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Characterization of the genes encoding carbonic anhydrase I of chimpanzee and gorilla: comparative analysis of 5′ flanking erythroid-specific promoter sequences

(Polymerase chain reaction; primate evolution; African hominoid trichotomy; orangutan; pigtail macaque; squirrel monkey)

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SUMMARY

The genes encoding carbonic anhydrase I (CA I) have been characterized for chimpanzee (*Pan troglodytes*) and gorilla (*Gorilla gorilla*). In addition, 44 nucleotides (nt) at the 5′ end of the noncoding first exon (exon 1a), which is unique to the erythroid *CA I* mRNA, together with 188 nt of the adjacent 5′ flanking regions, were sequenced for the corresponding positions of the *CA I* of orangutan, pigtail macaque, and squirrel monkey. When these 5′ flanking regions are compared, along with those published for human and mouse *CA I*, they were found to contain several conserved sequences that may bind factors involved in the erythroid-specific expression of *CA I*. Comparisons of the human, chimpanzee, and gorilla coding and noncoding *CA I* sequences do not significantly deviate from a pattern of trichotomy for the evolutionary origins of these three hominoid species.

INTRODUCTION

Comparative studies of the aa and nt sequences of the carbonic anhydrase (CA) isozymes have provided useful information on the evolutionary relationships of humans and other primates (Contel et al., 1981; Tashian et al., 1983; Hewett-Emmett et al., 1984; Hewett-Emmett and Tashian, 1991). Of the seven CA isozymes (CA I–CA VII) that have been described from amniotes (primarily mammals), only CA I is known to be under the control of two promoters (Fraser et al., 1989; Tashian et al., 1989; Brady et al., 1991; Hewett-Emmett and Tashian, 1991). When *CA I* mRNA is expressed in erythrocytes, it contains an additional, short (63 and 72 bp in mouse and human, respectively), 5′ noncoding exon (exon 1a) which replaces part of the untranslated region of exon 1. The *CA I* mRNA expressed in nonerythroid tissue (e.g., colon) lacks the noncoding exon 1a (Fraser et al., 1989; Tashian et al., 1989; Brady et al., 1991; Bergenhem et al., 1992b). As yet, only the *CA I* of human, pigtail macaque, and mouse have been characterized (Fraser et al., 1989; Lowe et al., 1990; Nicewander, 1990).

The question of the relative evolutionary relationships between humans and the African great apes, chimpanzee and gorilla, continues to be of special interest to students of human evolution. Evidence from DNA sequence studies have been reported supporting for the most part a closer evolutionary relationship between human and

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Abbreviations: aa, amino acid(s); bp, base pairs(s); CA, carbonic anhydrase; *CA*, gene encoding CA; Ch, chimpanzee; Hu, human; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); PCR, polymerase chain reaction; *tsp*, transcription start point(s).

chimpanzee than between either of these two species and gorilla by examination of the β-globin gene clusters (Miyamoto et al., 1987, 1988; Koop et al., 1989; Bailey et al., 1991, 1992) and mitochondrial genomes (Ruvolo et al., 1991; Horai et al., 1992). Nevertheless, certain inconsistencies exist, and the problem has yet to be resolved (Dijon and Green, 1990). The present report provides DNA sequence data for the *CA I* of chimpanzee (*Pan troglodytes*) and gorilla (*Gorilla gorilla*) that can be used to examine the evolutionary patterns of these hominoid species. Also, in order to compare the erythroid-specific promoter regions of the *CA I* of primates, we have determined the partial nt sequences of exons 1a and their 5′ flanking regions of orangutan (*Pongo pygmaeus*), pigtail macaque (*Macaca nemestrina*), and squirrel monkey (*Saimiri sciureus*).

EXPERIMENTAL AND DISCUSSION

(a) Structural and evolutionary aspects

The structures of the *CA I* of chimpanzee and gorilla have been determined by direct sequencing of double-stranded PCR products. Both genes consist of the 8 exons found to comprise the *CA I* of human, pigtail macaque, and mouse (Fraser et al., 1989; Lowe et al., 1990; Nicewander, 1990), beginning with the untranslated exon 1a plus the 7 exons that are also characteristic of the *CA II*, *CA III*, and *CA VII* of mammals (cf. Tashian, 1992). Since directly sequencing the PCR-amplified DNA allows both alleles to be sequenced simultaneously, polymorphisms can be readily detected by the presence of two bands at any specific position on the sequencing gel (Bergenhem et al., 1992a); no such polymorphisms were observed in any of the sequences studied. In Fig. 1, the sequences for the coding regions of chimpanzee and gorilla *CA I* are compared with those of the human *CA I*, and the differences in their nt and aa are highlighted. The intron splice junctions are shown in Fig. 2 along with flanking intron sequences which are identical with those of the human *CA I*, with the exception of 1 nt in the donor sequence of intron 1. The identification of a Phe residue at position 114 from an earlier study on the protein sequence of chimpanzee CA I suggests a Phe/Tyr polymorphism, or a rare variant, at this position (Contel et al., 1981).

The active site of CA I has been well-characterized by X-ray crystallography, and the aa residues involved in binding the active-site zinc ion, enzyme catalysis, and formation of the active-site cavity have been identified (Ericksson and Liljas, 1991; Fig. 6 in Tashian, 1992). The deduced aa sequence of *CA I* from chimpanzee and gorilla are identical to the human sequence at the posi-

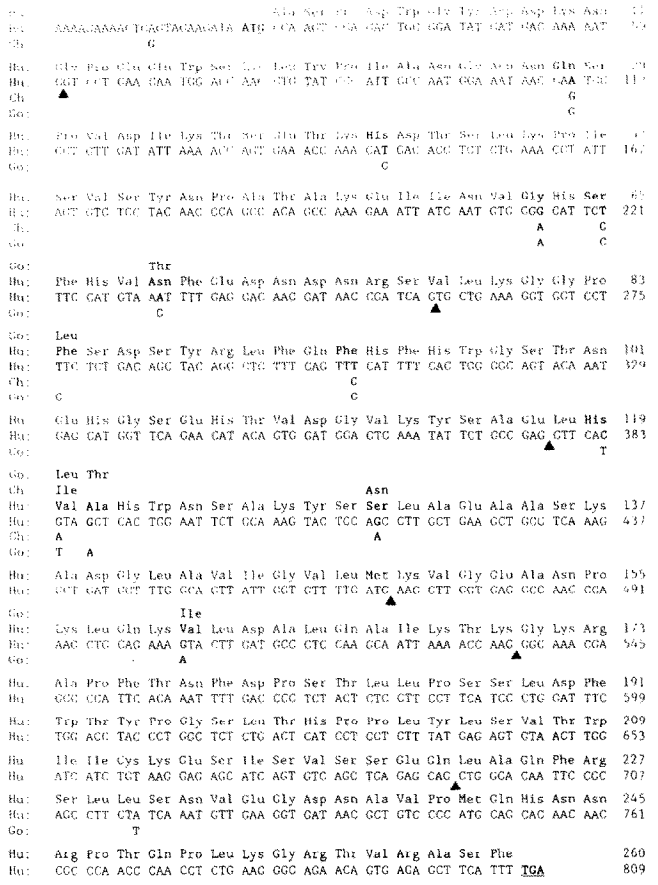

Fig. 1. The nt sequences of the coding regions of the *CA I* of chimpanzee (Ch) and gorilla (Go) compared with human (Hu) and deduced aa sequences. Only the differences from the human sequence are shown; where lines are omitted for chimpanzee and gorilla, the sequences are identical to the human sequence (Lowe et al., 1990). The positions of introns 1–6 are indicated (in order) by the arrowheads (see Fig. 2). The GenBank accession Nos. for the chimpanzee and gorilla *CA I* sequences are L11621 and L11622, respectively. **Materials:** Genomic DNA from chimpanzee (*Pan troglodytes*) and gorilla (*Gorilla gorilla*) was supplied by Dr. Jerry Slightom (Upjohn Co., Kalamazoo, MI). **Methods:** Specific amplification of DNAs by PCR was carried out in a Bellco DNA Pacer thermal cycler using the primer sets listed in Table I which are based on the human *CA I* sequence (Lowe et al., 1990). The basic protocol suggested by Perkin Elmer was followed, except that the PCR primer concentration was 0.1 μg/μl. Excellent amplification was generally obtained using the following thermal cycling program: 95°C, 14 s; 54°C, 15 s; 71°C, 90 s; 35–40 cycles. DNA generated by PCR amplification was purified by polyacrylamide gel electrophoresis and then eluted from gel slices by soaking overnight in 0.5 M ammonium acetate, precipitated, washed, and resuspended into 20 μl of TE. Sequencing of the double-stranded DNA was carried out using a modification by Bergenhem et al. (1992a) of the methods of Casanova et al. (1990) and Bachmann et al. (1990). Annealing of the sequencing primer was carried out as follows: to 7 μl of DNA solution in TE were added 1 μl 5% Nonidet P-40, 1 μl of 1 mg/ml primer, and 2 μl Sequenase Buffer (US Biochemical, Cleveland, OH, USA); in all cases, one of the primers used for PCR amplification was used as the sequencing primer. The sample was placed in a boiling water bath for 5 min, then snap-cooled by rapid transfer to a dry ice/ethanol bath. After 60 s in dry ice/ethanol, the tube containing annealed template was placed on wet ice for 8–10 min and then sequenced exactly as described in the protocol supplied by US Biochemical. In all cases, both strands of the DNA were sequenced.

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Go: GACAAAAATG]gtaagagttc  (intron 1, 2.8 kb)  ------tcctggtag[GTCCTGAACA
Ch:                                            tgtttg
Go: AACCGATCAG]gtgagctgaa  (intron 2, 1.0 kb)  tccctttcttccag[TGCTGAAAGG
Ch:                                            -----
Go: TTCTGCCGAG]gtaatgtaat  (intron 3, 3.6 kb)  tagtatcatttttag[CTTCACATAG
Ch:
Go: TTTGATGAAG]gtgtgttaca  (intron 4, 0.9 kb)  ---ttaaatctccag[GTTGGTGAGG
Ch:                 a                          ttc
Go: TAAAACCAAG]gtaaacacac  (intron 5, 3.0 kb)  attcttttcttccag[GGCAAACGAG
Ch:
Go: CTCAGAGCAG]gtagagttgt  (intron 6, 0.8 kb)  attttatccttctag[CTGGCACAAT
Ch:
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Fig. 2. The nt sequence at exon/intron splice junctions of the *CA I* of chimpanzee (**Ch**) and gorilla (**Go**). The brackets indicate the junctions, with the capital and lower-case letters indicating exon and intron sequences, respectively. The only difference between the gorilla and chimpanzee sequences is shown in the donor sequence of intron 4. Dashes indicate sequences that were not determined. The two g's (bold-face type) in the donor sequence of intron 1 were reported as c's by Lowe et al. (1990); sequences of several human samples determined in our laboratory show g's at these positions. The chimpanzee sequence is identical to that of human except for an A (bold-face type) at position 7 in exon 4 which is a G in the human sequence.

tions known to coordinate the zinc ion as well as almost all positions known to comprise the active site of the molecule. Gorilla CA I, however, differs at residues 69 and 121 in having a Thr residue at these positions in place of Asn (residue 69) and Ala (residue 121) found in chimpanzee and human. The gorilla CA I protein has been partially purified and shows levels of catalytic activity similar to human and chimpanzee CA I (Tashian, 1977; and unpublished results); therefore, any change in the active-site environment caused by these two replacements would appear to be very small.

A matrix that compares the aa and nt sequences of the *CA I* of human, chimpanzee, gorilla, and pigtail macaque is shown in Table II. As can be seen, the human sequence differs less from chimpanzee than from gorilla by about 1% at the aa level and about 0.6% at the nt level. However, when these relationships are examined statistically by constructing phylogenetic trees (with the pigtail macaque *CA I* sequence as the outgroup) by neighbor-joining (Saitou and Nei, 1987) or parsimony tree-building methods (Czelusniak et al., 1990), a trichotomous branching pattern is produced which does not significantly favor a closer relationship of human to chimpanzee than to gorilla (data not shown).

TABLE I

PCR and sequencing primers

Exon[a]	Sequence
1aD	5'-G GAATTCAATCCACACCCCAACCACTTC
1aU	5'-G GAATTCACAGCTCTGAATGAGAGAAGG
1D	5'-G TTGGAATCTTGAGTGTACAAG
1U	5'-G GGATCCGCAGACAGTTCAACAATTAACC
2D	5'-C AAACAGGTAACTACACTCCT
2U	5'-A ATGGGTGTCATGTTTCTCG
3D	5'-G GAATTCGCAAAGATAAGCTAGAGTTTG
3U	5'-G GGATCCAGGGTAATTATCTCTCACTTAC
4D	5'-G GAATTCCACTGGATAAAGGTTCACATA
4U	5'-C CTTCTATTTTGAGGTCTAATTGG
5D	5'-G CAGTGTTTGATTGACAATAATC
5U	5'-G GAATTCACCCCCAGTTTTAATACTTCA
6D	5'-G GAATTCAATGACTCTTAGCTAAAATCTC
6U	5'-A ATATTCCTGCTACTATATTCCC
7D	5'-T CAGTGCGTTAGTAATCCTGTAA
7U	5'-A AAGCTTGGGCTGTGTTCTTGAGGAAGG

[a]D denotes a downstream-directed primer and U an upstream-directed primer.

(b) Comparative aspects of the 5′ flanking sequences of exon 1a

In Fig. 3, the partial nt sequences for exons 1a and their upstream flanking regions are compared for the *CA I* of human, three great apes (chimpanzee, gorilla, orangutan), an Old World monkey (pigtail macaque), a New World monkey (squirrel monkey), and the house mouse. As can be seen in Table III, the human, great apes, and macaque sequences are highly conserved.

Several potential transcription factor consensus binding sequences common to primates and mouse (i.e., CACCC, GATA-1, Oct-1, TATA) are identified in Fig. 3 which may play a role in the erythroid-specific expression of *CA I* mRNA. Of these, the GATA-1 sequence at −190 has been implicated by footprinting analysis in the erythroid expression of the mouse *CA I* (Butterworth et al., 1991). Two DNase I hypersensitivity regions have been described upstream from exon 1a in the mouse and human genes (Thiefelder et al., 1991; Sowden et al., 1992). The most distal of these are 1.0 and 1.5 kb from the *tsp* of exon 1a in human and mouse, respectively, while the other two are about 200 bp from the cap site of exon 1a in both species. It is possible that the GATA-1 site at nt position −190 coincides with the distal DNase I hypersensitivity region. In addition, three sequences of 5 or more nt that have been conserved in all species are indicated in Fig. 3.

TABLE II

Comparison of aa and nt sequences of CA I from human (Hu), chimpanzee (Ch), gorilla (Go), orangutan (Or), and pigtail macaque (Pm)[a]

	Hu	Ch	Go	Or	Pm
Hu	–	2 (0.77)	5 (1.9)	7 (2.7)	12 (4.6)
Ch	6 (0.77)	–	6 (2.3)	7 (2.7)	12 (4.6)
Go	11 (1.4)	8 (1.0)	–	7 (2.7)	12 (4.6)
Or				–	17 (6.5)
Pm	29 (3.7)	24 (3.0)	23 (2.9)		

[a]Above diagonal, aa difference followed by the percentage difference in parentheses; below diagonal, nt difference followed by the percentage difference. The nt sequence is not available for orangutan. Sources: human CA I (Lowe et al., 1990), orangutan CA I (R.E. Ferrell in Tashian, 1977), pigtail macaque CA I (Nicewander, 1990).

TABLE III

Comparison of nt differences in exon 1a and 5′ flanking sequences for the *CA I* of human (Hu), chimpanzee (Ch), gorilla (Go), orangutan (Or), pigtail macaque (Pm), squirrel monkey (Sm), and mouse (Mo)[a]

	Ch	Go	Or	Pm	Sm	Mo
Hu	4 (1.7)	6 (2.5)	10 (4.2)	10 (4.2)	25 (12.4)	87 (37.1)
Ch	–	7 (2.9)	7 (2.9)	11 (4.7)	27 (13.4)	88 (37.6)
Go		–	7 (2.9)	11 (4.7)	22 (10.9)	88 (37.6)
Or			–	13 (5.5)	26 (11.1)	88 (37.6)
Pm				–	25 (12.4)	77 (32.9)
Sm					–	78 (38.8)

[a]The nt differences between species are given followed by the percentage difference in parentheses. Gaps and insertions are counted as a single mutation. Alignments are for the nt positions between the vertical arrows in Fig. 3 except for squirrel monkey, which lacks the first 25 5′ nt.

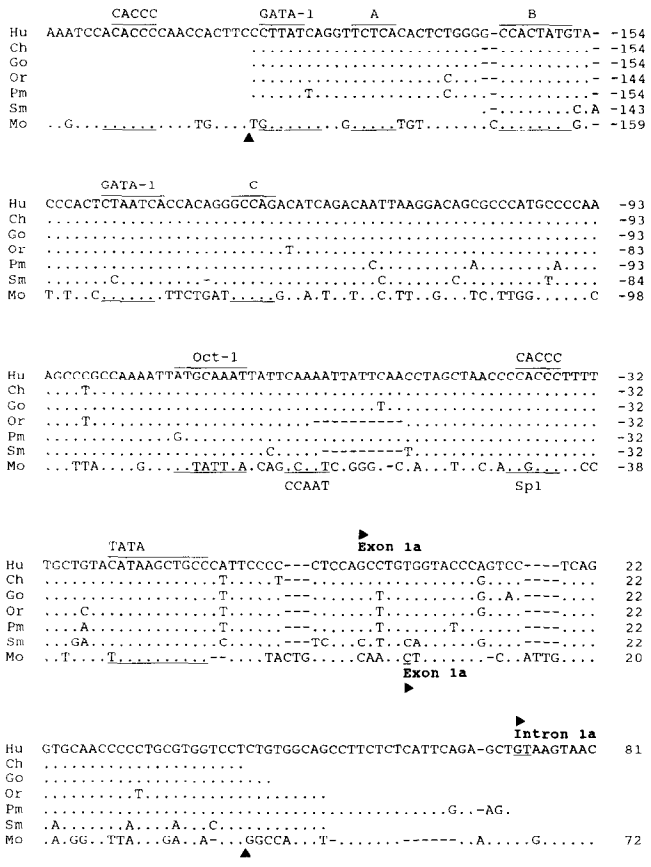

Fig. 3. Comparative nt sequences of exons 1a and 5′ flanking regions of the *CA I* of human (Hu), chimpanzee (Ch), gorilla (Go), orangutan (Or), pigtail macaque (Pm), squirrel monkey (Sm), and mouse (Mo). Dots indicate identical nt; dashes, deletions; blank spaces, regions not sequenced. **Materials:** Genomic DNA from chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), squirrel monkey (*Saimiri sciureus*), and orangutan (*Pongo pygmaeus*) was provided by Dr. Jerry Slightom (Upjohn Co., Kalamazoo, MI), and the DNA from pigtail macaque (*Macaca nemestrina*) was prepared in our laboratory from tissue samples provided by the University of Washington Regional Primate Center (Seattle, WA). **Methods:** PCR amplification from genomic DNA and direct sequencing of PCR products were as described in the legend to Fig. 1. Exon 1a from squirrel monkey was amplified by PCR primers with artificial linkers (Table I), and the PCR products were sequenced after purification and subcloning into pBSM13$^-$.

In Table III, the nt sequences at the 5′ end of exons 1a and adjacent 5′ flanking regions (i.e., nt positions between the vertical arrows in Fig. 3) are compared for the *CA I* of human, three great apes, an Old World monkey, a New World monkey, and house mouse. Although the human sequence differed less from chimpanzee than from gorilla by 0.8%, when all seven sequences were analyzed statistically by the tree-building computer programs discussed in section **a** above, a similar pattern of trichotomy was seen for the branching of the human, chimpanzee, and gorilla lineages from a common ancestor (data not shown).

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