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Short sequence-paper

Random nucleotide substitutions in primate nonfunctional gene for L-gulono-γ-lactone oxidase, the missing enzyme in L-ascorbic acid biosynthesis[1]

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Abstract

Humans and other primates have no functional gene for L-gulono-γ-lactone oxidase that catalyzes the last step of L-ascorbic acid biosynthesis. The 164-nucleotide sequence of exon X of the gene was compared among human, chimpanzee, orangutan, and macaque, and it was found that nucleotide substitutions had occurred at random throughout the sequence with a single nucleotide deletion, indicating that the primate L-gulono-γ-lactone oxidase genes are a typical example of pseudogene.

Keywords: L-Gulono-γ-lactone oxidase; Evolution; Nucleotide sequence; Polymerase chain reaction; Primate

Most higher animals can synthesize L-ascorbic acid; however, humans, other primates, and guinea pigs are exceptions that cannot do so and, as a consequence, are subject to scurvy if their diet is deficient in vitamin C. Their inability of the biosynthesis was traced to the deficiency in L-gulono-γ-lactone oxidase (GLO) that catalyzes the last step of L-ascorbic acid biosynthesis [1]. Our previous study by genomic Southern hybridization demonstrated that the human and guinea pig genomes contain nucleotide sequences that are homologous to that of the functional GLO gene of the rat [2]. Sequencing analysis of these GLO gene homologues revealed that their structural organization as well as their nucleotide sequence is altered to a great extent in comparison with the rat GLO gene, which consists of 12 exons and 11 introns [3,4]. We concluded, therefore, that the human and guinea pig GLO gene homologues currently exist as pseudogenes in their genomes. In this study, we have amplified, by PCR, fragments of GLO gene homologues from genomic DNAs of chimpanzee, orangutan, and macaque and analyzed their sequences to gain quantitative information about the nucleotide substitutions that occurred since the GLO gene of these species' ancestor lost its function and became free of functional restriction during evolution.

For amplification of the nucleotide sequence homologous to exon X of rat GLO gene from chimpanzee and macaque DNA, a pair of primers, 5′-CTGGGAACCTGTGCAGAGTCTTGAG-3′ as a sense primer and 5′-GAAAGATTGGAGAAGGAGGAGGTC-3′ as an antisense primer, were synthesized according to the sequence of the human

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[1] The nucleotide sequences reported in this paper have been submitted to GenBank under accession Nos. AB025719, AB025786, and AB025787.

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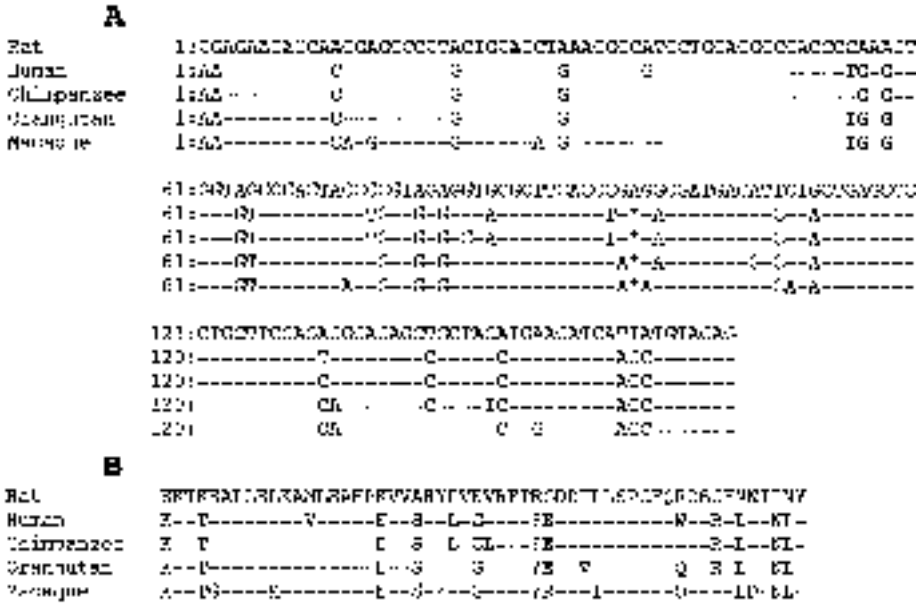

Fig. 1. Comparisons of nucleotide sequences of GLO gene exon X and their deduced amino acid sequences between rat and primates. (A) The sequences determined for chimpanzee, orangutan, and macaque are compared with the previously reported sequences of rat [3] and human [4]. A hyphen indicates that the nucleotide is the same as that of the rat sequence. An asterisk indicates deletion of a nucleotide. (B) The amino acid sequences deduced from the nucleotide sequences are aligned. An asterisk represents a stop codon. A question mark indicates the position of the single nucleotide deletion.

GLO gene homologue [4]. PCR was carried out in a 50 μl mixture containing 0.5 μg of heat-denatured genomic DNA (primate PCRable DNAs, BIOS Laboratories, New Heaven, CT), a 1 μM concentration of each primer, 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM ammonium sulfate, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 μg/ml bovine serum albumin, 1.25 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and a 200 μM concentration of each of dNTPs. In the amplification of orangutan DNA, the sense primer used was 5′-TATGAGGAAGGGGCCTTG-GAAGAG-3′. The first PCR cycle consisted of a denaturation step (94°C, 2 min), annealing step (55°C, 30 s), and elongation step (72°C, 2 min). In the subsequent 30 cycles, the denaturation was 30 s; the annealing 30 s; and the elongation 2 min. For the final step only, the elongation was 4 min. By this procedure, PCR products of the expected length, approx. 330 base pairs for human, chimpanzee, and macaque and approx. 410 base pairs for orangutan, were generated from genomic DNAs of the respective species (data not shown). Their nucleotide sequences were determined as follows. The PCR products were precipitated with isopropanol, and subcloned into the *Sma*I site of pUC 19 with *Escherichia coli* JM 109 as a host. The plasmids were prepared essentially as described by Kovalenko et al. [5], and the nucleotide sequences of their insert DNAs were read from both directions by use of a PRISM dye primer cycle sequencing kit (Applied Biosystems, Foster City, CA) on an automatic DNA sequencer (Model 373A, Applied Biosystems). The determined sequences were aligned together with the sequence of the rat gene as shown in Fig. 1A. Eighteen out of the

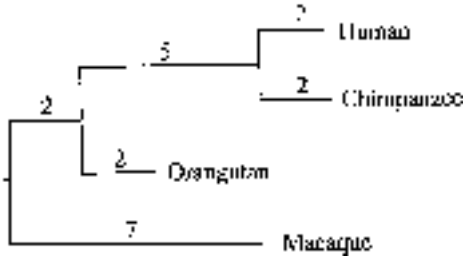

Fig. 2. The numbers of nucleotide substitutions that occurred in the exon X sequence of four primate GLO genes. Numbers represent the numbers of substitutions that were counted from the sequence data of Fig. 1A by regressing the indicated evolutional order of divergence.

Table 1
The number of synonymous and nonsynonymous substitutions between primate species

Species compared	Number of synonymous substitutions (substitutions/site)	Number of nonsynonymous substitutions (substitutions/site)
Human vs. chimpanzee	0.026	0.025
Human vs. orangutan	0.052	0.041
Human vs. macaque	0.054	0.092
Chimpanzee vs. orangutan	0.080	0.042
Chimpanzee vs. macaque	0.081	0.084
Orangutan vs. macaque	0.055	0.067

164 nucleotides compared are common to the primate species but different from the respective corresponding nucleotides of the rat sequence, indicating that the nucleotide substitutions at these positions occurred after the divergence of those primates from the rat. It is also noted that the nucleotide at position 97 of the rat sequence is deleted in all of the primates. Basing on the order of branching of the respective primates (4.9 million years (Myr) ago for the human-chimpanzee split [8], 13 Myr ago for the human/chimpanzee-orangutan split [9], and 20 Myr ago [10] for the human/chimpanzee/orangutan-macaque split), we counted the number of substitutions that had occurred after the ape-Old World monkey split, and found that there were nine, four, and seven substitutions for the human and chimpanzee lineages, the orangutan lineage, and the macaque lineage, respectively (Fig. 2). From these values, the evolutional rate of nucleotide substitutions were calculated to be 2.75×10^{-9}, 1.20×10^{-9}, and 2.15×10^{-9} substitutions/site/year, for the respective lineages. The values except for orangutan are close to the average synonymous (silent) substitution rate (2.3×10^{-9} substitutions/site/year) estimated by comparison of six kinds of genes between human and Old World monkey [11]. Thus, it is concluded that since the GLO gene had lost its function, the nucleotide substitutions in the lineages of human, chimpanzee, and macaque occurred at a neutral mutation rate. The small value for orangutan is possibly due to stochastic variance in the position of substitution because the number of nucleotides examined is small.

The above conclusion was substantiated by an analysis of the sequence data of Fig. 1A for the number of nucleotide substitutions per synonymous site and per nonsynonymous (amino acid-altering) site between pairs of the primate GLO gene homologues. For this calculation the method of Miyata and Yasunaga [6] was used and the obtained values were corrected for superimposed substitutions to obtain the corrected number of nucleotide substitutions for the respective sites [7] (Table 1). In the case of functional genes, nonsynonymous substitutions generally occur less frequently than the synonymous substitutions, because substitutions in the former are restricted by the selective pressure during evolution. However, since the GLO genes of the primate species became nonfunctional and free of functional restriction, the nonsynonymous substitutions would be expected to occur as frequently as the synonymous ones. This was found to be the case for the estimates between most of the pairs (Table 1).

Next, we compared the amino acid sequences deduced from exon X sequences of primate GLO gene homologues with the corresponding sequence of the functional gene of the rat (Fig. 1B). The result showed that many of the amino acid substitutions are nonconservative; in relation to this point, it should be emphasized that all of the primate species examined had a single nucleotide deletion at position 79 which causes a frameshift. These findings indicate again that the mutations in the primate GLO genes occurred without functional restriction after the loss of its function. In conclusion, the results presented in this study provide molecular evidence that the primates' loss of GLO [12] which resulted in the incapability of vitamin C synthesis goes back to dates at least before separation of apes and Old World monkeys, and that primate GLO gene homologues are regarded as a typical example of pseudogene in view of their nonfunctional state and the rapid evolutional rate.

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