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Review

Human pigmentation genes: identification, structure and consequences of polymorphic variation

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Abstract

The synthesis of the visible pigment melanin by the melanocyte cell is the basis of the human pigmentary system, those genes directing the formation, transport and distribution of the specialised melanosome organelle in which melanin accumulates can legitimately be called pigmentation genes. The genes involved in this process have been identified through comparative genomic studies of mouse coat colour mutations and by the molecular characterisation of human hypopigmentary genetic diseases such as OCA1 and OCA2. The melanocyte responds to the peptide hormones α-MSH or ACTH through the MC1R G-protein coupled receptor to stimulate melanin production through induced maturation or switching of melanin type. The pheomelanosome, containing the key enzyme of the pathway tyrosinase, produces light red/yellowish melanin, whereas the eumelanosome produces darker melanins via induction of additional TYRP1, TYRP2, SILV enzymes, and the P-protein. Intramelanosomal pH governed by the P-protein may act as a critical determinant of tyrosinase enzyme activity to control the initial step in melanin synthesis or TYRP complex formation to facilitate melanogenesis and melanosomal maturation. The search for genetic variation in these candidate human pigmentation genes in various human populations has revealed high levels of polymorphism in the *MC1R* locus, with over 30 variant alleles so far identified. Functional correlation of *MC1R* alleles with skin and hair colour provides evidence that this receptor molecule is a principle component underlying normal human pigment variation.

Keywords: Melanocyte; Melanin; Tyrosinase; *P*-gene; *MC1R*

1. Introduction

The derivation of the full complement of genes from the human genome sequencing project is but a beginning in the understanding of the genetic make-up of individuals (IHGSC, 2001; Venter et al., 2001). The relationship between having certain genetic characteristics and developing a phenotype or genetic disease is very complex and influenced by many factors including the developmental, environmental, stochastic and epigenetic events involved in gene expression. Additionally, the gene mapping details of the human genome only provide the template for understanding the role of specific genes in disease with the much more complicated task of translating this information into a knowledge of functional genomics.

Considering the general interest in the heritability of human physical features, it is perhaps unfortunate and disappointing that so few of them show clear-cut Mendelian pedigree patterns. The determinants of human skin, hair and eye colour fall into the quasi-Mendelian inheritance pattern of a polygenetic trait with a few major genes of dramatic effect and additional modifier genes. Visible pigment is synthesised by melanoctyes, the dendritic cells that lie at the junction of the dermis and epidermis of the skin, through an enzymatic pathway to produce the biopolymer melanin. During embryonic development melanocyte precursor cells (melanoblasts) migrate from the

Abbreviations: ACTH, adrenocorticotropic hormone; ASIP, agouti signalling protein; BOCA, brown oculocutaneous albinism; DOPA, 3, 4-dihydroxyphenylalanine; DHICA, 5, 6-dihydroxyindole-2-carboxylic acid; EGF, epidermal growth factor; *MC1R*, melanocortin-1 receptor; MITF, microphthalmia transcription factor; α-MSH, α-melanocyte stimulating hormone; OA, ocular albinism; OCA, oculocutaneous albinism; *PAR2*, protease activated receptor-2; PKA, protein kinase-A; POMC, proopiomelanocortin; RHC, red hair colour; *SILV*, silver; *TYRP*, tyrosinase related protein; *TYR*, tyrosinase

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neural crest to the skin, hair follicles and uvea of the eye and are thus directly responsible for these colour characteristics. To understand the genetic basis of pigmentation, a molecular understanding of the gene products expressed in the melanocytic cell and its cellular interaction with surrounding keratinocytes that absorb the pigment is required.

Classic genetic studies of inheritance have provided little understanding as to the molecular basis of normal variation in human pigmentation, beyond that intermarriage between Black and White peoples results in offspring of intermediate pigmentation (reviewed Robins, 1991; Sturm et al., 1998). With respect to hair colour intensity, dark hair dominates more or less over light and with respect to quality, brown/black dominates over red/yellow. Although hair and skin melanocytes arise from the same embryonic source, the genes affecting colour can be expressed independently with combinations of dark hair and fair skin or fair hair and tanned skin seen in different human populations. The influence of environmental and age-related factors that lead to modification of the pigmentation phenotype are confounding variables when attempting genetic analysis, hence evaluation of these traits can become subjective unless proper controls are in place.

In contrast to the study of human genetics, much has been learnt about human pigmentation from a comparative genomics approach (Jackson, 1997). Mouse coat colour mutants were some of the first traits to be subject to Mendelian analysis, and over 30 of the genes underlying these coat colour mutations have now been molecularly cloned with many found to have a corresponding human disease phenotype. A subset of these genes preferentially affect pigment function (Table 1), with several other genetic loci producing mutations that have more general developmental or physiological defects that accompany a pigmentation deficiency (see Albinism database www.cbc.umn.edu/tad/). This review will focus on those genes that have so far been shown to act in a melanocyte specific manner or possibly associated with normal variation in human pigmentation.

2. Melanocytes, melanosomes and the melanogenic pathway

The human pigmentary system is dependent on the production of the light absorbing biopolymer, melanin, within epidermal, ocular and follicular melanocytes (Nordlund et al., 1998). Melanocytes within the skin are situated on the basal layer between the dermis and epidermis and have a number of dendritic processes that interdigitate with the surrounding keratinocytes. While pigment synthesis occurs within the melanocyte, the majority of pigment within the skin is found in melanin laden vesicles known as melanosomes located within the keratinocytes. It has been the characterisation of proteins that are contained within and form the melanosomal organelle that has provided the biochemical understanding of some of the coat colour and human albinism conditions.

The melanin pigments are of no fixed molecular weight but are all derived by enzymatic oxidation of the amino acid tyrosine and eventually produce two types of melanin in mammalian skin. Major advances in the understanding of the chemistry and enzymology of the biosynthetic pathway involved in the synthesis of the eumelanin (black or brown) and pheomelanin (red or yellow) have now been made

Table 1
Human pigmentation-related genes

Mouse coat colour	Human locus	Human chromosome	Protein	*Mutation/Phenotype*	Function
Melanosome proteins					
Albino (c)	TYR	11q14-q21	Tyrosinase	OCA1	Oxidation of tyrosine, dopa,
Brown (b)	TYRP1	9p23	Gp75/TRYP1	OCA3	DHICA-oxidase, TYR stabilization
Slaty (slt)	DCT	13q32	TRYP2	?	Dopachrome tautomerase
Silver (si)	SILV	12q13-q14	gp100/pMel17/silver	?	DHICA-polymerization/stablin
Pinkeyed dilute (p)	OCA2	15q11.2-q12	P-protein	OCA2	pH of melanosome
Underwhite (uw)	LOC51151	5p14.3-q12.3	AIM-1	OCA4	Homology to Sugar Transporters
Signal proteins					
Agouti (a)	ASIP	20q11.2-q12	Agouti signal protein	?	MC1R antagonist
Extension (e)	MC1R	16q24.3	MSH receptor	Red Hair	G-protein coupled receptor
Pomc1	POMC	2p23.3	POMC, MSH, ACTH	Red Hair	MC1R agonist
Oa1 (oa1)	OA1	Xp22.3	OA1 protein	OA1	G-protein coupled receptor
Microphthalmia (mi)	MITF	3p12.3-14.1	MITF	Wardenburg syndrome type 2	Transcription factor
Melanosome transport/uptake by Keratinocyte					
Dilute (d)	MYO5A	15q21	MyosinVa	Griscelli syndrome	Motor protein
Ashen (ash)	RAB27A	15q21	Rab27a	Griscelli syndrome	RAS family protein
F2rl1	F2RL1	5q13	PAR2	?	G-protein coupled receptor

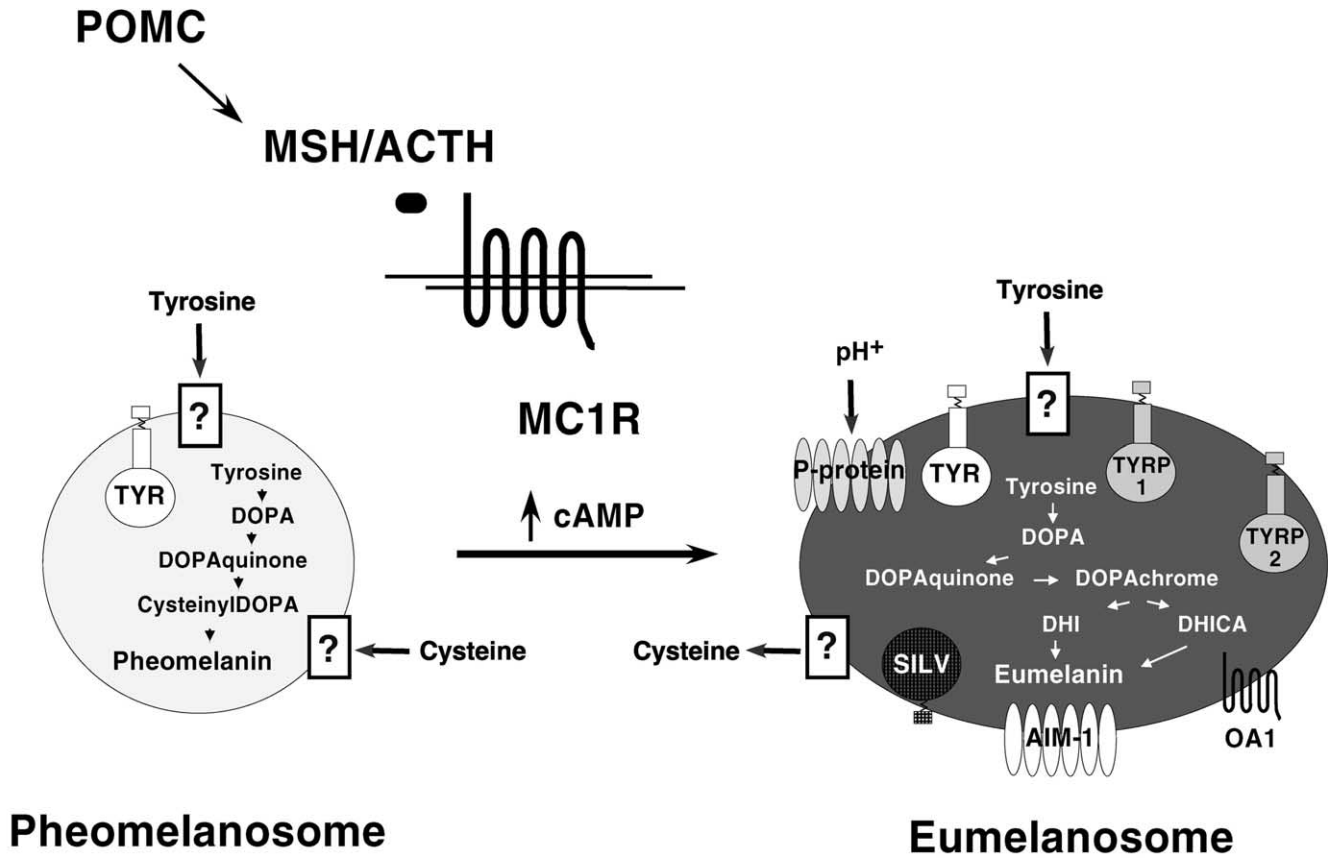

Fig. 1. MC1R control of pigment type switching. The POMC precursor is cleaved to give the α-MSH/ACTH ligands for the MC1R receptor which activates the PKA/cAMP pathway. MC1R signalling induces the maturation of the phenomelanosome containing the TYR molecule to the eumelanosome containing all the melanogenic enzymes, with the possible active transport of tyrosine or cysteine indicated by the boxed question marks.

(Prota, 1992; Nordlund et al., 1998). Tyrosinase can catalyse the first two steps, the hydroxylation of tyrosine to 3, 4-dihydroxyphenylalanine (DOPA), and oxidation of DOPA to DOPAquinone (Fig. 1). The eumelanins are derived from the metabolites of DOPAchrome, whereas the pheomelanins derive from metabolites of 5-S-cysteinylDOPA. The isomerization of DOPAchrome to 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) is catalysed by DOPAchrome tautomerase and the oxidation of DHICA is performed by a DHICA-oxidase enzyme. Mice with mutant forms of these enzymes have been found in the *albino*, *brown* and *slaty* coat colour loci proposed to produce tyrosinase, and tyrosinase related proteins TYRP1 and TYRP2, respectively, (del Marmol and Beermann, 1996), though the exact enzyme activities encoded at some of these loci are yet to be fully defined.

Identification of other melanogenic enzymes has been based on the study of mouse coat colour mutations through the culture of their melanocytes, extraction or expression of melanosomal proteins and testing for catalytic and functional activities. The *silver* mouse coat colour has been found to be due to a mutation that results in the progressive loss of pigment through the disappearance of melanocytes from their hair follicles. In vitro studies have found the *silver* encoded protein (mouse gp87; human GP100/PMEL17) to catalyse the polymerisation of DHICA to DHICA-melanin (Chakraborty et al., 1996; Lee et al., 1996). Another activity acting in melanoma cells upon the DHICA substrate has been termed stablin, which prevents the auto-oxidation of DHICA to melanin, this function has also been assigned to the *silver* protein, with the suggestion that there maybe a steady state relationship between DHICA-polymerisation and stablin activities (Solano et al., 2000).

The function of the P-protein encoded by the *pink-eyed(p)* dilute mutation has been studied in mouse melanocytes, and the fact that the phenotype could be partially corrected by higher concentrations of tyrosine led to the suggestion that it may be related to the transport of the tyrosine substrate into the melanosome to assist melanogenesis. Direct biochemical assay has shown no difference in tyrosine transport between normal and *p*-melanocytes (Gahl et al., 1995; Potterf et al., 1998), however antibodies against the P-protein demonstrated association with the melanosomal membrane and this intracellular location suggested it might transport a critical substance between the cytoplasm and the lumen of melanosomes. In situ tyrosinase activity requires an appropriate ionic environment, the melanosome

is known to be an acidic organelle however mammalian tyrosinases have demonstrated optimal activity at neutral pH. The potential role of the P-protein as a pH exchange membrane channel has been examined in normal and *p*-melanocytes by measuring acid sensitive stain incorporation, which was shown to be compromised in *p*-melanosomes as recognised using a TYRP1 antibody (Puri et al., 2000). The regulation of tyrosinase activity in human melanocyte cultures has also been proposed through the finding of differences in melanosomal pH of those cells cultured from Black and White skin. Staining of human cells with a fluorescent weak base showed that the pH of melanosomes from White melanocytes was acidic whereas those from Black melanocytes were more neutral, suggesting that an acidic environment causes suppression of human tyrosinase activity (Fuller et al., 2001). Neutralisation of melanosomal pH by vacuolar proton pump inhibitors leads to increased melanogenesis, melanosome maturation and eumelanin/pheomelanin ratio of treated White melanocytes (Ancans et al., 2001a). It has been suggested that the P-protein, which shares structural similarity with the *E. coli* Na+/H+ anti-porter, mediates neutralization of melanosomal pH by functioning as a channel to reduce the proton concentration inside melanosomes and in this way regulate tyrosinase activity (Ancans et al., 2001b). It is also possible that a neutralised melanosome is important for the formation of the high molecular weight melanogenic complex involved in eumelanogenesis (Section 4).

Tyrosine is not freely permeable through lipid bilayers and the mechanism for delivery of the initial substrate for melanogenesis to its catalytic compartment is unknown. The requirement for a melanosomal tyrosine transporter would seem obvious, as increasing intracellular concentrations of either tyrosine or DOPA both result in an increase in melanogenesis. Melanocytes do express receptors that bind tyrosine or DOPA (Gahl et al., 1995; Potterf et al., 1998), however the fact that the P-protein is not responsible for tyrosine transport still leaves an essential step of melanogenesis to be resolved. The transport of cysteine essential for pheomelanogenesis and its potential efflux from the melanosome that may stimulate eumelanogenesis is also yet to be investigated (Potterf et al., 1999).

The genetic study of the *B*-gene allele series associated with recessive orange-red colouration or hypopigmentation in makeda fish has resulted in the molecular identification of another integral melanosomal membrane protein known as AIM-1 (Fukamachi et al., 2001). The mouse and human gene orthologs were also reported in this study and the encoded proteins show strongest sequence and structural similarity to plant sucrose transporter proteins. Mutations of the AIM-1 protein (Newton et al., 2001) have now been found to map to the *underwhite* mouse mutation series (Sweet et al., 1998) which effects both eye and coat colour. The primary defect appears specific to the melanosome as electron microscopy of the retina showed irregular size, shape and reduction in the fraction of mature melanosomes. Moreover, melanocyte cells from *underwhite* mutant mice failed to pigment under conditions suitable for normal mouse melanocytes (Lehman et al., 2000), with epistatic interactions shown upon breeding with *p*-mutant mice producing a significant reduction in the level of eumelanin. The possible role of AIM-1 in melanosomal transport of a saccharide molecule(s) is yet to be assessed in relation to the biochemical pathway of melanogenesis, however glycosylation of the TYRP proteins is known to occur and affect their activity and intracellular organalle transport.

3. Melanosome ultrastructural components, maturation and translocation

Ultrastructural examination of melanocytes reveals that melanosomes are discrete membrane bound organelles related to lysosomes, produced through the action of the Golgi and rough endoplasmic reticulum that mature and pigment as they are transported along the dendritic processes and extruded into the surrounding keratinocytes of the skin and hair follicle. Melanocytes of the eye do not secrete their melanosomes but retain them within their cytoplasm (Nordlund et al., 1998). Two major types of melanosomes are produced and named according to the type of melanin they contain. The eumelanosome is large ($\sim 0.9 \times 0.3$ μm) and ellipsoidal with a highly ordered glycoprotein matrix that is integral to the production of the black or brown coloured eumelanin pigments whereas the red or yellow pheomelanins are produced within smaller and spherical (~ 0.7 μm diameter) pheomelanosomes that are composed of a loosely aggregated and disordered glycoprotein matrix.

There are quantifiable differences in the degree of melanization and distribution of melanosomes in individuals of diverse ethnic background (Nordlund et al., 1998). Although the distribution of melanocytes is essentially constant, the number, size and packaging of the melanosomes within the keratinocyte vary. In general, more deeply pigmented skin contains numerous large melanosomal particles, lighter pigmentation is associated with small and less dense melanosomes that are clustered in membrane bound groups. These distinct distribution patterns are present at birth and are not determined by sun exposure. Differences in the degree of melanisation, as well as chemical differences in the melanin pigments themselves are the determining factors in the visual gradation of skin and hair colour (Sturm et al., 1998).

The melanosome has four stages of maturation, Stage I is common to eumelanogenesis and pheomelanogenesis and derives from late endosomes from the endoplasmic reticulum. However, in the later stages eumelanosomes are always ellipsoidal in contrast to pheomelanosomes which retain a spherical shape. Stage II eumelanosomes demonstrate a well organised internal structure, in Stage III the regular and periodic deposition of opaque melanin is evident

upon the matrix with Stage IV melanosomes so melanized that all internal structure is obscured. Pheomelanosomes contain only granular material through all four stages of melanosomal maturation. Tyrosinase has a characteristic pattern of post-translational glycosylation and plays a role in the biogenesis of both eumelanosomes and pheomelanosomes, however TYRP1 and TYRP2 proteins are exclusive to the eumelanosomes. The intracytoplasmic vesicular transport and membrane trafficking to melanosomes has been well characterised (Jimbow et al., 2000).

The translocation process for melanosomes within the melanocyte is beginning to be understood but melanosomal transfer to the keratinocytes is not well characterised. Melanosomal movement from the perinuclear area toward the dendritic processes of the melanocyte is dependent upon microtubules. Melanocytes from the *dilute* mouse coat colour mutation have melanosomes that are concentrated in the centre of the cytoplasm due to a mutation in the myosinVa molecule (Wu et al., 1998). This compromises the ability of melanosomes to move along actin filaments, producing rapid bidirectional movements between the cell centre and the periphery, with the myosinVa defect leading to the failure to capture and retain the organelle at the ends of dendrites. Another mouse mutation *ashen* produces a similar phenotype indicating that it affects a related pathway and positional cloning has mapped a non-functional mutation within the murine *Rab27a* gene, a member of the RAS superfamily of GTPases (Wilson et al., 2000). In normal melanocytes the Rab27a protein is localised on the cytoplasmic face of melanosomes together with myosinVa; in melanocytes from *ashen* mice that lack Rab27a, myosinVa failed to attach to melanosomes in the periphery of the dendrites resulting in rapid bidirectional movement without retention in the ends of the dendrites (Hume et al., 2001; Wu et al., 2001). These results indicate that Rab27a provides a receptor binding site for myosinVa to melanosomes and a direct interaction between the two proteins has been shown by coimmunoprecipitation (Hume et al., 2001). In addition Rab27a can rescue melanosome transport in human melanocytes from Griscelli syndrome patients who have partial albinism associated with defects in melanosome distribution (Bahadoran et al., 2001).

Human OA1 is the commonest form of ocular albinism. It is characterised by the appearance of giant melanosomes in the melanocytes within the retinal pigmented epithelium and skin. The OA1 protein structure identifies it as a member of the seven transmembrane G-protein coupled receptor family and was detected as a melanosomal membrane glycoprotein (Schiaffino et al., 1999). These observations suggest that OA1 is involved in melanosomal organelle formation possibly as a sensor of an unidentified intra-melanosomal ligand regulating organelle biogenesis through activation of G-proteins on the cytoplasmic side of the melanosomal membrane. Analysis of Oa1 deficient mice generated by gene knockout produced animals anatomically indistinguishable by coat colour from normal but examination of the eye revealed hypopigmentation of the retina with melanosomes displaying a giant pigment granule phenotype (Incerti et al., 2000). Recent immunofluorescence cell staining of cultured mouse cells has localised Oa1 to enodolysosomes and revealed that it is not actually present in mature melanosomes (Samaraweera et al., 2001), raising the possibility that it functions in intracellular vesicle trafficking to melanosomes.

A major gap in the understanding of the pigmentary process is the determinants and mechanisms involved in the transfer of the melanosomes to the surrounding keratinocytes. Four theoretical processes have been proposed, phagocytosis, endocytosis, physical transfer of melanosomes through an inter-cellular communication gap or direct inoculation into keratinocytes. Whatever the mechanism, disruption of this process would be expected to have severe consequences for pigmentation. Experimental evidence implicates the keratinocyte receptor *PAR2* as being involved in melanosome transfer (Seiberg et al., 2000a,b). Activation of the PAR2 receptor can be effected through protease treatment or with a mimetic of its N-terminal tethered peptide ligand which induces pigmentation in cocultures of human keratinocytes and melanocytes. Inhibition of the PAR2 receptor with a serine protease inhibitor resulted in depigmentation when topically applied to the skin of swine and human skin xenotransplanted onto mice. Electron microscopy studies found an accumulation of immature melanosomes inside melanocytes of inhibitor-treated in vitro epidermal equivalents. No pigmentation effects of *Par2* mutant mice have been reported (Lindner et al., 2000), but fundamental differences in human and murine pigmentary systems are to be expected since human melanocytes localise to the epidermal-dermal junction whereas in mice they sit predominantly in hair follicles of the dermal compartment. It is intriguing to note that a variant allele of the human *PAR2* gene has recently been reported to display reduced sensitivity to trypsin and differential responses to PAR agonists, but any association with pigmentation defects are yet to be investigated (Compton et al., 2000).

4. Identification and genomic structure of human pigmentation genes: the *TYRP* and *SILV* families

The genes encoding the mouse coat colour mutations described have been molecularly cloned by a variety of methods and have provided a cornerstone for melanocyte gene function, moreover their utility is the identification of analogous phenotypes of human albinism and other pigmentary disorders. Perhaps the most dramatic examples of gene inactivation in human pigmentation are the albinism phenotypes of oculocutaneous albinism OCA1 and OCA2, the myriad of inactivating mutations responsible can be viewed at the International Albinism database hosted by the University of Minnesota (www.cbc.umn.edu/tad). Mutations in the

tyrosinase gene (*TYR*) are responsible for OCA1 with mutations in the *P*-locus underlying the tyrosinase-positive albinism OCA2 phenotype. The genes and mutations responsible for other forms of human albinism have also been determined and collated, OCA3 arising from genetic changes in *TYRP1*, and *OA1* gene defects in ocular albinism.

The human tyrosinase gene family consists of three members that produce the TYR, TYRP1 and TYRP2 proteins involved in the catalytic steps of melanogenesis (Fig. 1), and a tyrosinase pseudogene (*TYRL*) which is not active. To determine whether additional members of the tyrosinase gene family exist the total human genome sequence was analysed (IHGSC, 2001; Venter et al., 2001). While individual *TYRP* members were able to readily identify the other *TYRP* genes when the entire human genome was analysed using tblastn, they failed to detect any additional genes or sequences that showed significant homology. Based on this we conclude that no additional members of the *TYRP* family exist within the first draft of the human genome. *TYR* encodes a 529 aa copper binding protein with a molecular weight of 55 kD, following glycosylation the molecular weight of the mature protein shifts to 65–75 kD. Comparison of the TYR, TYRP1 and TYRP2 proteins reveals a common protein structure of approximately 50% similarity which is schematically shown in Fig. 2A (Cassady and Sturm, 1994). All three enzymes contain an N-terminal signal sequence, an epidermal growth factor (EGF) repeat and other conserved cysteine residues that may be involved in protein-protein interactions, two metal binding domains A and B that serve as the catalytic site, and a C-terminal transmembrane domain with a short cytoplasmic tail. These proteins share a common tertiary structure, have the potential to associate in a higher order melanosomal protein complex and contribute to the stability of complex formation (Jimenez-Cervantes et al., 1998; Kobayashi et al., 1998).

The human *TYR* (Giebel et al., 1991; Ponnazhagan et al., 1994), *TYRP1* (Box et al., 1998) and *TYRP2* (Sturm et al., 1995) genomic structures have previously been determined and show *TYR* to be encoded by five exons, *TYRP1* protein by seven of eight exons, and *TYRP2* by eight exons. Analysis of the exonic regions and splice junction phase shown in Fig. 2A reveals only one site that is identical in all three genes, with the final exon containing the C-terminal transmembrane region (Sturm et al., 1995). Both the *TYR*/*TYRP1*, and *TYRP1*/*TYRP2* gene pairs share another common splice junction. This exon-intron boundary study clearly indicates that the *TYRP* gene family has evolved from one common ancestral gene by duplication and subsequent divergence with analysis by sequence similarity and comparative genomic studies (Budd and Jackson, 1995) supporting the hypothesis that *TYR* gave rise to *TYRP1*, which duplicated to give rise to *TYRP2* (Sturm et al., 1995). These events appear to have at least predated the evolution of mammals with both *TYRP*-like genes existing in birds, fish and axolotl, however a single *TYRP1* like gene in ascidians suggests that triplication of the tyrosinase family occurred during the early radiation of chordates (Sato et al., 1999). Given the age of gene duplication, it is not surprising that there are no paralogous genes flanking the *TYRP* loci when the human genomic sequence is examined. The three *TYRP* gene structures with their repeat elements indicated are presented in Fig. 2B. The *TYR* locus has not yet been completely resolved with 105 kb so far assembled, but both the *TYRP1* and *TYRP2* genes are now complete with 37 and 60 kb encompassing each locus presented. The distribution of repetitive elements within these loci supports the notion that the bulk of the human genome is derived from the accumulation and decay of transposable elements (Smit, 1999).

The human *SILV* gene product PMEL17 was originally isolated from a cDNA library by screening with anti-tyrosinase antibodies and also as the GP100 protein a frequently recognised antigen on the surface of melanoma tumour cells by cytotoxic T lymphocytes, later it was found to be orthologous to the mouse *silver* locus. The deduced amino acid sequence revealed a 668 aa protein of 70 kD molecular weight, with a potential signal peptide sequence and a hydrophobic C-terminal region indicating that it is membrane bound, consistent with its identification as a melanosomal matrix protein. The protein contains a PKD domain (residues 237–307) found in the polycystic kidney disease protein, and an triplet repeat of a 26 aa motif in the centre of the protein (residues 315–392) found only twice in the mouse *silver* protein. Although there has been some initial discrepancy as to the structure of the *SILV* genomic locus, being reported to contain nine exons spanning 7.9 kb (Kim et al., 1996) or 11 exons spanning 9.1 kb (Bailin et al., 1996), it is recognised that two proteins are produced from the locus. PMEL17 and GP100 proteins are generated by alternative splicing of the gene product to two competing 3′ acceptor sites producting two proteins with potentially different catalytic activities (Solano et al., 2000). Scanning of the total human genomic sequence with *SILV* can detect one gene of high similarity using a tblastn search, this gene *GPNMB* has previously been cloned in a subtractive cDNA library screen of melanoma cells (Weterman et al., 1995), but any role in the pigmentation pathway is yet to be investigated. Interestingly the *SILV* locus is flanked quite closely on each side by the *CDK2* and *DGKA* genes at distances of 829 and 193bp, respectively, orientated head to head with *CDK2* in such a way as to allow sharing or overlapping of gene promoter elements. Any significance of this gene arrangement is yet to be tested.

The human *P*-gene transcript associated with OCA2 is divided into 24 exons with the Human Genome Project providing an incomplete assembly of 267 kb, again there are no other *P*-gene homologues revealed in a tblastn search of the total genome. The gene encodes an 838 aa open reading frame producing a 110 kD protein that contains 12 transmembrane spanning regions that has been located as an integral melanosomal membrane protein. In addition to

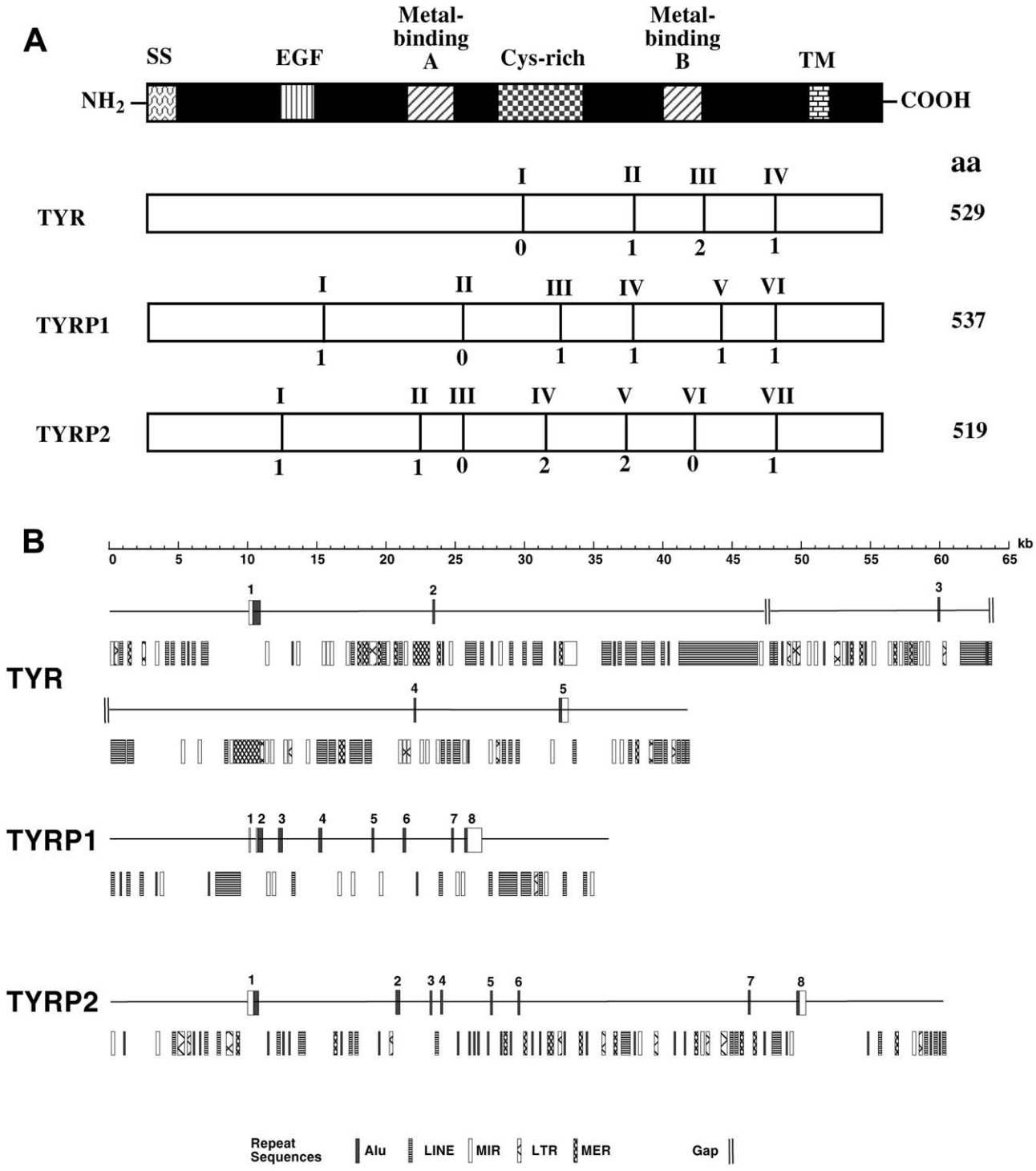

Fig. 2. (A) Chromosomal structure of the human *TYRP* family members. A schematic illustration of the generic structure of the *TYRP*-family showing the position of N-terminal secretory signal peptide (SS), epidermal growth factor-like region (EGF), two metal binding domains (A and B), cysteine rich region (Cys-rich), and C-terminal membrane spanning domain (TM). Below are representations of the TYR, TYRP1 and TYRP2 proteins with the exons boxed, intron numbers relative to the coding region are indicated by Roman numerals above the junctions, with the intron phases shown below. The phase interruptions are listed as 0 - between two codons, 1 - between the first and second nucleotides of a codon, 2 - between the second and third nucleotides of a codon. (B) The assembled sequence contig from the Human Genome Projects (IHGSC, 2001; Venter et al., 2001) of each *TYRP* loci are shown below a 5 kb ruler together with the relative location of exons and interspersed repeat sequences. The non-coding portions of the exons are indicated by open boxes, the coding region by closed boxes. The relative position of the repeat units identified (Smit, AFA & Green, P RepeatMasker at http://ftp.genome.washington.edu/RM/RepeatMasker.html) are shown by the key. IHGSC accession/Celera gene identification numbers *TYR*, 13639321/hCG19690; *TYRP1*, 13641579/hCG27256; *TYRP2*, 13627278/hCG32235.

its function regulating pH it may also be involved in the eumelanin-pheomelanin switch (Section 5). Recently the *P*-gene has also been shown to be the basis of a rare but distinct form of brown albinism (BOCA) in the South African population (Manga et al., 2001), however the molecular pathology of this pigment disorder has not been precisely determined. *P*-gene mutations have been reported to account for only 50% of tyrosinase-positive mutations suggesting that at least one other locus may be responsible for clinical manifestations of tyrosinase-positive albinism (Passmore et al., 1999). Alterations in the human orthologue of the AIM-1 protein have now been reported to be the basis of a condition termed OCA4 (Newton et al., 2001) which phenotypically resembles OCA2, and this may at least in part explain cases of tyrosinase-positive albinism in which *P*-gene mutations have not been found.

5. Regulation of melanogenesis by differential intracellular signalling

A unifying model of melanogenesis has emerged with the cloning of two genes that control melanocyte switching between eumelanin and pheomelanin production (Barsh, 1996). In mice, two interacting loci were identified that regulate switching between the formation of pheomelanosomes and eumelanosomes (Fig. 1), the coat colour mutants *Agouti* and *extension*. The wild type *Agouti* mouse produces coat hairs that are black at the tips and base but contain a yellow subapical band which corresponds to a discrete and pulsatile switch from eumelanin synthesis to pheomelanin during the anagen phase of the hair cycle. Allele series at both *Agouti* and *extension* may disrupt this wild-type coat phenotype. The human agouti gene homologue *ASIP* has been shown to inhibit the binding of the normal ligand for the gene product of the extension locus, the melanocortin-1 receptor (*MC1R*) and block pigmentation (Suzuki et al., 1997). In mouse, the pituitary hormone pro-opiomelanocortin (POMC) is the prohormone from which α-melanocyte stimulating hormone (α-MSH) and adrenocorticotrophic hormone (ACTH) are produced. Both of these peptide hormones interact with MC1R to stimulate melanin production through the cAMP/PKA signalling pathway (Busca and Ballotti, 2000), inducing changes in protein phosphorylation and gene expression, largely through the transcription factor gene product MITF of the *microphthalmia* locus. Ultimately this signal results in generation of a mature, eumelanogenic melanosome that contains along with TYR, the P-protein, TYRP1, TYRP2 and SILV proteins (Kobayashi et al., 1995; Lamoreux et al., 1995). In the absence of an MC1R signal such as through binding of the inhibitory ASIP protein, the eumelanogenic melanosome is unable to form and the immature pheomelanosome is produced.

The same *Agouti/extension* gene nexus controlling melanogenic complex formation may account for the natural red or yellow pigmentation seen in many mammalian species, with allele series at one or the other locus accounting for pheomelanic coat colours. In humans, red hair is the orthologous phenotype to the pheomelanic *extension* mouse coat colour, and also due to several variant alleles of the *MC1R* gene (Section 6). It seems unlikely that variation at the *ASIP* locus may account for any of the variation in red hair colour seen in human populations as there is no known analogous phenotype to the agouti banded hair pattern in humans. Given the lack of phenotypic evidence for the role of *ASIP* in human pigmentation, the large size of this genomic locus in man and the potential for regulatory sequence polymorphism, there has been little interest in searching for polymorphism at this locus that may influence human pigmentary phenotypes. Some polymorphism screens have been performed on the *ASIP* coding region but no functional variability has yet been identified (Voisey et al., 2001).

A report of a rare mutation within the *POMC* gene producing the MSH/ACTH ligands has suggested that variability at this gene could also account for a small proportion of red hair (Krude et al., 1998), however, the concurrent *MC1R* variant genotype status was not reported in this pedigree to exclude this locus as a cause of the red hair. A transgenic *Pomc* gene knock-out mouse ablating α-MSH/ACTH production has been generated (Yaswen et al., 1999), although the mice displayed a yellowish tinge to their coat this change was quite subtle, suggesting that there is substantial ligand-independent constitutive signalling from the murine *Mc1r* receptor.

6. Functional polymorphism in human pigmentation genes

Many alleles responsible for OCA1 albinism have been identified in the *TYR* locus, but ethnic differences in the tyrosinase protein are rare and the simplistic expectation that *TYR* polymorphism would be a principle component underlying normal variation of human pigmentation is unfounded. Results obtained from melanocytes cultured from Black and White skin do correlate melanin content with in situ tyrosinase activity, there being up to 10-fold higher catalytic activity in darker skin types (Iozumi et al., 1993), but despite this large difference in tyrosinase activity there is no difference in the amount of enzyme (Fuller et al., 2001). Activation of the tyrosinase enzyme within the melanosomal organelle must somehow explain these differences and in support of this hypothesis there are similar levels of activity once the enzyme is isolated from the melanosomal compartment implicating pH as a potential control mechanism (Ancans et al., 2001a,b).

The influence of TYRP1 and TYRP2 proteins on the stability of tyrosinase (Jimenez-Cervantes et al., 1998; Kobayashi et al., 1998) also present these loci as candidates to play a major role in normal variation in human pigmentation. Genetic variation within each locus has been screened in a collection of Australian Caucasian individuals selected

for different hair colours without any report of non-synonymous polymorphism (Box et al., 1998). Of the other genes identified as important in eumelanin versus pheomelanin synthesis, the *SILV* and *P*-genes remain as potential candidates for some of the common pigmentary differences in human populations. The *SILV* loci has not yet been screened for polymorphism or associated with any form of OCA. Although a number of polymorphisms have been reported in the *P*-gene that are apparently variable in frequency in some human populations (Lee et al., 1994), they are yet to be screened for association with common human pigmentation differences. The collective absence or low level of polymorphism within the *TYRP* gene family argues that differences in common patterns of melanisation are not produced by differences in the encoded catalytic activity of these enzymes, although different TYRP protein levels or enzymatic activities are integral to the pigmentary differences in human populations. It is the control of these proteins in the melanosome that acts as the chief determinant of pigmentation phenotype and it is this regulation that must be understood.

The *MC1R* locus has provided one of the most obvious examples of the power of comparative genomics for identifying candidate genes underlying human complex genetic traits such as pigmentation. Cloning and identification of the mouse *extension* locus as the *Mc1r* gene was followed soon after by the report that human *MC1R* genetic variation was associated with red hair colour (Valverde et al., 1995). Since this discovery, a number of studies have shown the *MC1R* gene to be highly polymorphic, with over 30 variant alleles so far reported in Caucasian populations from the British Isles, Holland and Australia (Valverde et al., 1995; Box et al., 1997; Smith et al., 1998; Flanagan et al., 2000; Palmer et al., 2000; Bastiaens et al., 2001; Box et al., 2001a,b) and these have been compiled in Table 2. Two additional studies have also examined the frequency of *MC1R* variants in Asian and African populations, which have shown little *MC1R* coding region variability in the African population (Rana et al., 1999; Harding et al., 2000).

Nine common amino acid missense changes with *MC1R* allele frequencies greater than 1% are shared in Caucasian populations. In each case allele frequencies were similar in the study populations, except for the Asp294His variant reported at 11% in the Scottish population examined by Valverde et al. (1995) which may be explained by ascertainment bias. In each study which has correlated genetic with phenotypic features, three of the common variants with amino acid substitutions Arg151Cys, Arg160Trp and Asp294His (RHC alleles) are strongly associated with red hair and fair skin.

In the largest study of human pigmentation to date, the *MC1R* genotype of 859 Caucasian individuals was determined and variant alleles correlated with pigmentation phenotype (Palmer et al., 2000). Over 60% of redheads carried two or three variants and none carried a wildtype *MC1R* genotype, all 71 redheads in this study carried at least one variant, and 45 carried two of the five variants examined. Furthermore, 66% of all study members carrying two RHC alleles were redheads, while only 8% of those carrying a single RHC allele were redheads. In this study Val60Leu and Asp84Glu were not associated with hair colour, though this is not entirely clear as Val60Leu has previously been reported at a higher frequency in those with fair/blonde or light brown hair colour (Box et al., 1997), and it may act as a partially penetrant recessive RHC allele (Flanagan et al., 2000) as may Asp84Glu (Flanagan et al., 2000; Bastiaens et al., 2001).

The associations between *MC1R* variants and skin colour in general reflect those seen for hair colour where the frequency of *MC1R* alleles increases as skin colours lighten. The common variant alleles Val92Met and Arg163Gln have failed to show association with any hair or skin colours (Box et al., 1997; Palmer et al., 2000; Box et al., 2001a), however the minor alleles Arg142His and Ile155Thr may also act as recessive RHC alleles when in combination with the three common RHC alleles (Flanagan et al., 2000; Bastiaens et al., 2001). In addition to the nine common *MC1R* variants present in Caucasian populations, numerous low frequency variants have been identified (Table 2).

The same three RHC alleles associated with pigmentation have also been associated with increased risk of melanoma and non-melanocytic skin cancers such as Basal Cell Carcinoma and Squamous Cell Carcinoma (Smith et al., 1998; Palmer et al., 2000; Bastiaens et al., 2001; Box et al., 2001a). This observation is perhaps not surprising given that pigmentary traits of fair skin, lack of tanning response and propensity to freckle have also been identified as risk factors for these forms of skin cancer. Nevertheless, these associations clearly point to the necessity for understanding the genetic component of multifactorial disease as an essential step in implementing targeted strategies for community disease management and prevention.

Although a clear model of MC1R signalling in eumelanin/pheomelanin switching is emerging in the mouse (Furumura et al., 1998; Furumura et al., 2001), few attempts have been made to assess the molecular basis and functional consequences of genetic variation at *MC1R* in humans, and an integrated understanding of the role of these variants in influencing melanocyte signal transduction, gene expression, pigmentation and proliferation is eagerly awaited. Culturing of human melanocytes of defined *MC1R* genotype may provide the best experimental approach to defining the functional consequences for pigmentation of each MC1R allele (Abdel-Malek et al., 2000). Expression of variant *MC1R* receptor alleles in heterologous cell lines have been used to examine the cAMP stimulating capacity in response to α-MSH (Frandberg et al., 1998; Schioth et al., 1999). These studies have suggested that the Val60Leu variant has a significantly reduced capacity to stimulate intracellular cAMP when compared to wild-type *MC1R*, and an ~2-fold lower affinity for α-MSH than the wild-type was demonstrated for the Val92Met variant. The

Table 2
Frequency of MC1R variant alleles in human populations

Variant[a]	Nucleotide change	Allele frequency in Caucasian population (%)					Allele frequency (%)		
		Australia[b]	N. Europe[c]	Scotland[d]	Ireland[e]	Holland[f]	Chinese[g]	African[h]	
Pro18Ala	52C > G					<0.5			
86insA		<0. 5	<0. 5			<0.5			
Val60Leu	178G > T	12.4	15		12.7	8.3			
Ala64Ser	190G > T			0. 7					
Arg67Gln	200G > A						1		
Phe76Tyr	227T > A			<0.5					
Asp84Glu	252C > A	1.1	1.5	0. 7	2	1			
Ala81Pro	241G > C					<0.5			
Val92Met	274G > A	9.7	8.7	7.4	7	7			
Thr95Met	284C > T		<0.5	< 0.5		<0.5			
Val97Ile	289G > A			< 0.5					
Ala103Val	308C > T			1.1					
Gly104Ser	310G > A					<0.5			
Leu106Gln	317T > A			< 0.5					
Leu106Leu	318G > A							1	
Arg142His	425G > A	0.9	0.6			0.8			
Arg151Cys	451C > T	11.1	9.9		1.2	4.8			
Arg151Arg	453C > G						<0.5		
Ile155Thr	464T > C	1	0.9		0.7	<0.5			
Arg160Trp	478C > T	7.1	8.7		8.5	10.5			
Arg163Gln	488G > A	5	4.8		2.8	5.2	70		
Val173del						<0.5			
Val174Ile	520G > A					<0.5			
537insC		<0.5	<0.5			<0.5			
Pro230Leu	689C > T					<0.5			
Pro230Pro	690G > A					<0.5			
Gln233Gln	699G > A					<0.5			
His260Pro	779A > C					0.5			
Ile264Ile	792C > T					<0.5			
Cys273Cys	819C > T							0. 9	
Lys278Glu	832A > G					<0.5			
Asn279Ser	836A > G					<0.5			
Asn279Lys	837C > A					<0.5			
Ile287Met	861C > G						1		
Asp294His	880G > C	2.8	3.6	11.1	3.4	0. 8			
Phe300Phe	900C > T							7	
Thr314Thr	942A > G		< 0.5				10	57	
Ser316Ser	948C > T								Other
Reference		1, 2, 3	4	5	6	7	1, 8	6, 8	6

(1) Box et al., 1997; (2) Sturm et al., 1998; (3) Box et al., 2001b; (4) Flanagan et al., 2000; (5) Valverde et al., 1995; (6) Harding et al., 2000; (7) Bastiaens et al., 2001; (8) Rana et al., 1999

[a] Individual variants presented without consideration of reported disequilibrium between some alleles.
[b] 1627 independent haplotypes derived from a collection of twins from Queensland schools (Box et al., 1997, 2001b); Sturm et al., 1998 first reports 537insC; T314T variant has been routinely observed but frequencies not reported.
[c] Haplotypes derived from 167 individuals from the British Isles and Northern Europe (Flanagan et al., 2000), also presented in part in Harding et al., 2000.
[d] Haplotype frequencies recalculated from the percentage of 135 individuals carrying variants taking into account homozygotes, presented in Valverde et al., 1995.
[e] Haplotype frequencies recalculated from the percentage of 71 or 102 (Asp84Glu and Asp294His) individuals carrying variants taking into account homozygotes, presented in Smith et al., 1998.
[f] Haplotype frequencies calculated from the percentage of 385 control individuals carrying taking into account homozygotes, presented in Bastiaens et al., 2001.
[g] Box et al., 1997 examined 10 Chinese individuals; Haplotype frequencies in Rana et al., 1999 include 60 individuals from East and Southeast Asia.
[h] Harding et al., 2000 calculated frequencies based on 106 African haplotypes; Rana et al., 1999 examined 25 African individuals.

Arg151Cys, Arg160Trp and Asp294His variants have also been shown to be totally unresponsive or severely impaired in their ability to stimulate cAMP production following α-MSH treatment. Although *MC1R* variants clearly impair cAMP signalling, these studies make little distinction between the RHC and non-RHC variants which is seen in

the genetic data now available. There is a definite need for further studies examining the cellular responses co-ordinated by this receptor and how they impact on skin colour, freckling, tanning, and melanocyte proliferation.

7. Conclusions and perspectives

Pigment gene identification in humans has proceeded largely through a comparative genomics approach based on analogous phenotypes seen in mouse coat colours (Jackson, 1997), combined with the study of severe hypopigmentation phenotypes characterising defective genes (Nordlund et al., 1998). Although there are numerous mutations affecting melanocyte and therefore pigmentary system function, the best candidates for the genetic variation that underlies the wide variety of pigmentation phenotypes in human populations are genes such as the *TYRP* and *SILV* gene family members and the *P*-gene that are restricted in expression to the melanocyte itself. Indeed, it is the characterisation of these genes as molecular components of the melanosomal organelle and its maturation which have provided a genetic foundation for present studies into the human pigmentary system. Unfortunately, not all genes directing human melanogenesis will be identified by either comparative genetic approaches or through examination of melanocyte cell defects. Fundamental differences are possible between the mouse and human pigmentary systems such as melanosomal pH stimulation of melanogenesis directed by the P-protein (Fuller et al., 2001; Ancans et al., 2001a,b), location of melanocytes in the mouse dermal follicles as opposed to the human epidermis, and the extrusion of melanosome particles to cutaneous keratinocytes as the basis for skin colour is not relevant for study in the mouse. There may well be keratinocyte-specific genes or pathways such as *PAR2* that control the fate of the melanosome once it has been formed within the melanocytes (Seiberg et al., 2000a,b).

The *MC1R* gene is the only pigmentation gene identified so far that plays a major role in human skin and hair colour phenotypes, to the extent of demonstrating almost Mendelian recessive inheritance in the case of red hair. Three RHC variant alleles of the receptor are strongly associated with red hair and fair skin when carried in the homozygous state. These alleles are at high frequency in the Caucasian population (Table 2), and it is notable that a recent study of skin sunsensitivity has also shown an association of *MC1R* genotype with degree of tanning after repeated sun exposure (Healy et al., 2000). Those individuals who carry a single *MC1R* variant allele were intermediate in their ability to tan when compared to those of wildtype genotype who tan well and do not sunburn, and those with two variant alleles with pale skin who do not tan and sunburn easily. This heterozygote effect indicates that dosage of *MC1R* variant alleles is important and that RHC alleles may not behave in a strictly recessive manner.

The evolutionary forces responsible for the spectrum of human skin tones are ambiguous, and probably reflect a relationship to climatic environment occupied by particular populations. Pigmentation as a well characterized phenotype provides a model system for connecting genetic variation to phenotypic variation in an evolutionary context, and by exploiting this knowledge it is possible to examine how genotypic and phenotypic processes have acted in modern human evolution. The influence of environment as a selective pressure for skin colour has led to the postulate that in Northern European populations there has been selection against dark skin in places where levels of ambient sunlight are low so as to prevent rickets when the diet is poor in vitamin D (Robins, 1991), whereas there is selection for dark skin in geographical areas of high UV-exposure to prevent sun-burn/skin cancer, photolysis of folate and protection of the sweat glands to ensure the integrity of somatic thermoregulation (Jablonski and Chaplin, 2000).

There have been conflicting molecular inferences made when using the *MC1R* variant coding region alleles with regard to the mechanism of selection operating at this locus during human evolution. In an early study the *MC1R* coding region was examined in 121 individuals sampled from several different geographical population groups, but with a focus on the Asian community (Rana et al., 1999). They reported changes in five non-synonymous sites and one synonymous site together with the consensus allele and concluded that as the pattern of *MC1R* diversity was lower in African populations than in those from Northern Europe that this was due to diversity selection. In contrast, a larger study (Harding et al., 2000) examining ten non-synonymous and six synonymous sites together with the wildtype allele in 776 samples from Africa, Asia and Europe, reported that the most common *MC1R* haplotype was the consensus allele, and that the root of all human *MC1R* diversity is a haplotype common in Africa and equatorial Asia but found at low frequency elsewhere. The low *MC1R* diversity in the African population was interpreted by this group as being due to the functional constraints operating to maintain cutaneous pigmentation, where any divergence from dark skin produced through *MC1R* gene mutation and corresponding loss of eumelanogenesis appears to be evolutionarily deleterious. However, comparison of the level of divergence between the chimp and human *MC1R* sequences together with selective ascertainment of functional mutations associated with red hair suggested that neutral selection has occurred in Europe, with a relaxation of the strong functional constraints that operate in Africa. There were several caveats to this conclusion, as nucleotide diversity at *MC1R* is several times higher than the average nucleotide diversity in human populations. High nucleotide diversity with common variation at non-synonymous sites suggests that *MC1R* variation is an adaptive response to selection for different alleles in different environments (Owens and King, 1999).

The characterisation of the *MC1R* locus and examination

of its polymorphic states in different human populations will provide insight into the selective forces operating in the evolution of modern humans. It is the first such human pigmentation gene to allow such a genotype-phenotype analysis and evolutionary study, however genes of equal importance may exist in the human genome, remain to be identified and examined. The availability of the total human genome sequence, combined with functional genomic analysis, will hopefully provide these tools in the near future.

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