Hum Genet (2001) 108 : 37–42
DOI 10.1007/s004390000439

ORIGINAL INVESTIGATION

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Human type I hair keratin pseudogene *φhHaA* has functional orthologs in the chimpanzee and gorilla: evidence for recent inactivation of the human gene after the *Pan-Homo* divergence

Received: 21 September 2000 / Accepted: 13 November 2000 / Published online: 15 December 2000

Abstract In addition to nine functional genes, the human type I hair keratin gene cluster contains a pseudogene, *φhHaA* (*KRTHAP1*), which is thought to have been inactivated by a single base-pair substitution that introduced a premature TGA termination codon into exon 4. Large-scale genotyping of human, chimpanzee, and gorilla DNAs revealed the homozygous presence of the *φhHaA* nonsense mutation in humans of different ethnic backgrounds, but its absence in the functional orthologous chimpanzee (*cHaA*) and gorilla (*gHaA*) genes. Expression analyses of the encoded cHaA and gHaA hair keratins served to highlight dramatic differences between the hair keratin phenotypes of contemporary humans and the great apes. The relative numbers of synonymous and non-synonymous substitutions in the *φhHaA* and *cHaA* genes, as inferred by using the *gHaA* gene as an outgroup, suggest that the human *hHaA* gene was inactivated only recently, viz., less than 240,000 years ago. This implies that the hair keratin phenotype of hominids prior to this date, and after the *Pan-Homo* divergence some 5.5 million years ago, could have been identical to that of the great apes. In addition, the homozygous presence of the *φhHaA* exon 4 nonsense mutation in some of the earliest branching lineages among extant human populations lends strong support to the "single African origin" hypothesis of modern humans.

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Introduction

The human hair keratin family, which comprises nine type I members, hHa1–hHa8 (including two hHa3 isoforms), and six type II members, hHb1–hHb6, is almost as complex as the large epithelial keratin family (Rogers et al. 1998, 2000). The genes encoding the members of the two hair keratin subfamilies are closely linked within the corresponding epithelial keratin gene clusters on chromosome 17q12-q21 (type I genes) and 12q13 (type II genes) respectively (Rogers et al. 1995, 1998, 2000). Both hair keratin gene clusters contain a number of pseudogenes that are interspersed between the functional genes (Rogers et al. 1998, 2000). Unusually, the type I pseudogene, *φhHaA* (*KRTHAP1*), which is part of a subcluster of three genes, *hHa7*, *hHa8*, *φhHaA*, being closely related in their coding domains but differing substantially in their flanking regions, has retained a completely normal exon-intron organization and is still transcribed (Rogers et al. 1998). However, all the differentially spliced *φhHaA* transcript variants identified in the human hair follicle contain an in-frame premature TGA stop codon in exon 4 and would therefore be predicted to encode rod-domain-truncated and, thus, functionally compromised hair keratins (Rogers et al. 1998, 2000).

In agreement with the notion that human and chimpanzee gene sequences are almost 99% identical (for a review, see Ruvolo 1997), human and chimpanzee hair keratin genes are highly conserved (Winter et al. 1996). Polymerase chain reaction (PCR) analysis of human and chimpanzee genomic DNA, with primers from any region of a given human hair keratin gene, consistently yield fragments of identical size and with only minor sequence differences, usually of the order of 1% (Winter et al. 1996).

We have investigated the chimpanzee and gorilla orthologs of the human type I pseudogene *φhHaA*. The two great apes have been found to lack the gene-inactivating mutation of *φhHaA*, and their orthologous genes therefore encode functional hair keratins cHaA and gHaA, respectively. Their expression is associated with dramatic differences in type I cortex keratin patterns between humans and the great apes.

Materials and methods

Genomic DNA and *φhHaA* genotyping

Human genomic DNA was isolated from peripheral blood lymphocytes of unrelated individuals of European (*n*=50), Chinese (*n*=10), and Afro-Caribbean origin (*n*=10) by using a DNA Isolation Kit for Mammalian Blood (Roche, Mannheim, Germany). Blood DNA samples from single members of eight sub-Saharan African populations (Biaka pygmy, Mbuti pygmy, Bamileke, Mandenka, Effik, Yoruba, Hausa, Ibo) were kindly provided by H. Kaessmann, Leipzig, Germany. Genomic DNA of six chimpanzees (*Pan troglodytes*, Biomedical Primate Research Centre, Rijswijk, The Netherlands) was prepared from plucked hair follicles by using a DNA Microextraction Kit (Stratagene Europe, Amsterdam, The Netherlands). Peripheral blood lymphocyte DNA from five additional chimpanzees, four bonobos (*Pan paniscus*), and seven gorillas (*Gorilla gorilla*) were kindly provided by W. Schempp, Freiburg, and C. Roos, Munich, Germany. Blood and hair collection was conducted according to the principles laid down in the Declaration of Helsinki.

An 818-bp *φhHaA* exon 4 fragment was generated by means of intron primers φa-in2*fo* (5'-CTACACAGGATAAGCCAACTCACA-3') and φa-in4*re (*5'-AGAGAAACATGGTCACCTACCTGC-3'). Amplifications were performed by using the Expand Long Template PCR System (Roche, Mannheim, Germany) under the following PCR conditions: 94°C for 2 min, then 30 cycles of 94°C for 10 s, 62°C for 30 s, and 72°C for 2 min. For each of the last 20 cycles, the elongation time was prolonged by 20 s. In most cases, the gel-purified PCR products (Agarose Gel DNA Extraction Kit, Roche, Mannheim, Germany) were sequenced directly by using a ^{33}P Chain Termination Cycle Sequencing Kit (Amersham, Braunschweig, Germany) and primer φa-in4*re* as the sequencing primer. Additionally, restriction endonuclease analysis was performed by *Mvn*I digestion and separation of the digestion products on 3% agarose gels stained in ethidium bromide.

Isolation of chimpanzee and gorilla hair follicle RNA and reverse transcription/PCR

RNA was extracted from 10–15 hair bulbs of freshly plucked chimpanzee or gorilla hairs by using the RNeasy System (Qiagen, Hilden Germany). The amount of cDNA generated with Supercript II Reverse Transcriptase (Gibco BRL, Karlsruhe, Germany) was sufficient to perform four to five PCR amplifications by means of the Expand Long Template PCR System, with primers φa-5'*fo* (5'-ACTGTGGGCAAAGCAGGATC-3') and φa-3'*re* (5'-CAGGAGGCAACAGAAGAGAG-3'), derived from the 5'- and 3'-non-coding regions of the *φhHaA* pseudogene. Reverse transcription/PCR (RT-PCR) conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 10 s, 60°C for 30 s, 68°C for 2 min, and 68°C for 10 min. The gel-purified PCR products were either subcloned into pCR-XL-TOPO (Invitrogen, Groningen, The Netherlands) and sequenced by using M13 and internal primers by means of an ABI 310 Sequence Analyzer, as described previously (Rogers et al. 1998, 2000), or sequenced directly with the corresponding PCR primers and internal primers as detailed above.

Extraction of hair keratins, one dimensional gel electrophoresis, and Western blots

Keratins were extracted from about 20–25 clipped human, chimpanzee, or gorilla hairs and separated by one-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). For Western blotting, gels were transferred to PDVF membranes (Immobilon-P, Millipore, Eschborn, Germany) by wet-blotting. The membranes were incubated with primary hair keratin antibodies and peroxidase-coupled secondary antibodies (see below), which were detected by chemoluminiscence (ECL, Amersham). Further details can be found in Langbein et al. (1999).

Antibodies and indirect immunofluorescence

Monoclonal mouse hHa1 antibody, LHTric-1, was used at a dilution of 1:2000 for Western blots and of 1:50 for indirect immunofluorescence (IIF; Langbein et al. 1999). The new polyclonal antibody against cHaA/gHaA was raised in guinea pigs by means of the synthetic peptide SDHCSSLLSGQVSE (see below), coupled to Keyhole limpet protein. Dilution was 1:10,000 for Western blots, and 1:2000 for IIF. The following secondary antibodies (all from Dianova, Hamburg, Germany) were used: peroxidase-coupled goat anti-mouse or anti-guinea pig IgG at a dilution of 1:10,000. For IIF, Cy3- or Cy2-coupled goat anti-mouse or anti-guinea pig IgG and IgM were used at a dilution of 1:50 to 1:200.

IIF was carried out on cryostat sections of samples taken from human scalp and from various body sites of a chimpanzee (which had died of natural causes at the Biomedical Primate Research Center, Rijswijk) as previously described (Langbein et al. 1999).

Accession numbers

The EMBL database accession numbers for the *gHaA*, *cHaA*, and *φhHaA* nucleotide sequences reported in this paper are AJ401055, AJ401054, and Y16795, respectively.

Mutation dating

In order to assess at what point in time after the *Pan-Homo* divergence the *hHaA* gene became inactivated, the 1293-bp DNA sequence comprising the *HaA* gene "coding" regions of human and chimpanzee were compared. Observed nucleotide differences were assigned to one or other of the two lineages, by using the *gHaA* sequence as an outgroup, and the resulting substitution numbers per lineage were modeled as Poisson variables with parameters λ_c (chimpanzee, synonymous), $3\lambda_c s$ (chimpanzee, non-synonymous), λ_h (human, synonymous) and $3\lambda_h \cdot [ts+1-t]$ (human, non-synonymous), respectively. Here, *s* denotes the relative fixation probability of non-synonymous vs synonymous mutations in both the human and chimpanzee lineage, and *t* is the proportion of time that has elapsed since the *Pan-Homo* divergence and since the inactivation of *hHaA*. Two models were employed: $\{\lambda_c=\lambda_h=\lambda\}$, which entails equal neutral substitution rates in the two lineages, and $\{\lambda_h=\lambda, \lambda_c=1.2\lambda\}$, which allows for a 20% higher neutral substitution rate in chimpanzee. For both models, maximum likelihood estimates of parameters λ, *s* and *t* were obtained by means of an in-house implementation of the Newton-Raphson method (Press et al. 1986).

Results

φhHaA genotyping

We genotyped *φhHaA* and its great ape orthologs in a representative number of unrelated humans from different eth-

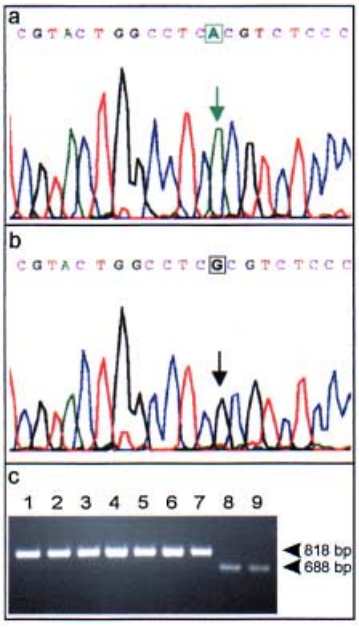


Fig. 1a–c DNA sequencing and restriction endonuclease analysis. DNA sequence excerpts of the PCR-amplified exon 4 of (**a**) the human *φhHaA* pseudogene and (**b**) the orthologous chimpanzee gene are shown in the antisense direction. *Arrows* Single nucleotide difference (T vs C on the sense strand) between two sequences. **c** *Mvn*I restriction endonuclease analysis of 818-bp PCR products encompassing exon 4 of *φhHaA*, *cHaA*, and *gHaA*. DNA samples were from individuals of seven different sub-Sahara African tribes (*lane 1* Biaka pygmy, *lane 2* Mbuti pygmy, *lane 3* Bamileke, *lane 4* Effik, *lane 5* Yoruba, *lane 6* Hausa, *lane 7* Ibo), and from chimpanzee (*lane 8*) and gorilla (*lane 9*). Fragment sizes were determined by using DNA standards. *Arrows* Undigested 818-bp fragment in humans and the restricted 688-bp fragment in chimpanzee and gorilla. A small 130-bp restriction fragment is not visible on the gel

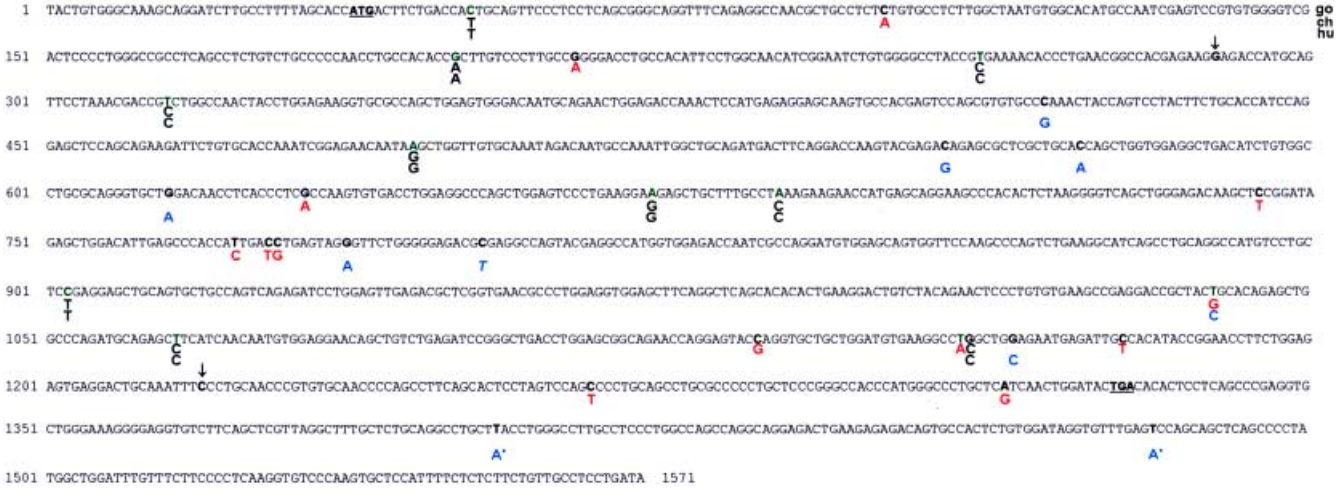
Fig. 2 Nucleotide sequence of gHaA (gorilla, *go*), cHaA (chimpanzee, *ch*), and φhHaA cDNAs (human, *hu*). *Arrows* Region encoding the rod domain; the initiation codon and the stop codon are *underlined*. Nucleotides specific for the gorilla (*green*), chimpanzee (*red*), or human (*blue*) cDNA sequences are indicated. The nucleotide substitution that creates a premature stop codon in the human cDNA is given in *italics*. *Asterisks* Two human-specific nucleotides in the 3′-non-coding region

nic backgrounds, including eight sub-Saharan African populations, and in unrelated chimpanzees (*Pan troglodytes*), bonobos (*Pan paniscus*), and gorillas (*Gorilla gorilla*). In each case, including the gorilla, amplification of exon 4 with *φhHaA* intron-specific PCR primers yielded PCR fragments of the expected size (818 bp). Direct sequencing of individual PCR fragments revealed that the premature exon 4 termination codon was present in the homozygous state in all humans (Fig. 1a). All ape sequences, by contrast, exhibited a single nucleotide difference (C vs T) at the first position of the human TGA stop codon, creating an in-frame CGA arginine codon (shown in Fig. 1b for the chimpanzee *cHaA* gene). Since the critical sequences in the great ape genes contain an *Mvn*I recognition site (CGCG), restriction endonuclease analysis could be used to confirm the sequencing data (Fig. 1c).

Characterization of cHaA and gHaA cDNAs and the encoded hair keratins

The *cHaA* and *gHaA* genes proved to be both transcribed and correctly spliced since we were able to generate both full-length cHaA and gHaA cDNAs by means of RT-PCR of RNA isolated from the bulbs of freshly plucked chimpanzee and gorilla hairs (Fig. 2). The coding region of the defective human sequence differs from the chimpanzee and gorilla sequences by 20 and 18 nucleotide changes, respectively. The coding sequences of the two great ape orthologs differ by 21 nucleotides. The number of nucleotides specific for each species are 11 (gorilla), 13 (chimpanzee), and 8 (human; Fig. 2). While two synonymous and six non-synonymous changes were found to have occurred in the human lineage, five synonymous and seven non-synonymous substitutions were noted in the chimpanzee line.

The deduced cHaA and gHaA hair keratin sequences are shown in Fig. 3 in comparison with the hypothetical human hair keratin. Both cHaA and gHaA contain 430 amino acid residues. They have calculated molecular weights of 48,313 Da (cHaA) and 48,285 Da (gHaA) and exhibit the typical accumulation of cysteine residues at both the head (8 residues) and tail domains (5 residues).



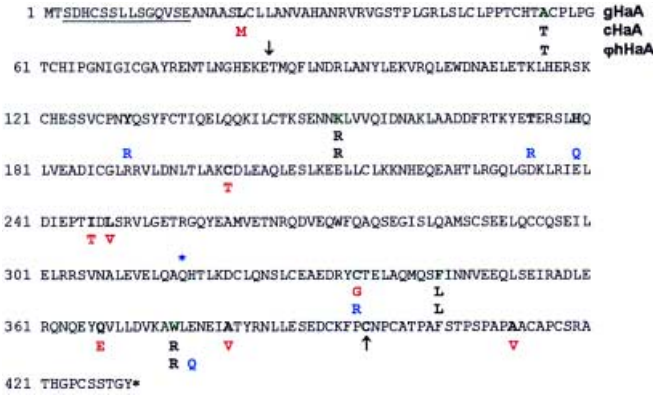


Fig. 3 Amino acid sequences of gHaA, cHaA, and φhHaA. *Arrows* Rod domain, *asterisk* premature translational arrest of the human sequence. The amino-terminal peptide sequence used for antibody generation in *underlined*. Gorilla-specific (*green*), chimpanzee-specific (*red*), and human-specific (*blue*) amino acid residues are indicated

The gorilla sequence differs from the chimpanzee sequence by 12 amino acid residues, whereas the human sequence differs from the gorilla and chimpanzee sequences by 9 and 12 amino acid residues. The number of amino acid residues specific for each of the sequences are 5 (φhHaA), 5 (gHaA) and 7 (cHaA).

Expression studies of cHaA and gHaA

Figure 4a shows that Coomassie-stained hair keratin patterns from human (lane 1), chimpanzee (lane 2), and gorilla (lane 3) are identical with respect to migration in one dimensional SDS-PAGE of type II (54–57 kDa) and type I (44–47 kDa) hair keratins (Langbein et al. 1999). Western blots with an antibody against human type I hair cortex keratin hHa1 (Langbein et al. 1999) revealed the presence of orthologous chimpanzee (cHa1) and gorilla (gHa1) hair keratins, although the cHa1 and gHa1 bands were consistently less intense than hHa1 (Fig. 4b). By contrast, an antibody against an N-terminal amino acid sequence motif, shared by cHaA and gHaA and also present in the hypothetical human hair keratin (SDHCSSLLSGQVSE, underlined in Fig. 3), reacted exclusively with hair keratins of the great apes (Fig. 4c). This confirms that the mutated mRNAs transcribed from *φhHaA* are not translated into detectable protein.

Comparative IIF studies with the cHaA/gHaA and hHa1 antibodies on longitudinal chimpanzee skin sections or human scalp sections indicated that, in chimpanzee hair follicles, cHaA and cHa1 are each expressed obviously in only one half of the hair cortex (Fig. 5a, b). As expected, the cHaA/gHaA antibody did not react with human hairs (results not shown), whereas the hHa1 antibody evenly decorated the entire hair cortex (Fig. 5c; Langbein et al. 1999). Both types of expression pattern were most readily discernible in cross-sectioned hair follicles (Fig. 5a', b', c'). Double label IIF with the cHaA/gHaA antibody (red) and the hHa1 antibody (green) confirmed a bipartite and mutually exclusive cHaA/cHa1 expression pattern in the cortex of chimpanzee hairs (Fig. 5d, d').

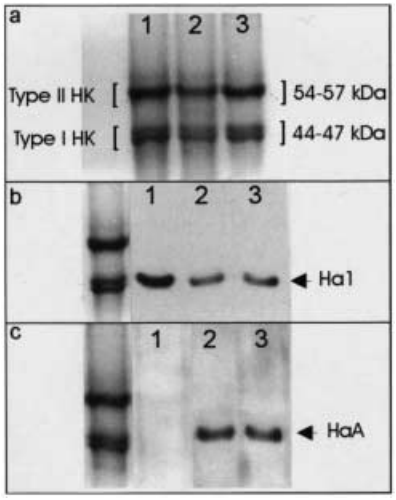


Fig. 4a–c One-dimensional Western blots of human, chimpanzee, and gorilla hair keratins. **a** Coomassie-stained human (*lane 1*), chimpanzee (*lane 2*), and gorilla (*lane 3*) hair keratins. The collectively migrating type II and type I hair keratins and their molecular weight ranges are indicated. **b** Western blot with hHa1 antibody. **c** Western blot with cHaA antibody. Coomassie-stained human hair keratins are shown as a reference pattern *left* in **b** and **c** (10% SDS-PAGE)

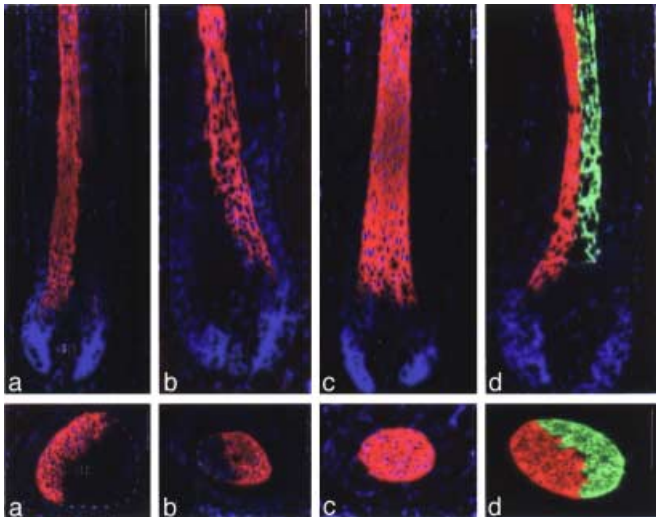


Fig. 5a–d Segmental hair keratin expression in chimpanzee hair. **a, a'** Chimpanzee hair follicle stained with cHaA antibody. **b, b'** Chimpanzee hair follicle stained with hHa1 antibody. **c, c'** Human hair follicle stained with hHa1 antibody. **d, d'** Chimpanzee hiar follicle stained by double label IIF with cHaA antibody (*red*) and hHa1 antibody (*green*). Note that both bipartite and non-segmental expression of the hair keratins are best seen in cross-sections of hair follicles. *Bar* 200 μm

Recent origin of the inactivating φhHaA mutation

The observed human to chimpanzee ratio of synonymous mutations (viz., 2:5) is not significantly different from 1:1

(two-sided tail probability of a binomial distribution, P=0.45) and does not therefore imply different rates of neutral substitution in the two genes. Furthermore, for the two 5'-most changes (one synonymous, one non-synonymous), the human *HaA* sequence is identical to that of the gorilla, whereas the chimpanzee sequence is identical to the closely related, and physically linked, human *hHa7* and *hHa8* genes (Rogers et al. 1998). These two changes in the chimpanzee line could, at least in principle, have resulted from gene conversion templated by the *cHa7* and/or *cHa8* gene(s), in which case their consideration would be misleading. Finally, the numbers of synonymous substitutions per lineage were also found to match their expectation based upon an identical neutral nucleotide substitution rate of 1.5×10^{-9} per site per year (Cooper 1999): assuming a divergence time between human and chimpanzee of 5.5 million years (Kumar and Hedges 1998), the expected number of synonymous substitutions per lineage is $1293\times 0.25\times(1.5\times10^{-9})\times(5.5\times10^{6})=2.7$ (note that, because of the structure of the genetic code, some 25% of "random" nucleotide substitutions are predicted to be synonymous).

The above considerations suggest that parameters λ_h and λ_c, which reflect the neutral substitution rates in the two lineages (see Materials and methods), are equal, resulting in maximum likelihood estimates of 3.5 for $\lambda=\lambda_h=\lambda_c$, 0.619 for *s*, and 0.0 for *t*. Under the assumption that the molecular clock has been running at the same rate in the human and chimpanzee genes, it may thus be concluded that the human gene copy became inactivated very recently in primate evolution (t~0). The observation that the *φhHaA* promoter is still active is not inconsistent with this assertion. However, a slowdown in nucleotide substitution rate has been reported for primates compared with other mammals, and this effects appears to be most pronounced in hominoids (Cooper 1999). This notwithstanding, reasonable estimates of the neutral substitution rate in the chimpanzee lineage are not more than 20% higher than for humans (Easteal 1991). Under this assumption, i.e., adopting the model $\{\lambda_h=\lambda, \lambda_c=1.2\lambda\}$, maximum likelihood estimates became 3.179 for λ, 0.612 for *s*, and 0.044 for *t*. It therefore appears likely that the inactivation of *hHaA* occurred less than approximately $0.044\times(5.5\times 10^{6})=240{,}000$ years ago.

Discussion

Humans, as members of the great ape family, share almost 99% of their gene-coding DNA sequence with chimpanzees, and both species are slightly more distant genetically from the gorilla (Ruvolo 1997). Indeed, human and chimpanzee genes are often so similar that differences between them fall within the range of intra-species DNA sequence variations (Winter et al. 1996; Young et al. 1998; Kaessmann et al. 1999; Clark 1999; Pääbo 1999). It has therefore been suggested that the main features distinguishing humans from their closest primate relative are attributable to a relatively small number of changes in the function or expression of certain key genes. Although this assumption was put forward some 25 years ago (King and Wilson 1975), as yet only a few examples of such variations have been described (Cooper 1999). Thus, humans have lost activity of the enzyme CMP-N-acetylneuraminic acid hydroxylase because of a 92-bp deletion in the corresponding *CMAH* gene. The normal mammalian enzyme converts the sialic acid derivative N-acetylneuraminic acid into N-glycolylneuraminic acid, which is generally found on the cell surface. The loss of enzyme activity in humans could have had far-reaching consequences, e.g., in the modulation of interactions of cells with pathogens that use sialic acid derivatives as receptors (Chou et al. 1998; Muchmore et al. 1998). Another genetic alteration that distinguishes humans from the great apes has recently been reported in the elastin gene. The human elastin gene (*ELN*) lacks the entire exon 34, which was lost possibly via an *Alu*-mediated recombination event (Szabó et al. 1999).

Here, we report for the first time a variation in the expression of a gene product related to one of the more obvious phenotypic differences between humans and the great apes, namely body hair. We have previously shown that humans possess a transcribed type I hair keratin pseudogene *φhHaA*, the inactivation of which resulted from a point mutation that introduced a premature stop codon into exon 4 (Rogers et al. 1998). In the present study, we have demonstrated that this defect is absent in chimpanzee and gorilla, and that the *φhHaA* orthologs of these species represent active genes that encode functional type I hair keratins. Unpublished data from our laboratory indicate that orthologs of virtually all functional human hair keratin genes are expressed in the chimpanzee hair follicle. Therefore, the expression of cHaA in chimpanzee hairs represents an addition to the other type I hair keratins, rather than a replacement of one of them. cHaA belongs to the cortex keratins, which constitute the most abundant genes of the hair keratin family (Rogers et al. 1998, 2000). However, compared with the uniform expression patterns seen in human hair cortex for the major type I cortex keratins (Langbein et al. 1999), cHaA expression in chimpanzee hair cortex is markedly different; a vertical restriction of cHaA synthesis to one half of the cortex was observed, concomitant with the expression of cHa1 in the cHaA-free cortical moiety. This left-right symmetry of cHaA/cHa1 expression in chimpanzee hairs explains why, in Western blots of human and chimpanzee hair keratins with the hHa1 antibody, the intensity of the cHa1 band was consistently weaker than that of hHa1 (Fig. 4b, lanes 1, 2). Since the same observation was also made for the gHaA hair keratin (Fig. 4b, lanes 1,3), it is likely that a segmental and mutually exclusive cortical gHaA/gHa1 expression pattern is also a feature of gorilla hair. Thus, one significant difference between the hair of contemporary humans and the great apes is the non-segmental cortical expression of hHa1 in humans, a change that may thus have arisen concomitantly with the evolutionary inactivation of the *hHaA* gene.

Two controversial hypotheses are currently discussed regarding the origin of modern humans. Both concepts entail an expansion approximately 1.5 million years ago

of *Homo erectus* from Africa into the Old World (Tattersall 1997; Disotell 1999). The "multiregional continuity" hypothesis maintains that our lineage, from then on, consisted of a continual stream of populations evolving in all areas of the Old World, constantly linked by gene flow that allowed evolutionary changes in one region to spread into others (Tattersall 1997; Disotell 1999; Wolpoff 1998). By contrast, the "single African origin" hypothesis contends that all modern humans descended from a single ancestral founder population, which consisted of approximately 10,000 breeding adults, and which emerged in Africa about 200,000 years ago, staying relatively isolated for probably thousands of years before expanding into the Old World and thereby gradually replacing more primitive forebears from the first migrational wave (Cann et al. 1987; Stringer and Andrews 1988; Klein 1995; Ruvolo 1997; Harpending et al. 1998; Harris and Hey 1999; Disostell 1999; Seielstad 1999). Undoubtedly, our results appear to be more plausible under the single African origin hypothesis. Studies of mtDNA suggest that the oldest human populations currently residing in Africa date back to 125,000–165,000 years (Chen et al. 1995, 2000). This includes the Biaka pygmies which, like all other sub-Saharan populations and non-African populations analyzed here, carry the *φhHaA* nonsense mutation in the homozygous state. This implies that the exon 4 mutation and its associated hair keratin phenotype should have become fixed in an ancestral African population prior to the divergence of all modern human populations. On the other hand, on the basis of our estimate of the time elapsed since *φhHaA* inactivation (≤240,000 years), the multiregional continuity hypothesis would require that the causative nucleotide change entered into all existing ancient populations and replaced existing alleles in a process of massive and highly specific gene flow over vast distances. In our view, such a scenario appears unlikely unless a very strong selective advantage were to be ascribed to the newly arisen hair keratin phenotype.

Acknowledegements We thank Henrik Kaessmann (Max Planck Institute for Evolutionary Anthropology, Leipzig), Walter Schempp (Institute of Human Genetics, University of Freiburg), Christian Roos (Gene Bank of Primates, University of Munich) for providing us with human, chimpanzee and gorilla blood DNA, Sandra Reichler (Zoological Garden, Heidelberg) for plucked gorilla hairs, and Bernard Cribier (Department of Dermatology, University of Strasbourg, France) for human scalp samples. We are grateful to Hans Richard Rackwitz (German Cancer Research Center, Heidelberg) for peptide synthesis and, last but not least, to Werner W. Franke (German Cancer Research Center, Heidelberg) for his continued interest and support. This work was supported in part by the Deutsche Forschungsgemeinschaft (Schw 539/1–3 and Kr 1093/5–2).

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