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Two Independent Mutational Events in the Loss of Urate Oxidase during Hominoid Evolution

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Summary. Urate oxidase was lost in hominoids during primate evolution. The mechanism and biological reason for this loss remain unknown. In an attempt to address these questions, we analyzed the sequence of urate oxidase genes from four species of hominoids: human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), orangutan (*Pongo pygmaeus*), and gibbon (*Hylobates*). Two nonsense mutations at codon positions 33 and 187 and an aberrant splice site were found in the human gene. These three deleterious mutations were also identified in the chimpanzee. The nonsense mutation at codon 33 was observed in the orangutan urate oxidase gene. None of the three mutations was present in the gibbon; in contrast, a 13-bp deletion was identified that disrupted the gibbon urate oxidase reading frame. These results suggest that the loss of urate oxidase during the evolution of hominoids could be caused by two independent events after the divergence of the gibbon lineage; the nonsense mutation at codon position 33 resulted in the loss of urate oxidase activity in the human, chimpanzee, and orangutan, whereas the 13-bp deletion was responsible for the urate oxidase deficiency in the gibbon. Because the disruption of a functional gene by independent events in two different evolutionary lineages is unlikely to occur on a chance basis, our data favor the hypothesis that the loss of urate oxidase may have evolutionary advantages.

Key words: Urate oxidase — Evolution — Mechanism of inactivation — Mutations — Hominoids

Introduction

Urate oxidase, or uricase (EC 1.7.3.3), is a purine metabolic enzyme that converts uric acid to allantoin in most mammals. The distribution of urate oxidase activity in primates varies substantially depending on species (Keilin 1959; Shnitka 1966; Christen et al. 1970; Fanelli et al. 1970; Nakajima and Bourne 1970; Friedman et al. 1985). All five genera of hominoids (Hominoidea, including *Homo, Pan, Gorilla, Pongo,* and *Hylobates*) were shown to have no detectable urate oxidase activity and their serum uric acid levels were increased about 10-fold compared to nonprimate mammals. Old World monkeys (Cercopitheecoidea) and Prosimians contain a significant amount of the enzyme activity, therefore, they have low serum uric acid levels. Most of the New World monkeys possess the functional urate oxidase and for those that do not show detectable urate oxidase activity, they have comparable uric acid levels to that of man. Because Old World monkeys retain the urate oxidase activity and New World monkeys diverged from the hominoids earlier than Old World monkeys, it was suggested that the loss of urate oxidase in hominoids and in some New World monkeys is likely the result of independent mutational events (Wu et al. 1989).

Although considerable studies have been carried out on urate oxidase enzyme activity in primates, the genetic events that resulted in the loss of urate oxidase during primate evolution remain unclear. Based upon the fact that urate oxidase is labile and the enzyme activity is reduced in some species of Old and New World monkeys, and that a few species of the New World monkeys lacked the enzyme, it was proposed that the loss of urate oxidase during primate evolution was a multistep process: genetic mutations in a primate ancestor resulted in reduced and/or labile urate oxidase activity and a subsequent mutation silenced the urate oxidase gene in a hominoid ancestor (Christen et al. 1970). An alternative hypothesis was derived from microradiochemical analysis of the urate oxidase in primates (Friedman

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et al. 1985). As stable urate oxidase was found in several New and Old World monkeys, it was suggested that the inactivation of urate oxidase during the evolution of primates occurred after the divergence of Old World monkeys from hominoids and a single mutational event inactivated the urate oxidase gene in a hominoid ancestor.

The biological function for the loss of urate oxidase activity in humans and certain other primates is an interesting question in human evolution. It has been debated whether this loss provided evolutionary advantages or it was simply an evolutionary accident leading to a common cause of renal stones and gouty arthritis in humans (Orowen 1955; Keilin 1959; Proctor 1970; Ames et al. 1981).

We have previously reported that two nonsense mutations were found in two exons of the human urate oxidase gene. This confirmed that the human gene is nonfunctional (Wu et al. 1989). To further investigate the mechanism for the loss of urate oxidase in hominoids, we have completed the characterization of the human urate oxidase gene and extended the DNA analysis to other hominoids—the chimpanzee, orangutan, and gibbon. We have identified two deleterious mutations that could inactivate the urate oxidase gene in the hominoid family independently. Our data also imply that the loss of urate oxidase could be the result of natural selection.

Materials and Methods

Materials. The human genomic library (EMBL 3) was provided by Dr. John Weis. The baboon DNA was isolated from a liver biopsy. Other primate DNAs were isolated from cell lines (from ATCC). All oligonucleotide primers for the polymerase chain reactions (PCR), sequencing, and hybridizations were synthesized on an Applied Biosystems (ABI) 380B DNA synthesizer.

Isolation and Sequencing of the Human Urate Oxidase Gene. The genomic clones containing the human urate oxidase gene were isolated from the human genomic library using baboon cDNA as a probe according to the procedure of Benton and Davis (1977). The hybridization was carried out at 5× standard saline citrate (1× SSC: 0.15 M sodium chloride, 0.15 M sodium citrate), 1% SDS, and 50% formamide at 42°C. Each individual exon was identified and subcloned into pTZ 18 and 19 (Pharmacia) and sequenced by the Sanger dideoxy sequencing method (Sanger et al. 1977).

In Vitro DNA Amplification and Direct DNA Sequencing. The PCR was performed in a total volume of 100 μl on 0.5 μg of the genomic DNA isolated from either tissue or cell culture. The reaction contained 50 pmol of each primer in 50 mM of KCl, 10 mM Tris HCl, pH 8.0, 1.5 mM $MgCl_2$, 0.01% gelatin, dATP, dCTP, dGTP, and dTTP at 300 μM each, and 2.5 units of Taq polymerase (Perkin-Elmer/Cetus). Amplification was achieved after 30 cycles of denaturation (94°C, 30 s), polymerization (68°C, 3 min), and annealing (61°C, 30 s). One microliter of the double-strand PCR products was used to initiate the second PCR containing 100 pmol of one primer only at the same condition as the first PCR. The single-strand PCR products were purified by NH_4OAc/ethanol precipitation and sequenced by using 5′-end radioactively labeled primer and dideoxynucleotide termination as described (Gibbs et al. 1989).

Sequence-Specific Oligonucleotide Hybridizations. The oligonucleotides were labeled with γ-^{32}P-ATP by T4 polynucleotide kinase as described (Carothers et al. 1989). After gel electrophoresis of the PCR products and transferring to a Zeta Probe membrane (Bio-Rad), hybridization was performed at 37°C in 5× SSC and 1% SDS. The membrane was washed once in 1× SSC and 0.1% SDS at 37°C for 30 min, and then at 50°C for 15 min. The sequence-specific oligonucleotides are: ASO H1 5′ CCATATTCAGTGAGATGG 3′; ASO B1 5′ CCATATTCAGCGAGATGG 3′; ASO H2 5′ TGAAGGACTGATGCTTTG 3′; ASO B2 5′ TGAAGGACCGATGCTTTG 3′. The differences between each pair of oligonucleotides are indicated by underlines.

Results

Three Deleterious Mutations in the Human Urate Oxidase Gene

The nonfunctional human urate oxidase gene was isolated from a human genomic library using the baboon cDNA as a probe. Eight exons were identified and their sequences were determined (Fig. 1). There were 304 codons in the sequence that corresponded to the entire open reading frame of the baboon gene. The exon sequences of the human urate oxidase gene showed a 15% divergence compared to the mouse cDNA and only 3.4% divergence compared to the baboon cDNA sequences. In addition to the two nonsense mutations at codon positions 33 and 187 that we reported earlier (Wu et al. 1989), an aberrant splice acceptor sequence was identified in exon 3. In this case, the wild type sequence *ag* was mutated to *aa*.

Occurrence of the Three Mutations in Other Hominoids

If the loss of urate oxidase in hominoids was caused by a single mutational event in a common ancestor, this event would likely be conserved in all five living genera of hominoids. The three deleterious mutations identified in the human sequence are candidates for this original event. To determine which mutation is responsible for the loss of urate oxidase in the hominoid family, we examined the three mutation sites in three other species of hominoids: the chimpanzee, orangutan, and gibbon.

Sequence-specific oligonucleotide hybridization was used to determine whether the two nonsense mutations were present in the chimpanzee, orangutan, and gibbon. Two sets of primers were used to amplify the nonsense mutation sites at codon po-

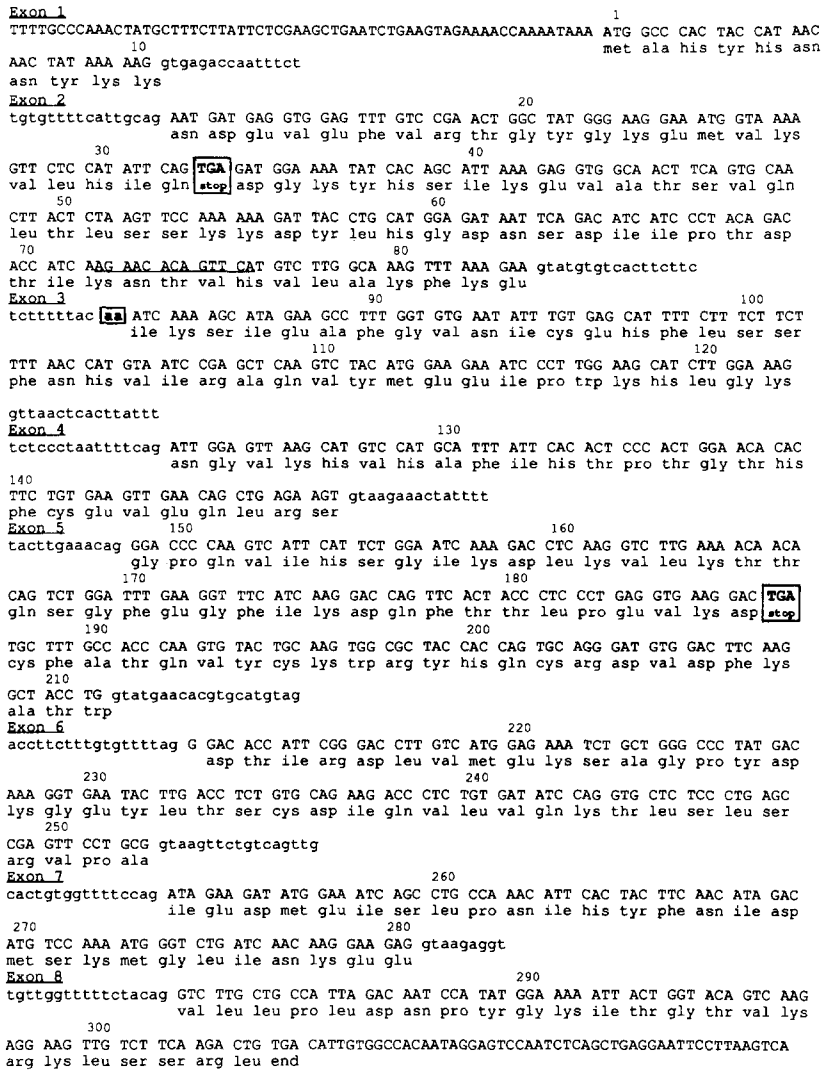

Fig. 1. Exon and exon–intron boundary sequences of the human urate oxidase gene. Exon sequences are shown as capital letters and intron sequences in lowercase letters. The deduced amino acid sequences are listed below the DNA sequence. The numbers above the sequence are the codon positions based on the baboon open reading frame (Wu et al. 1989). A total of 304 codons are present in the sequence. Three disruptive mutations are shown as open boxes. The 13-bp sequence between codon 72 and 76 (underlined) in exon 2 is deleted in the gibbon urate oxidase gene.

sition 33 and 187, respectively and simultaneously. The multiplex PCR amplification generated two products: the 2.3-kb band across exons 2 and 3 contained the nonsense mutation site at 33 and the 2.0-kb band that spanned exons 5 and 6 contained the nonsense mutation site at 187. The sequence-specific oligonucleotides were designed based on both the human and baboon sequences at the mutation sites. Each pair of oligonucleotides differed by only one nucleotide. The multiplex PCR products were hybridized to these sequence-specific oligonucleotides under the condition that only the perfectly matched oligonucleotide would hybridize. Therefore, the baboon-specific probes (ASO B1 and 2) detected only the wild type sequences, and the human-specific probes (ASO H1 and 2) were specific for the mutations.

By using this method, it was found that both mutations were conserved in four different human ethnic groups (Blacks, Caucasians, Hispanics, and Orientals), indicating that the mutations were generated in or before ancestral humans (data not shown). The nonsense mutation at codon position 33 (detected by ASO H1) in the human was also found in the orangutan and chimpanzee, whereas the nonsense mutation at codon position 187 (detected by ASO H2) existed only in the chimpanzee. The wild type sequences were found in the baboon and gibbon at both positions as they only hybridized to ASO B1 and ASO B2. The orangutan also showed the wild type sequence at position 187 as detected by probe ASO B2 (Fig. 2).

The splice acceptor sequences of exon 3 in the chimpanzee, orangutan, and gibbon were examined by direct sequencing of the PCR products containing the splice junctions (Fig. 3). Both the orangutan and gibbon showed the wild type splice acceptor signal *ag*, whereas the chimpanzee gene displayed the same aberrant splice sequence *aa* as in the human gene. In summary, the chimpanzee, like humans, had all three mutations, only one nonsense mutation existed in the orangutan at codon position 33, and none of the mutations was present in the gibbon (Table 1).

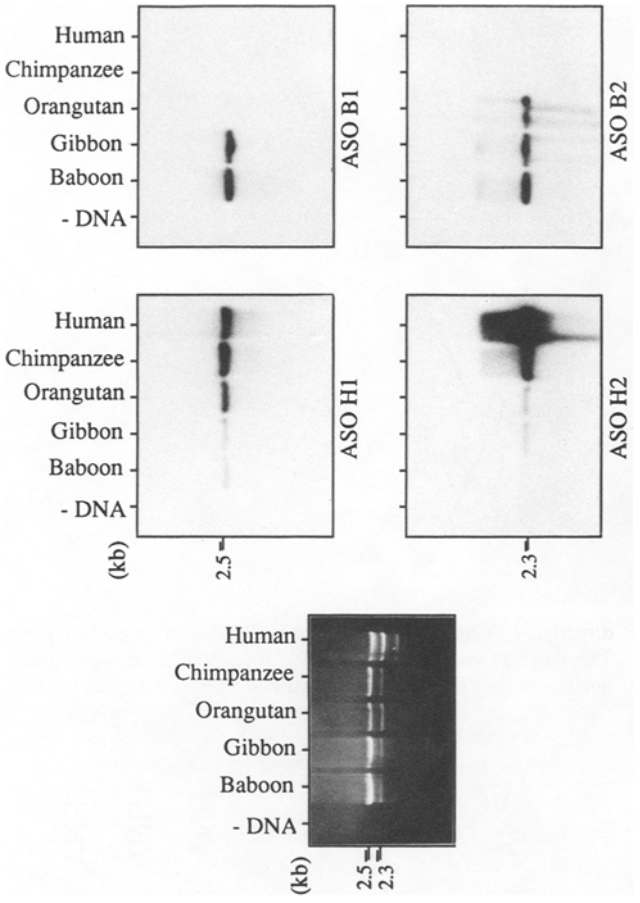

Fig. 2. Occurrence of two nonsense mutations in the chimpanzee, orangutan, and gibbon. Genomic DNA samples from chimpanzee, orangutan, and gibbon were amplified across the two nonsense mutation sites by PCR and hybridized to sequence-specific oligonucleotides. The 2.5-kb band and the 2.3-kb band contain the nonsense mutation sites at codon position 33 and 187, respectively (left panel). The sequence-specific oligonucleotide probe used in each experiment is indicated under each panel. ASO H1 detects the human sequence at codon 33 and ASO B1 detects the baboon sequence at the same region. ASO H2 and ASO B2 are specific for the human and baboon sequences at codon 187, respectively. Each pair differs only by one nucleotide.

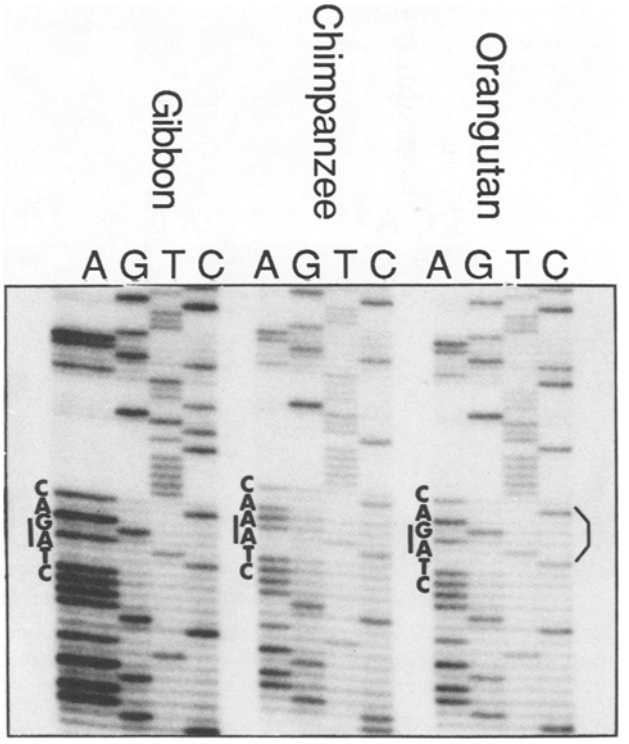

Fig. 3. PCR direct sequencing of the 5′ splice junction of exon 3 of the urate oxidase genes from gibbon, orangutan, and chimpanzee. The splice junction was amplified by PCR and sequenced by ^{32}P-labeled antisense primer. The sequences from the bottom to the top correspond to the direction of 5′ to 3′. The sequences at the splice site for each species are shown and the splice acceptor sequence is indicated by a line.

Table 1. Distribution of three deleterious mutations in hominoids

	Human	Chimpanzee	Orangutan	Gibbon
Nonsense mutation at 33	+	+	+	−
Nonsense mutation at 187	+	+	−	−
Splice site mutation	+	+	−	−

A 13-bp Deletion in the Gibbon Gene

It is known that the gibbon has lost its urate oxidase activity. However, none of the three mutations found in the human was present in the gibbon gene. In order to identify the mutation that was responsible for the loss of the urate oxidase activity in the gibbon, PCR direct sequencing of exons of the gibbon urate oxidase gene was performed. Direct sequencing of the PCR product containing exon 2 of the gibbon gene identified a new mutation not found in other hominoids: a 13-bp deletion between codons 72 and 76 that not only would delete four amino acids but also lead to a frameshift (Fig. 4). This deletion could possibly silence the urate oxidase gene in the gibbon.

Expression of Urate Oxidase Gene in the Human

It was previously reported that the urate oxidase mRNA was not detectable in humans by northern analysis (Reddy et al. 1988; Lee et al. 1989). Due to the low detection sensitivity of the method, the northern results could not exclude the possibility that a low level of urate oxidase transcripts may be present in the human. When a more sensitive PCR method was used, human urate oxidase gene transcripts were readily detected after 50 cycles of amplification (Fig. 5). This is unlikely to be caused by the contamination of genomic DNA as the two primers used in the amplification were separated by a 3-kb intron and no amplification was observed on the RNA without being reverse-transcribed. The possibility of amplification on cDNAs from other species was also excluded by hybridization with the

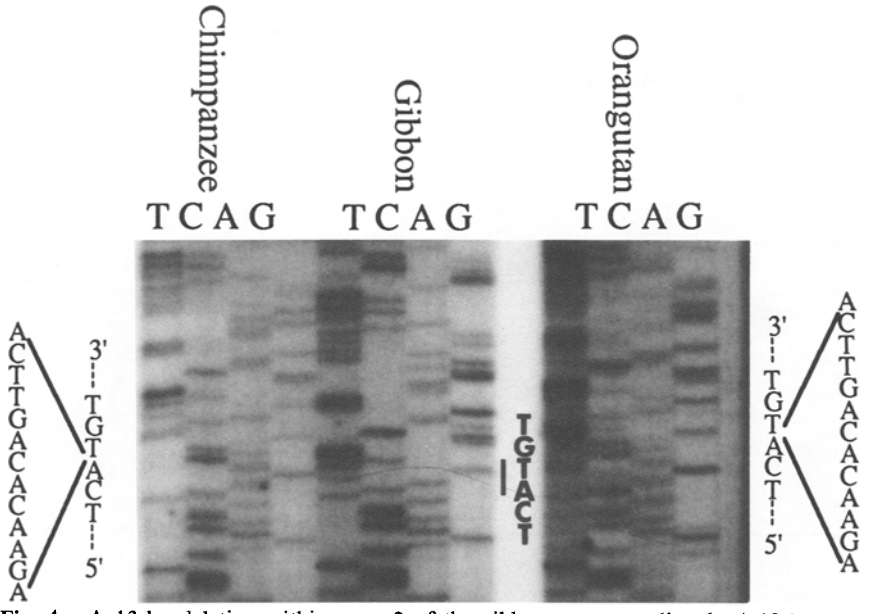

Fig. 4. A 13-bp deletion within exon 2 of the gibbon urate oxidase gene. Exon 2 of the urate oxidase genes from chimpanzee, orangutan, and gibbon were amplified by PCR and sequenced directly. A 13-bp sequence was found deleted in the gibbon gene. The deletion site is indicated by a line. Neither the orangutan nor the chimpanzee had the deletion.

human-specific oligonucleotide ASO H1 under the condition that only detects the human sequence.

Dating the Mutations in Hominoids

The phylogenetic tree for hominoids was established by various studies (Fitch and Margoliash 1967; Chiarelli 1972; Brown et al. 1982; Goodman et al. 1984; Sibley and Ahlquist 1984). Based upon the distribution of the mutations of urate oxidase genes among hominoids, it was possible to position each event on the tree (Fig. 6). The nonsense mutation at codon 187 and the aberrant splice signal were observed only in the chimpanzee and human, indicating that they were generated in their common ancestor. The nonsense mutation at codon 33 was present in the orangutan, chimpanzee, and human. Thus, this event occurred in a common ancestor of these three species. The 13-bp deletion was unique to the gibbon; therefore, this deletion occurred after the divergence of the gibbon lineage from the rest of the hominoids.

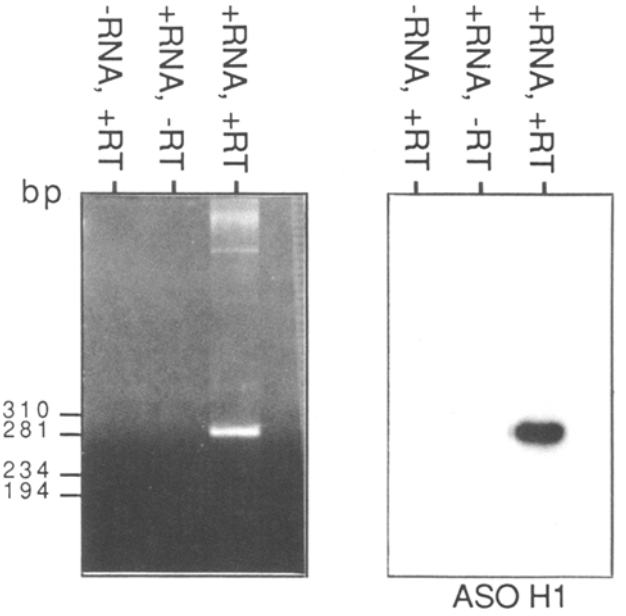

Fig. 5. PCR detection of the human urate oxidase mRNA. Total RNA was isolated from human liver and first strand cDNA was synthesized by M-MuLV-reverse transcriptase using 20 μg of total RNA as template and oligo dT as primer. One-fifth of the cDNA was used in the PCR. After 50 cycles of amplification, the PCR products were separated on a Nusieve agarose gel, and a distinct band at predicted size was apparent (left panel). The band was proven to be human specific by hybridizing with human-specific oligonucleotide probe ASO H1 under the condition that only perfectly matched sequences would hybridize (right panel). Two controls were included: reverse transcription without RNA (−RNA, +RT) and RNA without reverse transcriptase (+RNA, −RT).

Discussion

We have isolated the human urate oxidase gene and sequenced eight exons that contained the entire coding region. Three deleterious mutations were found in the sequence. Because a simultaneous occurrence of the three mutations is unlikely during the evolution of primates, we presume that they were acquired sequentially and at least two of them were generated after the urate oxidase gene was inacti-

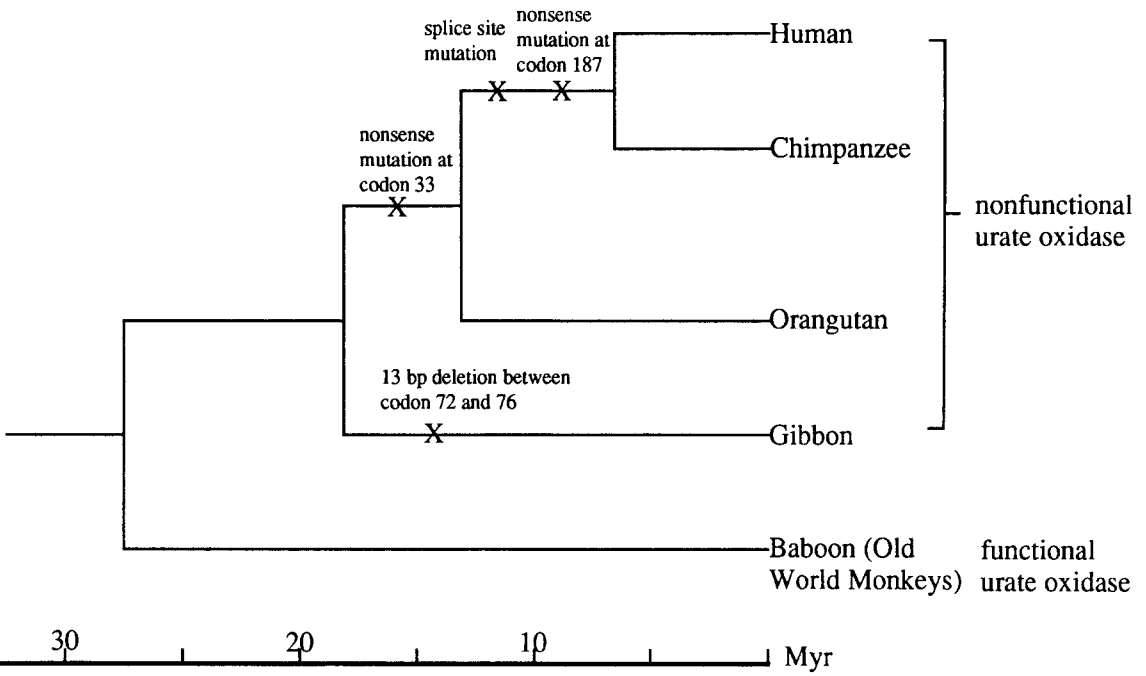

Fig. 6. The evolution of the urate oxidase gene in hominoids. The phylogenetic tree and the divergence time of the primate evolution is adopted from the work of Sibley and Ahlquist (1984). No urate oxidase activity is found in the hominoids, however Old World monkeys retain the enzyme activity (Christen et al. 1970; Friedman et al. 1985). The approximate time and site of mutations are indicated by X. The order of the splice site mutation and the nonsense mutation at codon 187 is not determined.

vated. We sought to define the distribution of these mutations in the hominoid family and to determine whether there is an original mutation in their common ancestor by analyzing the existence of the mutations in three other hominoids: the gibbon, orangutan, and chimpanzee.

The evolution of hominoids is a relatively short period, and multiple mutations at the same nucleotide site are unlikely. Therefore, it is reasonable to assume that the original mutation is conserved in all living hominoids if the loss of urate oxidase in hominoids was caused by a single mutational event in their common ancestor. Although we have identified a common nonsense mutation in the human, chimpanzee, and orangutan, none of the three deleterious mutations found in the human gene is present in the gibbon. Because the gibbon is also devoid of urate oxidase activity, it could be that either there is an unidentified mutation in the common ancestor of the hominoids that led to the loss of urate oxidase activity in the living apes and humans, or, alternatively, the loss of urate oxidase in the gibbon was the result of a different mutation after its divergence from the remainder of hominoids.

The three mutations identified in the human exon sequences could silence the urate oxidase gene. Mutations at other regions of the gene, such as the promoter, were also capable of disrupting the normal gene function. To determine whether the human urate oxidase gene promoter is still functioning, we examined urate oxidase gene expression in human liver. Although human urate oxidase mRNA could not be detected by northern analysis (Reddy et al. 1988; Lee et al. 1989), we showed that low level of transcripts is present using PCR. Because nonsense mutations could lead to low steady-state levels of mRNA (Baserga and Benz 1988; Urlaub et al. 1989), the human urate oxidase gene promoter may be functional and the low steady-state mRNA level was probably caused by the two inframe nonsense mutations.

We have not completely excluded the possibility that there is a single original mutation in the common ancestor of humans and apes, but the available evidence suggests that a different mutation event was likely responsible for the loss of urate oxidase activity in gibbons. The 13-bp deletion identified in the gibbon urate oxidase sequence but not in other species is consistent with this hypothesis. As gibbons are the first to branch out from the rest of the hominoids, it may well be that the loss of urate oxidase in hominoids was the consequence of two independent events; the nonsense mutation at codon position 33 resulted in the loss of urate oxidase activity in the human, chimpanzee, and orangutan, whereas the 13-bp deletion was responsible for the urate oxidase deficiency in the gibbon. The two additional mutations found in the human and chimpanzee likely occurred after the gene was inactivated. We have also examined mutations in the urate oxidase gene in gorilla (*Gorilla gorilla*). In agreement with our hypothesis, all three mutations found in

human were present in the gorilla gene (data not shown).

Although we have not explored the possibility of additional mutations in the gibbon, our data so far suggest that gibbons and the rest of the hominoids lost urate oxidase activity by independent mutational events. If this is indeed the case, it may suggest that these independent events were the result of natural selection as the gibbon has evolved along a different lineage from the rest of the hominoids and it is unlikely to disrupt a functional gene in two evolutionary lineages by two mutational events without selection. Thus, the loss of urate oxidase might have selective advantages in the evolution of hominoids, much like the mutations seen at the β-globin locus in different human populations (Antonarakis et al. 1982).

It is known that urate oxidase has also been lost in some New World monkeys (Christen et al. 1970). Although the mutations in these species have not been examined, it is likely that the loss of urate oxidase in hominoids and in New World monkeys was the result of independent mutational events, as Old World monkeys retained urate oxidase activity and New World monkeys diverged from the hominoids earlier than did Old World monkeys. Our present study showed that the loss of urate oxidase in hominoids was also likely caused by independent mutations. This result suggests that independent mutation could be the general mechanism for the loss of urate oxidase in primates.

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References

Ames BN, Cathcart R, Schwiers E, Hochstein P (1981) Uric acid provides an antioxidant- and radical-caused aging and cancer: a hypothesis. Proc Natl Acad Sci USA 78:6858–6862

Antonarakis SE, Boehm CD, Giardina PJV, Kazazian HH Jr (1982) Nonrandom association of polymorphic restriction sites in the β globin gene cluster. Proc Natl Acad Sci USA 79: 137–141

Baserga SJ, Benz EJ Jr (1988) Nonsense mutations in the human beta globin gene affect mRNA metabolism. Proc Natl Acad Sci USA 85:2056–2060

Benton W, Davis R (1977) Screening lambda-gt recombinant clones by hybridization to single plagues in situ. Science 196: 180–182

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18:225–239

Carothers AM, Urlaub G, Mucha J, Grumberger D, Chasin LA (1989) Point mutation analysis in a human gene: rapid preparation of total RNA, PCR amplification of cDNA, and Taq sequencing by a novel method. Biotechniques 7:496–499

Chiarelli B (1972) Taxonomic atlas of the living primates. Academic Press, London

Christen P, Peacock WC, Christen AE, Wackker WEC (1970) Urate oxidase in primate phylogenesis. Eur J Biochem 12: 3–5

Fanelli GM, Bohn DL, Russo HF (1970) Renal clearance of uric acid in nonhuman primates. Comp Biochem Physiol 33: 459–464

Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 155:279–284

Friedman TB, Polanco GE, Appold JC, Mayle JE (1985) On the loss of uricolytic activity during primate evolution—I. Silencing of urate oxidase in a hominoid ancestor. Comp Biochem Physiol 81B:653–659

Gibbs RA, Nguyen P-N, Caskey CT (1991) Direct DNA sequencing of complementary DNA amplified by the polymerase chain reaction. In: Mathew C (ed) Methods in molecular Biology. Humana (in press)

Goodman MB, Koop BF, Czelusniak J, Weiss ML, Slightom JL (1984) The η-globin gene: its long evolutionary history in the β-globin gene family of mammals. J Mol Biol 180:803–823

Keilin J (1959) Biological significance of uric acid on guanine excretion. Biol Rev 34:265–296

Lee CC, Wu X, Caskey CT (1989) Cloning the full-length cDNA for the porcine urate oxidase by the MOPAC generated probe. In: Mikanagi K, Nishioka K, Kelly WN (eds) Purine and pyrimidine metabolism in man VI. Plenum, New York, pp 499–505

Nakajima Y, Bourne GH (1970) Histochemical studies on urate oxidase in several mammals with special reference to uricolytic ability of primates. Histochemistry 22:20–24

Orowen E (1955) The origin of man. Nature 175:683–684

Proctor P (1970) Similar function of uric acid and ascorbate in man? Nature 228:868

Reddy PG, Nemali MR, Reddy MK, Reddy MN, Yuan PM, Yuen S, Laffler TG, Shiroza T, Kuramitsu HK, Usuda N, Chrisholm RL, Rao MS, Reddy JK (1988) Isolation and sequence determination of a cDNA clone for rat peroxisomal urate oxidase: liver specific expression in the rat. Proc Natl Acad Sci USA 85:9081–9085

Sanger R, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Shnitka TK (1966) Comparative ultrastructure of hepatic microbodies in some mammals and birds in relation to species differences in uricase activity. J Ultrastruct Res 16:598–625

Sibley CG, Ahlquist JE (1984) The phylogeny of the hominoid primates, as indicated by DNA–DNA hybridization. J Mol Evol 20:2–15

Urlaub G, Mitchell PJ, Ciudad CJ, Chasin LA (1989) Nonsense mutations in the dihydrofolate reductase gene affected RNA processing. Mol Cell Biol 9:2868–2880

Wu X, Lee CC, Muzny DM, Caskey CT (1989) Urate oxidase: primary structure and evolutionary implications. Proc Natl Acad Sci USA 86:9412–9416

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