

ORIGINAL INVESTIGATION

Lori A. Passmore · Barbara Kaesmann-Kellner  
Bernhard H. F. Weber

## Novel and recurrent mutations in the tyrosinase gene and the P gene in the German albino population

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**Abstract** Albinism is a heterogeneous group of genetic disorders resulting from deficiencies in pigmentation. Clinically, it is divided into ocular (OA) and oculocutaneous albinism (OCA). OCA involves lack of pigment in the skin, hair, and eyes and results from mutations in the tyrosinase gene or in the P gene. OA mainly affects pigmentation in the visual system and may be a mild form of OCA or may be caused by other genetic defects. Clinical diagnosis of albinism type is difficult, because of the observed range of phenotypic variation. Thus, genetic analysis may be helpful with respect to a more accurate diagnosis. Here, we report the mutational profile, determined by genetic analysis of the tyrosinase and P genes, of a large German albino population. We have revealed a total of 42 distinct mutations, 19 of which are novel. Of the 74 unrelated patients screened, 32 (43%) had mutations in the tyrosinase gene, 16 (22%) had P gene mutations, and 26 (35%) patients had no detectable genetic abnormalities. This defines a population of albino patients who are tyrosinase-gene- and P-gene-negative and who thus may represent a good study group for searching for additional genes associated with albinism.

### Introduction

Albinism is a rare autosomal recessive disorder that affects approximately 1 in 20,000 persons and results from

defects in melanin production. It is a heterogeneous disorder comprised of oculocutaneous albinism (OCA) with reduced pigmentation in the skin, hair, and eyes, and ocular albinism (OA) with pigment deficiencies mainly limited to the eye, although the skin is always involved to some extent. Visual pathways develop abnormally, resulting in the loss of binocular vision, strabismus, decreased visual acuity, nystagmus, and photophobia (for a review, see Spritz 1994).

The clinical phenotype resembling OCA1 (OMIM 203100) is caused by absent (OCA1A) or residual (OCA1B) catalytic activity of tyrosinase (monophenol monooxygenase; monophenol, L-dopa:oxygen oxidoreductase; EC 1.14.18.1) and is the most severe form of albinism. Tyrosinase is a 529-amino-acid copper-binding protein catalyzing at least three steps in the conversion of tyrosine to melanin (Lerner et al. 1949; Lerner and Fitzpatrick 1950; Tripathi et al. 1992a). The first 18 amino acids act as a hydrophobic leader peptide and are cleaved to form mature tyrosinase, whereas the amino acids at the C-terminus are involved in anchoring the protein to the membrane (Kwon et al. 1987). In addition, there are two proposed copper-binding domains. The tyrosinase gene has five exons spanning approximately 50 kb on chromosome 11q14-q21 (Giebel et al. 1991a). Approximately 90 mutations in the tyrosinase gene have been reported so far, with many of these mutations occurring either at the amino terminus between codons 21–89 or in the region of the proposed copper-binding site B (CuB) at codons 371–448 (Tripathi et al. 1992b; Oetting and King 1999; see also the Appendix for electronic database information: ADB and HGMD).

The clinical phenotype suggestive of OCA2 (OMIM 203200) is generally less severe than OCA1 with some affected patients accumulating pigment later in life. It is caused by mutations in the P gene, which codes for an 838-amino-acid integral membrane protein with 12 putative transmembrane domains (Rinchik et al. 1993). The function of the P protein is still unknown but it may be involved in the transport of tyrosine or some other small molecule into the melanosome (Rinchik et al. 1993). It

L. A. Passmore  
Department of Biochemistry and Molecular Biology,  
University of British Columbia, Vancouver, Canada V6T 1Z3

L. A. Passmore · B. H. F. Weber (✉)  
Institut für Humangenetik, Biozentrum, Universität Würzburg,  
Am Hubland, D-97074 Würzburg, Germany  
e-mail: bweb@biozentrum.uni-wuerzburg.de,  
Tel.: +49 931 888 4062, Fax: +49 931 888 4069

B. Kaesmann-Kellner  
Department of Ophthalmology, University of Saarland,  
Campus, 66421 Homburg (Saar), Germany

has significant homology to several prokaryotic transporters and is the human homolog of the mouse pink-eyed dilution gene (Lee et al. 1995). The human P gene contains 25 exons, the first of which is non-coding, spanning 250–600 kb on chromosome 15q11-q13 (Lee et al. 1995). Previous reports have indicated that fewer than 50% of Caucasians clinically diagnosed with tyrosinase-positive albinism actually have a mutation in the P gene, suggesting that a third locus may be a major contributor to OCA2 (Spritz 1994).

OA may be autosomal recessive (AROA) or X-linked (OAI). The gene for the X-linked form has been cloned (Bassi et al. 1995), and disease-causing mutations have been identified (Schiaffino et al. 1995; Schnur et al. 1998). AROA is probably clinically heterogeneous with at least some cases representing mild presentations of OCA with mutations in either the P gene or the tyrosinase gene (Lee et al. 1994; Fukai et al. 1995).

Although a biochemical assay for tyrosinase activity has been developed, the test is inaccurate and not commonly used (Spritz 1994). For this reason, the clinical classification of albinism relies on the observation of skin color, hair color, pigment accumulation, and ocular examination. It has been shown that approximately one third of all clinical diagnoses of OCA2 are indeed caused by mutations in the tyrosinase gene and thus should genetically be classified as OCA1 (Tripathi et al. 1992c). This emphasizes the importance of genetic analysis in the diagnosis of albinism.

In this paper, we report the spectrum of tyrosinase gene and P gene mutations in a population of 79 German albino patients, 74 of whom are unrelated. These results provide a mutational profile of the German albino population and facilitate a gene-based classification of albinism. Finally, our analyses suggest that there is at least one additional

locus responsible for the clinical manifestations of albinism in the Caucasian population.

## Subjects, materials and methods

### Patients

A total of 79 German patients, 74 of whom were unrelated, were included in the mutational analysis. All the patients have been seen by one of the authors (B. K.-K.), and the clinical classification was performed according to a scheme summarized in Table 1. In addition to our own experience, longitudinal follow-up studies have shown that, by itself, hair color at birth cannot be taken as a reliable indicator for the type of albinism, as many children with P gene mutations show white hair at birth and are indistinguishable from OCA1, whereas during infancy, hair color may change to yellowish white or blond. Therefore, the clinical evaluation was performed by using a combination of clinical characteristics and weighing up whether the manifestations were more suggestive of OCA1, OCA2, or AROA (Tables 2, 3, 4). Iris translucency as observed by slit lamp biomicroscopy under confocal light was described by using the following criteria: grade 1, punctate transillumination defects; grade 2, moderate amount of iris pigment and greater amount of iris transillumination; grade 3, minimal iris pigment mostly at the collarette and near to complete iris translucency; grade 4, full iris translucency. The appearance of the fundus was graded for hypopigmentation of retinal pigment epithelium and macular transparency: grade 1, moderate peripheral hypopigmentation, annular reflex detectable; grade 2, marked peripheral hypopigmentation, annular reflex incomplete; grade 3, peripheral and central hypopigmentation, macular hypoplasia; grade 4, peripheral and central marked hypopigmentation, choroidal vessels easily detectable in the macular region. Optic nerve pathology was graded as: grade 1, normal; grade 2, pale; grade 3, small and pale; grade 4, dysplastic.

### Polymerase chain reaction amplification

Genomic DNA was extracted from peripheral blood leukocytes by using standard techniques. Oligonucleotide primers flanking each

**Table 1** Criteria for clinical classification of patients with OCA

Clinical manifestation	Degree of phenotypic manifestation	Clinical phenotype
Hair color	Snow white, silvery or yellowish	OCA 1
	All darker aspects	OCA 2
Color of eye lashes	Resembling scalp hair in cases of snow-white silvery or yellowish hair	OCA 1
	Definitely darker than scalp hair, even in patients with white hair	OCA 2
Pigment accumulation of scalp hair	None or only discrete	OCA 1
	Moderate to distinct	OCA 2
Skin pigmentation and pigment accumulation	White, no or only minimal accumulation of pigment	OCA 1
	White with definite pigment accumulation, all darker types and tanning types	OCA 2
Iris translucency	Grade 4	OCA 1
	Grade 3 and lower	OCA 2
Macular and retinal hypopigmentation	Grade 4	OCA 1
	Grade 3	OCA 2
Optic nerve pathology	Grade 3 and 4	OCA 1
	Grade 2 and lower, grade 3 in cases of darker skin/hair complexion	OCA 2

**Table 2** Mutations of the tyrosinase gene and clinical phenotype

Patient ID <sup>a</sup>	Hair color (pigment accumulation <sup>b</sup> )	Skin color (pigment accumulation <sup>b</sup> )	Iris color (translucency <sup>c</sup> )	Fundus <sup>c</sup>	Optic nerve	Clinical type	Tyrosinase mutations (nucleotide change)	Reference
1	Snow white (-)	White (-)	Pale blue (4)	4	Dysplastic	OCA1	C289R (T865C), G446S (G336A)	ADB, Tripathi et al. 1992b
9	Silvery white	White (-)	Deep blue			OCA1	T373K (C1118A) <sup>d</sup>	Spritz et al. 1990
18	Yellowish white (-)	White (+)	Pale blue (4)	4	Small, pale	OCA1	A355P (G1063C), T373K (C1118A), P406L (C1217T)	Spritz et al. 1997a, Spritz et al. 1990, Giebel et al. 1991c
21	White-blond (+)	White (+)	Deep blue (3)	3	Pale	OCA2	G446S (G1336A) <sup>d</sup>	Tripathi et al. 1992b
22	Silvery-white	White (+)	Pale blue	4		OCA1	C36Y (G107A) <sup>d</sup>	Novel
27	Snow white (-)	White (-)	Blue			OCA1	Frameshift (731delGT), IVS2-7T→A	Oetting et al. 1991, Gershoni-Baruch et al. 1994
32*	Snow white (-)	White (+)	Pale blue (4)	4	Small, pale	OCA1	W272 C (G817 C), R422Q (G1265 A)	Novel, Giebel et al. 1991b
33*	Snow white (-)	White (-)	Blue (4)	4	Pale	OCA1	W272C (G817C), R422Q (G1265A)	Novel, Giebel et al. 1991b
34	Snow white (-)	White (-)	Pale blue (4)	4	Small, pale	OCA1	W236S (G707C), F439V (T1315G)	Novel, novel
43	Pale blond (+)	White (-)	Blue (4)	4	Small, pale	OCA1	IVS2-7T→A <sup>d</sup>	Gershoni-Baruch et al. 1994
45	Silvery white (-)	White (-)	Pale blue (4)	4	Small, pale	OCA1	C289R (T865C), D448N (G1342A)	ADB, Tripathi et al. 1992b
47	Silvery white (-)	White (-)	Pale blue			OCA1	Homozygous G419R (G1255A)	King et al. 1991
49	White-blond (-)	Pale, tans (+)	Deep blue (3)	4	Small, pale	OCA2	R77Q (G230A), IVS2-7T→A	Kikuchi et al. 1990, Gershoni-Baruch et al. 1994
51°	Yellowish white (+)	White (+)	Gray-blue			OCA2	Frameshift (338delCA), IVS2-7T→A	Novel, Gershoni-Baruch et al. 1994
52°	Pale blond (+)	White (+)	Pale blue			OCA2	Frameshift (338delCA), IVS2-7T→A	Novel, Gershoni-Baruch et al. 1994
65	White-blond (-)	White (+)	Blue			OCA2	Frameshift (338delCA) <sup>d</sup>	Novel
67	Snow white (-)	White (-)	Pale blue (4)	4	Small, pale	OCA1	G97R (G289C), T373K (C1118A)	ADB, Spritz et al. 1990
69	Snow white (-)	White (-)	Pale blue (4)	4		OCA1	Homozygous T373K (C1118A)	Spritz et al. 1990
72	Yellowish white (+)	White (-)	Pale blue (4)	4	Small, pale	OCA1	P81L (C242T), G446S (G1336A)	Giebel et al. 1990, Tripathi et al. 1992b
73	Snow white (-)	White (-)	Pale blue (4)	4		OCA1	Homozygous frameshift (344delGA)	ADB
75	White-blond (-)	White (-)	Pale blue			OCA2	R217W (C649T), frameshift (649delC)	Tripathi et al. 1992b, Gershoni-Baruch et al. 1994
80	Snow-white (-)	White (-)	Pale blue			OCA1	Frameshift (459insT), IVS2-7T→A, T373K (C1118A)	Novel, Gershoni-Baruch et al. 1994, Spritz et al. 1990
81	Snow white (-)	White (-)	Pale blue			OCA1	A355P (G1063C), P406L (C1217T), T373K (C1118A)	Spritz et al. 1997a, Giebel et al. 1991c, Spritz et al. 1990
82	Yellowish white (-)	White (-)	Deep blue			OCA1	R217Q (G650A), E294K (G880A)	Oetting and King 1993, Gershoni-Baruch et al. 1994
90	Yellowish white (+)	White (-)	Blue			OCA1	Homozygous P406L (C1217T)	Giebel et al. 1991c

**Table 2** (continued)

Patient ID <sup>a</sup>	Hair color (pigment accumulation <sup>b</sup> )	Skin color (pigment accumulation <sup>b</sup> )	Iris color (translucency <sup>c</sup> )	Fundus <sup>c</sup>	Optic nerve	Clinical type	Tyrosinase mutations (nucleotide change)	Reference
97	Medium brown (++)	White (-)	Deep blue			OCA2	Frameshift (1164delT) <sup>d</sup>	Tripathi et al. 1992b
98	White-blond (+)	White (+)	Deep blue			OCA2	R77W (C229T) <sup>d</sup>	Spritz et al. 1997a
99	Snow white (-)	White (-)	Pale blue (4)	4	Small, pale	OCA1	Homozygous frameshift (841delG)	Novel
101	Yellowish white (+)	Pale, tans (+)	Pale blue			OCA2	A355P (G1063C), P406L (C1217T), R403S (G1209T)	Spritz et al. 1997a, Giebel et al. 1991c, Tripathi et al. 1992b
104#	Snow-white (-)	White (-)	Pale blue pink (4)	4	Dysplastic	OCA1	R77Q (G230A), frameshift (731delGT)	Kikuchi et al. 1990, Oetting et al. 1991
105#	Snow-white (-)	White (-)	Pale blue pink (4)	4	Dysplastic	OCA1	R77Q (G230A), frameshift (731delGT)	Kikuchi et al. 1990, Oetting et al. 1991
109	Yellowish white (+)	White (+)	Deep blue			OCA2	Frameshift (731delGT), N371Y (A1111T)	Oetting et al. 1991, novel
113	Snow white (-)	White (-)	Pale blue (4)	4	Small, pale	OCA1	E294G (A881G), R403S (G1209T)	Novel, Tripathi et al. 1992b
114	Yellowish white (-)	White (-)	Pale blue			OCA1	Frameshift (344delGA), R403S (G1209T)	ADB, Tripathi et al. 1992b
119	Yellowish white (-)	White (+)	Blue			OCA1	Homozygous D448N (G1342A)	Tripathi et al. 1992b

<sup>a</sup>Siblings are denoted with \*, °, # symbols, respectively

<sup>b</sup>Amount of pigment accumulation is represented by: - none, + some, ++ yes, +++ significant

<sup>c</sup>The grades of iris translucency and hypopigmentation of the fundus are given in parentheses

<sup>d</sup>A second mutation was not identified

**Table 3** Mutations of the P gene and clinical phenotype

Patient ID <sup>a</sup>	Hair color (pigment accumulation <sup>b</sup> )	Skin color (pigment accumulation <sup>b</sup> )	Iris color (translucency <sup>c</sup> )	Fundus <sup>c</sup>	Optic nerve	Clinical type	P gene mutations	Reference
3	Yellowish white (+)	White (+)	Blue			OCA2	V443I (G1327A) <sup>d</sup>	Lee et al. 1994
8	Yellowish white (-)	White (+)	Blue			OCA2	Homozygous del 2.7 kb	Durham-Pierre et al. 1994
11	Medium blond (+)	Pale, tans (+)	Deep blue (3)	3	Small, pale	OCA2	Frameshift (163delG), delV833 (2498delTGG)	Novel, novel
15	White-blond (+)	White (+)	Blue			OCA2	R720C (C2158T) <sup>d</sup>	Novel
20	Silvery white (-)	White (-)	(4)	4	Small, pale	OCA1	Homozygous R290G (A868G)	Novel
30	White-blond (+)	White (+)	Blue			OCA2	V443I (G1327A) <sup>d</sup>	Lee et al. 1994
41	Medium blond (++)	White (-)	Green-brown (3)	4		OCA2	V443I (G1327A), IVS16+2T→C	Lee et al. 1994, novel
57	White-blond (+)	White (-)	Blue			OCA2	Frameshift (2336delG) <sup>d</sup>	Novel
71	Yellowish white (-)	White (-)	Blue			OCA2	V443I (G1327A) <sup>d</sup>	Lee et al. 1994
77	Yellowish white(+)	White (-)	Deep blue			OCA1	K614E (G1840A), W679C (G2037C)	Novel, novel
85	Dark blond (++++)	White (+)	Pale blue with green			OCA2	V443I (G1327A) <sup>d</sup>	Lee et al. 1994
86	Medium blond (+)	Pale, tans (+)	Aniridia	4	Dysplastic	OCA2	R290G (A868G) <sup>d</sup>	Novel
89	Medium blond (-)	Pale, tans (+)	Deep blue (2)	3	Normal	OCA2	G795R (G2383C) <sup>d</sup>	Novel
91	Yellowish white (+)	White (-)	Deep blue			OCA2	Homozygous V443I (G1327A)	Lee et al. 1994
96	Yellowish white (-)	White (-)	Pale blue			OCA1	R290G (A868G) <sup>d</sup>	Novel
117*	Snow white (-)	White (-)	Deep blue (1)	3	Normal	OCA2	I617L (A1849T) <sup>d</sup>	Novel
118*	Snow white (-)	White (-)	Deep blue (1)	3	Normal	OCA2	I617L (A1849T) <sup>d</sup>	Novel

<sup>a</sup>Siblings are denoted with the \* symbol

<sup>b</sup>Amount of pigment accumulation is represented by: - none, + some, ++ yes, +++ significant

<sup>c</sup>The grades of iris translucency and hypopigmentation of the fundus are given in parentheses

<sup>d</sup>A second mutation was not identified

**Table 4** Clinical phenotype of individuals in which no mutations were identified (note: no history is available for patients 19, 100 and 107)

Patient ID <sup>a</sup>	Hair color (pigment accumulation <sup>b</sup> )	Skin color (pigment accumulation <sup>b</sup> )	Iris color (translucency <sup>c</sup> )	Fundus <sup>c</sup>	Optic nerve	Clinical type
2	Yellowish white (+)	White (+)	Pale blue			OCA2
10	Dark blond (++)	Pale, tans (++)	Deep blue			OCA2
14	Silvery white (-)	White (-)	Blue (4)			OCA1
17	Medium blond (+)	Pale, tans (+)	Deep blue (3)	3	Dysplastic	OCA2
28	White-blond (+)	White (+)	Pale blue			OCA2
29	Medium blond (+)	Pale, tans (+)	Deep blue (3)	3	Small, pale	OCA2
35*	Dark brown (++)	Normal (+)	Dark brown (3)	3	Small, pale	ARO
37*	Dark brown (++)	Normal (+)	Dark brown (2)	2	Normal	ARO
44	Medium blond (+)	Pale, tans (-)	Deep blue (3)	3	Pale	OCA2
46	Silvery white (-)	White (-)	Pale blue			OCA1
48	Pale brown (++)	Pale, tans (++)	Green-brown (3)	3	Pale	OCA2
50	Dark brown (++)	Normal (+)	Green-brown (3)	3	Pale	ARO
58	Yellowish white (+)	White (+)	Blue			OCA2
59	Dark blond (++)	White (+)	Blue			OCA2
68	Medium blond (+)	Pale, tans (+)	Green-brown (2)	3	Pale	OCA2
70	Dark blond (+)	Pale, tans (+)	Deep blue (2)	3	Normal	OCA2
74	Yellowish white (+)	White (+)	Blue			OCA2
76	Medium blond (+)	Pale, tans (+)	Deep blue (3)	3	Pale	OCA2
78	Dark brown (+)	Normal (+)	Green-brown (3)	3	Pale	ARO
87	Yellowish white (+)	White (-)	Blue (4)	4	Small, pale	OCA2
102	Dark brown (-)	Pale, tans (+)	Blue (2)	3	Normal	ARO
103	Yellowish white (+)	White (+)	Pale blue (4)	4	Dysplastic	OCA1
110	Dark blond (+)	Pale, tans (++)	Blue (2)	3	Pale	OCA2
112	Silvery white	White				OCA2

<sup>a</sup>Siblings are denoted with the \* symbol<sup>b</sup>Amount of pigment accumulation is represented by: - none, + some, ++ yes, +++ significant<sup>c</sup>The grades of iris translucency and hypopigmentation of the fundus are given in parentheses**Table 5** PCR primers and conditions used for mutation analysis of the tyrosinase gene

Exon	Primer	Sequence (5'[[minus]]3')	Restriction enzyme	Fragment size (bp) <sup>a</sup>	TA <sup>b</sup>
1	Tyr1.1f	CCTTGTGAGGACTAGAGGAA	<i>MspI</i>	250+277	56°C
	Tyr1.1r	CGTTAAACATGGGTGTTGAT			
	Tyr1.2f	CAGCTCAGACTATGTCATC	<i>HinfI</i>	196+222	
	Tyr1.2r	CCTCCCTACTCTGACATCGT			
2	Tyr2f	TCCTACTGACTGGTGGTGAC	<i>DdeI</i>	196+140	55°C
	Tyr2r	GGACTTTGGATAAGAGACTG			
3	Tyr3f	AATCACATAGGTTTTTCAGTCA	-	256	55°C
	Tyr3r	TTTTAAATCCAATGAGCACGT			
4	Tyr4f	ATGTTTCTTAGTCTGAATAACC	-	257	55°C
	Tyr4r	ACTAGATTCAGCAATTCCTCT			
5	Tyr5f	ATCGTAACAATGGTGGTAAC	<i>DpnII</i>	106+242	55°C
	Tyr5r	CTGGGAACCTGGACATTACT			

<sup>a</sup>PCR products were digested with the indicated restriction enzymes to obtain appropriate fragment sizes for optimal sensitivity in SSCA<sup>b</sup>PCR conditions were 94°C for 5 min, 30 cycles at 94°C for 30 s, exon-specific annealing temperature (TA) for 30 s, 72°C for 30 s, and finally 72°C for 5 min

coding exon of the tyrosinase gene and the P gene were designed from published sequences (accession nos.: tyrosinase gene, M63235-M63239; P gene, U19152-U19175) and are shown in Tables 5 and 6. Tyrosinase exon 1 was amplified as a pair of overlapping fragments. Exon 1 of the P gene, which is noncoding, was not analyzed. The polymerase chain reaction (PCR) was performed in the presence of <sup>32</sup>P-dCTP (<3,000 Ci/mmol) by using *Taq*-polymerase (Gibco BRL) and 1×PCR buffer supplied by the manufacturer in a 25-μl reaction volume.

#### Mutational analysis and sequencing

Mutational analysis was carried out by using the technique of single-stranded conformational polymorphism (SSCP; Orita et al. 1989). PCR products of exon fragments were separated electrophoretically on a 6% nondenaturing polyacrylamide gel with 10% glycerol at 4°C. DNA fragments exhibiting mobility shifts were directly sequenced by using the PRISM Ready Reaction Sequencing Kit (Perkin Elmer-Cetus) and analyzed on an ABI 310 automated sequencer. For individuals in whom only one mutant tyrosinase allele was revealed by SSCP, the entire coding region of the tyrosinase gene was sequenced.

**Table 6** PCR primers and conditions used for mutation analysis of the *P* gene

Exon	Primer	Sequence (5'[[minus]]3')	Fragment size (bp)	TA <sup>a</sup>
2	Pex2f	AGTCTCAGTGTGCCTTTCAT	154+183 <sup>b</sup>	55°C
	Pex2r	TGAAGTCCACATTTACAAGAT		
3	Pex3f2	ATTATTTCTGTGTTGGTGATTC	200	52°C
	Pex3r2	TCTCAAGTTCCTCCAGCATACA		
4	Pex4f	AGCTTGCTTTGTAGCCATTA	273	58°C
	Pex4r	GGTGGGCACCCCAAGTC		
5	Pex5f	ACTGCTGCCCTTGCCACTA	140	61°C
	Pex5r	ACGGACCCAACAGTAGTGCT		
6	Pex6f	CTGTCATTCAACGTTTCTGA	129	55°C
	Pex6r	GGCCATCTCAGAGTGGATT		
7	Pex7f	GGACATGGGGTTTCTCCTGT	253	55°C
	Pex7r	GAGATTTACAAATTCCTTTCAA		
8	Pex8f	CAGATGGTGTCTCAGGTGAA	167	55°C
	Pex8r	CCCCAACACCTCACTCACT		
9	Pex9f	TCCCCTAACTGTTGACCTTG	227	55°C
	Pex9r	GGGGAGCAGGTGTGAAAGT		
10	Pex10f	GCACTGGAACGCGGTAATT	140	55°C
	Pex10r	TAAGCCAGGGATTGGGACT		
11	Pex11f	TTAGGCTCATCACTGGGA	149	56°C
	Pex11r	CACGGGGAGAGCTGTAAT		
12	Pex12f	TCGTTTTAATATGGTGGCCT	141	56°C
	Pex12r	ATTATTAAATGCAACATCATACC		
13	Pex13f	CCTGCTCACTGGCTTGTGA	192	56°C
	Pex13r	CACGGCAGAGGTGCTTTG		
14	Pex14f	TCACGATGTGTATAGTGGGA	238	56°C
	Pex14r	AAGTGGAGGTGTGCGTTTAC		
15	Pex15f	TGGGATTACAGGCGTGAGC	222	50°C
	Pex15r	TACATGAGGTTGCACTTGTACT		
16	Pex16f	GTCATGGGAGACCCAAGCT	228	60°C
	Pex16r	CTGCACACCAAGCACAGTCT		
17	Pex17f	CGTGACCAGGGAAGTAATGA	161	57°C
	Pex17r	CATCACTCACTCCCCTTCTTG		
18	Pex18f	CAGGAGTCAGAAGGTTGTGC	195	57°C
	Pex18r	CAAAGCCTATGAACCAAAGC		
19	Pex19f	GTTATGTATTTGCAGCCCCT	172	60°C
	Pex19r	ACAAAATCGTTACCCTCCA		
20	Pex20f	AAGAAATGAATCGGTGTGTTA	235	57°C
	Pex20r	AAAAATCAGGTAAAAATGCCA		
21	Pex21f	TATATTTTCGGTTCTAAACTGA	159	55°C
	Pex21r	AAAATCAAAGAACAGTGGCT		
22	Pex22f	GGTCTGACCCTAAGTGCATG	191	58°C
	Pex22r	AGCCCTCGACATGGACATG		
23	Pex23f	ATTGGTCACAGTATGGCAGC	231	60°C
	Pex23r	CTGTTGCTTTGGGCTGAA		
24	Pex24f	AAGATGAACTGGGATTTTGT	245	55°C
	Pex24r	TCTCTACTTGCTAAAAATATGC		
25	Pex25f	GAGCTTATCCAGATTTTCAGAT	214	55°C
	Pex25r	GGGGTCAGGGTAGTTTTATG		

<sup>a</sup>PCR conditions were 94°C for 5 min, 30 cycles at 94°C for 30 s, exon-specific annealing temperature (TA) for 30 s, 72°C for 30 s, and finally 72°C for 5 min

<sup>b</sup>Exon 2 was digested with enzyme *Hinf*I to obtain appropriate fragment sizes for SSCP

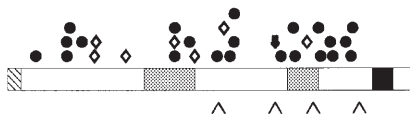
#### Restriction enzyme analysis

The following tyrosinase mutations were confirmed by restriction enzyme analysis: A355P creates a *Msp*I site; P81L, S192Y, R403S, and P406L eliminate *Hae*III, *Mbo*I, *Ban*I, and *Mn*I restriction sites, respectively. The respective exons were amplified by PCR, and the fragments were digested with the indicated enzymes. Digested PCR products were analyzed on 2% agarose gels.

#### Results

Mutational analyses of the tyrosinase gene and the P gene were performed by SSCP with oligonucleotide primers flanking the individual exons (Tables 5 and 6). All 79 patients were analyzed with respect to the tyrosinase gene. Only those patients with no mutations in the tyrosinase

## Tyrosinase



## P protein



**Fig. 1** Tyrosinase and P polypeptide structure and locations of mutations. *Box* The 529-amino-acid tyrosinase protein and the 838-amino-acid P polypeptide, *hatched region* the 18-amino-acid leader peptide, *shaded region* the proposed copper-binding domains (CuA, CuB), *black region* the transmembrane domains, *triangles below boxes* positions of intervening sequences, *dark bar* the 2.7-kb deletion including exon 7 of the P gene identified in patient 8. Mutations found within the German albino population are indicated by *circles* (missense mutations), *diamonds* (frameshift mutations), *double arrows* (splice site mutation) and a *black triangle* (amino acid deletion)

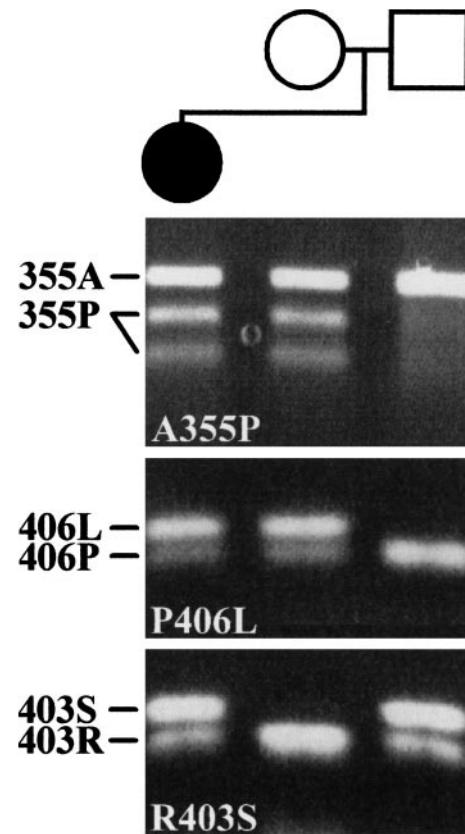
gene were further analyzed with respect to the P gene. PCR products that demonstrated aberrant band shifts were directly sequenced.

## Mutational analysis of the tyrosinase gene

Thirty distinct mutations were identified in the tyrosinase gene in 35 patients, of whom 32 were unrelated by genealogy. These included 22 missense mutations (C36Y, R77Q, R77W, P81L, G97R, R217Q, R217W, W236S, W272C, C289R, E294K, E294G, A355P, N371Y, T373K, R403S, P406L, G419R, R422Q, F439V, G446S, and D448N), one splice site mutation (IVS2-7T→A), and seven frameshift mutations (338delCA, 344delGA, 459insT, 649delC, 731delGT, 841delG, and 1164delT; Table 2, Fig. 1). Six of the missense and three of the frameshift mutations are novel (Table 2). In eight patients, SSCP revealed only one mutant allele. Therefore, the entire coding region of the tyrosinase gene was subsequently sequenced for these individuals. This revealed one additional mutation, P81L, in patient 82. As P81L eliminates an *Hae*III restriction enzyme site (Giebel et al. 1990), all patients were tested for this mutation by restriction enzyme digest. No further P81L mutations were found in our albino population.

Two tyrosinase mutations, P81L and T373K, have been reported to be common within Caucasian OCA1 patients, each with an allele frequency of approximately 0.10 (reviewed in Spritz 1994). We have found seven alleles containing the T373K mutation, resulting in an allele frequency of 0.05, whereas the P81L mutation was found only once (allele frequency of 0.007).

Patients 18, 80, 81, and 101 each had three different nucleotide alterations. Additional DNAs were available from the unaffected parents of patients 80, 81, and 101. Segregation patterns of the nucleotide changes were de-



**Fig. 2** Mutation analysis of the tyrosinase gene in family S (family of patient 101). Restriction enzyme digests showing segregation of the A355P, R403S, and P406L mutations demonstrating that the A355P and P406L mutations segregate on a single allele

**Table 7** Polymorphisms detected in the tyrosinase and the P gene and their frequencies in the German albino population

Nucleotide change	Amino acid change	Number of alleles (frequency)
Tyrosinase gene		
C575A	S192Y	43/114 (0.38)
G1205A	R402Q	38/146 (0.26)
P gene		
C913T	R305W	11/106 (0.10)
G1065A	None	83/106 (0.78)
IVS11-4A→G	None	89/106 (0.84)
G1256A	R419Q	8/106 (0.08)
IVS13-15T→C	None	30/106 (0.28)
C1551T	None	31/106 (0.29)
T2328C	None	37/106 (0.35)
G2364A	None	20/106 (0.19)

termined by restriction enzyme digests or direct sequencing (Fig. 2, plus data not shown). Patient 18 was heterozygous for A355P, T373K, and P406L. Patient 80 was heterozygous for a frameshift (459insT), a splice site mutation (IVS2-7T→A), and T373K. The frameshift (459insT) and splice site mutation (IVS2-7T→A) were both inherited on the maternal allele, whereas T373K was inherited

on the paternal allele (data not shown). Patient 81 was heterozygous for A355P, T373K, and P406L, with A355P and P406L being inherited on the maternal allele (data not shown). Patient 101 was heterozygous for A355P, R403S, and P406L, with A355P and P406L also being maternally inherited (Fig. 2).

The two nonpathological polymorphisms, S192Y and R402Q, were determined for 57 and 73 patients, respectively (Table 7). S192Y was determined by restriction enzyme digest, and the allele frequency in our albino population was 0.38 (43/114 alleles). R402Q was determined by a combination of sequencing and examination of SSCP mobility shifts. The allele frequency of the R402Q polymorphism was 0.26 (38/146 alleles). These findings are consistent with previously reported allele frequencies of 0.48 for S192Y and 0.15 for R402Q (Spritz 1994).

### Mutational analysis of the P gene

SSCP and sequencing of the P gene revealed 12 distinct mutations in 17 patients, 16 of whom were unrelated. These included seven missense substitutions (R290G, V443I, K614E, I617L, W679C, R720C, and G795R), one splice site mutation (IVS16+2T→C), two frameshifts (163delG and 2336delG), and one amino acid deletion (delV833; Table 3, Fig. 1). For patient 8, exon 7 failed to amplify, and subsequent *Eco*R1 digestion and Southern blot analysis revealed homozygosity for a 2.7-kb deletion including exon 7. This deletion is common in the African albino population (Durham-Pierre et al. 1994; Stevens et al. 1997), and a review of the family history of patient 8 confirmed his African ancestry. All mutations in the P gene were novel, except for the 2.7-kb deletion and the recurrent V443I missense substitution (Lee et al. 1994).

In 10 unrelated patients, only one mutant allele was identified. However, because of the size of the gene and the finding that the sequencing of the tyrosinase gene only revealed one additional alteration that was not detected by SSCP, the P gene was not further analyzed by direct sequencing.

SSCP revealed several polymorphisms in the exonic or the nearby intronic sequences in the P gene, and the frequencies were determined for 53 unrelated patients (Table 7). Two of the exonic polymorphisms led to amino acid changes (R305W, R419Q), whereas the remaining ones were silent (Table 7).

### Discussion

We have performed a mutational analysis on 79 German albino patients with regard to the tyrosinase gene and the P gene. Mutations were found in 48 of the 74 unrelated patients (65%), providing a detailed mutational profile for the German albino population. Thirty two unrelated patients (43%) had mutations within the tyrosinase gene, 16 (22%) had mutations in the P gene, and 26 (35%) had no detectable genetic defect in the two genes analyzed.

By correlating genotype and phenotype, we show that there is a significant overlap in the range of phenotypes between OCA1 and OCA2. In approximately 15% of the patients, the clinical phenotype does not appear to correlate with the genetic data. Indeed, revision of the clinical manifestations after knowing the results of the mutation analyses in the tyrosinase and the P gene confirmed the original clinical classification. Therefore, we conclude that OCA1 patients may have the so-called classical aspects of OCA2 and vice versa. This makes genetic analysis indispensable for determining the type of albinism in an individual patient.

Common tyrosinase mutations found in the German population include IVS2-7T→A (five alleles), T373K (seven alleles), and P406L (five alleles). Of the 30 distinct mutations in the tyrosinase gene, nine mutations (C36Y, W236S, W272C, E294G, N371Y, F439V, 338delCA, 459insT, 841delG) have not been found in any of the various ethnic populations analyzed so far (Oetting and King 1999) and thus may be specific to German albino patients. The frameshift mutations would be expected to produce null alleles, completely abolishing tyrosinase activity. Some of the frameshift mutations (338delCA, 344delGA, 731delGT) occur within dinucleotide repeats and may have arisen because of slipped mispairing (Cooper et al. 1995). The novel mutations C36Y, W236S, and W272C involve the substitution of tryptophane or cysteine residues. Since these residues are usually important in protein structure, the mutations would be expected to be deleterious to protein function. E294G occurs at the same position as E294K, a previously reported missense mutation (Gershoni-Baruch et al. 1994). N371Y occurs at the same position as N371T, a mutation previously reported by Oetting et al. (1993) in a patient with OCA1. F439V occurs in the mutation cluster corresponding to the CuB site where about 30% of the missense mutations have been reported (Tripathi et al. 1992b).

By segregation analysis, we have shown that both patients 81 and 101 inherited two missense substitutions, A355P and P406L, on a single allele. There have been no previous reports of such a complex allele, providing evidence for an ancestral founder in the German population. In addition, three missense substitutions, viz., A355P, T373K, and P406L, were found in patient 18. Unfortunately, no parent DNA was available for further analysis. Nevertheless, similar to the findings in patients 81 and 101, we can expect the A355P and P406L mutations to segregate on a single allele. It is interesting to note the differences in the phenotypes of the three patients. Whereas patients 18 and 81 have severe phenotypes, patient 101 was clinically diagnosed as having OCA2. These findings indicate that the double mutant allele and the T373K mutation have no residual tyrosinase activity. At present, it is unknown whether the two mutations of the double mutant allele produce the nonfunctional tyrosinase protein independently or as a consequence of a cis-acting effect. The phenotype of patient 90 who is homozygous for P406L and has a severe form of albinism suggests that P406L alone is sufficient to produce an inactive protein. This

conclusion is in contrast to a previous study that has associated the P406L allele with residual tyrosinase activity (Giebel et al. 1991c). Another discrepancy in the genotype/phenotype correlation becomes evident with the R403S mutation in patient 101; this mutation is associated with a milder form of albinism, OCA2, which should be the result of residual enzyme activity of the paternally inherited R403S allele. In a previous report, this mutation has been correlated with a severe albino phenotype (Tripathi et al. 1992b).

We report 12 distinct P gene mutations, two of which (V443I, del 2.7 kb) have been previously published (Lee et al. 1994; Durham-Pierre et al. 1994). Of these, the V443I missense mutation represents a significant proportion of the P gene mutations (seven alleles) in our German albino population. A previous study has found that most missense substitutions occur within the loops between the transmembrane domains (Spritz et al. 1997b). Of the seven missense substitutions that we have detected, two are within these loops, and five occur at or close to the interface between the transmembrane domains and the intra- or extracellular regions. The novel splice site mutation (IVS16+2T→C) is expected to eliminate splicing following exon 16, as the invariant GT dinucleotide of the donor splice consensus sequence is affected by the mutation. Alignments of mouse and human P polypeptide sequences with several prokaryotic transporter proteins show that several mutations occur at highly conserved residues (Lee et al. 1995). These include K614E, I617L, W679C, R720C, G795R, and V833D.

Out of 29 unrelated patients (79%) clinically diagnosed as having OCA1, 23 do indeed have mutations in the tyrosinase gene. Of the remaining six patients, three have mutations in the P gene. Interestingly, two of these patients have the same missense substitution, R290G. Patient 20 is homozygous for R290G; patient 96 is heterozygous for R290G and has no other detectable genetic abnormalities. This suggests that the R290G mutation, located in the extracellular region of the protein, severely impairs its function.

Of the 38 unrelated patients classified as having OCA2, nine (24%) have mutations in the tyrosinase gene. It is not uncommon for patients with mild forms of OCA1 to be clinically diagnosed as having OCA2 because of their overlapping phenotypes (Tripathi et al. 1992c). The tyrosinase missense mutations found in patients clinically diagnosed as having OCA2 probably result in decreased tyrosinase activity rather than a complete loss of function. Of the unrelated OCA2 patients, 16 were found to have mutations in the P gene. Results from conventional methods of mutational analysis suggest that mutations in the P gene are more difficult to locate than those in the tyrosinase gene. Consequently, in 10 of the 16 unrelated patients with mutations in the P gene, we have not identified the second mutation.

Previous studies have reported cases of AROA associated with P gene mutations and tyrosinase mutations (Lee et al. 1994; Fukai et al. 1995). Interestingly, in the case of tyrosinase gene mutations, it has been proposed that the

AROA disease phenotype results from compound heterozygosity for a pathologic mutation and the R402Q polymorphism (Fukai et al. 1995). In our study, we have been unable to find mutations in either the tyrosinase or P genes in the four unrelated patients with AROA, and the R402Q polymorphism was present in only one of these patients (data not shown).

There are several possible explanations for our inability to find genetic defects in 26 patients (35% of our unrelated patient population). First, they may have mutations in the promoter regions of either the tyrosinase gene or the P gene. Furthermore, the noncoding first exon of the P gene has not been analyzed but may nevertheless contain mutations (e.g., splice site mutations) with functional consequences. Second, they may have larger rearrangements in the tyrosinase gene or P gene, undetectable by our screening methods. Third, a frequent founder mutation may have escaped identification and thus may account for some or most of the 26 patients. Fourth, the patients may be affected by a type of OCA caused by mutations in other genes. In addition to the tyrosinase gene and the P gene, three other genes have thus far been associated with OCA, namely the tyrosinase-related protein-1 (TYRP1) gene (Boissy et al. 1996) and two genes associated with syndromic forms of OCA, Hermansky-Pudlak syndrome (HPS; Oh et al. 1996) and Chediak-Higashi syndrome (CHS; Nagle et al. 1996; Barbosa et al. 1996). However, since mutations in the TYRP1 gene have only been reported in the African albino population so far (Boissy et al. 1996; Manga et al. 1997), and since our patients show no syndromic manifestations reminiscent of HPS or CHS, it does not appear likely that a large fraction of the 35% of undetected mutations can be accounted for by alterations in these three genes. In addition, the lack of an indication of an X-linked inheritance of the defect in our patient sample and the finding that almost 50% of patients in whom we did not detect a mutation are female make the X-linked OA1 gene an unlikely candidate for explaining the large number of genetically undefined patients. Therefore, in agreement with previous notions (Spritz 1994), our results indicate that other, as yet unidentified, genes could be associated with albinism. This is also supported by the finding that our mutation-negative patients generally have much milder forms of albinism than those who have mutations in either the tyrosinase or the P genes. We conclude that our group of albino patients may be helpful in further investigating the genetic causes of albinism.

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## Electronic database information

- ADB, Albinism DataBase, <http://www.cbc.umn.edu/tad>
- HGMD, Human Gene Mutation Database Cardiff, <http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html>
- OMIM, Online Mendelian Inheritance in Man, Center for Medical Genetics, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), <http://www.ncbi.nlm.nih.gov/omim/>

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