The Region Coding for the Helix Termination Motif and the Adjacent Intron 6 of the Human Type I Hair Keratin Gene hHa2 Contains Three Natural, Closely Spaced Polymorphic Sites

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Mutations in distinct sites of epidermal keratins, in particular in the helix initiation and termination regions, cause human genodermatoses due to faulty intermediate filament formation. Extension of this observation to human hereditary hair and nail diseases includes population analyses of human hair keratin genes for natural sequence variations in the corresponding sites. Here we report on a large-scale genotyping of the short helix termination region (HTR) of the human type I cortical hair keratins hHa1, a3-I, and a3-II, and the cuticular hair keratin hHa2. We describe two polymorphic loci, P1 and P2, exclusively in the cuticular hHa2 gene, both creating dimorphic protein variants. P1 is due to a C→T mutation in a CpG element leading to a threonine→methionine substitution; P2 concerns a serine codon AGT that also occurs as an asparagine coding variant AAC. A third polymorphism, P3, is linked with a C→T point mutation located at the very beginning of intron 6. The three polymorphic sites are clustered in a 39-nucleotide sequence of the hHa2 gene. Both allelic frequency calculations in individuals of different races and pedigree studies indicate that the two-allelic hHa2 variants resulting from P1 and P2 occur ubiquitously in a ratio of about 1:1 (P1) and 2:1 (P2) respectively in our survey, and are clearly inherited as Mendelian traits. A genotype carrying both mutations simultaneously on one allele could not be detected in our sampling, and there was no association of a distinct allelic hHa2 variant with the known ethnic form variations of hairs. Sequence comparisons of the HTR of hHa2 with those of other type I hair keratins including the hHa2-ortholog from chimpanzee provide evidence that the P1- and P2-linked mutations must have occurred very early in human evolution and that the two P2-associated codon variants may be the result of two independent point mutations in an ancestral AGC serine codon. These data describe natural polymorphisms in the HTR of a member of the keratin multigene family.
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Intermediate filaments (IF) are polymeric aggregates of different but related proteins that exhibit cell type–specific expression and share a common secondary structure. Each of the proteins possesses non–α-helical head and tail portions that vary in size and sequence, and a central α-helical domain highly conserved in length and sequence. The first step in IF assembly is the formation of a coiled coil rod-like molecule composed of two compatible chains aligned in parallel and exact axial register. For keratin IF, this initial molecule represents a heterodimer obligatorily consisting of a basic to neutral type II keratin and an acidic type I keratin (Steinert *et al*, 1994; Fuchs and Weber, 1994; Parry and Steinert, 1995). Molecular mutagenesis studies and keratin gene transfections aimed at identifying the sequences important for IF formation have shown that the most critical regions are the short, highly conserved helix initiation and termination sequences where even subtle point mutations led to faulty IF assembly *in vitro* (Bader *et al*, 1991; Coulombe *et al*, 1990; Hatzfeld and Weber, 1991; Letai *et al*, 1992; Steinert *et al*, 1992; Wilson *et al*, 1992). Moreover, transgenic mice expressing mutated human epidermal keratin genes exhibited a disturbed keratin network either in basal or in suprabasal cells along with tissue abnormalities resembling the autosomal dominant human skin diseases Epidermolysis bullosa simplex or epidermolytic hyperkeratosis both typified by skin blistering in basal (epidermolysis bullosa simplex) or differentiating (epidermolytic hyperkeratosis) cells (Vassar *et al*, 1991; Fuchs *et al*, 1992). The link between these *in vitro* and *in vivo* observations was then established by sequence analyses of the genes for the basal epidermal keratins K5 and K14 and the suprabasal keratins K1 and K10 in epidermolysis bullosa simplex and epidermolytic hyperkeratosis patients, respectively, which led to the first genetic evidence that both diseases arise from point

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Abbreviation: IF; intermediate filament(s).

mutations in the corresponding keratin genes (Bonifas *et al*, 1991; Coulombe *et al*, 1991; Lane *et al*, 1992; Chipev *et al*, 1992; Rothnagel *et al*, 1992).

The multitude of cases investigated since then indicates that these disease-causing mutations are not random along the keratin sequence but clustered around certain foci, among which the helix initiation and termination regions are the most frequently affected ones (Fuchs and Weber, 1994; Lane, 1994; Parry and Steinert, 1995; Rothnagel and Roop, 1995). Recently, three further epidermal fragility syndromes, epidermolytic palmoplantar keratoderma, ichthyosis bullosa of Siemens, and pachyonychia congenita, were related to the faulty expression of the genes of keratins K9, K2e, K16, K17, and K6, respectively. Again, in all cases the critical mutations were located either at the very beginning or the end of the α-helix (Bonifas *et al*, 1994; Kremer *et al*, 1994; McLean *et al*, 1994; Reis *et al*, 1994; Bowden *et al*, 1995; McLean *et al*, 1995; Navsaria *et al*, 1995; Rothnagel *et al*, 1995).

Given the large number of keratin genes, it would be amazing if beyond the documented epidermal keratin mutations, keratin filament diseases were not found in other epithelia including those expressing the so-called hard α-keratins or hair keratins (i.e., the subset of the keratin multigene family involved in the formation of hard keratinized structures such as wool, hairs, and nails). At present, eight major hair keratins, four type I and four type II, are known and are designated Hal-4 (type I) and Hbl-4 (type II) (Heid *et al*, 1986; Lynch *et al*, 1986). Both molecular properties and patterns of expression of hair keratins have been best studied in sheep and mouse (for summary, see Winter *et al*, 1994). In the latter, it was recently shown that the expression of the four type I keratins in the hair follicle is compartmentalized. Three structurally related keratins, mHa1, a3, and a4, are synthesized in the cortex of the hair shaft, whereas the forth, the structurally distinct member, mHa2, only occurs in cells of the peripheral hair cuticle (Winter *et al*, 1994).

Considering the large number of hereditary diseases that affect hair and nails in humans (Dawber *et al*, 1992a; Dawber *et al*, 1992b), we have decided to explore whether these diseases are causally related to the expression of mutated hair keratins. To this purpose we have isolated cDNA clones for the human type II hair keratin hHb1 (Rogers *et al*, 1995a), the human type I hair keratins hHa1 (Fink *et al*, 1995), hHa2 (Rogers *et al*, 1995a), and two isoforms of hHa3 (Rogers *et al*, 1995b; Yu *et al*, 1993), and have localized type II and type I hair keratin genes on chromosome 12q13 and chromosome 17q12–21, respectively (Rogers *et al*, 1995a). To appreciate reliably mutations in hair keratin genes as a cause of hereditary diseases, however, the normal sequence variations of these genes in the human population must be known. As a first approach, we have therefore subjected the helix termination region of the four known human type I hair keratins to a large-scale genotyping. We show that this region is sequentially inconspicuous for the cortex keratins hHa1, hHa3-I, and hHa3-II. In contrast, the cuticular keratin gene hHa2 contains two natural closely spaced polymorphisms in this short α-helical sequence segment, which are followed by a third polymorphic site immediately at the beginning of the adjacent intron 6 of the gene.

MATERIALS AND METHODS

Subjects Fifty-seven unrelated individuals of different races were recruited either at the German Cancer Research Center, Heidelberg, or at the Department of Experimental Dermatology, London Hospital Medical College, London. After informed consent, blood was drawn for direct DNA extraction.

Pedigree studies involved three 3- to 4-generation families from different parts of Germany. These families were referred to the Institute of Human Genetics, Heidelberg, for the evaluation of a Duchenne Muscular Dystrophy carrier risk. In addition, blood DNA of nine unrelated chimpanzees of the species Pan troglodytes and Pan paniscus was kindly provided by Dr. W. Schempp, Institute of Human Genetics, University of Freiburg, Germany.

DNA Extraction Genomic DNA was prepared from individual blood samples using either the Qiagen Blood and Cell Culture Kit or the conventional high-salt method (Millar *et al*, 1988).

Polymerase Chain Reaction The following polymerase chain reaction (PCR) primers were used for the amplification of the helix terminating sequence of the human hair keratins hHa1, hHa2, hHa3-I, and hHa3-II. hHa1 (Fink *et al*, 1995): forward primer, 5′-TCA CCA ACG TGG AGT CCC AG-3′ (nucleotide positions 1,006–1,025); reverse primer, 5′-TGC ATC CTT GCT CCT CTG GCA-3′ (nucleotide positions 1,316–1,336); length of the fragment, 1.0 kb. hHa3-I and hHa3-II (Rogers *et al*, 1995b): forward primer, 5′-CGG GTG GAG TGT GAG ATC AA-3′ (nucleotide positions in hHa3-II 917–936); reverse primer, 5′-GTA CCC AAA GGT GTT GCA AGG-3′ (nucleotide positions in hHa3-II 1067–1087). For the amplification of the two hHa3 isoforms, the same primers were used which led to a 0.38-kb fragment (hHa3-I) and a 1.0-kb fragment (hHA3-II), respectively. hHa2 (Rogers *et al*, 1995a): a 2,926-bp DNA fragment of the hHa2 gene was amplified using the following 20 *mer* oligonucleotide primers: forward primer, 5′-CAGATGCAGTGCATGATCAC-3′; reverse primer, 5′-GCCACTGAATACCAGGCTGC-3′ (for positioning of the primers in the hHa2 gene, see also **Fig 1*A*** below).

Two hundred nanograms of genomic DNA was used in 50-μl PCR amplification reactions containing 5 μl 10 × PCR-buffer (500 mM Tris-HCl, pH 9.2, 140 mM $(NH_4)_2SO_4$, 17.5 mM $MgCl_2$), 7 μl dNTPs (10 mM each), 50 pmol of each primer, and 0.75 μl enzyme mix containing thermostable Taq and PwO DNA polymerases (Expand Long Template PCR System, Boehringer, Mannheim, Germany). Samples were processed in a DNA Thermal Cycler (Prem, LEP Scientific, Manchester, UK). After an initial denaturation for 2 min at 93°C, 25 temperature cycles were carried out consisting of 10 s at 93°C, 30 s at 63°C, 3 min at 68°C. For each of the last 15 cycles the elongation time was prolonged for 20 s. Amplification products were separated on and excised from 1.5% LMP agarose gels and purified with Gelase (Biozym Hess. Oldendorf, Germany) followed by ethanol-precipitation.

Direct Sequencing of PCR Products In most instances, purified PCR products from human and chimpanzee DNA were directly sequenced using the Sequenase-PCR Sequencing Kit (USB, Cleveland, OH) according to the manufacturer's instructions. The amplification products were sequenced from both ends using the two PCR primers as sequencing primers.

Cloning and Sequencing of PCR Products Purified PCR products from heterozygous individuals were cloned into pMos-Blue-Vector (Amersham, Braunschweig, Germany). For the identification of clones containing different alleles, plasmid DNAs from multiple single colonies were prepared and sequenced by using the U19 and T7 primers in conjunction with the Pharmacia T7 Sequencing Kit.

RESULTS

Based on the previously published cDNA sequences of four human type I hair keratins, hHa1, hHa2, hHa3-I, and hHa3-II (Rogers *et al*, 1995a, 1995b; Fink *et al*, 1995), specific primers were used for the amplification of the gene region coding for the terminal part of the α-helix and the carboxyterminus of each individual keratin. Genomic DNA from a large number of randomly selected unrelated individuals was used as template for the PCR-based amplification of fragments, which were sequenced directly. This systematic screening revealed only rare, individual point mutations, which were randomly scattered throughout the amplified gene region of hHa1, hHa3-I, and hHa3-II. In no case did they involve amino acid substitutions (results not shown). In contrast, three closely spaced polymorphic sites were detected in the hHa2 gene.

Figure 1*A* shows the DNA and amino acid sequence of the terminal part of the previously published hHa2 cDNA-clone (Rogers *et al*, 1995a), which besides the 3′-noncoding region comprises the region coding for both the α-helical 2B subdomain (exon 6) and the carboxyterminus (exon 7). By using the two sequence-specific PCR primers indicated and human genomic DNA as template, a 2,926-bp fragment was amplified that contained the 2,584-bp-long intron 6, whose position is marked by an arrowhead in **Fig 1*A***.[1] Direct sequencing of the amplification products of DNA from 57 individuals from both ends about 50 bp into the respective intron 6 sequences showed virtually no sequence alterations in the region coding for the carboxy terminus, but revealed two closely spaced polymorphic sites P1 and P2 in the region encoding the helix termination motif **(Fig 1*A*)**. P1 concerned triplet 1,083–1,085 for

[1] The complete genomic sequence of hHa2 will be published elsewhere.

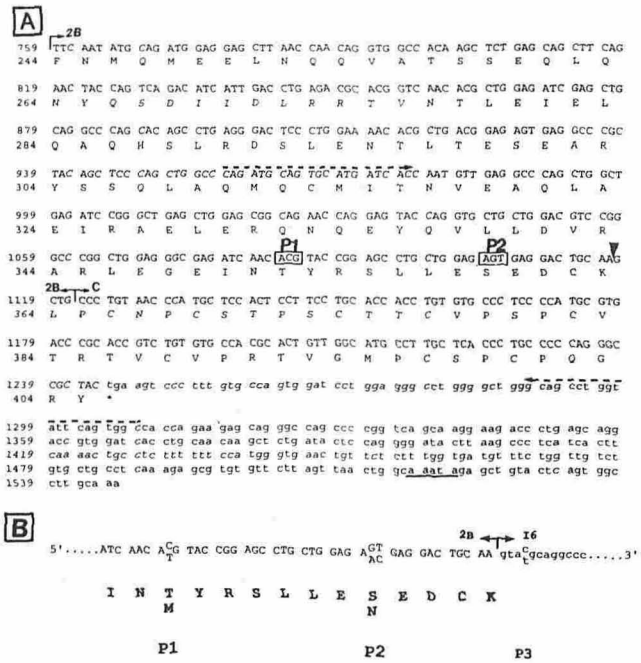

Figure 1. Nucleotide sequence and deduced amino acid sequence of the 3′-terminal part of the hHa2 keratin. *A*) ⌐, the boundary of the α-helical subdomain 2B and the carboxy terminus (C) of the hHa2 keratin protein, ▶, the position of intron 6; —, the polyadenylation signal; ---→, oligonucleotide sequences used as PCR and sequencing primers; □, the codon triplets involved in polymorphisms P1 and P2. *B*) Part of the sequence of *Panel A* showing the exon 6/intron 6 boundary (⌐) and the initial nucleotides of intron 6 (*lowercase letters*). The nucleotide and amino acid variants of the polymorphic sites P1, P2, P3 are indicated. The hHa2 nucleotide sequence is available from the EMBL Data Library, accession number X 81419.

which either a threonine codon ACG or a methionine codon ATG was observed **(Fig 1*B*)**. This alteration was accompanied by the loss of a *Mae*II site (recognition sequence ACGT) and the creation of a *Nsp*I site (recognition sequence PuCATGPy). P2 affected triplet 1,105–1,107 which occurred as a serine codon AGT or as an asparagine coding version AAC **(Fig 1*B*)**. Remarkably, on the nucleotide level, the mutation underlying this polymorphism appears as a dinucleotide substitution.

When analyzed separately, both P1 and P2 showed up as two-allelic polymorphisms; the allele frequencies calculated from 114 haplotypes are indicated in **Table I**. Since our survey comprised individuals of different races, the P1- and P2-associated allele frequencies were also listed according to this criterion. As can be seen from **Table I**, the distribution of the various alleles within both the total sampling and the ethnic subgroups did not exhibit significant differences and occurred at ratios approaching 1:1 for P1

Table I. Allele Frequencies of Polymorphism P1- and P2-Associated hHa2 Variants

		Allele Frequencies			
		P1		P2	
Sampling	Haplotypes	ACG	ATG	AGT	AAC
Total	114	0.56	0.44	0.65	0.35
Subgroups					
Caucasian	52	0.52	0.48	0.69	0.31
Negroid	20	0.70	0.30	0.50	0.50
Indian	20	0.55	0.45	0.60	0.40
Japanese	22	0.55	0.45	0.73	0.27

Table II. Frequency of Genotypes for the P1/P2 Variants

Genotypes (n = 57)		
P1 ACG-ATG	P2 AGT-AAC	*Frequency*
C/C	GT/GT	0.02
C/C	AC/GT[a]	0.19
C/C	AC/AC	0.12
T/T	GT/GT	0.23
T/T	AC/GT[a]	—
T/T	AC/AC	—
C/T	GT/GT	0.18
C/T	AC/GT[a]	0.26
C/T	AC/AC	—

[a] For identification of the alleles cloned PCR products were used.

and 2:1 for P2, except for the negroid subgroup in which these ratios seem to be reversed.

The frequencies of the genotypes resulting from the different possible combinations of the P1- and P2-associated allelic hHa2 variants are given in **Table II**. In this context, it is worth mentioning that the genotype exhibiting both the P1 variant ATG and the P2 variant AAC in one allele could not be detected in our survey. Thus, on the protein level this finding excluded the occurrence of a hHa2 keratin variant containing methionine 352 and asparagine 359 simultaneously in its helix termination motif.

To establish the mode of inheritance of the various hHa2 alleles, their occurrence was analyzed in three unrelated German three- to four-generation families. All pedigrees, one of which is shown representatively in **Fig 2**, revealed that the allele distribution within the families can be explained without exception by genetic transmission as an autosomal Mendelian trait.

In addition to polymorphisms P1 and P2, sequencing of each of the 57 amplification products revealed a third polymorphic site, P3, four bases downstream the exon 6–intron 6 boundary which showed up as a C→T point mutation **(Fig 1*B*)**. The frequency of the allele exhibiting a cytosine residue in the critical position was found to be 0.89, that of its thymidine-containing counterpart was 0.11.

DISCUSSION

In this study we have subjected the gene region spanning from exon 6 to the end of exon 7 of the four known human type I hair keratins, hHa1, hHa2, hHa3-I, and hHa3-II (Rogers *et al*, 1995 a,b; Fink *et al*, 1995), to a large-scale genotyping. This analysis resulted in the detection of three closely spaced polymorphic loci exclusively in the cuticular hHa2 gene. Polymorphisms P1 and P2 are located in the gene region that codes for the penultimate part of the α-helical rod domain of the hHa2 keratin. While P1 is due to a C→T point mutation, P2 is apparently linked to a 2-bp GT→AC alteration; however, both mutations cause conservative amino acid substitution. The third polymorphism is encountered in the noncoding region at the very beginning of intron 6 and, similar to P1 consists of a single base pair alteration. Remarkably, the three polymorphic sites are clustered within a gene region comprising only 39 nucleotides. In this context, it should be mentioned that ongoing analyses in our sampling do not indicate the occurrence of sequence variations in the region encoding the helix initiation motif of either the hHa2 gene or the genes of the human type I cortex keratins.

Mechanistically, polymorphisms P1 and P3 can be explained on the basis of the presence of a CpG dinucleotide in the respective sites of the hHa2 gene **(Fig 1*A,B*)**. It is well known that eukaryotic 5-methyl cytosine methylases can operate at CpG elements and, if a methylated cytosine is deaminated (Cooper and Youssoufian, 1988; Shen *et al*, 1992), this gives rise to the C→T transitions observed for P1 and P3. In the case of P1, this interpretation of the

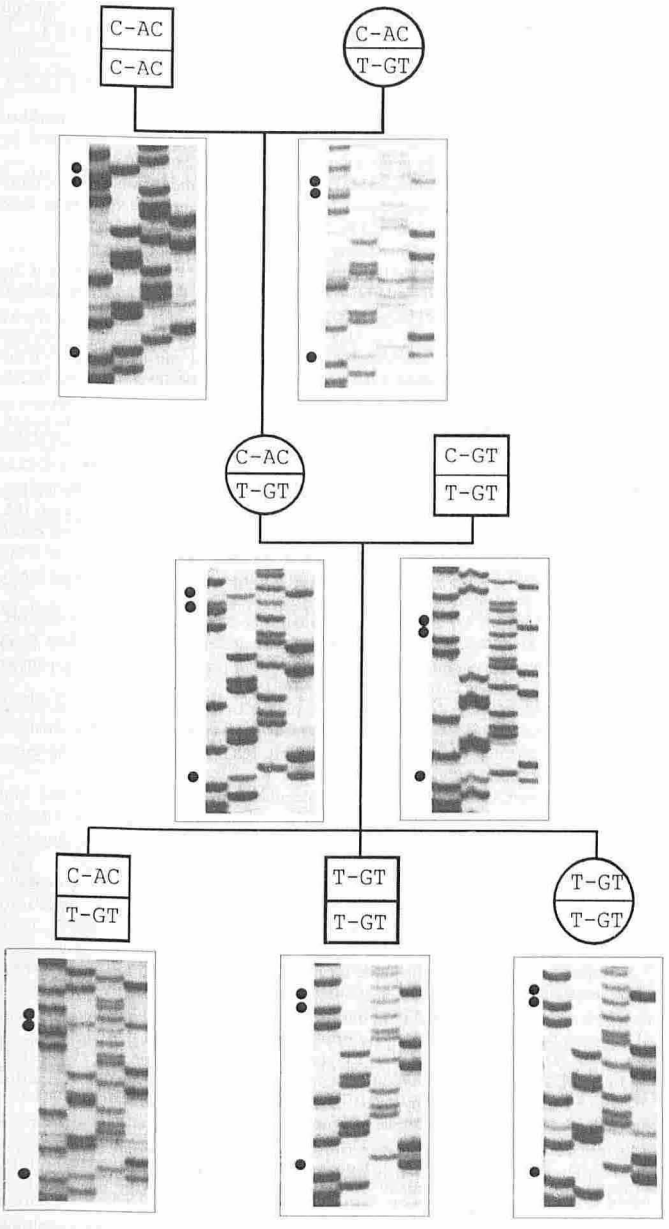

Figure 2. The various hHa2 alleles in a three-generation family are transmitted as a Mendelian trait. For each member of the family that part of the sequencing gel harboring the variable nucleotides of P1 and P2 is shown. The single base pair variants of P1 (ACG-ATG) are marked by the *lower dot*; the dinucleotide variants of P2 (AGT-AAC) are indicated by the two *upper dots*. The reading scheme of the sequencing gel is (from left to right): A, C, G, T. In the pedigree, genotypes are given in *circles* (females) and *squares* (males), respectively.

			P1							P2					
hHa2	ATC	AAC	ACG	TAC	CGG	AGC	CTG	CTG	GAG	AGT	GAG	GAC	TGC	AAG	CTG
hHa1	---	---	--A	---	---	---	---	---	---	--C	---	---	---	--T[a]	---
hHa3-I	---	---	---	---	---	---	---	---	---	--C	---	---	---	---	--C[b]
hHa3-II	---	---	--A	---	---	---	---	---	---	--C	---	---	---	---	---
chHa2	---	---	---	---	---	---	---	---	---	--C	---	---	---	---	---
Amino acid sequence	I	N	T	Y	R	S	L	L	E	S	E	D	C	K	L
Heptad position	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a

Figure 3. Sequence comparison of the region coding for the helix termination motif of hHa2. The hHa2 sequence was compared with the corresponding regions of human type I hair keratins hHa1, hHa3-I, and hHa3-II (Fink *et al*, 1995; Rogers *et al*, 1995a, 1995b) and with Ha2 of chimpanzees (chHa2). The positions of the individual amino acids within the α-helical heptad repeats are indicated below the amino acid sequence. □, the codon triplets of the polymorphic sites P1 and P2 of hHa2. [a]hHa1 triplet coding for asparagine (Fink *et al*, 1995); [b]nucleotide alteration in the hHa3-I triplet not leading to an amino acid change (Rogers *et al*, 1995b).

mutational event implies that the threonine codon ACG is the wild-type codon of the hHa2 gene. Indeed, the corresponding nucleotide positions within the region coding for the helix termination motif of the murine hHa2 gene are also occupied by an ACG threonine codon (Winter *et al*, 1994). Further confirmation was gained from the successful amplification of genomic DNA from chimpanzees by means of the PCR primers used for the amplification of the end region of the hHa2 gene. Not only were the amplified fragments the same size as their human counterparts (results not shown), but their sequence revealed a nearly 100% homology with that of the corresponding human fragments including the critical ACG threonine codon **(Fig 3)**. Moreover, the sequence homology extended into intron 6 and the chimpanzee gene also comprised the CpG element involved in polymorphism P3 in humans (results not shown). Despite analysis of genomic DNA of nine unrelated chimpanzees, however, neither site was subject to C→T transition in this primate species, thus indicating that the Ha2 gene polymorphism is specific to humans.

The mutational event leading to polymorphism P2 (i.e., the alternate occurrence of either a serine codon AGT or an asparagine codon AAC in the respective allelic variants of the hHa2 gene) is more difficult to deduce. Comparison of the helix termination peptide motifs of all known type I hair keratins from sheep (Dowling *et al*, 1986; Wilson *et al*, 1988), mouse (for summary, see Winter *et al*, 1994), and humans (Rogers *et al*, 1995 a, 1995b; Fink *et al*, 1995; Yu *et al*, 1993) clearly shows that the critical site is invariably occupied by a serine residue, so that the asparagine coding variant of the hHa2 gene should have originated from an allele containing a serine codon at the respective position. Conceptually, a GT→AC 2-bp substitution involving the AGT serine codon is highly improbable. Except for one reported case (Winnard *et al*, 1992), there is virtually no concrete evidence for direct 2-bp substitutions as mutational event (Cooper and Krawczak, 1993). On the other hand, as shown in **Fig 3**, unlike hHa2, the respective serine residues in hHa1, hHa3-I, and hHa3-II are encoded by the triplet AGC, which is invariably followed by a glutamic acid coding GAG triplet so that the last and first bases of the two codons form a C→T mutation sensitive CpG element. Remarkably, this AGC GAG codon constellation is also encountered in the Ha2 gene of the chimpanzee **(Fig 3)**. Based on these data, it is tempting to hypothesize that the AGT serine codon 359 in the human Ha2 gene may already constitute the result of an evolutionary early methylation-deamination–mediated C→T transition at the terminal cytosine residue of an ancestral serine codon AGC that has also served for the generation of the asparagine coding variant AAC by a central G→A point mutation.

Independent of the mode of generation of polymorphism P2, our data show that the mutational scenario to which the hHa2 gene has been subject did not lead to a haplotype containing the two mutated α-helical codons simultaneously. In other words, out of the four theoretically possible variants of the hHa2 keratin, only three are encountered in our survey, two of them exhibiting one amino acid substitution each. To our knowledge, this is the first report of natural polymorphisms in the helix termination region of a member of the keratin multigene family. A survey of all known IF proteins reveals that not only can the critical sites in the helix termination region of hHa2 be occupied by a variety of other neutral amino acids but they also accept negatively and positively charged amino acid residues (Hatzfeld and Weber, 1991; Sawada *et al*, 1995). This has been confirmed by competitive keratin IF disassembly studies with synthetic helix termination region peptides, in which each amino acid position along the consensus sequence was substituted

both conservatively and nonconservatively (Steinert *et al*, 1993). This tolerance in amino acid occupancy may explain why the conservative threonine-methionine and serine-asparagine substitutions in the helix termination region of hHa2 apparently occur without macroscopically recognizable phenotypic consequences. On the other hand, it cannot be overlooked that threonine 352 of hHa2 is positionally strictly conserved on soft and hard keratins of both types and that serine 359 of hHa2 is maintained in all known type I hair keratins. It can therefore not be excluded that the substitution of these hydroxy amino acids, which both occupy c-positions at the outer surface of the coiled coils (see **Fig 3**), entails subtle alterations in the filament properties of cuticular cells that may for instance explain the individually varying sensitivity of hairs to changes in ambient temperature and humidity.

Beyond the importance of the knowledge of specific hHa2 sequences variations among humans for the evaluation of potentially pathogenic mutations in this gene, the availability of a well-characterized polymorphic marker directly within the cluster of type I keratin genes on chromosome 17q12–21 is certainly of great utility for the improvement of further linkage analyses of suspected keratin alterations and a given epithelial disease phenotype. Moreover, besides its general significance for population genetics and evolutionary studies, the polymorphic hHa2 hair keratin will also be a helpful tool in forensic medicine.

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