These findings also undermine the role of c-AMP-dependent mechanism in mediating melanogenesis and suggest that MSH may act via cell cycle-

dependent mechanism. Thus, these recent findings which have shown that MSH may act via the calmodulin pathway or the cell cycle are important because they suggest multiple pathways for the control of melanogenesis by MSH and further show that the classic c-AMP pathway may not necessarily be the only transduction mechanism that MSH utilizes.

1.7.2 Does MSH acts at a pre- or post-translational level?

The question of whether MSH acts at a pre- or post-translational level remains to be elucidated. Reports from various laboratories on this subject have so far been conflicting. Some studies suggest that MSH-stimulated tyrosinase activity is due to de novo synthesis of the enzyme (Hirobe & Takeuchi, 1977; Fuller & Viskochil, 1979; Fuller et al., 1987; Jiménez et al., 1988; and Kwon et al., 1988), while other reports suggest that MSH and activators of the c-AMP system enhance enzyme activity through post-translational events (Lee et al., 1972; Wong & Pawelek, 1975; Korner & Pawelek, 1977). In this review, studies on the post-translational as well as the pre-translational regulation of tyrosinase activity will be described in depth beginning with the former control mechanism.

Wong & Pawelek (1975) used synchronized cell cultures of Cloudman S91 melanoma to investigate the effect of MSH on

tyrosinase activity in the presence of cycloheximide (a protein synthesis inhibitor) or actinomycin D (RNA synthesis inhibitor). Results showed that even in the presence of either cycloheximide or actinomycin D, MSH increased tyrosinase activity 15- to 30-fold compared to untreated cells. From these results, it was not clear whether this increase in tyrosinase activity in the presence of MSH was due to the direct activation of the enzyme or a decrease in the degradation rate of the enzyme. To investigate the latter possibility, melanoma cells were cultured for 48 hours in DMEM with or without MSH. After this period of culture, protein synthesis was blocked and tyrosinase activity measured at different intervals. No significant difference in tyrosinase activity was found between MSH-treated and untreated cells, suggesting that the hormone did not alter the degradation rate of tyrosinase. In addition, melanoma cells were cultured for 48 hours in [^{3}H]-leucine-supplemented DMEM with or without MSH. Since the levels of [^{3}H]-leucine incorporation in MSH-treated cells was the same as in control cultures, this suggested that MSH did not promote the synthesis of new enzyme molecules. On the basis of these results, the main conclusion proposed in this study was that the increase in tyrosinase activity in the presence of MSH was due to the activation of pre-existing enzyme.

In a later study, Korner & Pawelek (1977) investigated the effect and mechanism of MSH action in PST-HGPRT-1 melanoma cells (a cell line with low basal tyrosinase activity). The relationship between MSH, c-AMP-dependent protein kinase and tyrosinase activity was investigated. A 30 000g supernatant fraction was prepared from these melanoma cells and incubated with c-AMP-dependent protein kinase. It was found that tyrosinase activity markedly increased in response to this protein kinase. However, when the 30 000g supernatant was prepared from cells that were pre-exposed to MSH in culture, the addition of c-AMP dependent protein kinase did not result in a further activation of tyrosinase. These results suggested that the mechanism of action of MSH and c-AMP-dependent protein kinase in activating tyrosinase could be similar.

In subsequent experiments aimed at understanding the mechanism by which c-AMP dependent protein kinase could activate tyrosinase, an unidentified protein kinase modulator (an inhibitor of c-AMP dependent protein kinase) was used. As expected, it was found that the presence of protein kinase modulator in the 30 000g fraction inhibited both the protein kinase and the activation of tyrosinase, but it had no effect on the enzyme that was activated already. Later, the 30 000g fraction was shown to contain an inhibitor of tyrosinase which disappeared on the addition of c-AMP dependent protein

kinase. The suppression by the kinase of this inhibition correlated with the disappearance of acid-precipitable [^{32}P]-counts. This decay of acid-precipitable [^{32}P]-counts did not result from proteolytic degradation since [^{3}H]-leucine-labelled proteins added to the same assay system were not degraded. These results clearly indicated that the disappearance of [^{32}P]-counts was due to the inactivation of an inhibitor.

In summary, the major findings of this study were that (a) c-AMP-dependent protein kinase mediated the inactivation of a tyrosinase inhibitor through phosphorylation events and (b) since the kinase failed to further activate tyrosinase in cells pre-treated with MSH, it was assumed that the effect of MSH was also to remove this inhibitor. On the basis of these results, a model was proposed that MSH mediated the post-translational activation of tyrosinase by phosphorylating a tyrosinase inhibitor in order to release this inhibitor.

In contrast to the previous results indicating that MSH acts at a post-translational level, Fuller & Viskochil (1979) showed that both RNA and protein synthesis mediate the short-term activation of tyrosinase by MSH. In this study, the increase in tyrosinase activity in S91 melanoma cells occurred 6-9 hours after MSH administration. One of the questions that needed to be answered was: What happened

during this 9-hour period which these investigators called 'the silent period'? Subsequent results showed that within this lag period, there was first a transient increase in intra-cellular levels of c-AMP within 20 minutes, followed by an increase in RNA and protein synthesis 4-6 hours before the increase of tyrosinase activity. To gain further insight into the role of transcription and translation on MSH-mediated tyrosinase activation, the effects of cycloheximide (0.28 μg/ml) and actinomycin D (0.05 μg/ml) were tested on tyrosinase activity stimulated by MSH (10^{-7} M). The addition of either cycloheximide or actinomycin D in the culture media resulted in the 3- to 4- fold decrease in tyrosinase activity. These results demonstrated that protein and RNA synthesis were an absolute requirement for the short-term activation of tyrosinase by MSH (i.e. over a period of 24 hours). Furthermore, to confirm the level of MSH action, a double labelling technique was used. Melanoma cells were first cultured for 4 days in media containing [^{3}H]-leucine and [^{14}C]-tyrosine, then in the presence of MSH for an additional 30 hour period. It was confirmed that MSH increased tyrosinase activity by promoting the <u>de novo</u> synthesis of the enzyme, since the ratio of [^{14}C]-tyrosine to [^{3}H]-leucine in MSH treated cells was found to be two fold higher than in untreated cells.

In a similar study, Fuller & Visckochil (1987) extended their investigation by studying the effect of α-amanitin (an RNA polymerase II inhibitor) and cordycepin (an inhibitor of polyadenylation) on tyrosinase activity in Cloudman S91 melanoma cells. Both α-amanitin (10 μg/ml) and cordycepin (1 μg/ml) effectively suppressed α-MSH-stimulated tyrosinase activity. The results of this study also showed that MSH-mediated increase in tyrosinase activity was due to increased transcriptional rate of the enzyme in melanoma cells.

In an attempt to resolve the controversy about the level of MSH action, Halaban et al. (1984) investigated the effect of this hormone, dibutyryl c-AMP and isobutylmethylxanthine (IBMX) on tyrosinase activity in S91 melanoma cells. The relationship between the changes in tyrosinase activity and its abundance in response to MSH was investigated. Results demonstrated that MSH, IBMX and dibutyryl c-AMP caused an increase in tyrosinase activity that was directly proportional to the abundance of the enzyme. In fact, it was found that MSH stimulated a four fold increase in the activity as well as in the synthesis of tyrosinase. In addition, the model proposed by Korner & Pawelek (1977), (i.e. that MSH mediated the phosphorylation and the inactivation of a tyrosinase inhibitor) was also re-investigated in this work. Melanoma cells, cultured in the presence and absence of MSH, were incubated with

[^{32}Phosphorus]. Analysis of immunoprecipitated tyrosinase from MSH-treated melanoma cells showed no [^{32}Phosphorus] incorporation. These results do not disprove the hypothesis of Korner and Pawelek (1977) since they proposed that it is the tyrosinase inhibitor that is phosphorylated, not the enzyme itself. In addition, Fuller & Visckochil (1987) also found that c-AMP-dependent protein kinase had no effect on tyrosinase activity in melanoma cells. Although the work done by these investigators (Halaban and co-workers; Fuller & Viskochil), did not confirm the model proposed by Wong & Pawelek (1977), they did not propose an alternative model of MSH action.

Due to the recent success in the cloning and sequencing of tyrosinase cDNA's from human (Kwon et al., 1987) and mouse (Yamamoto et al., 1986; Kwon et al., 1988; Müller et al., 1988; Hoganson et al., 1989), the changes in the levels of mRNA in melanocytes and various melanoma cell lines in response to MSH can now be directly quantitated by means of these probes. Kwon et al. (1988) used a tyrosinase cDNA to determine the levels of mRNA in S91 Cloudman melanoma cells treated with β-MSH or IBMX or β-MSH plus IBMX. Melanoma cells were cultured for 4 days in the presence and absence of β-MSH (0.2 μM) or IBMX (0.02 mM) or both agents. The presence of either β-MSH or IBMX increased tyrosinase activity six-fold above control. When β-MSH was added together with IBMX,

tyrosinase activity increased 20 times above control. Northern blot analysis revealed that this increase in tyrosinase activity stimulated by either β-MSH or IBMX was accompanied by a proportional increase in the levels of tyrosinase mRNA. The quantity of mRNA in cells treated with both β-MSH and IBMX was six-fold higher than those treated with either β-MSH or IBMX. These results demonstrate that the increase in the expression of tyrosinase activity is due to an increased rate of transcription as shown by levels of mRNA and confirm previous reports on this subject (Fuller & Viskochil, 1979; Halaban et al., 1984; Fuller et al., 1987).

In support of the above results reported by Kwon et al. (1988), another mouse cDNA has been used to explore the transcriptional regulation of tyrosinase activity in amelanotic (AM-7AS) and melanotic (MEL-11AS) melanoma cell lines (Hoganson et al., 1989). Treatment of the amelanotic and melanotic cell lines with MSH resulted in a 2- and 3.7-fold increase in tyrosinase mRNA respectively. These results together with those of Kwon et al. (1988) clearly demonstrate that the activation of tyrosinase by MSH involves the synthesis of tyrosinase mRNA transcripts and therefore show a transcriptional control of tyrosinase activity by the hormone.

1.8 THE EFFECT OF TEMPERATURE ON TYROSINASE ACTIVITY

Temperature is known to affect the kinetics of most enzyme-catalyzed reactions and tyrosinase is no exception. A study by Chen (1975) provided useful information regarding the effect of temperature on tyrosinase activity in a variety of vertebrates ranging from amphibians to mammals and in melanoma cells. Tyrosinase activity was measured in crude homogenates of skins and melanoma cells by means of a radiometric assay which measures [^{14}C]-tyrosine incorporation into melanin. He showed that maximal tyrosinase activity in most vertebrates and in melanoma cells occurred between 35°C and 45°C. It was further shown that the lowering of skin temperature reduced tyrosinase activity, suggesting that optimal activity of mammalian tyrosinase occurred at normal body temperature.

In contrast, maximal synthesis of melanin in mutant mammals such as the Himalayan rabbit (Chaudhuri, 1929) and Himalayan mouse (Green, 1961) occurs below the normal body temperature. Further histological examination of Himalayan hair follicles also confirmed that there is more melanin deposition below body temperatures than at 37°C (Kidson & Fabian, 1979). At this stage, it was not clear whether this was due to the direct increase in tyrosinase activity or post-tyrosinase factors. In order to resolve this issue, Himalayan

tyrosinase activity was measured in a crude homogenate at various temperatures between 16°C and 60°C and compared to that of the wild type brown mouse (Kidson & Fabian, 1981). By using the [^{14}C]-tyrosine and [^{3}H]-tyrosine assays described by Hearing and Ekel (1976) and Pomerantz (1964) respectively, it was shown that maximal melanin deposition occurring below 37°C, corresponded with maximal tyrosinase activity at ± 25° C.

The next question addressed in the above study was whether the inactivation of the Himalayan enzyme at 37° C was due to endogenous tyrosinase inhibitors. By running a crude homogenate prepared from the Himalayan skin on a Sephadex G-25 column, it was shown that at 37°C, the removal of a low molecular weight fraction from the homogenate resulted in a 27% to 96% increase in tyrosinase activity. The addition of this low molecular weight fraction to the original homogenate from which it was isolated, resulted in a 6% to 34% decrease in tyrosinase activity. However, when the mixture was assayed at 25°C, there was no reduction in tyrosinase activity. These results suggested the presence of a tyrosinase inhibitor in the Himalayan skin that was tightly bound to the enzyme at normal body temperature.

While Himalayan tyrosinase activity had been measured in a crude homogenate by Kidson and Fabian (1981), it was

impossible then to carry out this investigation in isolated melanocytes due to the inability, at that stage, to establish pure melanocyte cultures in vitro. Recently, a technique developed by Tamura et al (1987) alleviated this problem. Using this technique, Halaban et al. (1988) cultured a variety of mouse melanocytes including those of normal wild type (B10.BR-C/C & C57BL/6J-C/C) and Himalayan (c^h/c^h) strains. In this study, the effect of temperature (30°C to 45°C) and DOPA (0.1 μM to 1000 μM) on tyrosinase activity were investigated. In support of Kidson & Fabian (1981), it was also found that the Himalayan tyrosinase activity was higher at 30°C than at 37°C. Furthermore, the response of the Himalayan and B10.BR tyrosinase to various concentrations of DOPA at 30°C were similar, whereas at 37°C, 20 times less DOPA was required to maximally activate the Himalayan tyrosinase when compared to the B10.BR enzyme, further confirming low levels of tyrosinase activity at 37°C. Most importantly, the reduction of the Himalayan tyrosinase activity at 37°C was found to be accompanied by a significant reduction in the ability of this mutant to incorporate radioactive mannose and N-acetylglucosamine than in the wild type. In addition, the molecular weight of the immunoprecipitated Himalayan tyrosinase corresponded to the underglycosylated enzyme. Since carbohydrate residues are known to regulate the stability, turnover and the proteolytic resistance of glycoproteins, it was proposed that the lower

Himalayan tyrosinase activity at 37°C resulted from an unstable enzyme that was susceptible to proteolytic degradation.

1.9 OUTLINE AND AIM OF INVESTIGATION

It is clear from the evidence presented in this review that some of the key factors that determine the activity of tyrosinase are; glycosylation, MSH and environmental temperature. Very little is known about how these factors interact in activating tyrosinase and their mechanisms of action are still poorly understood. In particular, it is not known precisely whether the short-term regulation of tyrosinase activity by these agents is controlled at the level of transcription or post-transcription.

The present study has used wild type and mutant mice at the C locus and transformed melanocytes to investigate the control of melanin synthesis in pigment cells. The mice used are; the normal wild type (C57BL/6J-C/C) (which optimally synthesizes melanin at normal mammalian body temperature), the Himalayan (C57BL6J-c^h/c^h) mutant (which optimally synthesizes melanin at temperatures below normal mammalian body temperatures) and albino (Balb C-c/c) mutant (which does not synthesize melanin). The Himalayan mouse is particularly

interesting because of conflicting reports with regard to identification of the actual mutation.

As a preliminary step in resolving these issues, the temperature sensitivity of the Himalayan melanocytes was confirmed by housing these mice under different temperature conditions and visually observing the resulting color of the hair (see Fig. 1.6). In chapter 2, an attempt was made to investigate the general mechanism of α-MSH action by studying the effect of this hormone on wild type, Himalayan and albino tyrosinase activity and to determine whether α-MSH acts at a pre- or post-translational level. In chapter 3, the possibility that the Himalayan tyrosinase could be underglycosylated compared to the wild type enzyme was investigated. Finally, the role of translation and transcription as additional control mechanisms of tyrosinase activity was explored (Chapter 4).

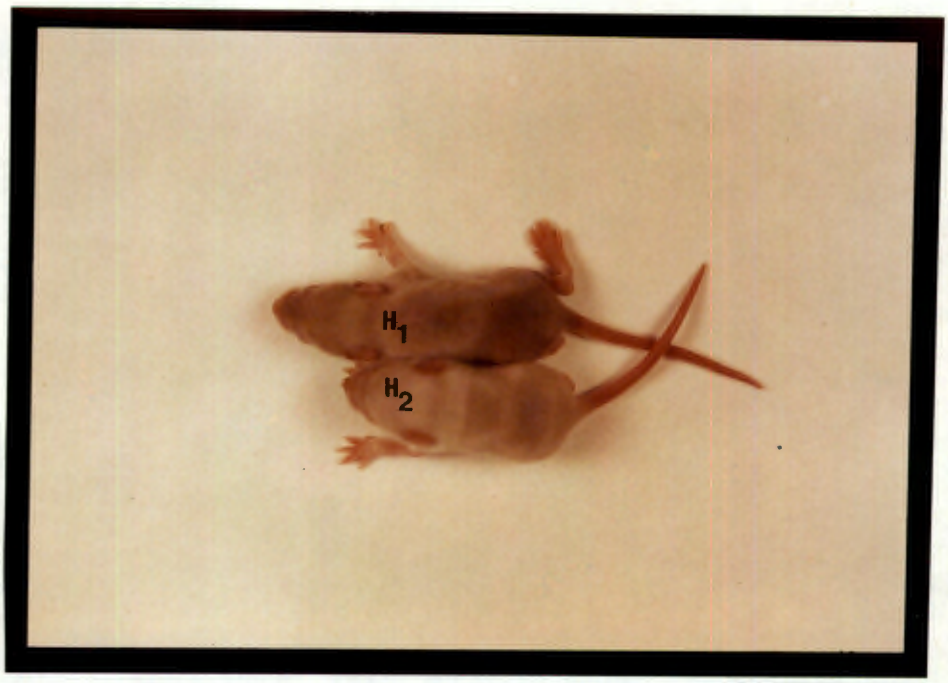

Fig. 1.6: The coat color of Himalayan mice housed under different temperature environments. H_1, mouse housed under cold temperature conditions (15°C); H_2, mouse housed at room temperature (22°C). Note that the Himalayan moused at 15°C appears darker than that at 22°C

CHAPTER II

EFFECT OF α-MSH ON TYROSINASE ACTIVITY IN CULTURED SKIN EXPLANTS

2.1 INTRODUCTION

Melanocyte stimulating hormone (MSH), synthesized and secreted by the pars intermedia of the pituitary gland, modulates integumental pigmentation of many vertebrates. This neuropeptide has previously been shown to increase tyrosinase activity in mouse hair follicle melanocytes (Hirobe & Takeuchi, 1977; Weatherhead & Logan, 1981; Burchill & Thody, 1986; Burchill et al., 1988; Seerchin et al., 1988) and mouse melanoma cells (Wong & Pawelek, 1973, 1975; Pawelek 1976; Hu et al., 1977; Korner & Pawelek, 1977; Fuller & Viskochil, 1979; Halaban et al., 1984; Abdel-Malek et al., 1985,1986; Levine et al., 1987; Kwon et al., 1988; Hill et al., 1989). However, the question of whether the hormone acts at a pre- or post-translational level still remains to be elucidated. Reports on this subject have so far been conflicting; some studies suggest that MSH-stimulated tyrosinase activity is due to de novo synthesis of the enzyme (Hirobe & Takeuchi, 1977; Fuller & Viskochil, 1979; Fuller et al., 1987; Jiménez et al., 1988; and Kwon et al., 1988), while others suggest that MSH and activators of the c-AMP system enhance enzyme activity through post-translational events (Wong & Pawelek, 1973; Wong et al., 1974; Wong & Pawelek, 1975; Korner & Pawelek, 1977). This subject has been reviewed in detail in the General introduction.

The primary aim of this study was to investigate the effect of MSH on tyrosinase activity of the following mice; normal wild type (C/C) (which maximally synthesizes melanin at normal body temperature), Himalayan (c^h/c^h) (a mutant which maximally synthesize melanin at temperatures << 37°C) and albino (a mutant which does not synthesize melanin). Earlier studies carried out on Himalayan tyrosinase showed that the reduced enzyme activity at 37°C is possibly due to a strong enzyme-inhibitor affinity (Kidson and Fabian, 1981). On the other hand, more recent evidence indicates that the temperature-sensitivity of the Himalayan tyrosinase is due the underglycosylation of this enzyme (Halaban et al., 1988). In addition, the analysis of the Himalayan cDNA has revealed that the mutation in this mouse is due to a change of a histidine to an arginine residue at the amino acid 420 (Kwon et al., 1989). The present study has investigated the mechanism of MSH action by studying the effect of the hormone on the Himalayan tyrosinase activity at a temperature that normally inhibits melanin synthesis (i.e. 37°C). If α-MSH does activate the Himalayan tyrosinase under these conditions, this would provide some clues about this mutant enzyme and the general MSH action.

As a starting point, it was necessary to establish an assay that would make it possible to quantitate the levels of tyrosinase activity due to MSH action in vitro. Most studies

on MSH action have been carried out on cultured melanoma cells with one or two on whole skin. The latter studies were carried out by directly injecting MSH into the skins of living mice (Gershwind 1966; Gershwind et al., 1972), but this procedure has practical limitations because:

(a) It is difficult to quantitate and control the amount of hormone injected and therefore impossible to measure the tissue levels of this hormone.

(b) It is also not possible to quantitate the level of enzyme activity due to MSH action in vivo, so evaluation of injected hormone depends on the subjective visual observation of pigment formation.

A number of radiometric assays have been shown to be sensitive enough to measure tyrosinase activity in vitro (Pomerantz, 1966; Hearing & Ekel, 1976). The main disadvantage with these assays is that they measure tyrosinase activity in a crude homogenate and therefore unsuitable for hormonal studies since hormones act by binding to cell surface receptors of target cells (Varga et al., 1974; de Robertis & de Robertis, 1987). To overcome the problems of quantitation inherent with in vivo studies and a crude homogenate, the next viable option was to use cultured mouse skin melanocytes. In an attempt to isolate and culture

mouse skin melanocytes, a method recently developed by Tamura et al. (1987) was used. The major obstacle encountered with this technique was the lack of success of eliminating fibroblast contamination. Finally therefore, a radiometric assay developed by Gatsios and Kidson (1986) from that originally described by Kitano and Hu (1971) was used. This assay measures tyrosinase activity of skin explants cultured on glucose-agar medium in the presence of cycloheximide (a protein synthesis inhibitor). It was shown that the inhibition of protein synthesis by cycloheximide (0.9 μM) does not affect the levels of tyrosinase activity intended to be measured. Furthermore, this assay has two major advantages over other conventional assays used in studies involving melanoma cells because:

(a) Concentrations of exogenous MSH can easily be varied and the effect of this hormone on tyrosinase activity can be measured by quantitating the incorporation of radioactive tyrosine into melanin during α-MSH treatment.

(b) It preserves the normal histological condition of skin in vitro and therefore possibly provides a reasonable model for the events that occur in vivo.

The limitation of this assay technique is that skin removed from its natural environment is subject to gradual autolysis. A measure of this rate of autolysis can be estimated by studying its protein synthesis as function of time. By measuring [^{3}H]-leucine uptake over a 48 hour period, Gatsios and Kidson (1986) showed that skin can be cultured up to 24 hours with minimal tissue deterioration. They also assessed the reliability of this assay by comparing [^{14}C]-tyrosine uptake in highly pigmented agouti and non-pigmented albino skins. Agouti tyrosinase activity was, as expected, found to be 85% to 90% higher than that of the albino mutant. Taken together, these factors show that this radiometric assay is the best `tool' to use in studying the effect of MSH <u>in vitro</u>.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Himalayan mice (C57BL/6J-c^h/c^h) were obtained from the Jackson Laboratory, Bar Habour, USA; Black mice (C57BL/6J-C/C) and albino mice (Balb $C/^J$-c/c) were obtained from the Animal Unit, University of Cape Town. Four to five day old mice were used in all experiments unless otherwise stated.

2.2.2 In vitro radiometric assay

2.2.2.1 Enzyme preparation

Mouse skins were homogenized using a Polytron ultra-homogenizer in 0.1 M phosphate buffer (pH 7.4) containing 0.1% Triton X-100. Phenyl-methyl sulfonyl-fluoride (a protease inhibitor) was added to a final concentration of 10^{-4} M. The homogenate was centrifuged at 20 000g for 20 minutes and the resulting supernatant used to determine enzyme activity. Protein determination was carried out using biuret reaction according to Bradshaw (1966).

2.2.2.2 Measurement of tyrosinase activity

This assay was carried out according to the method of Chen & Chavin (1965) as modified by Hearing & Ekel (1976). The assay specifically measures the incorporation of [^{14}C]-tyrosine into acid insoluble melanin. Each assay was carried out in duplicate (final volume 25 µl) and contained 10 µl of skin homogenate in phosphate buffer, 5 µl of [^{14}C]-tyrosine (0.5 µCi/ml, specific activity 375 µCi/mmole) (ICN Radiochemicals), 5 µl of solution containing chloramphenicol (1mg/ml), cycloheximide (0.9 µM), penicillin G (1000 units/ml) and 5 µl of 0.05 M dihydroxyphenylalanine (dopa). For the determination of non-enzymatic incorporation of [^{14}C]-tyrosine, the homogenate was substituted with 0.1 M phosphate buffer (pH 7.4).

After five hours of incubation in a water bath at 37°C, the mixture was micropipetted onto GFC Whatmann discs, washed twice with 10% trichloroacetic acid (TCA) for 5 minutes, then successively rinsed in 90% ethanol and acetone for 5 minutes and air-dried. Finally, the proteins were solubilized with 0.1 ml Soluene (Packard) and counted in 5 mls of toluene scintillator (Packard) in a Packard Tricarb liquid scintillation counter. Tyrosinase activity was expressed as cpm/µg of protein of homogenate.

2.2.3 Skin culture radiometric assay

2.2.3.1 Preparation of skin explants

Four to five day old mice were killed and immediately rinsed in 70% alcohol and in phosphate buffer saline (PBS) (0.5 M NaCl; 2.7 mM KCl; 3 mM KH_2PO_4 and 28 mM $Na_2HPO_4.12H_2O$, pH 7.4) in quick succession. Skin explants of approximately 2 mm^2 were cut from the dorsal area and subcutaneous fat removed.

2.2.3.2 Measurement of tyrosinase activity in skin explants

Skin explants were cultured with dermis facing down at 37°C for 18 hours on glucose albumin agar (GAA) medium containing cycloheximide (0.9 μM) and [^{14}C]-tyrosine (0.5 μCi/ml, specific activity 513 μCi/mmole) (Amersham) in a humidified chamber. GAA medium was prepared as follows: Thin albumin from an unfertilized chick egg was mixed in a 1:1 ratio with sterile glucose agar (2 g bacto-agar and 1 g dextrose) in 100 mls of mammalian saline (0.18 M NaCl; 5mM KCl and 2.2 mM $NaHCO_3$; pH 7.4) in a water-bath maintained at 50°C. Included in the culture medium were 2.1 mg/ml penicillin G, 4.3 mg/ml streptomycin-sulfate and 150 unit/ml mycostatin.

After culture, explants were washed in 0.4 M perchloric acid (PCA) for 18 - 24 hours, and rinsed twice in 0.2 M PCA for 10 minutes, dehydrated in alcohol and ether in succession for an hour and dried in an oven at 37°C overnight. After drying, the tissues were weighed, placed in glass scintillation vials and solubilized in 200 µl Soluene (Packard). Radioactivity was counted in a Packard Tricarb liquid scintillation counter in 5 mls toluene scintillator (Packard). Tyrosinase activity was expressed as cpm/mg of dry tissue weight.

2.2.4 Measurement of α-MSH-stimulated on tyrosinase activity in skin explants

Wild type, albino and Himalayan mouse skin explants were prepared as described in paragraph 2.2.3.1. They were cultured for six hours on glucose agar albumin (GAA) medium containing α-MSH (Boehringer-Mannheim) at a concentration ranging from 10^{-9} M to 10^{-4} M (as specified in each experiment) then assayed for tyrosinase activity as previously described (paragraph 2.2.3.2.). In order to measure the effect of α-MSH when protein synthesis was blocked, cycloheximide (0.9 µM) was added during the culture period as well as during the assay period.

2.2.5 Culturing of normal murine melanocytes

2.2.5.1 Preparation for melanocyte culture

All glassware was autoclaved and dissecting instruments were sterilized with 70% alcohol. Human placental extract (HPE) was prepared as follows; whole placenta was first washed with sterile PBS and homogenized using a Polytron ultra-homogenizer. The homogenate was centrifuged at 10 000g for 20 minutes and the resulting supernatant was used as placental extract. Dulbecco's Minimal Essential Medium (DMEM) and HPE were sterilized using a 0.45 μm filter. HPE and foetal calf serum (FCS) were inactivated for 30 minutes in a water-bath maintained at 50°C. Mice were killed with chloroform, rinsed in 70% alcohol and sterile PBS and the skin was collected from the dorsal area and processed as described below.

2.2.5.2 Isolation of melanocytes from skin

Method 1

Skins of 4-5 day old mice were treated with 0.25% trypsin and 0.02% collagenase in phosphate buffer (0.1 M, pH 7.4) for 1 hour at 37°C. The tubes containing the skins were vortexed after every 15 minutes so as encourage disaggregation of

cells. After digestion, released cells and tissue debris were spun down at 2 000g and resuspended in TIP medium (DMEM containing 48 nm 12-O-tetradecanoyl-phorbol-13-acetate (TPA); 0.1 mM isobutylmethylxanthine (IBMX); human placental extract (50 μg protein/ml); penicillin G (200 units/ml, Sigma); 100 μg/ml streptomycin) and plated onto tissue culture dishes. Cells were cultured in an incubator at 5% CO_2 and 70% humidity. Tissue debris were sucked out after 2 days of culture.

Method 2

The second method used for the isolation of skin melanocytes was modified from that developed by Tamura et al (1987). Newborn to 4 day old mouse skins from the dorsal area were incubated in 0.25% trypsin in DMEM at 37°C for 3 hours or at 4°C overnight. The dermis was carefully separated from the epidermis by means of forceps and placed separately into TIP medium, teased apart with jeweler's forceps and transferred to 25 cm^2 culture dishes, then suspended in a small volume of culture medium to facilitate optimal attachment of melanocytes.

After 48 hours of culture, the debris were removed and fresh TIP medium was supplemented with Geneticin (100 μg/ml). After treatment with Geneticin for two days, melanocytes were

cultured in Geneticin-free TIP medium. Melanocytes were then cultured either at 30°C or 37°C at 70% humidity and 7% CO_2. After 5 days in culture, the melanocytes were detached with 0.05% EDTA-0.02% trypsin, homogenized in a 1 ml mini homogenizer and homogenate prepared according to the procedure in 2.2.2.1. Tyrosinase activity was measured by means of an *in vitro* radiometric assay (see paragraph 2.2.2.2.). Mouse 3T3 cells were used as a source of tyrosinase-negative cells.

2.2.6 Statistical Analysis

The statistical significance of all results was determined by Student's t-test unless otherwise specified. Values of $P \geq 0.05$ were considered as being insignificant.

2.3 RESULTS

2.3.1 Tyrosinase activity in cultured skin explants

The radiometric assay used in this study to measure tyrosinase activity in cultured skin explants has previously been tested and shown to be reliable as a quantitative measure of tyrosinase activity *in vitro* (Gatsios and Kidson, 1986). Nevertheless, this method was put to the test by determining the incorporation of [^{14}C]-tyrosine into explants of black mouse skin cultured in the presence of 0.9 μM cycloheximide and 1 mM phenylthiourea (a specific tyrosinase inhibitor). Approximately 92% of [^{14}C]-tyrosine incorporation was reduced in the presence of cycloheximide and a further 6% by phenylthiourea. The remaining 2% in the presence of both inhibitors was considered as background uptake of [^{14}C]-tyrosine uptake. These results showed that the inhibition of general protein synthesis by cycloheximide does not affect the incorporation of [^{14}C]-tyrosine due to melanin synthesis. Thus, the 6% reduction in [^{14}C]-tyrosine incorporation caused by phenylthiourea represents tyrosinase activity.

The sensitivity of this skin culture radiometric assay was further put to test by using it to measure levels of tyrosinase activity in skin explants of Himalayan mice housed

at different temperatures. Himalayan mice housed at 15°C and at room temperature (± 22°C), albino and wild type skin explants were assayed for tyrosinase activity for 18 hours in the presence of [^{14}C]-tyrosine and cycloheximide (0.9 μM). This experiment was carried out twice and results are presented in Table 2.1. Since albino skins are known to have no tyrosinase activity, the incorporation into these tissues was considered as background. This background incorporation was subtracted from the activity measured in wild type and Himalayan skins. The incorporation of [^{14}C]-tyrosine into explants of Himalayan mice housed at 15°C was found to be 31-47% higher than skins from those housed at room temperature. The incorporation of [^{14}C]-tyrosine in the wild type strain was 1.5 to 2.8 fold higher than that of the Himalayan mice housed at room temperature.

2.3.2 In vitro radiometric assay

Skins of Himalayan and wild type mice were used to prepare crude homogenates as described in paragraph 2.2.1.1.. (homogenates were prepared from Himalayan mice housed at both 15°C and 22°C). Tyrosinase activity was assayed for 5 hours in the presence of [^{14}C]-tyrosine. It was found that tyrosinase activity of Himalayan mice housed at 15°C was 55-117% higher than those raised at room temperature (Table 2.1). It is interesting to note that in the in vitro assay,

Table 2.1

Skin culture radiometric assay and in vitro radiometric assay

	[^{14}C]-tyrosine incorporation (cpm/mg dry wt) cultured skin[a]	[^{14}C]-tyrosine incorporation (cpm/μg of protein of homogenate homogenate[b]
Experiment 1		
$H^{R.T}$	8 175 ± 870	10 112 ± 644
$H^{L.T}$	10 682 ± 562	15 755 ± 801
W.T.	13 876 ± 492	138 088 ± 2 781
Experiment 2		
$H^{R.T}$	8 889 ± 1 013	8 125 ± 941
$H^{L.T}$	13 116 ± 883	17 665 ± 571
W.T.	24 996 ± 1 069	142 556 ± 4 064

[a] Mouse skin explants were assayed for tyrosinase activity for 18 hour in the presence of [^{14}C]-tyrosine (0.5 μCi/ml) and cycloheximide (0.9 μM) as described in materials and methods. Values represent means of three measurements ± S.D.

[b] Crude homogenate was prepared from mouse skins as detailed in materials and methods and tyrosinase activity assayed for 5 hours in the presence of [^{14}C]-tyrosine. Each data point represent an average of duplicate measurements ± S.D. $H^{R.T}$, Himalayan mice housed at room temperature; $H^{L.T}$, Himalayan mice housed at 15°C; W.T, wild type mouse

the tyrosinase activity of the wild type homogenate was 13-16 times higher than the Himalayan skin, whereas in the skin culture assay wild type activity was 1.5 to 2.7 fold greater. The implications of these results is addressed in the discussion.

2.3.3 Melanocyte culture

Melanocytes were isolated from mouse skins, cultured in TIP medium and assayed *in vitro* for tyrosinase activity as detailed in materials and methods. Tyrosinase activity in Himalayan melanocytes cultured at 30°C for 5 days was approximately 45% higher than those cultured at 37°C (Fig. 2.1). Tyrosinase activity in melanocytes cultured either at 30°C or 37°C, was found to be significantly higher than the incorporation in 3T3 cells ($P < 0.05$).

2.3.4 The effect of α-MSH on tyrosinase activity

In order to determine the effect of α-MSH on tyrosinase activity, the skin culture radiometric assays was used. Wild type, Himalayan and albino skin explants were cultured for six hours in media containing various concentrations of MSH and then assayed for tyrosinase activity for 18 hours. This experiment was repeated at least three times with similar results which are presented in Figure 2.2. The background

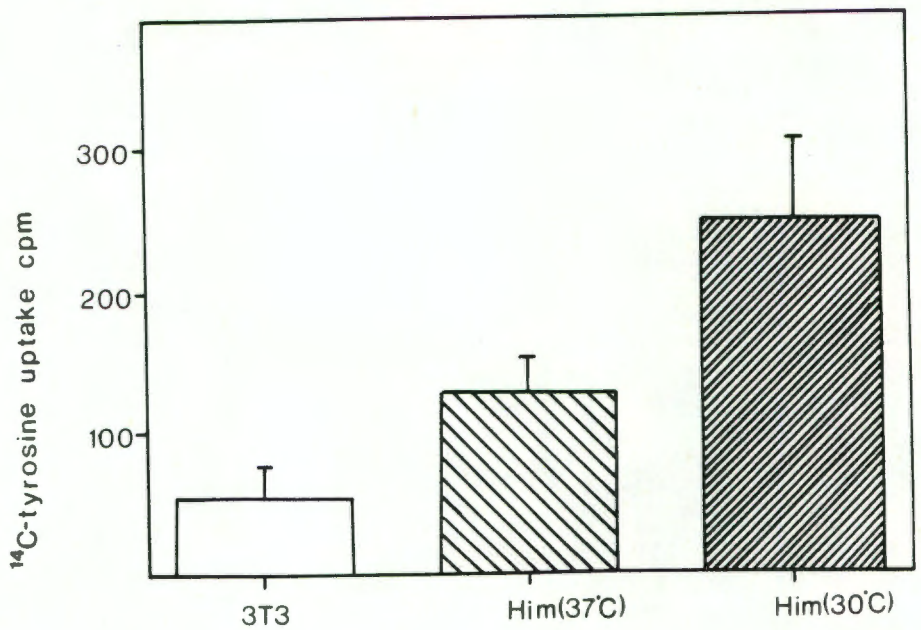

Fig. 2.1: Tyrosinase activity in cultured melanocytes. Himalayan melanocytes were isolated from newborn to 4 day old mouse skins, cultured at either 30°C or 37°C in TIP medium and assayed for tyrosinase activity (see materials and methods). Each value represents a mean of two measurements. All values are significantly different from control (3T3) ($P < 0.05$). 3T3, fibroblasts cells; Him (37°C), Himalayan melanocytes cultured at 37°C; Him (30°C), Himalayan melanocytes cultured at 30°C.

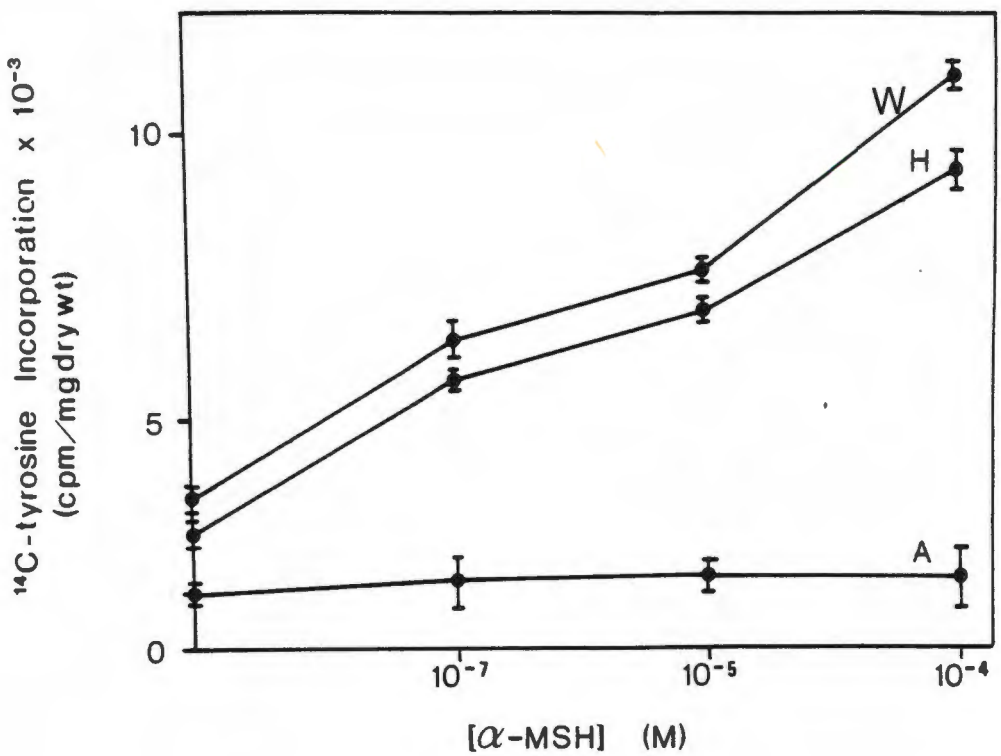

Fig. 2.2: Effect of α-MSH on tyrosinase activity. Skin explants were cultured for 6 hours in medium containing various α-MSH concentrations. They were assayed for tyrosinase activity for 18 hours in the presence of [^{14}C]-tyrosine (0.5 μCi/ml) and cycloheximide (0.9 μM) (see materials and methods). Data points are averages of triplicate measurements and represent one of the three experiments performed. A, albino; H, Himalayan, W, wild type.

incorporation into albino skins was subtracted and the results are presented in Table 2.2. The presence of α-MSH in the culture medium increased tyrosinase activity of both the Himalayan and wild type explants in a dose-dependent manner. At 10^{-9} M, α-MSH increased the Himalayan tyrosinase activity 1.3 fold above control (i.e. [MSH]=0), which increased to 4.1 fold at a concentration of 10^{-4} M. Similarly, wild type tyrosinase activity increased from 1.5 fold at 10^{-9} M to 5.2 fold at 10^{-4} M. It is interesting to note that the extent to which the wild type tyrosinase was activated by α-MSH was similar to that of the Himalayan mouse.

2.3.5 The level of α-MSH action in cultured skin explants

To determine whether the short-term activation of tyrosinase by α-MSH was depended on protein synthesis, cycloheximide (0.9 μM) was added in the culture medium prior to and together with the treatment with α-MSH. At this concentration (i.e. 0.9 μM), cycloheximide has been shown to effectively inhibit protein synthesis by 85% to 90% without affecting the incorporation of [^{14}C]-tyrosine due to tyrosinase activity (Gatsios and Kidson, 1986). The values representing the relative increase of tyrosinase activity at a particular α-MSH concentration were obtained by first

Table 2.2

The effect of α-MSH on wild type and Himalayan tyrosinase activity

[MSH] (M)	[^{14}C]-tyrosine incorporation (cpm/mg of dry weight)	
	Himalayan	wild type
Experiment 1		
0	3 626 ± 237	5 271 ± 772
10-9	6 758 ± 196	9 325 ± 115
10-7	9 504 ± 484	11 402 ± 340
10^{-5}	11 033 ± 808	15 846 ± 834
Experiment 2		
0	1 616 ± 379	2 731 ± 439
10^{-7}	2 186 ± 526	4 719 ± 119
10^{-5}	4 528 ± 73	7 374 ± 471
10^{-4}	6 092 ± 602	9 668 ± 830
Experiment 3		
0	1 047 ± 300	1 643 ± 404
10^{-7}	3 666 ± 568	4 346 ± 80
10^{-5}	4 850 ± 517	5 553 ± 423
10^{-4}	11 233 ± 518	15 846 ± 536

Mouse skin explants were cultured for six hours on glucose-agar media containing various concentrations of α-MSH (as specified in each experiment). They were then assayed for tyrosinase activity for 18 hours in the presence of α-MSH, [^{14}C]-tyrosine (0.5 μCi/ml) and cycloheximide (0.9 μM). Each data point represents the mean ± range of activities from triplicate measurements which have been corrected for background incorporation in the albino skin.

subtracting the background incorporation of the albino skin, then dividing this value by control values. The results obtained show that α-MSH, at a concentration of 10^{-5} M (which was the only tested concentration), caused a relative 4.6- and 3.7-fold increase in the wild type and Himalayan tyrosinase activity respectively (Table 2.3). Similarly, the relative increase of tyrosinase activity in the wild type skin in the absence of cycloheximide was found to be 3.9-fold while that in the Himalayan skin was 3.2-fold. These findings suggest that the increase in tyrosinase activity in response to α-MSH in wild type and Himalayan mice is not due to de novo synthesis of new enzyme molecules.

TABLE 2.3

The effect of α-MSH on tyrosinase activity in the presence of a protein synthesis inhibitor

[MSH] (M)	Relative increase of tyrosinase activity above control			
	Himalayan		wild type	
	$^{+}$CH[a]	$^{-}$CH	$^{+}$CH	$^{-}$CH
0	1.0	1.0	1.0	1.0
10^{-9}	N.D[a]	1.3[b]	N.D.	1.5
10^{-7}	N.D.	2.1	N.D.	2.5
10^{-5}	3.7	3.2	4.6	3.9
10^{-4}	N.D.	4.1	N.D.	5.2

Mouse skin explants were cultured for 6 hours on agar medium containing [^{14}C]-tyrosine (0.5 μCi/ml) with or without cycloheximide (0.9 μM) and various concentrations of α-MSH. They were assayed for tyrosinase activity for 18 hours and processed as described in materials and methods. The values presented here are averages of relative increase of tyrosinase activity above control from three separate experiments. [a] CH, cycloheximide N.D., not determined. [b], value does not significantly differ from control ($P > 0.05$).

2.4 DISCUSSION

2.4.1 Development of a sensitive radiometric assay

The aim of the work presented in this chapter was to investigate the effect of MSH on tyrosinase activity in mouse skin melanocytes *in vitro*. In order to carry out this investigation, a suitable assay had to be established. The assay should make it possible to:

(a) Monitor precisely the concentration of exogenous MSH and to

(b) Detect different levels of tyrosinase activity due varying hormonal concentrations.

The skin culture radiometric assay developed by Gatsios and Kidson (1986) satisfies the conditions stated above. Before using this assay, it was first necessary to asses the sensitivity of this assay. To do this, the temperature-sensitivity of the Himalayan tyrosinase was exploited. Tyrosinase activity was measured in cultured skin explants of Himalayan mice previously housed under different temperature conditions (i.e. 15°C and 22°C). As expected with the Himalayan mutant, this assay showed a 31% to 47% increase in tyrosinase activity in mice housed at 15°C compared to those at 22°C. However, when the radiometric assay described by Hearing and Ekel (1976) was used, tyrosinase activity in a

crude homogenate of Himalayan mice housed at 15°C was found to be 55% to 117% above those at 22°C. Considering the fact that in cultured skin, the measurement of tyrosinase activity (i.e. [^{14}C]-tyrosine uptake) depends on the diffusion rate of substances dissolved in agar, this assay compares well with that of Hearing and Ekel (1976). The sensitivity of this assay and the fact that one can concurrently measure tyrosinase activity during the treatment with hormones has made this assay suitable to fulfil the aims of this study.

2.4.2 Differences in tyrosinase activity in cultured skin and crude homogenate

A striking observation made in this study is that in cultured skin (in vivo), the wild type tyrosinase activity was found to be twice that of the Himalayan mouse, whereas in a crude homogenate (in vitro), the ratio of wild type to Himalayan tyrosinase activity was in most cases found to be approximately 13:1 (Table 2.1). In a similar study, the wild type (C57BL6J-C/C) tyrosinase activity measured by the assay method of Pomerantz (1966) in a crude homogenate, was found to be 100 fold higher than that of the Himalayan mutant (Halaban et al., 1988). Considering the result that in skin culture, the ratio of the wild type to Himalayan tyrosinase activity was found to be 2:1, it would be logical to expect the same ratio even in vitro. Since the actual ratio

(≈ 13:1) significantly deviated from the expected ratio (2:1), what do these results suggest?

First, it should be noted that the de novo form of tyrosinase, T_3, as well as the glycosylated form, T_1, are inhibited by the indole blocking factor in the cytoplasm (Hearing et al., 1982) and are therefore inactive. Thus, in vivo, melanin formation is restricted to the T_4 form which is found in the melanosome (Seiji, 1967; Miyakazi, 1971; Hearing et al., 1982; Hearing & Jiménez, 1987). The skin culture radiometric assay, which mimics the in vivo situation, measures only T_4 form of the enzyme. However, in the in vitro assay, all isozymes (T_3, T_1, and T_4) are released into the homogenate and have been shown to be active (Hearing et al., 1981). This might explain the difference between the results obtained when comparing the in vitro and skin culture assays and suggests that the wild type skin contains a higher level of accumulated precursor forms of T_3 and T_1. This proposition justifies the much higher levels of wild type tyrosinase activity in a crude homogenate as all the isozymes are active. These results are important because they provide some clue to the effect that the de novo processing of the wild type tyrosinase could be different to that of the Himalayan mutant.

2.4.3 The effect of α-MSH on tyrosinase activity in skin explants

The injection of α-MSH into agouti and brown mice has previously been shown to stimulate melanin synthesis and deposition into the growing hair of these animals (Gershwind & Huseby, 1966; Gershwind *et al.*, 1972; Tamate & Takeuchi, 1984; Thody *et al.*, 1984; Burchill & Thody, 1986; Burchill *et al.*, 1986; 1988). A pilot study carried out in our laboratory revealed that the injection of α-MSH into living Himalayan mice also results in the darkening of hair. In order to study the mechanism of MSH action in depth, which is the key aim of this study, it was of absolute necessity to quantify more accurately this effect of α-MSH on the Himalayan hair *in vitro*. To meet this condition, the sensitive skin culture radiometric assay fully described earlier was used to measure the levels of skin tyrosinase activity due to α-MSH stimulation. In addition to the Himalayan mouse, the effect of α-MSH was also investigated in the wild type and the albino mice.

Results showed that over a period of 24 hours, α-MSH stimulated the wild type and Himalayan tyrosinase activity in a dose-dependent manner, but had no effect on the albino tyrosinase (Table 2.2; Fig. 2.2). This activation of wild type and Himalayan tyrosinase by α-MSH was found to be

exclusively post-translational, since this response was not reduced in the presence of cycloheximide (Table 2.3). These results are in agreement with previous reports on melanoma cells (Wong & Pawelek, 1975; Korner & Pawelek, 1977) and in conflict with reports that MSH stimulates the de novo synthesis of tyrosinase (Fuller & Viskochil, 1979; Halaban et al., 1984; Fuller et al., 1987; Kwon et al., 1988; Honganson et al., 1989). The post-translational activation of the Himalayan tyrosinase by α-MSH is particularly interesting, since the enzyme in this mutant has previously been shown to be less active at normal mammalian body temperatures than at lower temperatures (Kidson & Fabian 1979, 1981). How can the post-translational activation of the Himalayan tyrosinase by α-MSH be explained?

The first possibility is that MSH acts through the post-translational removal of an endogenous tyrosinase inhibitor. Support for this hypothesis is provided by the following evidence. In the first place, Kidson and Fabian (1981) proposed that the lower tyrosinase activity in the Himalayan mutant is due to a temperature-induced conformational change of tyrosinase, resulting in increased affinity between the enzyme and its inhibitor. The presence of an inhibitor in pigment cells has also been reported for human melanocytes (Quevedo & Isherwood, 1960; Chian & Wilgram, 1967; Vijiyan, 1982) and melanoma cells (Cooper & Mishima, 1967; Hamada &

Mishima, 1972; Satoh & Mishima, 1967; Korner & Pawelek, 1977; Fuller et al., 1988). Treatment of mouse melanoma cells with α-MSH has been shown to result in very low levels of indole blocking factor (an endogenous tyrosinase inhibitor) and increased levels of dopachrome conversion factor (DCF) when compared to untreated cells (Hearing et al., 1982). Furthermore, recent evidence from studies in which dopachrome conversion factor and dopa oxidase activities were separated by sucrose density centrifugation, clearly demonstrated that MSH enhances DCF activity in melanoma cells (Pawelek, 1989). Similarly, Korner & Pawelek (1977) have demonstrated a MSH-mediated phosphorylation of a tyrosinase inhibitor in S91 melanoma cells, resulting in an inactivation of that inhibitor. It appears therefore that in general, α-MSH stimulates the activity of DCF which may in turn displace the IBF. Interestingly, the results of the present study have shown that the increase of the wild type and Himalayan tyrosinase activity caused by α-MSH was similar (Fig. 2.2), which suggests the presence an inhibitor even in the skin of the wild type. The binding affinity of this inhibitor to the wild type tyrosinase appears to be lower than that of the Himalayan as shown by a higher basal enzyme activity in the former mouse. Furthermore, the failure of α-MSH to activate the albino tyrosinase suggest that the absence of enzyme activity in this tissue is not caused by endogenous inhibitors. On the basis of these findings, it is possible

that at 37°C, α-MSH could activate the Himalayan tyrosinase through the removal of an endogenous inhibitor (the indole blocking factor) and facilitate the binding of the dopachrome conversion factor which promotes melanogenesis via some unknown mechanism (see Fig. 2.3).

A second hypothesis for the mechanism of MSH action in the Himalayan mouse is provided by the recent finding that the lower tyrosinase activity at 37°C in this mutant, could be due to an underglycosylated enzyme (Halaban et al., 1988). The implication of this result may be that the hormone could also promote the glycosylation of the enzyme in this mutant. In light of this, the extent to which glycosylation could influence the activational status of the Himalayan tyrosinase is investigated in the following chapter.

2.4.4 Difficulties in culturing murine melanocytes

Culturing of mouse non-malignant melanocytes in vitro, was for a long time unsuccessful due to:

(a) The difficulty to isolate melanocytes from skin caused by the abundance of acellular tissue in mouse skin.
(b) Failure to select effective mitogens.
(c) Inability to eradicate contaminating fibroblasts.

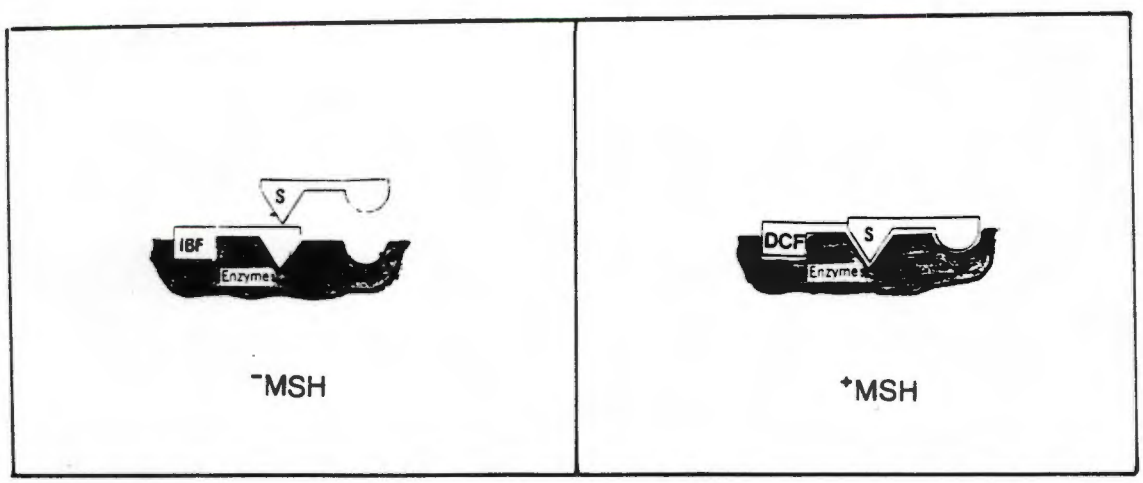

Fig. 2.3: Digrammatic representation of the mechanism of MSH action involving the removal of indole blocking factor. This scheme shows a possible mechanism of MSH whereby the hormone mediates the removal of an endogenous inhibitor (IBF) and facilitates the binding of dopachrome conversion factor (DCF).

Just recently, Tomita et al (1987) developed a technique that overcomes these problems. Using this technique, the digestion of skin with 0.25% trypsin in DMEM was shown to an effective procedure to optimally release melanocytes. In addition, the combined mitogenic action of TPA, IBMX and human placental extract was demonstrated to be necessary for the continued survival of mouse melanocytes in vitro. Finally, the use of Geneticin, an antibiotic which selectively kill fibroblasts, was recommended as a reliable way of eliminating fibroblasts.

In the present study, initial attempts to isolate skin melanocytes using the trypsin-collagenase procedure described earlier in this chapter, were found to be unsuccessful because:

(a) Not many cells were released from skin after digesting with trypsin and collagenase for an hour.

(b) When the digestion period was increased to 2 hours, very few cells survived.

To overcome these problems, the method developed by Tamura et al. (1987) was refined and used to isolate skin melanocytes. This method proved to be effective since a reasonable proportion of viable cells was released from the skin. Isolated melanocytes were subsequently grown in TIP medium originally proposed by (Tamura et al., 1987). The successful culturing of melanocytes in this culture medium confirmed the

effectiveness of mitogens it contains in supporting the growth of murine melanocytes *in vitro*. However, as noted by Tamura *et al.* (1987), it was also confirmed in this study that only dermal melanocytes survived in the TIP medium as epidermal melanocytes routinely died after five days in culture. These observation could suggest that, in addition to mitogens present in this culture medium, the dermal tissue debris could be a source of other mitogens that are necessary for the survival of melanocytes. The surviving dermal melanocytes were treated with 100 μg/ml of Geneticin, a dose recommended to be effective in killing contaminating fibroblasts. Despite the use of Geneticin, it was not possible to be sure that pure melanocytes were ultimately obtained. In conclusion, it is worth emphasizing that this technique was difficult to master.

CHAPTER III

THE EFFECT OF UNDERGLYCOSYLATION AND ENHANCEMENT OF GLYCOSYLATION ON TYROSINASE ACTIVITY IN NORMAL AND TRANSFORMED MELANOCYTES

3.1 INTRODUCTION

In the previous chapter, an hypothesis that α-MSH activates Himalayan and wild type tyrosinase by removing an endogenous inhibitor (the indole blocking factor) was proposed. This inhibitor could be more tightly bound to the Himalayan tyrosinase than the wild type as shown by higher basal enzyme activity in the latter. In addition to endogenous inhibitors, it has also been recently reported that reduced Himalayan tyrosinase activity could be due the deficiency of N-linked glycosylation (Halaban et al., 1988). In the light of this, the present chapter has investigated the possibility that the activation of the Himalayan tyrosinase by α-MSH could involve glycosylation. Based on the assumption that low Himalayan tyrosinase activity results from under-glycosylation, this chapter examines whether the missing sugars are core or terminal residues and to what extent these sugar residues affect tyrosinase activity

Tyrosinase is known to be a glycoprotein with four to six N-linked glycosylation sites (Hearing & Jiménez, 1987; Kwon et al., 1988, 1989), but not much is known about the functional role of the sugar residues. For many glycoproteins, carbohydrate moieties confer important physical properties such as protease resistance (Schwartz et al., 1976; Older et al., 1978), charge and water-binding capacity (Paulson,

1989), conformational stability (Stanely, 1987), intracellular signal for localization to specific organelles (von Figura et al., 1979; Smith & Baenzinger, 1988) and enzyme activity (Paulson et al., 1989).

Although the biosynthesis of glycoproteins is complex, the unique nature of the linkages between the protein core and the carbohydrate side chains (i.e. N- or O- linked sugars), have simplified the study on the role of these sugars of glycoproteins. The N- or asparagine- linkages involve the amide group of the amino acid, asparagine, with the carbon-1 of N-acetylglucosamine (Fig. 3.1). The O-linkages are formed via the reaction of the hydroxyl group of serine or threonine and the reducing carbon of the sugar C_1 or C_2 (Fig.3.1). The availability of drugs such as tunicamycin and glucosamine which preferentially inhibit the synthesis of N-linked core sugars, has recently spurred a number of investigators to endeavor to unravel the functions of the carbohydrate moieties of glycoproteins. As shown in Figure 3.2, tunicamycin blocks the addition of N-acetylglucosamine to the carrier molecule (dolichol phosphate), the first step in the formation of the core oligosaccharide. Glucosamine acts at a later step in this biosynthetic pathway by inhibiting the transfer of mannose from dolicholmannose and guanosine diphosphomannose (GDP-mannose) to the growing carbohydrate chain.

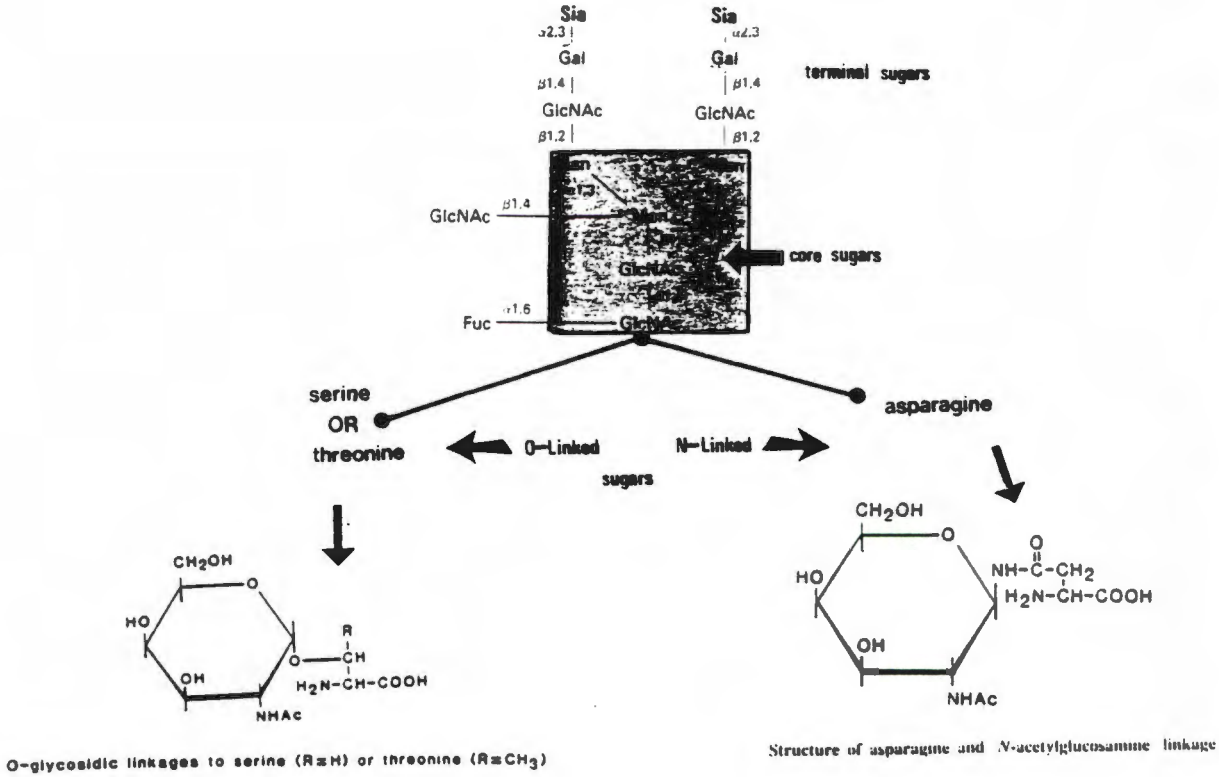

Fig. 3.1: Scheme showing the nature of O- and N-linkages of glycoproteins. Note the core sugars which are shown in the dark background and terminal sugars in the light background. GlcNAc, N-acetylglucosamine; Man, mannose; Gal, galactose; Sial, sialic acid residues.

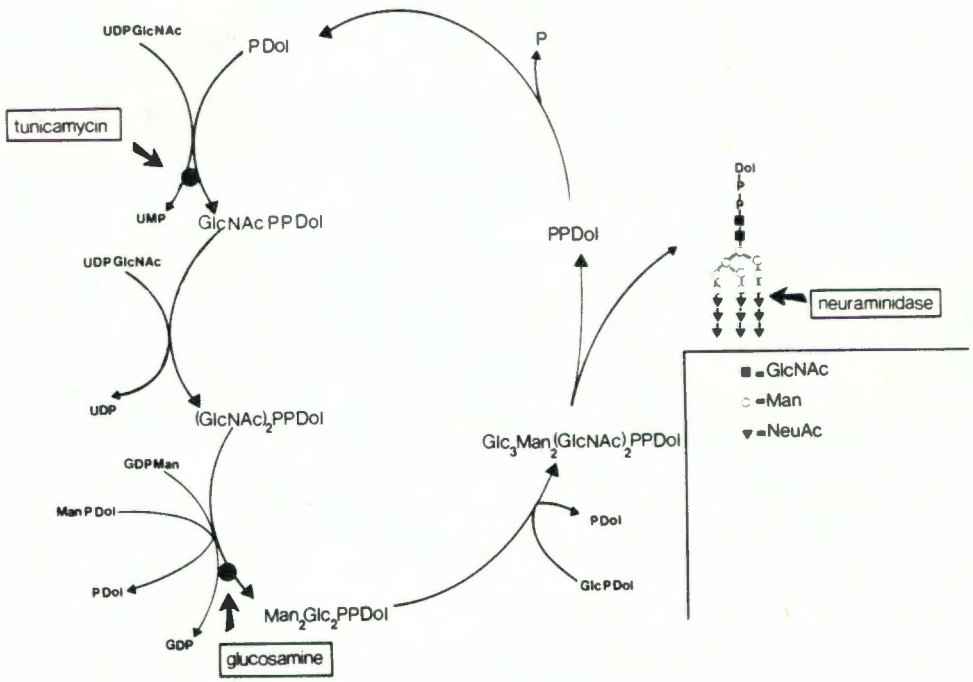

Fig. 3.2: Scheme showing the inhibition sites of glycosylation inhibitors (After Imokawa & Mishima, 1988). GlcNAc, N-acetylglucosamine; Man, mannose; NeuAc, N-acetylneuraminate (sialic acid).

Lectins, which are carbohydrate-binding proteins from plants, are widely used to investigate the biological function of a broad spectrum of glycoproteins (Stanley, 1983). The concanavalin A lectin affinity chromatography (which specifically binds to internal and non-reducing α-mannosyl residues) has been used to confirm the known effects of glucosamine and tunicamycin on tyrosinase (Imokawa & Mishima, 1985). Tyrosinase prepared from melanoma cells cultured in the presence of either glucosamine (1 mg/ml) or tunicamycin (0.4 μg/ml) was shown not to bind to lectins. These results showed that these drugs inhibited the synthesis of N-linked core sugars which may be involved in the binding of tyrosinase to the melanosome (Hearing et al., 1981). Furthermore, the ultra-structural examination of glucosamine- or tunicamycin-treated melanoma cells revealed a selective loss of melanin in premelanosomes, suggesting that the N-linked core sugars were responsible for the intracellular localization of tyrosinase. This subject is reviewed in detail in Chapter 1.

Since it is presently not known whether reduced tyrosinase activity in the Himalayan mutant is due to the deficiency in core or terminal glycosylation, this study has attempted to dissect and study each component separately. The ability of the Himalayan tyrosinase to incorporate mannose or N-

acetylglucosamine (which are core sugars) has been shown to be significantly lower than that of the wild type enzyme (C57BL/6J and B10.BR strains) (Halaban et al., 1988). In this study, the effect of core glycosylation on wild type (C57BL/6) and Himalayan tyrosinase activity was first studied. To carry out this study, the specificity of glucosamine was exploited to investigate the inhibition of mannose incorporation (see Fig. 3.2) on tyrosinase activity of these mice. If diminished levels of Himalayan tyrosinase activity results from a deficiency in core sugars, glucosamine should have a differential effect in reducing the Himalayan and wild type tyrosinase activity.

In addition to core glycosylation, the role of terminal sugars on the wild type and Himalayan tyrosinase activity was also explored. As a means of investigating this possibility, the sialic acid residues were chosen for initial investigation because, by virtue of being charged, they are likely to influence the conformation of any glycoprotein and thereby alter its enzymatic activity. Neuraminidase (an enzyme which cleaves the terminal sialic acid sugars) (Fig. 3.2), has been used to investigate the role of these sugars on wild type and Himalayan tyrosinase activity. If the Himalayan tyrosinase is inherently devoid of terminal sialic acid sugar residues, one can expect neuraminidase to cause a differential decrease on the activity this enzyme in these

mice. On the other hand, the addition of sialic acid residues should cause a differential increase of the Himalayan and wild type tyrosinase activity. Retinoic acid, a vitamin A derivative, has previously been shown to increase the activity of tyrosinase in melanoma cells (Lotan et al., 1978; Lotan & Lotan, 1980), by stimulating the activity of sialyltransferases (enzymes that mediate the addition of sialic acid residues) (Lotan et al., 1984). Based on these findings, the possibility that retinoic acid could reverse the effect of neuraminidase on wild type and particularly on the Himalayan tyrosinase activity was explored.

3.2 MATERIALS AND METHODS

3.2.1 Measurement of basal and α-MSH-stimulated tyrosinase activity in the presence of glucosamine

Wild type and Himalayan skin explants were cultured for six hours on agar medium with or without α-MSH (10^{-5} M, Boehringer-Mannheim) or/and glucosamine (5 mM) and tyrosinase activity was assayed for 18 hours using the skin culture radiometric assay described previously (paragraph 2.2.2.2.). Glucosamine was added in the culture medium during the culture and assay period.

3.2.2 Measurement of tyrosinase activity in the presence of neuraminidase

Neuraminidase (EC 3.2.1.18) (from Clostridium perfringens, Boerhringer-Mannheim) was added to a 20 000g supernatant of wild type and Himalayan crude homogenates to a final concentration of 0.25 µg/ml. Tyrosinase activity was concurrently measured for 5 hours by means of an in vitro radiometric assay described in paragraph 2.2.1.2.

3.2.3 Quantitation of tyrosinase activity in the presence of retinoic acid in wild type and Himalayan skin

Wild type and Himalayan skin explants were culture for six hours on agar medium containing retinoic acid (10 μM) and with or without cycloheximide (0.9 μM). Tyrosinase activity was assayed for 18 hours in the presence of retinoic acid, [^{14}C]-tyrosine (0.5 μCi/ml) and cycloheximide as described previously (see skin culture radiometric assay, paragraph 2.2.2.2.).

3.2.4 Determination of retinoic acid-stimulated tyrosinase activity and cell density in B16 melanoma cells

B16 melanoma cells were cultured for 72 hours in DMEM with or without various concentrations of retinoic acid (10^{-6} M to 10^{-5} M), as specified in each experiment. No cycloheximide was added during this culture period. After culture, cells were lifted with 0.05% EDTA-0.02% trypsin and a crude homogenate prepared as described in paragraph 2.2.2.1. Tyrosinase activity of the 20 000 g supernatant was measured using the *in vitro* radiometric assay. Cell density was determined by means of a haemocytometer. Percentage decrease in cell density was calculated in relation to control cells using the following formula:

$[(C_C - C_{R.A})/ C_C \times 100]$, where C_C, cell density in control cells and $C_{R.A.}$, cell density at a particular retinoic acid concentration.

3.3 RESULTS

3.3.1 The effect of glucosamine on tyrosinase activity in cultured skin explants

Wild type and Himalayan skin explants were cultured for 6 hours in media with or without α-MSH and/or glucosamine and assayed for tyrosinase activity as described in materials and methods. This experiment was repeated twice and the results of the representative experiment are shown in Figure 3.3. The basal and α-MSH stimulated tyrosinase activity was effectively reduced by glucosamine after 24 hours in culture in both the Himalayan and the wild type. The average relative decrease in basal tyrosinase activity in the Himalayan mutant was 32% and that of the wild type was found to be 35%. Similarly, the reduction of α-MSH stimulated tyrosinase activity by glucosamine was 44% and 51% in the Himalayan mutant and wild type respectively. The activity in the albino skin was not affected by either glucosamine or α-MSH.

3.3.2 The role of neuraminidase on wild type and Himalayan tyrosinase activity

Neuraminidase (0.25 μg/ml) was added to 20 000 g supernatant of a crude homogenate of wild type and Himalayan skins, then

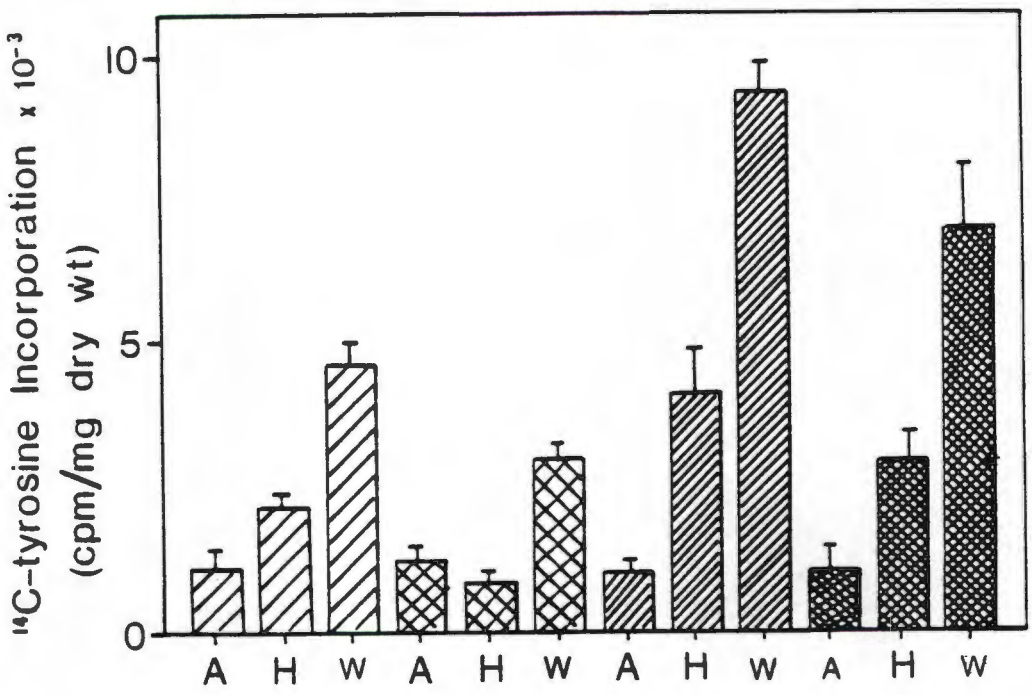

Fig. 3.3: Effect of glucosamine on basal and α-MSH-stimulated tyrosinase activity. Skin explants of 4-5 day old mice were cultured on agar media with or without α-MSH (10^{-5} M) or/and glucosamine (5.5 mM) for six hours and assayed for tyrosinase activity for 18 hours (see materials and methods). This experiment was carried out twice with similar results. Each value is a mean ± S.D. from three measurements and all values are significantly different from each other ($P \leq 0.04$). A, albino; H, Himalayan; W, wild type. (▨), control; (▩), + glucosamine - α-MSH; (▨), - glucosamine + α-MSH; (■), + glucosamine + α-MSH.

assayed for tyrosinase activity 5 hours (see materials and methods). This experiment was carried out twice with similar results. It was found that the average decrease in wild type tyrosinase activity in the presence of neuraminidase was 21% (Table 3.1). Similarly, the average reduction of the Himalayan tyrosinase activity was found to be 15%. These results showed that neuraminidase reduced the activity of wild type and Himalayan tyrosinases to the same extent.

3.3.3 The effect of retinoic acid on tyrosinase activity and cell density in B16 melanoma cells

It has been reported that retinoic acid caused an increase in tyrosinase activity and a simultaneous inhibition of growth in human (Hs 939) and mouse (S91) melanoma cells (Lotan et al., 1978; Lotan & Lotan, 1980). In this study, this effect of retinoic acid on tyrosinase activity and cell density was confirmed using B16 mouse melanoma cells. Melanoma cells were cultured for 72 hours in cycloheximide-free DMEM medium containing various concentrations of retinoic acid (see materials and methods). Under these conditions, retinoic acid markedly increased tyrosinase activity in a dose-dependent manner (see Fig. 3.4). At a concentration of 10^{-8} M, retinoic acid caused a 26% increase in tyrosinase activity above control and at a molar concentration of 10^{-6}, the drug

Table 3.1

Effect of neuraminidase on wild type and Himalayan tyrosinase activity

	[^{14}C]-tyrosine incorporation (cpm/μg of protein of homogenate)	
	wild type	Himalayan
Experiment 1		
homogenate	1 497 ± 57	1 345 ± 6
homogenate + neur.	1 140 ± 85	1 168 ± 87
Experiment 2		
homogenate	10 020 ± 860	971 ± 150
homogenate + neur.	8 090 ± 195	802 ± 143

Neuraminidase was added to wild type and Himalayan crude homogenates t a final concentration of 0.25 μg/ml and tyrosinase activity assayed fo 5 hours using *in vitro* radiometric assay (see materials and methods). Each data point is a mean two measurements ± S.D. All control values representing non-enzymatic incorporation were subtracted. neur., neuraminidase.

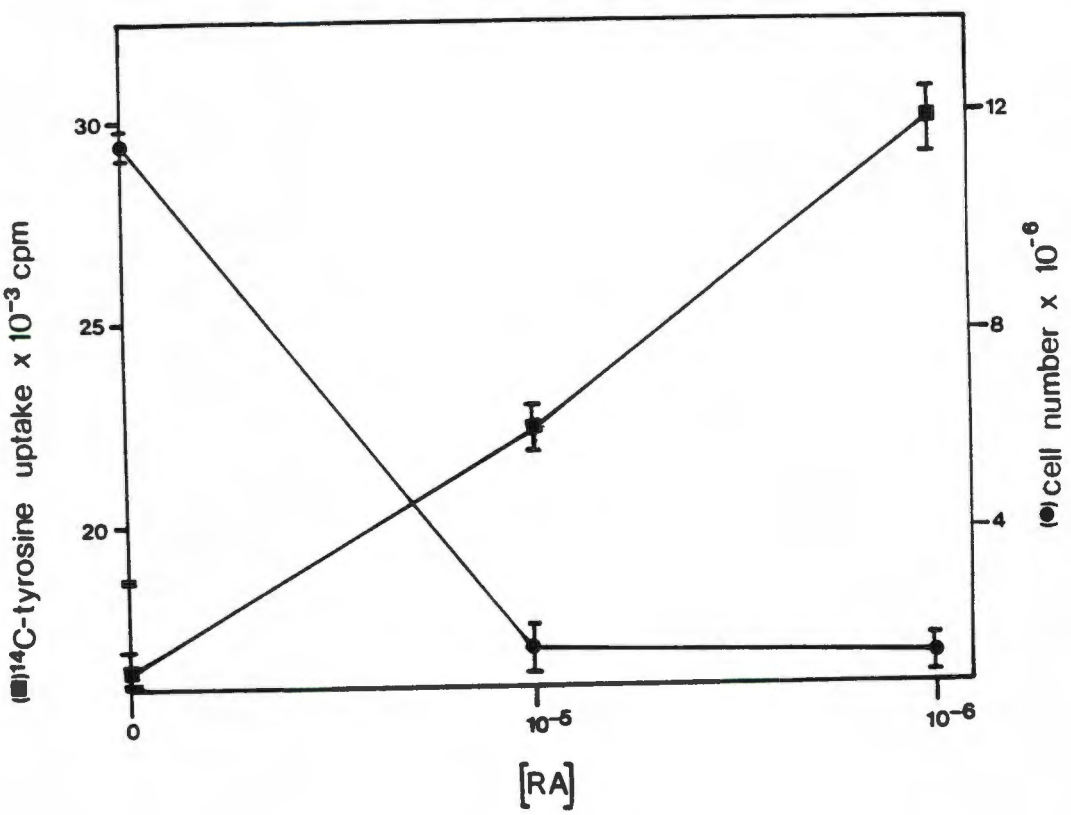

Fig. 3.4: Effect of retinoic acid on tyrosinase activity and cell density in melanoma cells. B16 melanoma cells were cultured in DMEM for 72 hours with various concentrations of retinoic acid. Tyrosinase activity and cell density were determined as described in materials and methods. Values are means of three measurements ± S.D.

increased tyrosinase activity by 45%. As expected, results showed that in addition to an increase in tyrosinase activity, retinoic acid also caused a dose-dependent inhibition of growth. The results are presented in Table 3.2 and Figure 3.4. At a concentration of 10^{-6} M and 10^{-5} M, retinoic acid induced a 32% and 47% growth inhibition respectively.

3.3.4 The effect of retinoic acid on tyrosinase activity in skin explants.

This experiment was carried to investigate the possibility that retinoic acid could reverse the effect of neuraminidase by differentially increasing the wild type and Himalayan tyrosinase activity. Skin explants of wild type and Himalayan mice cultured for six hours on retinoic acid-supplemented agar medium with and without cycloheximide and were assayed for tyrosinase activity as described in materials and methods. In the presence of cycloheximide, there was no significant difference in wild type and Himalayan tyrosinase activity between retinoic acid-treated and control skin explants ($P \geq 0.06$) (Fig. 3.5). In the absence of cycloheximide, retinoic acid still failed to increase the Himalayan tyrosinase activity significantly when compared to control ($P > 0.05$),

Table 3.2

Effect of retinoic acid on cell density in B16 melanoma cells.

[RA] (M)	cell density		
	exp 1	exp 2	average
0	12.3×10^6	10.5×10^6	$11.4 \pm 0.9 \times 10^6$
10^{-6}	8.0×10^6	7.3×10^6	$7.65 \pm 0.4 \times 10^6$
10^{-5}	5.8×10^6	6.1×10^6	$5.95 \pm 0.2 \times 10^6$

Cells were cultured for 72 hours in DMEM containing various concentrations of retinoic acid and cell density was determined using haemocytometer as described in materials and methods. Average values represent a mean of two measurements ± S.D. All experimental values significantly different from control at $P \leq 0.04$..

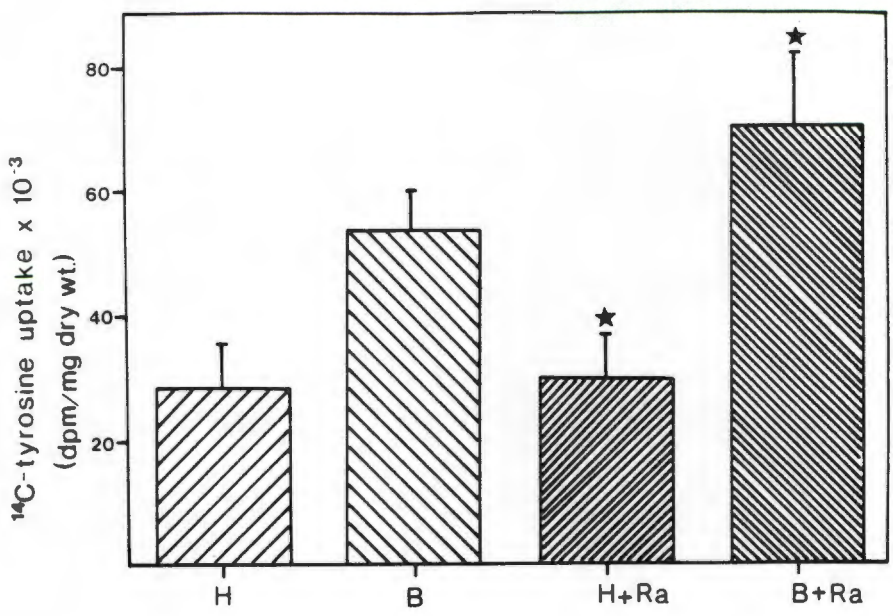

Fig. 3.5: Effect of retinoic acid on tyrosinase activity in skin explants in the presence of cycloheximide. Wild type and Himalayan skin explants were cultured six hours on agar medium with or without retinoic acid (10 μM) and cycloheximide (250 μg/ml). Tyrosinase activity was assayed for 18 hours in the presence of [^{14}C]-tyrosine (0.5 μCi/ml) and cycloheximide as detailed in materials and methods. The background incorporation in the albino skin has been subtracted. ★, values not significantly different from control ($P > 0.06$). H, Himalayan; W, wild type; R.a., retinoic acid.

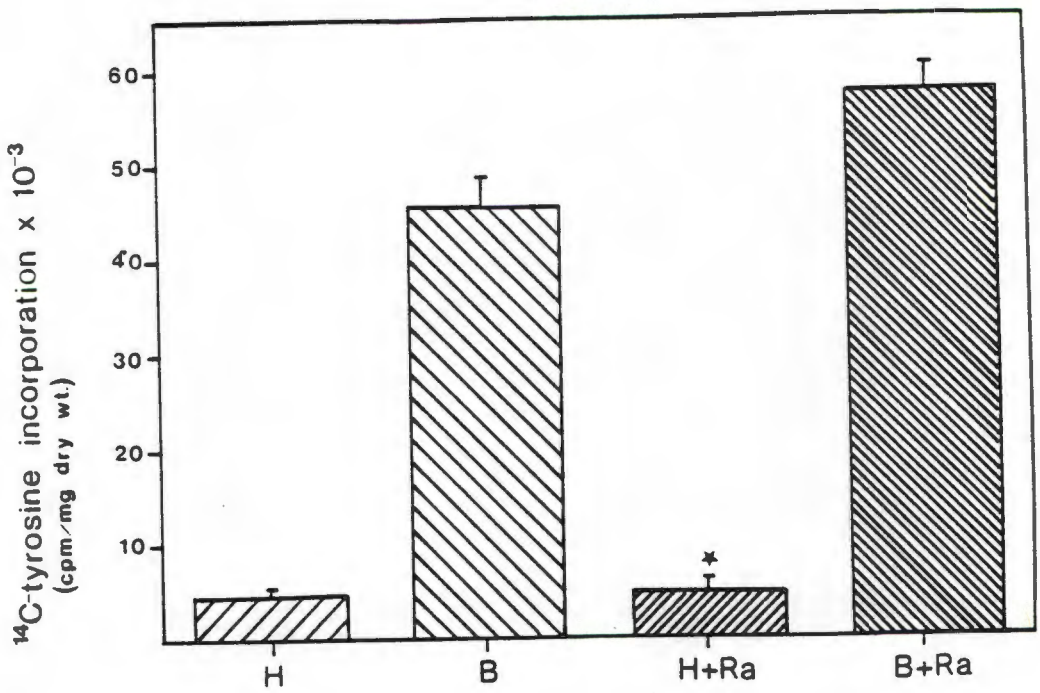

Fig. 3.6: Effect of retinoic acid on tyrosinase activity in skin explants in the absence of cycloheximide. Wild type and Himalayan skin explants were cultured on cycloheximide-free agar medium with or without retinoic acid (10 μM) and assayed for tyrosinase activity as described in materials and methods. The background incorporation in the albino skin has been subtracted. ✱, value not significantly different from control ($P > 0.05$). H, Himalayan; W, wild type.

whereas the wild type tyrosinase activity increased by 1.3-fold (Fig.3.6).

3.4 DISCUSSION

In chapter two, the activation of the wild type and Himalayan tyrosinase by α-MSH was demonstrated and a theory was put forward that this activation involved the removal of endogenous inhibitor. In this chapter, another mechanism by which α-MSH could activate the Himalayan tyrosinase is considered. Recently, Halaban et al. (1988) reported that the Himalayan tyrosinase is deficient in N-linked glycosylation and proposed that this results in rapid degradation of this molecule at normal body temperature. This hypothesis was based on their finding that immunoprecipitated Himalayan tyrosinase corresponded to the molecular weight of an underglycosylated enzyme, whereas the wild type tyrosinase was predominantly in the fully glycosylated form. Secondly, the ability of Himalayan tyrosinase to incorporate [^{3}H]-glucosamine and [^{3}H]-mannose significantly less than the wild type.

If α-MSH is able to activate Himalayan tyrosinase as shown in the preceding chapter, the above results raise the possibility that α-MSH could act via glycosylation to activate the Himalayan and wild-type tyrosinase.

In the hope of gaining a more detailed understanding of tyrosinase glycosylation, work in this chapter analyses the

role of the core and terminal sugars on tyrosinase activity. To investigate core glycosylation, the synthesis of N-linked core sugars was inhibited by treatment with glucosamine, then the activity of tyrosinase measured. It was found that at a concentration of 5.5 mM, glucosamine reduced basal and α-MSH-stimulated tyrosinase activity to approximately the same degree in both the wild type and Himalayan mutant (Fig. 3.3). These results may be explained in the following way: As N-linked core sugars determine the intra-cellular destination of tyrosinase, reduced tyrosinase activity in the presence of glucosamine results in the interruption of tyrosinase translocation to the melanosome (Imokawa & Mishima, 1985). Since tyrosinase activity in melanocytes is, in part, regulated by the rate of turnover of the enzyme (Jiménez et al., 1988), in the presence of glucosamine, no tyrosinase would be transported to the melanosome to replace the degraded enzyme. If the Himalayan tyrosinase is inherently deficient in N-linked core sugars, less enzyme would transferred to the melanosome as compared to the wild type. Under these conditions, further inhibition in the transport of wild type and Himalayan tyrosinase induced by glucosamine should reduce tyrosinase activity of these mice differentially. Since this was not found to be the case, the results suggest that the mutation in the Himalayan mouse does not involve core sugars.

It is interesting to note that the decreased activity of tyrosinase resulting from the inhibition of intra-cellular transport of the enzyme by glucosamine is similar to that described in the chinchilla mutant *in vivo*, which has an inherent defect to transfer tyrosinase to melanosomes (Imokawa & Mishima, 1988). In this study, it was shown by organ culture technique and electron microscopy that theophylline and dbc-AMP enhanced the transfer of tyrosinase to the melanosome *in vitro*, whereas MSH failed to do so. The possible mechanism of action of theophylline and dbc-AMP was not investigated. These findings are in accord with those of the present study, as α-MSH did not increase the wild and the Himalayan tyrosinase activity in the presence of glucosamine.

Since the inhibition of the synthesis of N-linked core sugar equally reduced the Himalayan and wild type tyrosinase activity, the role of terminal sugars were subsequently investigated. In order to investigate this possibility, the role of sialic acid residues on the Himalayan and wild type tyrosinase activity was investigated using neuraminidase (which hydrolyzes terminal sialic acid residues). The key reason behind the use of sialic acid was that these sugars are charged and their removal as a consequence of a mutation *in vivo* or *in vitro* is likely to alter the nett charge of tyrosinase, which can in turn change the conformational dynamics of the enzyme. Results showed that in the presence

of neuraminidase, the average reduction of the wild type and Himalayan tyrosinase was 21% and 15% respectively. These results are in perfect agreement with those Hearing et al (1981), whereby neuraminidase was shown to reduce purified tyrosinase from melanoma cells by 10% to 20%. The similar reduction of the wild type and Himalayan tyrosinase activity suggest that reduced tyrosinase activity in the latter mouse is not caused by sialic acid sugar residues.

Due to the fact that the action of neuraminidase did not result in the differential reduction of the wild type and tyrosinase activity, the effect of retinoic acid (which facilitates the addition of sialic acid residues) was next investigated. The main reason of using retinoic acid was to investigate the possibility that it might activate the Himalayan tyrosinase to a higher level that the wild type enzyme. Previously, retinoic acid has been shown to activate human (Lotan & Lotan, 1982) and mouse (Lotan et al., 1980) melanoma cells. As a preliminary study, this effect of retinoic acid was verified by demonstrating its dose-dependent increase of tyrosinase activity in B16 melanoma cells (Fig. 3.4). Having confirmed these results, the effect of retinoic acid was then investigated on the wild type and Himalayan tyrosinase activity. Skin explants of wild type and the Himalayan mouse were cultured in the presence of retinoic acid (10 μM) with or without cycloheximide.

Retinoic acid had no effect on the wild type and Himalayan tyrosinase activity in the presence of cycloheximide ($P \geq 0.06$) (Fig. 3.5), whereas in the absence of cycloheximide, the wild type tyrosinase activity slightly increased (i.e. 1.3-fold) (Fig. 3.6). The finding that retinoic had no effect on the Himalayan tyrosinase activity and slightly activated the wild type tyrosinase may be incorrect due to inherent limitations of the skin culture assay. More specifically, the effectiveness of retinoic acid at this concentration on the distant and scattered skin melanocytes is much less than that in the culture medium, since its action is depended on the diffusion rate through the agar and the dermis and secondly, retinoic acid does not only stimulate the activity of sialyltransferases of tyrosinase, but also of other glycoproteins and glycolipids present in skin.

Considering these conditions in cultured skin, it likely that our failure to detect the effect of retinoic acid could be due to the fact that it is diluted. Nevertheless, the finding that only the wild type tyrosinase activity was increased in the absence of cycloheximide suggests a protein synthesis-dependent activation of this enzyme. There is strong evidence in literature showing that retinoic acid exerts its effect by binding to a nuclear receptor (Giguere et al., 1987; Petkovich et al., 1987; Benbrook et al., 1988;

and Brand et al., 1988) which has recently been cloned and sequenced in B16 melanoma cells (Dally & Redfern, 1989). In support of this evidence, is the reported nuclear localization of retinoic acid (Chytil, 1984; Lotan, 1984) Thus, a possibility exists that in mouse skin, retinoic acid could stimulate the transcriptional synthesis of new tyrosinase molecules that are subsequently glycosylated de novo. If that is the case, why is it that an increase in Himalayan tyrosinase activity could not be detected even when protein synthesis was not blocked? A small increase in tyrosinase activity due to the synthesis of new enzyme like that measured in the wild type skin (i.e. 1.3-fold), could not have been detected in the Himalayan mutant since at 37°C, the enzyme is more tightly bound to inhibitors (see Chapter two).

Although retinoic acid may increase tyrosinase activity by promoting the synthesis of new enzyme molecules, its mechanism of action still remains unclear. The retinoic acid-stimulated tyrosinase activity in melanoma cells has been shown in the present and other studies (Lotan et al., 1978; Lotan & Lotan, 1980), to be concomitant with growth inhibition (see Fig. 3.4). This inhibitory effect of retinoic acid on growth has also been demonstrated in human epidermal cells (Hashimoto et al., 1985) and embryonal carcinoma cells (Plet et al., 1985). The elucidation of the

mechanism of action of retinoic acid is further complicated by its diverse effects including promotion of growth (Christophers, 1974; Marhle et al., 1981; Marhle & Gray, 1983), morphological changes in melanoma cells (Lotan et al., 1983) cancer prevention (Moon et al., 1983) and many other effects. However, there is substantial evidence in literature indicating that the inhibition of growth caused by retinoic acid in melanoma cells (Niles & Logues, 1979) and other cell types (Lee et al., 1976; Byus et al., 1977; Costar et al., 1978; Plet et al., 1985) could involve the type II regulatory subunit of c-AMP dependent kinase. In the presence of retinoic acid, this kinase has been shown to stimulate the activity of ornithine decarboxylase, an enzyme that contributes to the accumulation of cells in the G_1 phase of the cell cycle (Jetten, 1984). These results suggest that retinoic acid may act via ornithine decarboxylase and the cell cycle to inhibit the growth of melanoma cells. Even if the activation of tyrosinase in melanoma cells, which is accompanied by inhibition of growth, has been shown to be cell cycle-dependent (Abdel-Malek et al., 1989), the relationship between retinoic acid, c-AMP dependent kinase, ornithine decarboxylase and cell growth remains to be investigated. Despite the fact that the precise mechanism of retinoic acid remains unknown, the possibility that it could share the same pathway with α-MSH may be ruled out because: (a) Unlike α-MSH, no rise in intra-cellular levels of

adenosine-3,5-monophosphate in response retinoic acid has been detected (Lotan et al., 1978). and (b) Even human Hs 939 melanoma cells that are unresponsive to α-MSH, demonstrate a rise in tyrosinase caused by retinoic acid (Lotan & Lotan, 1980).

In summary, under the experimental conditions of this study, it could not be confirmed that the Himalayan tyrosinase was underglycosylated compared to the wild type enzyme since: (a) Neuraminidase did not differentially reduce the Himalayan and wild type tyrosinase activity. and (b) Retinoic acid failed to reverse the effect of neuraminidase by activating the Himalayan tyrosinase more than that of the wild type.

In view of the existing limitations of the assay method used in this study, these results need to be confirmed by directly testing the effect of retinoic acid on cultured Himalayan melanocytes. More importantly, the use of retinoic acid has provided some insight to the effect that the activation of wild type and Himalayan tyrosinase could involve the synthesis of new enzyme molecules. Thus, the role of transcription and translation of tyrosinase on the activity of this enzyme is investigated in depth in the following chapter.

CHAPTER IV

TRANSLATIONAL AND TRANSCRIPTIONAL CONTROL OF TYROSINASE ACTIVITY

4.1 INTRODUCTION

The evidence presented so far in this study has clearly demonstrated that the activation of tyrosinase in mouse skin melanocytes is at the level of post-translation. In chapter 2, the post-translational activation of tyrosinase by α-MSH was demonstrated. In chapter 3, glycosylation was shown to be an additional parameter that regulates tyrosinase activity. The questions that the present chapter attempts to answer are: (1) Is the post-translational processing of tyrosinase in skin melanocytes the only control mechanism by which this enzyme is regulated? and (2) Does translation and/or transcription play a part in the regulation of tyrosinase activity in these melanocytes?

When the present project was initiated, it was not known whether tyrosinase activity of normal and mutant mice used in this study was controlled at the level of translation or transcription. While this study was in progress, investigators in other laboratories were studying the same issues. The results presented in this chapter satisfactorily agree with some of these studies and others disagree.

Earlier studies on melanoma cells have indirectly studied the role of translation and transcription on tyrosinase activity by making use of protein and RNA inhibitors respectively

(Wong & Pawelek, 1975; Fuller & Visckochil, 1979; Halaban et al., 1984; Fuller et al., 1987). Recently, Halaban et al.(1988) have investigated the translational regulation of tyrosinase by directly quantitating the total amount of enzyme using an anti-tyrosinase antibody. The levels of tyrosinase were measured in a variety of cultured mouse melanocytes including the wild type (BR10.BR), Himalayan (c^h/c^h) and pink-eyed chinchilla (c^{ch}/cp) strains. Equal levels of immunoprecipitated tyrosinase were measured in the wild type and the Himalayan melanocytes, which were found to be four times higher than the quantity of albino and chinchilla enzyme. Although these results suggested the possible involvement of translation in the regulation of tyrosinase activity, it was not clear at this stage whether the levels of tyrosinase measured in these four mice were due to the differential expression of the tyrosinase gene. The answer depended on the accurate quantitation of mRNA levels in these mice, which can now be done due to the recent success in the sequencing and cloning of tyrosinase cDNA's (Shibahara et al., 1986; Yamamoto et al., 1987; Kwon et al., 1987, 1988).

Using the PTY-1 tyrosinase cDNA (Kwon et al., 1988), Halaban et al. (1988) continued their study by investigating the role of transcription on tyrosinase activity. This probe was used to measure levels of mRNA in cultured melanocytes of the wild

type, Himalayan and chinchilla mutant mice which were found to be the same in all the three genotypes. At the same time, Takeuchi et al. (1988) used the Tyrs cDNA (Yamamoto et al., 1987) to measure the amount of mRNA in the wild type (C57BL/6J-C/C) and albino skins. They also measured equal levels tyrosinase mRNA in both these mice. In another related study in which the pMT4 cDNA (Shibahara et al., 1986) was used, similar levels of mRNA were also found in the skins of the black and albino mice (Shibahara, 1988). More recently, the findings of Halaban et al (1988) were verified since the levels of RNA in cultured Himalayan melanocyte were shown to be the same as in the wild type (Kwon et al., 1989). Most importantly, in all of the above-mentioned studies, the same size of the hybridization signal was found (about 2.3 kb), suggesting the lack of gross deletions in the tyrosinase gene of the mutant mouse strains used. In support of this idea, is the recent identification of a point mutation in the Himalayan mouse (Kwon et al., 1989).

This study has made an attempt to investigate the extent to which the translation of tyrosinase is involved in the regulation of its activity. To carry out this aim, an anti-tyrosinase antibody has been used to quantitate the abundance of tyrosinase in the skins of the normal wild type (C57BL/6J), Himalayan and albino mutant mice. In order to gain additional insight on how tyrosinase activity could be

regulated *in vivo*, the relationship between translational and transcriptional control mechanisms was investigated in these mice. To explore the role of the latter mechanism on the activity of tyrosinase, the levels of mRNA transcripts were measured in the skins of these three mice using tyrosinase cDNA's.

4.2 MATERIALS AND METHODS

4.2.1 Purification of tyrosinase

4.2.1.1 DEAE ion exchange chromatography

The column was pre-washed with 10 mls of NaOH (pH 13). A crude cell homogenate was applied to 1.5 cm x 8.0 cm DEAE Sephadex ion exchange column, equilibrated with low salt buffer (20 mM Tris, pH 8) and allowed to run under the influence of gravity at a flow rate of 1 ml/min. The column was then washed with the low salt buffer until the effluent measured at $O.D._{280}$ was ≈ 0. The bound material was eluted with the high salt buffer (20 mM Tris + 0.3 M NaCl, pH 8) and 1 ml fractions were collected. Fractions containing high tyrosinase activity were identified using the spectrophotometric assay at $O.D._{475}$ (0.1 ml of each fraction was mixed with 0.9 ml of L-DOPA (1mg/ml) in phosphate buffer, then incubated for 2 hours at 37°C and tyrosinase activity measured hourly). Fractions with high tyrosinase activity were pooled and concentrated against polyethyleneglycol (PEG 6000) and subjected to gel electrophoresis.

4.2.1.2 Polyacrylamide gel electrophoresis without detergents

Electrophoresis was carried out by the method originally described by Davis (1964). Crude homogenate proteins from B16 melanoma cells were run in a 7.5% acrylamide gel in a 0.025 M Tris/glycine buffer at 100 V for 3½ hours. After electrophoresis, gels were neutralized in phosphate buffer (0.5 M, pH 7.4) for ½ an hour and thereafter stained with 1 mg/ml of L-DOPA in phosphate buffer (0.1 M, pH 7.4). Tyrosinase stained as a dark band which appeared after 10 minutes of incubation. Bands corresponding to various tyrosinase isozymes (T_1, T_3 and T_4) were cut out, kept for raising antibodies while some were further analysed on an SDS gel.

4.2.1.3 Analysis of purified tyrosinase isozymes on SDS gels

SDS gel electrophoresis was carried out utilizing the discontinuous buffer system originally described by Laemmli (1970). Gel pieces of approximately 3 mm^2 containing various tyrosinase isozymes were mixed with 20 μl SDS sample buffer, layered into the wells of the gel and eletrophoresed for 3 hours at 120 V. Standard molecular weight markers (Pharmacia) were run concurrently with the samples for

molecular weight determination. Tyrosinase and other contaminating proteins were visualized by staining with Comassie blue.

4.2.1.4 Molecular weight determination of purified tyrosinase

The distance migrated by tyrosinase and the standard molecular weight protein markers were measured. These values were fed onto the Enzfitter non-linear regression data analysis computer programme (Elsevier, 1987) which was used to calculate the molecular weight of tyrosinase.

4.2.2 Immunization of animals

Immunization of rabbits and guinea pigs was carried out at the Animal Unit, University of Cape Town Medical School. Non-immune sera was drawn before injecting with tyrosinase. The T_4 tyrosinase isozyme contained in acrylamide, was subcutaneously injected into guinea pigs. Rabbits were injected with all isozymes (T_3, T_1 & T_4). Both guinea pigs and rabbits were boost-injected 2 weeks after the initial injection. The immune sera was drawn a week after the boosting and tested for the presence of tyrosinase antibodies using the western blot technique.

4.2.3 Western immunoblot technique

Wild type, Himalayan, albino and melanoma crude homogenate proteins mixed with SDS sample buffer in 2:1 ratio, were separated on an SDS gel and transferred to the nitrocellulose membrane (0.45 μM, Schleicher & Schull) according to Towbin et al. (1979). The proteins were transferred using a B2300-protein transfer chamber (Consort) filled with blotting buffer (190 mM glycine; 50 mM Tris, 0.2% methanol, pH 8.3) at 100 mA, 14 V overnight. Sheets containing transblotted molecular weight markers were washed for ½ an hour in 0.1 M phosphate buffer (pH 7.4), stained with Indian ink for 1½ hour and destained in distilled water. Sheets onto which crude homogenate proteins were transblotted, were incubated for 2 hours at room temperature in a mixture of Tris buffer saline (TBS), 3% Tween, 3% fat-free milk and 1:100 goat serum. After washing with 3% fat-free milk in TBS (2 x 5 min), the sheets were incubated overnight at room temperature with 5 mls of TBS containing a tyrosinase polyclonal antibody in serum (1:250). After this step, the sheets were processed using either the 4-chloro-1-napthol/hydrogen peroxide or immunogold staining procedures as described below.

4.2.3.1 The 4-chloro-1-napthol/hydrogen peroxide staining method

After the primary antibody reaction, the sheets were washed in 0.05% tween-20 in TBS (2 x 15 minutes), then incubated with peroxidase-conjugated goat-antirabbit secondary antibody (1:500) for an hour. After rinsing twice for 5 minutes each in TBS, sheets were finally incubated with 0.5 mg/ml 4-chloro-1-napthol, 16% methanol, 0.015% hydrogen peroxide in TBS. The reaction was terminated by washing with water and the sheets were air-died and stored until photographed. Quantitation of positive staining bands was determined by densitometric scanning at 500 nm in a Cliniscan densitometric equipment (Helena laboratories, Texas)

4.2.3.2 The immunogold staining method

This immuno-staining procedure originally described by Moeremans et al. (1984) is reported to be at least ten times more sensitive than the 4-chloro-1-napthol/hydrogen peroxide staining method. After incubation with the primary antibody, the nitrocellulose sheets were washed in TBS (3 x 10 minutes), then incubated with a gold labelled secondary antibody (1μg/ml) diluted in TBS supplemented with 0.4% gelatin. Thereafter, the strips were washed with TBS (2 x 10 min) on a rocking table and air-dried. Subsequently, they

were washed in distilled water (2 x 10 min) and in 0.2 M citrate buffer (pH 3.85) for 2 minutes to remove chloride ions then incubated with the physical developer protected from light with aluminium foil. The physical developer was prepared as follows: 60 ml of H_2O was mixed with 10 ml stock citrate buffer (2 M, pH ± 4.85), 0.85 g hydroquinone in 15 ml H_2O, 0.11 g of silver lactate in 15 ml). Hydroquinone and silver lactate were prepared just before use in a vessel protected from light.

4.2.4 Northern blot technique

4.2.4.1 RNA extraction

All glassware (i.e corex tubes, pasteur pipettes, etc.) were siliconized with 4% dichloro-dimethyl-silan in carbon tetrachloride, treated with DEP (diethylpyrocarbonate) (an RNAse inhibitor) and autoclaved. All buffers were treated with diethylpyrocarbonate and autoclaved before use. The ultra-homogenizer was dipped in 70% alcohol for 24 hours before use. Gloves were worn at all times.

Wild type, Himalayan and albino mouse skins or epidermis were homogenized in 10 parts of NETS buffer (v/v) in a polytron ultra-homogenizer. The homogenate was treated with 10 µg/ml proteinase K at 37°C for 30 minutes. RNA was extracted three

times with phenol/chloroform and precipitated with 0.3 M sodium acetate and 2½ volumes of ice cold absolute ethanol overnight at -20°C. Precipitated RNA was spun down in a high speed centrifuge at 20 000 g for 20 minutes, then washed with 70% alcohol and dried. RNA was resuspended in sterile water and its quantity determined at A_{260}. The purity was determined by the $A_{260/280}$ ratio.

4.2.4.2 Purification of poly A+ mRNA on an Oligo-dT-cellulose column

The column containing ± 1 ml of oligo-dT-cellulose was washed with 5 ml of NaOH (0.1 M). Total RNA (20 A_{260} units/ml) was dissolved in 1 ml high salt buffer (0.01 M Tris; 0.5 M NaCl, pH 7.5), then applied to the column (0.5 cm x 30 cm) equilibrated with 10 ml of the same buffer. In order to facilitate the binding of poly A^+ mRNA, the effluent was loaded to the column twice before being washed with the high salt buffer until the reading at $O.D._{260} \approx 0$. Bound poly A^+ mRNA was eluted with the low salt buffer (0.01 M Tris-HCl, pH 7.5) and precipitated with 0.3 M sodium acetate and 2½ volumes of ice cold absolute alcohol overnight at -20°C. To encourage precipitation, transfer RNA was added to the mixture.

4.2.4.3 RNA blot analysis

All procedures were performed according to Davis et al. (1986). 10 or 20 micrograms of total or poly A^+ RNA were fractioned by electrophoresis on 1.1% agarose denaturing gels, blotted onto nitrocellulose filters (Hybond-N, Amersham) and baked for 2 hours at 80°C . The filters were prehybridized for 2 hours at 42°C in hybridization mix (40% formamide, 10% dextran sulphate, 4 x SSC, 0.02 M Tris (pH 7.4); and 20 μg/ml salmon sperm DNA in water). They were hybridized for 24 hours at 42°C to either the tyrosinase pMT4 cDNA (Shibahara et al., 1986) or the tyrosinase MTY811 cDNA (Kwon et al., 1988) cloned into pBR322 and pGEM respectively. After hybridization, the filters were washed in high salt mixture (20 ml 20 x SSC; 178 ml H_2O; 2 ml 10% SDS) twice for 20 minutes at room temperature followed by two 20 minutes washes in low salt mixture (1 ml 20 x SSC, 197 ml H_2O; 2 ml 10% SDS). After the washing, filters were air-dried, loaded onto cassettes and exposed for 5-10 days at -70°C.

4.3 RESULTS

4.3.1 Purification of tyrosinase

A crude homogenate prepared from B16 melanoma cells was loaded onto a DEAE column and eluted as described in materials and methods. As shown in Fig. 4.1, unbound material formed a single small peak (peak "a"). When the bound material was eluted, it emerged as a larger sharp peak (peak "b"). Fractions with high protein content as measured at 280 nm were pooled and assayed for tyrosinase activity by measuring dopachrome formation at 475 nm. Figure 4.2 shows fractions from peak "a" (i.e. A_1, A_2,& A_3) with low levels of tyrosinase activity, compared to those from peak "b" (i.e B_1 & B_2) with higher levels of tyrosinase activity. These two samples from peak "b" were combined, concentrated against polyethelylene glycol and electrophoresed on a non-SDS gel. The DOPA staining of this gel revealed the presence of three tyrosinase isozymes (T_1, T_3 and T_4) (Fig. 4.3). In agreement with previous reports (Hearing et al., 1981; Hearing & Jiménez, 1987), the T_1 isozyme was also found to have migrated further that T_3 due to the presence of charged sialic acid sugar residues. Furthermore, these results are consistent with those of Hearing et al., (1981) since the T_2 isozyme which has been reported to be an artifact due to the deamination of T_3 on SDS gels was also not detected.

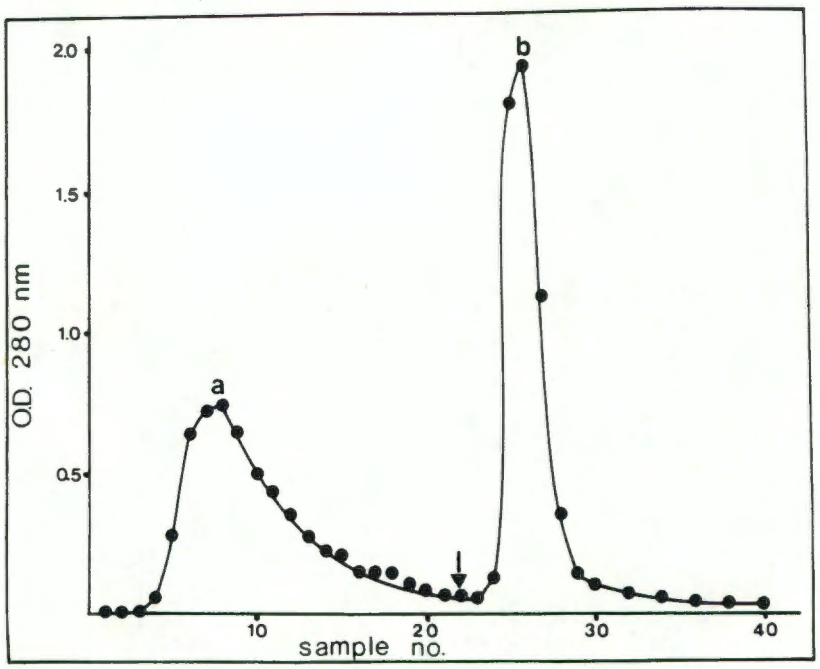

Fig. 4.1: Purification of tyrosinase. A crude homogenate from B16 melanoma cells was purified by DEAE ion exchange column chromatography as detailed in materials and methods. The homogenate was applied to the column with low salt buffer (20 mM Tris, pH 8). Bound material, eluted with high salt buffer (20 mM Tris, 0.3 M NaCl, pH 8), emerged as a single peak-(peak b). ↓ changed low to high salt buffer.

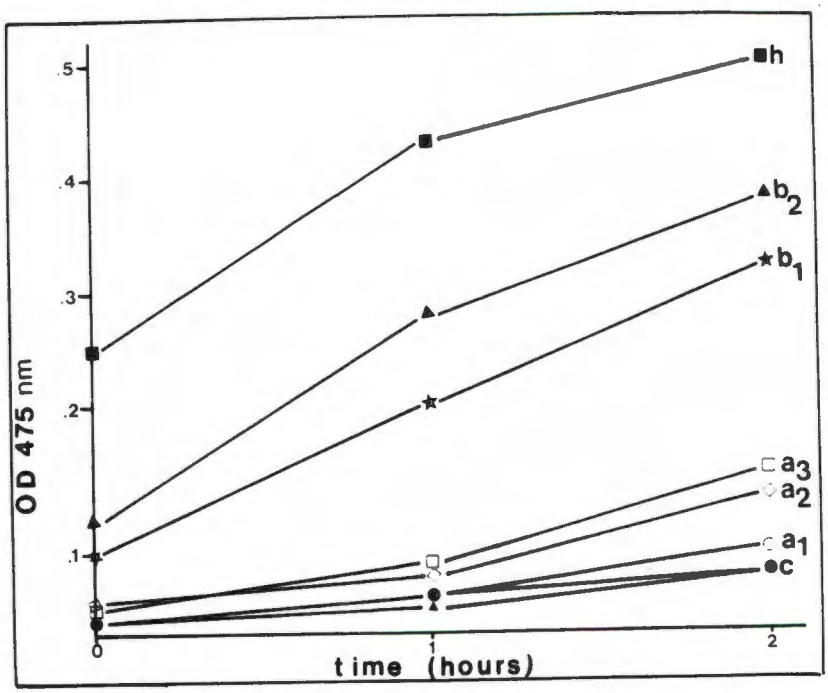

Fig. 4.2: Determination of tyrosinase activity of fractions eluted from the DEAE column. Fractions from peaks "a" & "b" (Fig. 4.1), as measured at A_{280} were pooled and assayed for tyrosinase activity. C, control (homogenate substituted with phosphate buffer). A_1, A_2, & A_3, fractions from peak "a" with low tyrosinase activity; B_1 & B_2, fractions from peak "b" with high tyrosinase activity. H, homogenate.

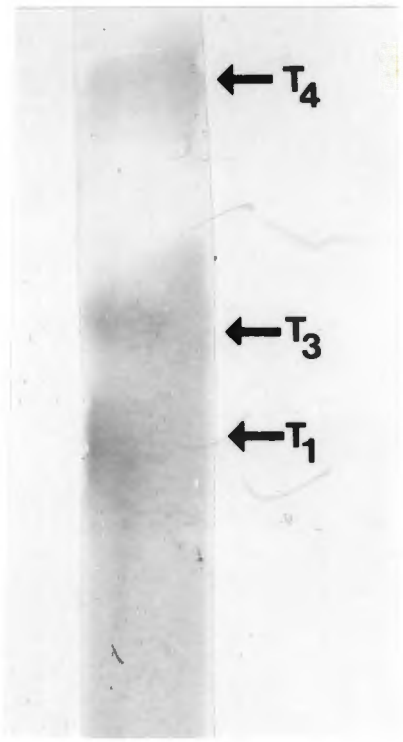

Fig. 4.3: Separation of tyrosinase isozymes by gel electrophoresis. Fractions with high tyrosinase activity (see Fig. 4.2), pooled and concentrated against poly ethylene glycol (PEG 600), were electrophoresed on a non-denaturing polyacrylamide gel. The gel incubated with DOPA (1 mg/ml) in phosphate buffer (0.1 M, pH 7.4) shows dark bands corresponding to various tyrosinase isozymes; T_3, de novo form of tyrosinase, T_1, glycosylated form of tyrosinase and T_4, matured form of tyrosinase which complexes with melanosomal membrane components.

4.3.2 Molecular weight determination of tyrosinase

Each of the three tyrosinase isozymes was cut out and electrophoresed on an SDS gel and proteins visualized by staining with Comassie blue. As shown in Fig. 4.4, all the isozymes were reasonably pure and formed two distinct bands. Using the Enzfitter non-linear regression data analysis programme (see materials and methods), the molecular weight of these isozymes were calculated to be about 77 kd and 42 kd (Fig. 4.5).

4.3.3 Raising of a tyrosinase polyclonal antibody

T_4 tyrosinase isozyme purified from B16 melanoma cells (see materials and methods), was subcutaneously injected into guinea pigs and boost injected after 2 weeks. A week after the boost injection, sera were drawn and tested for the presence of tyrosinase antibodies using the western blot technique. No positive signal was detected suggesting the absence of tyrosinase antibodies.

In view of the fact that guinea pigs failed to raise antibodies, rabbits were subsequently immunized with T_1 and T_3 isozymes, purified as described in paragraph 5.2.1.2. It was also found that rabbits immunized with T_1 and T_3 failed

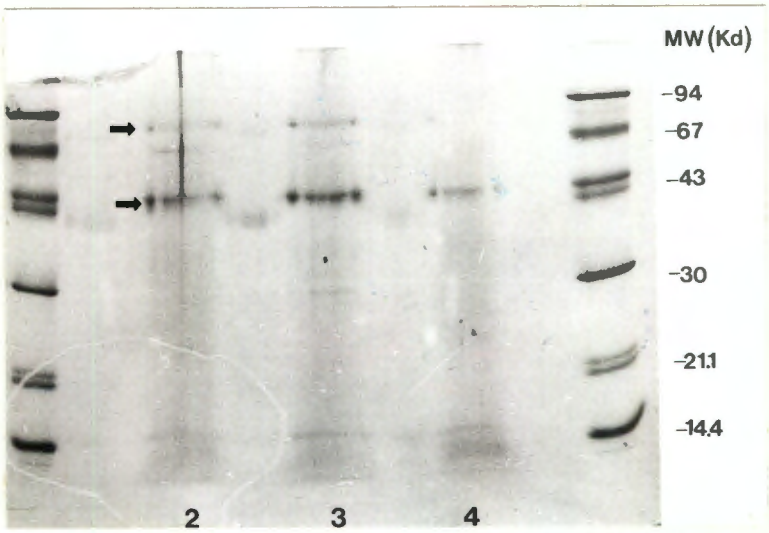

Fig. 4.4: SDS gel electrophoresis analysis of purified tyrosinase. Tyrosinase isozymes (see Fig. 4.3), were further electrophoresed on an SDS polyacrylamide gel and stained with Commassie blue (see materials and methods). Lane 2, T_1; lane 3, T_3; lane 4, T_4. Note the presence of two distinct proteins; the molecular weight calculation for each of these is shown in Fig. 4.5.

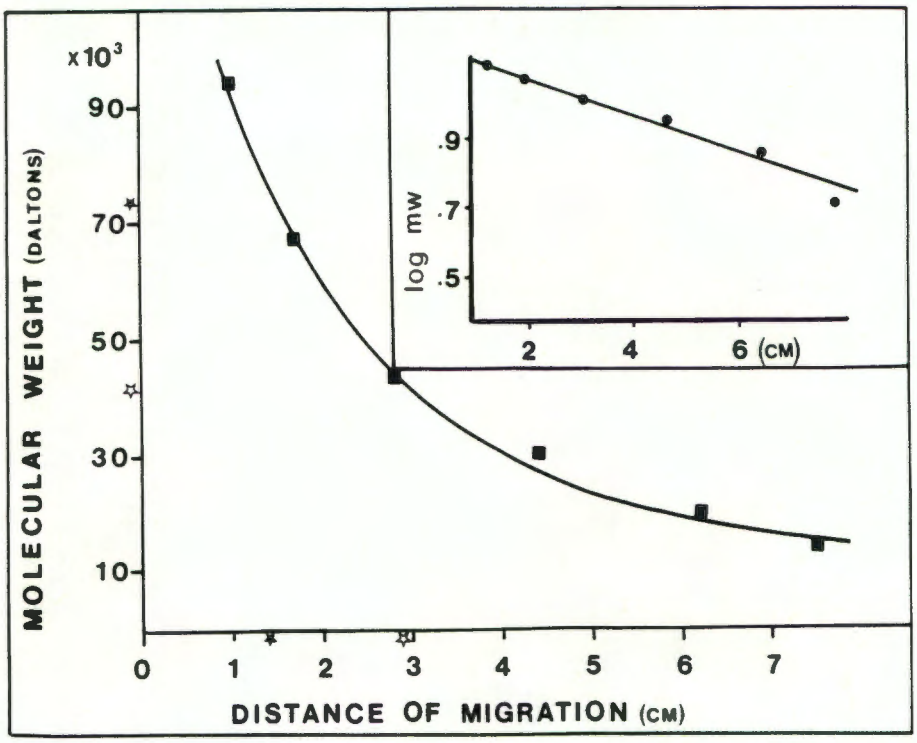

Fig 4.5: Molecular weight determination of tyrosinase. Isozymes of tyrosinase (Fig. 4.3), analysed by SDS gel electrophoresis resolved into two proteins (see Fig. 4.4). Distance migrated by proteins of molecular weight markers and also these two proteins were loaded on to Enzfitter non-linear regression data analysis programme (Elsevier 1987), and used to calculate molecular weights. (★), molecular weight of the upper protein was calculated to be 77.82 kD, and that of the lower protein (☆), was 42.66 kD.

to generate antibodies against these two isozymes and subsequent attempts to raise an antibody against the T_4 isozyme in rabbits were also unsuccessful. The implication of these results is addressed in the discussion.

4.3.4 Quantitation of tyrosinase levels by immunoblot technique

It was shown in Chapter 2 that tyrosinase activity in wild type skin, measured in the presence of cycloheximide was 1.6 to 2.7 fold higher than in the Himalayan skin, suggesting that the activity of tyrosinase is regulated post-translationally. To test whether this activity of tyrosinase correlated with its abundance, an anti-tyrosinase antibody (which binds active and inactive form of tyrosinase) was used to quantitate the total levels of this enzyme in the skins of wild type, Himalayan and albino mice. Crude homogenate proteins from skin were electrophoresed on a SDS gel slab (Fig. 4.6) and blotted onto nitrocellulose, then reacted with a tyrosinase antibody. As mentioned in paragraph 4.3.2, the sera drawn from both guinea pigs and rabbits did not give any signals when tested using the 4-chloro-1-naphthol/hydrogen peroxide staining procedure. In an attempt to solve this problem, a number of options were tried: (1) different antibody titers from 1:250 to 1:10), (2) Various blocking times and (3) Deliberate overloading of gels. Since all

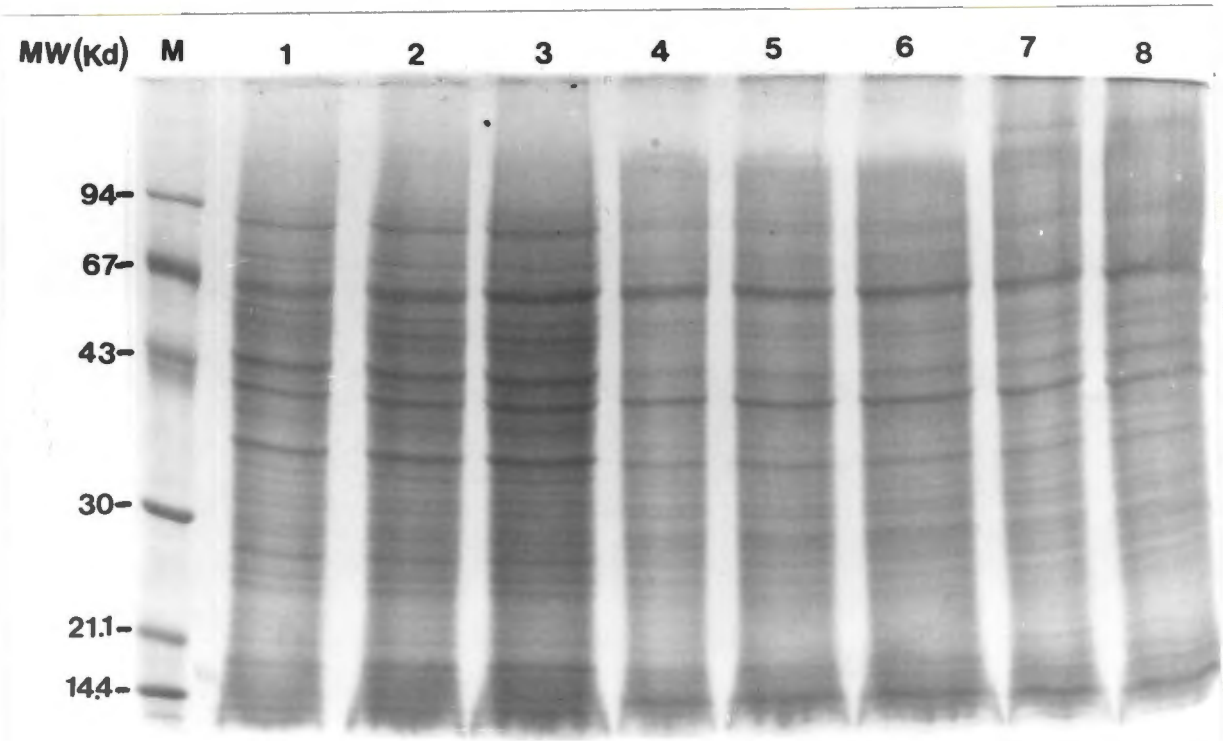

Fig. 4.6: Electrophoresis of proteins on SDS polyacrylamide gels. Melanoma, albino, Himalayan, and wild type crude homogenate proteins were run on an SDS gel slab and stained as described in materials and methods. Lanes 1-3, B16 melanoma cells; lanes 4 & 5, albino; lane 5, Himalayan; lanes 7 & 8, wild type.

these attempts were unsuccessful, a more sensitive method (the immunogold staining procedure) was used. To our disappointment, this method was found to be too sensitive, which then made it impossible to distinguish between the genuine tyrosinase signal and the background reaction. Due to the lack of success to measure the levels of tyrosinase using our antibody, a tyrosinase polyclonal antibody which was a gift from Dr V.J. Hearing was finally used. Using this antibody, it was found that the total quantity of tyrosinase is higher in the wild type than in the Himalayan skin (Fig. 4.7). The densitometric analysis showed that the wild type skin tyrosinase is twice that of the Himalayan skin (Fig. 4.8). Interestingly, the albino tyrosinase, which is known to be inactive, did not react to this tyrosinase antibody.

4.3.5 Determination of mRNA levels

4.3.5.1 Hybridization of total RNA with pMT4 cDNA

Results presented in the preceding section showed that the abundance of the wild type tyrosinase was twice that of the Himalayan, suggesting a translational control of this enzyme. The next logical step was to determine whether this abundance of tyrosinase was the result of transcriptional regulation. This was done by measuring the levels of endogenous mRNA transcripts in wild type, Himalayan, and albino mouse skins.

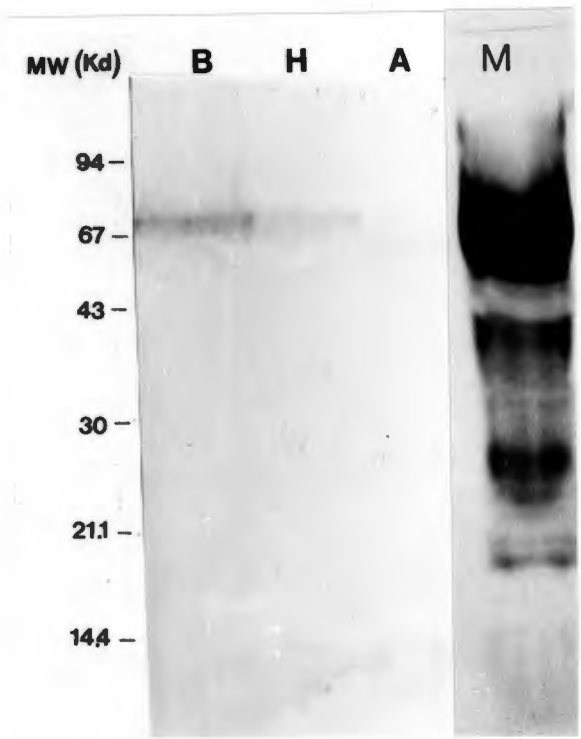

Fig. 4.7: Immunoblot quantitation of skin tyrosinases. Proteins prepared from crude homogenates and eletrophoresed under SDS denaturing conditions, were transblotted overnight onto nitrocellulose membrane at 100 mA, 14 V overnight. Transblotted proteins were incubated with polyclonal antibody 3819 (1:250) (from Dr. V.J. Hearing) overnight, reacted with peroxidase-linked antirabbit secondary antibody (1:500) for an hour and incubated with 4-chloro-1-napthol/hydrogen peroxide mixture as described in materials and methods. B, black (wild type); H, Himalayan; A, albino; M, melanoma. Note that albino mutant shows no reaction, and also that the antibody reacts to all melanoma tyrosinase isozymes.

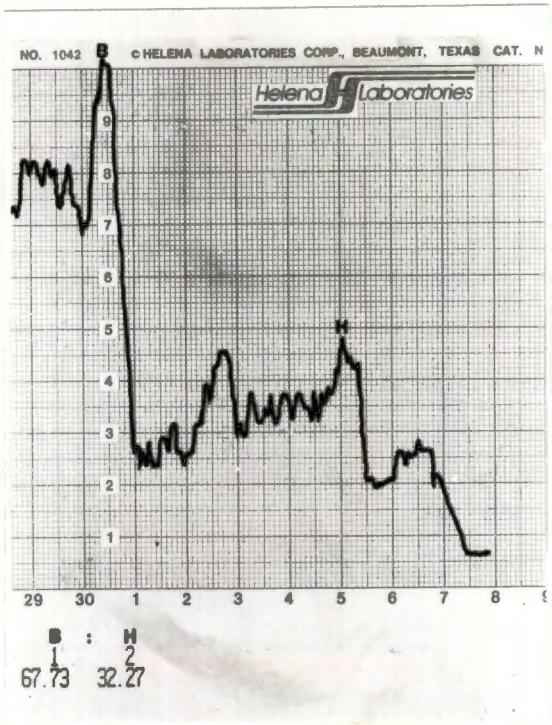

Fig. 4.8: Densitometric analysis of immunoblotted tyrosinase. The total levels of wild type and Himalayan tyrosinase determined by immunoblot technique (see Fig. 4.7) were analysed on a Cliniscan densitometric equipment (Helena Laboratories, Texas) at 500 nm by horizontally scanning. The intregration of the two peaks showed that the amount of tyrosinase in the wild type skin is twice more than in the Himalayan skin. B, back wild type; H, Himalayan.

RNA was extracted from mouse skins, fractioned on a 1.1% formaldehyde denaturing gel, transblotted to nitrocellulose membrane and hybridized to a [^{32}P]-nick-translated radioactively labelled pMT4 cDNA (Shibahara et al., 1988). This experiment was repeated at least five times and it was consistently found that the pMT4 cDNA did not hybridize to RNA extracted from skin melanocytes of these mice. This failure to detect the signal was thought to be due to the probe that is not optimally labelled. To overcome this problem, the cDNA was then labelled using the multiprime method. Again, no signal was detected even if the gels were deliberately overloaded. However, when B16 melanoma cells were used as a source of RNA, a signal corresponding to the molecular weight of tyrosinase mRNA (about 2.3 kb) was detected (Fig. 4.9).

4.3.5.2 Hybridization of poly A+ mRNA with pMT4 cDNA

Unsuccessful attempts to detect mRNA levels extracted from mouse skins were thought to be due to the low concentration of mRNA since total RNA was loaded onto gels. In an attempt to overcome this problem, poly A^+ mRNA was separated on an oligo(dT)-cellulose column and fractioned on a 1.1% formaldehyde denaturing gel, blotted and hybridized to the pMT4 cDNA as detailed in materials and methods.

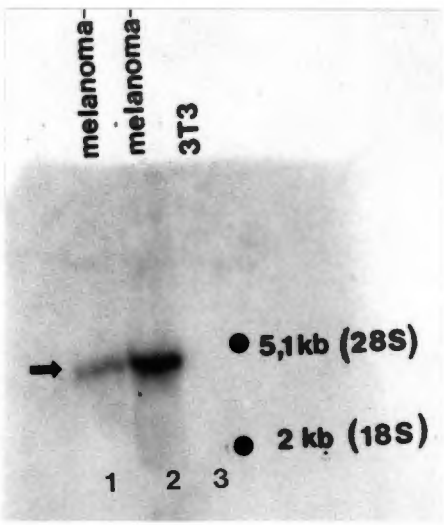

Fig. 4.9: Northern blot analysis of melanoma mRNA hybridized to pMT4 cDNA. Total RNA was extracted from B16 melanoma cells, then transblotted to nitrocellulose membrane and hybribrized to a ^{32}P-labelled probe as described in materials and methods. Lane 1, 5 μg of melanoma RNA loaded; lane 2, 20 μg of melanoma RNA loaded. Note that no hybridization signal was detected in lane 3 (RNA from fibroblast cells)

Unfortunately, three attempts to hybridize purified poly A^+ mRNA to this probe were not successful.

4.3.5.3 Hybridization of mRNA with MTY811 cDNA

Due to futile attempts to detect tyrosinase mRNA using the pMT4 probe, the MTY811 cDNA (Kwon et al., 1988) was used as the next option. Total RNA extracted from the skins of wild type, Himalayan and albino mice was hybridized to the MTY811 cDNA and processed as previously described. It was found that this cDNA also did not hybridize to the mRNA extracted from the three mice. The next strategy employed to solve this problem was to eliminate RNA from acellular dermal debris by using the epidermis as a source of RNA extraction (see materials and methods). Under these conditions, a hybridization signal of the expected length (i.e. 2.3 kb) was detected in all the three mice (Fig. 4.10). Although the levels of mRNA were low, densitometric scanning revealed that the level of RNA transcripts in the wild type skin was 1.2 times higher than in the Himalayan mouse and 3 times more than in the albino mutant mice (Fig. 4.10)

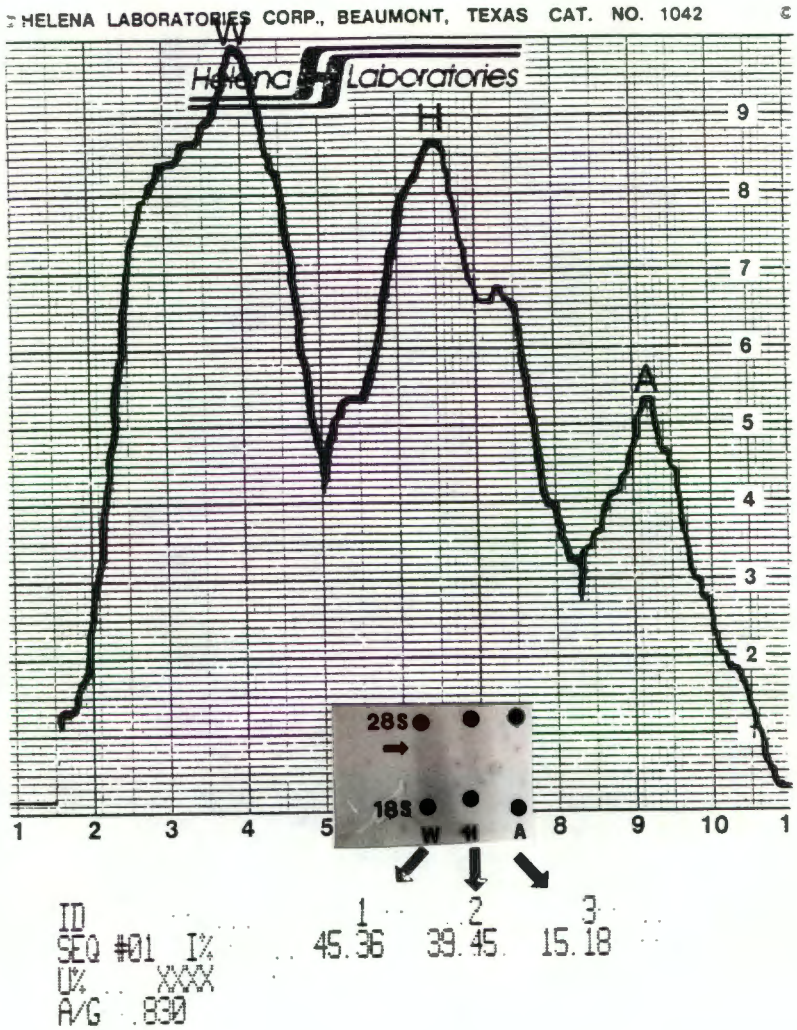

Fig. 4.10: Northern blot analysis and densitometric quantitation of wild type, Himalayan and albino mRNA hybridized to MTY 811 cDNA. Total RNA extracted from the epidermis, transblotted onto nitrocellulose membrane and hybridized to a ^{32}P-labelled probe (see materials and methods). The quantity of RNA in the wild type (W) skin was found to be 1.2- and 3-fold more than in the Himalayan (H) and albino (A) skins respectively.

4.4 DISCUSSION

4.4.1 Translational and transcriptional control of tyrosinase activity

There is substantial evidence that tyrosinase activity is regulated at a post-translational level (see Chapter 1). In Chapter 2 and 3 of this study, further evidence in support of this was provided.

This chapter explores the possibility that in addition to the post-translational regulation of tyrosinase activity, translation and transcription might also play a role in the control of tyrosinase activity. If tyrosinase activity was exclusively post-translationally controlled and if the Himalayan tyrosinase was underglycosylated, similar quantities of newly synthesized T_3 would be measured in both the wild type and Himalayan skins, but they would differ in the amount of T_1 (glycosylated form). This is what Halaban et al. (1988) found using immunoprecipitation. In this study however, the amount of newly synthesized tyrosinase (M.W.≈ 65 kD, Fig. 4.7) of the wild type was shown by immunoblot technique to be twice that of the Himalayan mutant (Fig. 4.8). The possible reasons for these different results could be due to either the different techniques or different specificities of the antibodies used. No tyrosinase was

detected in the albino skin, suggesting that this enzyme was not translated.

At the same time as the above studies were being carried out, the levels of tyrosinase mRNA were measured to see whether transcription of the tyrosinase gene also played a role in the regulation of Himalayan tyrosinase activity. If tyrosinase activity was not regulated at the level of transcription, equal quantities of mRNA would be measured. Using the MTY811 cDNA, the levels of mRNA transcripts in the wild type skin were found to be 1.2- and 3-fold higher than in the skins of the Himalayan and albino mice respectively (Fig. 4.10). These results suggest that the transcription of the tyrosinase gene is yet another control mechanism that regulates the activity of skin tyrosinase in these mice. Interestingly, these findings conflict with reports that there are equal levels of mRNA in the skins and cultured melanocytes of the wild types (C57BL/6J & B10.BR), Himalayan, chinchilla and albino mutant mice (Halaban et al., 1988; Shibahara, 1988; Takeuchi et al., 1988; Kwon et al., 1989). Why are these results different from those of the current study? A possibility exists that in the present study, the different levels of mRNA transcripts measured in the skins of these mice could be incorrect if different amounts of RNA were loaded on the gel. Since these results are considered as preliminary, the repeats of this experiment should include

an internal control probe to ensure that equal amounts of RNA are loaded.

4.4.2 Raising of a tyrosinase polyclonal antibody

In the initial attempt aimed at raising an anti-tyrosinase polyclonal antibody, purified T_4 tyrosinase isozyme was injected into guinea pigs. On testing the serum for the presence of antibodies, it was found that this isozyme did not trigger an immune response in these animals. All subsequent attempts to raise a tyrosinase antibody against T_4 were not successful. As an alternative source of raising an antibody, rabbits that were immunized with the de novo (T_3) and glycosylated (T_1) forms of tyrosinase also did form antibodies against these isozymes. The question to answer is: Why is it that all attempts were unsuccessful? Futile attempts to raise tyrosinase monoclonal antibodies against the T_1 and T_3 isozymes were also reported by Tomita et al., (1985 and others, personal communication). As suggested by Hearing and Jiménez (1989), these findings confirm that precursor forms of tyrosinase are in general highly conserved in nature and as a result immunologically inert. Tomita et al. (1985) did, however, succeed to raise monoclonal antibodies against various epitopes of the T_4 isozyme. Subsequent tests revealed that these monoclonal antibodies did not cross-react with T_1 and T_3 isozymes, but they reacted

with the neuraminidase-treated T_4 isozyme. As suggested by these results, the immunogenic properties of mammalian tyrosinase seem complicated since they appear be independent of the presence of sialic acid residues, but on other post-translational modifications of the enzyme which are currently not known. Whatever epitope is involved, it appears not to change drastically in various mammalian species. In support of this view is the fact that although the present study has failed to generate a tyrosinase antibody against T_4 in guinea pigs, a chick anti-tyrosinase polyclonal antibody against this isozyme has been raised in these animals (Kidson et al., unpublished results).

Other factors that make the raising of tyrosinase antibodies notorious are that:

(a) The ultra-purification of tyrosinase is difficult due to the presence of sticky melanin which drags along contaminants which co-purify with the enzyme (Hearing & Jiménez, 1989).

(b) Tyrosinase is generally a highly conserved protein in nature which, in addition, has a common molecular weight which makes it difficult to be isolated it from other housekeeping proteins (Hearing & Jiménez, 1989).

(c) Due to the microheterogeneity of tyrosinase, the various isozymes are difficult to separate (Hearing et al., 1981; Hearing & Jiménez, 1989).

Considering these facts, it is therefore not a surprise that the present study has not succeeded to raise antibodies against various tyrosinase isozymes.

4.4.3 Molecular weight determination of murine tyrosinase

To date, there have been wide variations concerning the molecular weights of tyrosinase reported in the literature The reported molecular weight of the de novo form of tyrosinase ranges from 55 kD to 68 kD, meanwhile that of the matured form ranges from 70 kD to 94 kD (Burnett, 1971; Pomerantz, 1973; Hearing et al., 1978, 1981; Tomita et al., 1985; Fuller et al., 1987; Halaban et al., 1988; Jiménez et al., 1988; Hearing & Jiménez 1989; Mc Ewan et al., 1988; Yamasaki et al., 1988; Halaban & Straight, 1989; Moody et al., 1989).

In the light of these differences, the present study has investigated this issue by determining the molecular weight of tyrosinase isolated from B16 melanoma cells. To carry out this investigation, tyrosinase isozymes were purified as described in materials and methods, the separated on a non-SDS gel and localized by DOPA staining (Fig. 4.3). Further electrophoresis of these isozymes under SDS denaturing conditions consistently revealed the presence of two proteins

whose molecular weights were calculated to be 77,82 kD and 42,66 kD (Fig. 4.4). The molecular weight of the 77 kD protein seems reasonable, since it corresponds to the average molecular weight of the matured form of tyrosinase, but the nature of the 42 kD protein remains to be elucidated. The possibility that this could be a contaminating protein can be discounted, since it was present in the T_1, T_3, and T_4 isozymes, which originated from different positions on the gel slab (Fig. 4.3). Furthermore, this protein may not represent partially glycosylated tyrosinase, since the de novo form of mammalian tyrosinase, which is unglycosylated, has an apparent molecular weight of about 55 kD (Hearing et. al., 1981). A recent study by Halaban et al. (1988) has provided some insight relating to how this 42 kD protein could arise. In this study, the 42 kD and 47 kD proteins were predominantly immunoprecipitated in the chinchilla melanocytes in addition to the matured form of tyrosinase. The 47 kD protein was also immunoprecipitated in the wild type (BR10.BR) melanocytes which were left for longer in the absence of protease inhibitors. These results suggest the existence of protease-dependent cleavage products of the matured form of mammalian tyrosinase which is also present in the chinchilla mutant. On the basis of these reports by Halaban et al. (1988), it is concluded that even in B16 transformed melanocytes used in this study, the 42 kD is the cleavage product of the 77 kD matured form of tyrosinase

which did not change when these cells were transformed. However, this proposition needs to be substantiated further by experimental work.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

Differentiation is a process by which cells and tissues become different from one another by virtue of their ability to synthesize specific structural and functional end products. The synthesis of these end products is directed by the activity of tissue-specific genes. The factors that regulate the `switching on' of eukaryotic genes are now beginning to emerge. The process of differentiation is very complex indeed and cannot be fully explained by differential gene activity per se, since the parameters that regulate the repression of those unexpressed genes are still not understood. Thus, it appears that we are still far from understanding the complex events that direct the differentiative pathway of cells.

Those active genes that are normally expressed in a particular differentiated system are characterized by a unique nucleotide sequence of RNA which is the result of accurate transcription of the particular gene. The production of proteins in differentiated cells is further regulated at translational and post-translational levels. Any gross deviations in any of these regulatory mechanisms or in the normal sequence of the gene itself, may result in a mutation or transformation of that particular cell or tissue. These mutant and transformed cells are in turn valuable `tools' that may be used in understanding the complex mechanisms that operate in differentiated cells.

The present study has made use of a normal, mutant and transformed melanocytes to explore the mechanisms that regulate the synthesis of melanin. The first agent used in this study to investigate the regulation of melanin synthesis was melanocyte stimulating hormone (MSH). This study was initiated as a consequence of an earlier observation made in our laboratory that the injection of α-MSH into the skins of Himalayan living mice resulted in the darkening of the coat color. This finding suggested that the hormone could override the inhibitory effect of temperature on the activity of the Himalayan tyrosinase. In order to verify this subjective evaluation of the effect of α-MSH, an assay was established that made it possible to measure the activity of tyrosinase in the presence of the hormone.

Using this skin culture radiometric assay, it was possible to show that at 37°C, MSH (10^{-9} M to 10^{-4} M) activated both the wild type and the Himalayan tyrosinase in a dose-dependent manner. This activation of the Himalayan and wild type tyrosinase by α-MSH was found to be similar even in the presence of cycloheximide, suggesting that the hormone was acting at a post-translational level. α-MSH did not, as expected activate the albino tyrosinase. By increasing the concentration of α-MSH one would expect to reach an optimal concentration beyond which no further increase of tyrosinase activity would occur. Considering the situation <u>in vivo</u>, where MSH is transported in blood vessels and diffuses from

the sub-epidermal vessels to melanocytes, it is possible to saturate MSH receptors at a lower concentration of the hormone than in our skin culture assay where MSH supply to melanocytes relies on the diffusion through agar and the dermis. This might explain why it was not possible to reach a saturating concentration.

Despite this technical limitation, it has been possible to show a dose-dependent activation of wild type and Himalayan tyrosinase in culture. Is there a link or relationship between activation of the Himalayan tyrosinase by low temperature and the activation of this enzyme α-MSH? It has recently been shown that in the leopard frog, Rana chiricahuensis, the lowering of the skin temperature from 25°C to 10°C results in its darkening (Fernandez & Bagnara, 1989). By radioimmunoassay technique, it was further shown that at 10°C, the amount of α-MSH in the blood plasma of this frog was 62% higher than at 25°C. Although these findings were demonstrated using a cold-blooded vertebrate, they raise the possibility that in vivo, temperature could activate the Himalayan tyrosinase by triggering the release of MSH.

How does α-MSH activate the Himalayan tyrosinase? This may be explained in terms of two theories. Firstly, the presence of tyrosinase inhibitors has been shown in the Himalayan skin (Kidson & Fabian, 1981) and other pigment cell types (Quevedo & Mishima, 1960; Chian & Wilgram, 1967; Korner & Pawelek,

1977; Fuller et al., 1988). The removal of a tyrosinase inhibitor by phosphorylation-dependent events has been demonstrated in S91 mouse melanoma cells (Korner and Pawelek, 1977; Pawelek, 1989). It is possible therefore that α-MSH might act to remove the Himalayan inhibitor via phosphorylation (see Fig. 5.1)

The second theory that could account for the stimulation of the Himalayan tyrosinase activity in the presence of α-MSH involves the glycosylation of this enzyme. The Himalayan tyrosinase has been shown to be deficient in N-linked glycosylation (Halaban et al., 1988). It was shown in this study that the Himalayan tyrosinase has a reduced ability incorporate tritiated mannose and N-acetylglucosamine and that the bulk of immunoprecipitated tyrosinase corresponds to the molecular weight of an underglycosylated enzyme. The low molecular weight form of tyrosinase has been shown to be predominant in pigment cells with low tyrosinase activity and is degraded faster than the fully processed tyrosinase in cells with high enzyme activity (Halaban, 1984). The fully glycosylated tyrosinase is abundant and correlates with melanin content in normal human melanocytes derived from Caucasian and Black foreskin, further confirming the role of glycosylation on melanin synthesis (Halaban & Straight, 1989). Thus, reduced activity of the underglycosylated Himalayan tyrosinase is thought to be caused by the susceptibility of this enzyme to proteolytic degradation. In

the light of the fact α-MSH can activate the Himalayan tyrosinase, a possibility therefore exists that the hormone might do so via glycosylation and thereby making this enzyme more stable and active (see Fig. 5.1 & Appendix)

In order to investigate whether α-MSH activates tyrosinase by enhancing glycosylation, the effect of the hormone on mannose incorporation was examined. Due to difficulties involved in the culturing of normal mouse melanocytes and also the inability to control for sugars incorporated into glycolipids in cultured skin, it was impossible to directly test this hypothesis on the Himalayan mouse. In the light of these problems, the effect of α-MSH on the incorporation of mannose was investigated using B16 mouse melanoma cells.

As a preliminary step, it was necessary to confirm that our stock of α-MSH could activate melanoma tyrosinase and then under the same experimental conditions measure the incorporation of tritiated mannose. B16 melanoma cells were cultured for 24 hours in DMEM containing MSH (2×10^{-7} M) and with or without cycloheximide. α-MSH was found to stimulate a 9-fold increase of tyrosinase activity above control in the absence of cycloheximide and a marginal

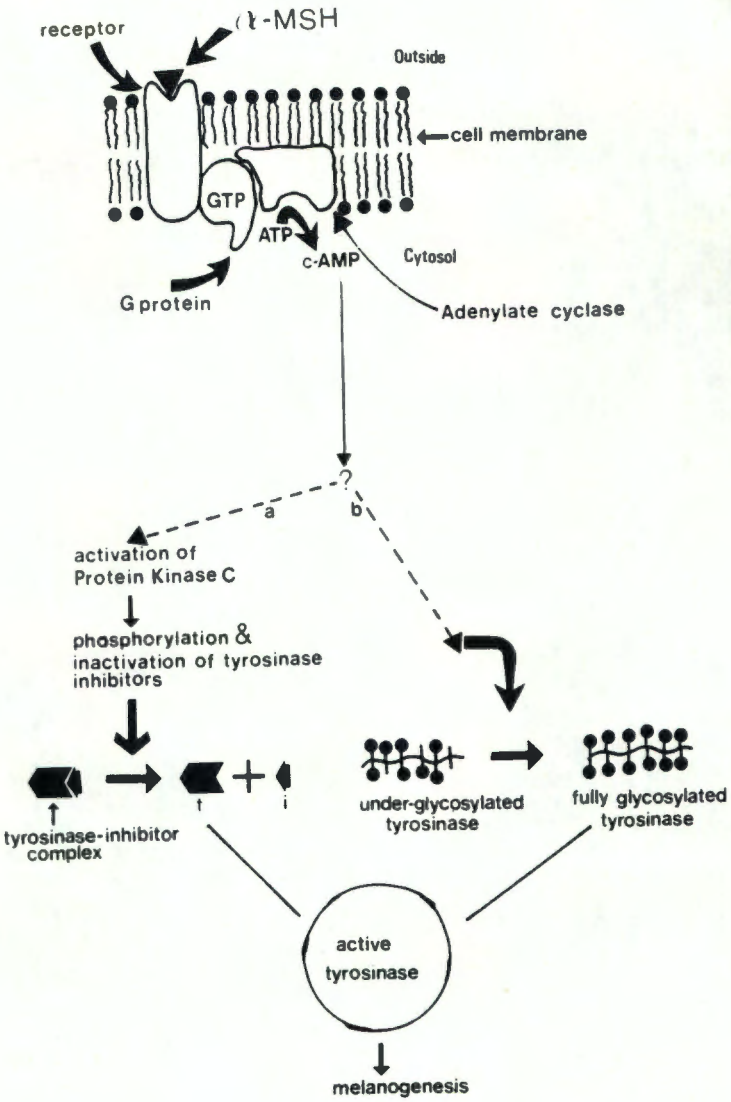

Fig. 5.1: Scheme showing the possible mechanisms of α-MSH action. Note that mechanism **a** shows the phosphorylation and inactivation of a tyrosinase inhibitor by α-MSH, while mechanism **b** shows an alternative mechanism of the hormone which involves glycosylation. α-MSH, melanocyte stimulating hormone; GTP, Guanosine triphosphate; c-AMP, cyclic -3,5-adenosine monophosphate.

increase (i.e. 1.3-fold) in the presence of cycloheximide (Fig. 5.2). Subsequently, the effect of α-MSH on D-[2-^{3}H]-mannose incorporation was investigated. If α-MSH promotes glycosylation, which is an exclusively post-translational event, it should do so even when protein synthesis is blocked. It was found that α-MSH increased the incorporation of tritiated mannose 1.3-fold above control in the absence of cycloheximide, whereas in the presence of this protein synthesis inhibitor, the incorporation of mannose was 3.5-fold lower than in the control (Fig. 5.3). These results suggest that the post-translational activation of tyrosinase by α-MSH does not involve glycosylation directly. Thus, the hormone may act to increase the de novo synthesis of tyrosinase molecules which are then glycosylated. Since α-MSH was shown to activate the Himalayan tyrosinase post-translationally (Chapter 2), it is unlikely that the hormone might exert its effect via glycosylation in this mutant.

In addition to the proposal that the Himalayan tyrosinase is under-glycosylated, a point mutation has recently been identified in this mouse (Kwon et al., 1989). In this study, the analysis of the Himalayan tyrosinase cDNA has revealed that this mutant contains an A to G change at nucleotide 1259 that changes a histidine to an arginine

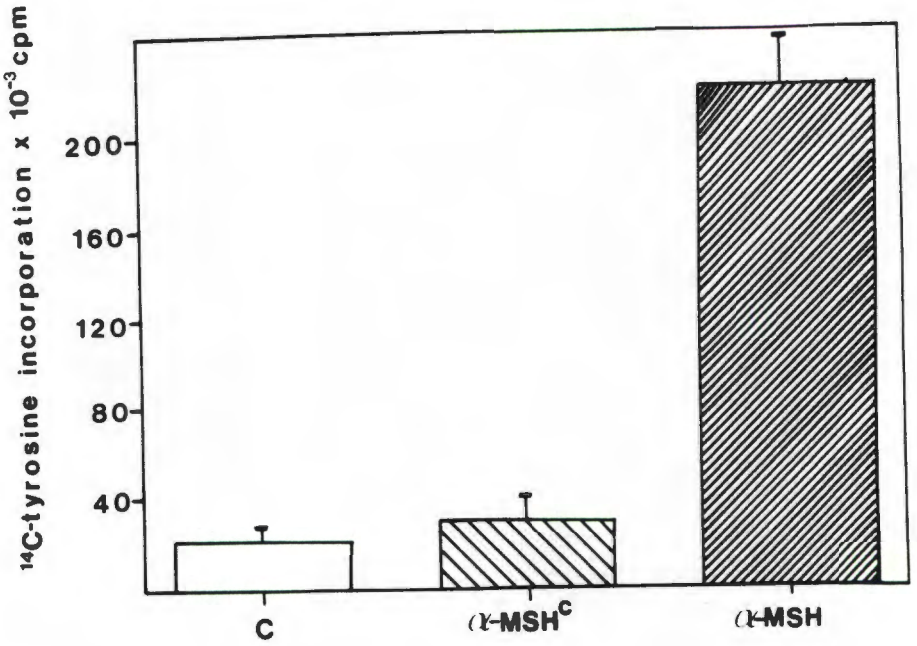

Fig. 5.2: The effect of α-MSH on B16 melanoma tyrosinase activity. Melanoma cells were cultured for 24 hours in DMEM containing α-MSH (2 x 10^{-7} M) and with or without cycloheximide (0.25 μg/ml). Tyrosinase activity was measured using the <u>in vitro</u> radiometric assay. This experiment was repeated twice with similar results. Each value represents the mean of two measurements ± S.D. The values are significantly different from control at $P < 0.05$. C, control; α-MSH, melanocyte stimulating hormone; c, cycloheximide.

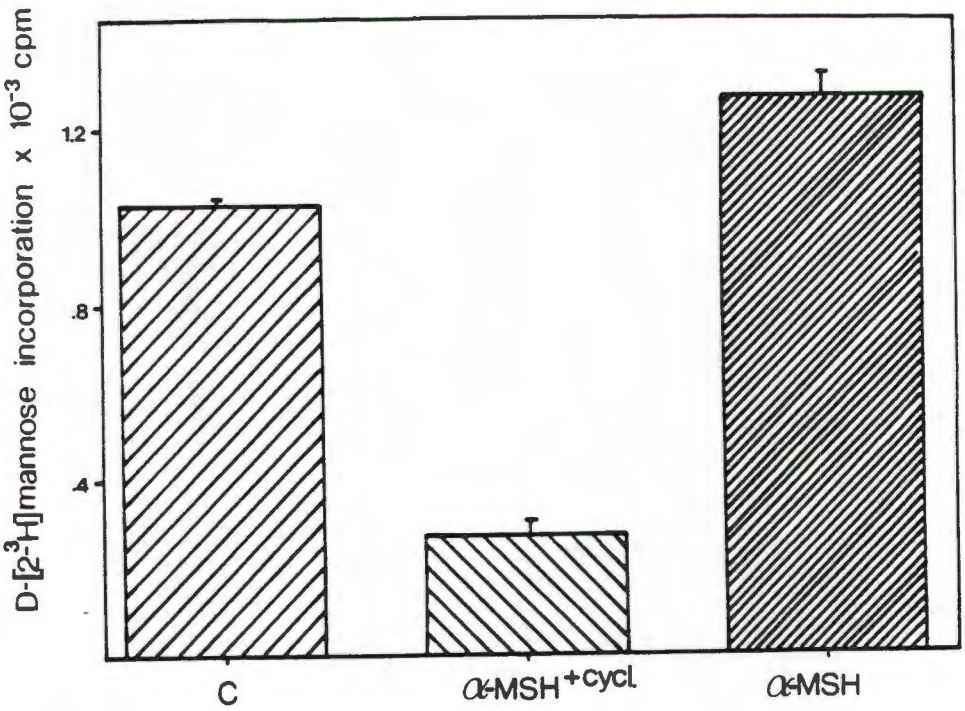

Fig. 5.3: The effect of α-MSH on mannose incorporation. B16 melanoma cells were cultured for 24 hours in DMEM containing MSH (2×10^{-7} M) and with or without cycloheximide (0.25 μg/ml). D-[2-^{3}H]-mannose was added in the culture medium to a final concentration of 0.5 μCi/ml. Cells were collected with 0.05% EDTA-0.02% trypsin. The cell lysate was micropipetted onto GFC Whatmann discs, washed twice in 10% trichloroacetic acid for 5 minutes, then rinsed in 90% ethanol for 5 minutes and twice in acetone for 5 minutes. The discs were air-dried and counted in 5 mls of toluene scintillator (Parkard) in a Parkard Tricarb liquid scintillator. This experiment was repeated twice with similar results. Each value represents the mean of two measurements ± S.D. α-MSH, melanocyte stimulating hormone; cycl., cycloheximide.

residue at amino acid 420. Histidine residues are known to be copper binding sites (Lerch, 1983), which affect the activity of this enzyme (Martinez et al., 1987). Since not all amino acid substitutions have destabilizing effects on enzymes (Alber, 1989), the histidine substitution can only affect the Himalayan tyrosinase activity if it is located at a critical position such as at or near the active site. The altered histidine of the Himalayan tyrosinase has been shown by Kwon et al. (1989) not to be proximal to the active site, but to be located next to the transmembrane region. Furthermore, only Hist 191 and Hist 200 of the mouse tyrosinase are involved as copper ligands at the active site of this enzyme (Lerch, 1988). Thus, the amino acid substitution may not affect the activity of the Himalayan tyrosinase directly, but can change the conformation so that the enzyme has a higher affinity for inhibitors (Kidson & Fabian) or is underglycosylated (Halaban et al., 1988) or inappropriately transported to melanosomes.

In conclusion, the results presented in this thesis have shown that in addition to the post-translational control mechanism, the activity of wild type and Himalayan tyrosinases is regulated at the level of translation and transcription. In particular, they show that reduced activity of the Himalayan tyrosinase at normal body temperature may be due to the lower expression of the tyrosinase gene. Since the transcription of eukaryotic genes

is influenced by regulatory elements with the promoters (Johnson & Mc Knight, 1989), it is possible that the Himalayan tyrosinase gene has a temperature-sensitive element within its promoter which somehow inactivates the gene at normal body temperature. Analysis of the promoter activity of the wild type and Himalayan tyrosinase genes might shed some light on this idea.

Having attempted in the current study to use a melanocyte to shed some light on the process of differentiation, where are we in the understanding of this complex process? Although this study has indeed provided some insight on the regulation of melanin synthesis, a glance at living cells under the microscope is still a sobering reminder of how little we understand.

APPENDIX

In vitro stimulation of murine melanogenesis by MSH

Nkosana C. Nkabinde and Susan H. Kidson

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In vitro stimulation of murine melanogenesis by MSH

N.C. Nkabinde and S.H. Kidson
Department of Anatomy and Cell Biology, University of Cape Town Medical School, Observatory, 7925.

Melancocyte stimulating hormone (MSH) controls melanogenesis in the integument of vertebrates, although its mechanism of action on tyrosinase, the rate limiting enzyme in this pathway, is still unclear. Numerous reports suggest that MSH increases tyrosinase activity by activating pre-existing enzyme. In contrast to this view, studies with protein and RNA inhibitors have demonstrated that MSH may act to stimulate tyrosinase synthesis as well as to activate pre-existing enzyme molecules through cAMP-mediated events.[1]

Because of the difficulties of measuring the effect of MSH *in vitro*, we have developed an assay to measure the effect of MSH on tyrosinase activity *in vitro*. We used this assay to determine whether protein synthesis is necessary for the short-term stimulation of tyrosinase activity in black (C57BL/6J) and Himalayan mice by MSH. Albino (Balb C) mouse skin served as a negative control. The skin explants of 4 – 5-day-old mice were cultured at 37°C on agar medium with or without MSH and cycloheximide (a protein synthesis inhibitor), then assayed for tyrosinase activity.

Our results show that an increase of MSH in the culture medium induces a proportional increase in tyrosinase activity in black and Himalayan mice. No activity was detected in the albino mouse. When protein synthesis was blocked prior to the addition of MSH, the level of stimulation of tyrosinase was the same as in those cultures without cycloheximide. These findings suggest that the activation of tyrosinase by MSH is not dependent on protein synthesis.

Previous studies have shown that in the Himalayan mutant, tyrosinase activity is considerably reduced at 37°C,[2] and that this is possibly due to a deficiency in the post-translational glycosylation of the enzyme.[3] In view of our finding that at 37°C MSH enhances tyrosinase activity in this mutant, we suggest that MSH might possibly activate tyrosinase by stimulating post-translational glycosylation.

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