Evolutionary Conservation of 5' upstream Sequence of Nine Genes between Human and Great Apes

Takashi Kitano[†] and Naruya Saitou*

Division of Population Genetics, National Institute of Genetics Mishima, 411-8540, Japan

Nucleotide sequences of nine 5' upstream gene regions for human, chimpanzee, gorilla, and orangutan were determined. We estimated nucleotide differences (d) for each region between human and great apes. The overall d was 0.027 (ranged from 0.004 to 0.052). Rates of nucleotide substitution were estimated by using d and divergence times of human, chimpanzee, gorilla, and orangutan. The overall rate of nucleotide substitution between human and other hominoids was estimated to be 0.52–0.85×10^{-9}. This rate in 5' upstream regions was lower than that of synonymous sites, suggesting that 5' upstream regions have evolved under some functional constraints. Because lower rates have been reported for coding sequences in primates compared to rodents, we also estimated the rate (1.17–1.76×10^{-9}) of nucleotide substitutions for the corresponding 5' upstream regions in rodents (mouse/rat comparison). Thus the primate rate was lower than rodent rate also for the 5' upstream regions.

Key words: human, great apes, rodents, upstream region, evolutionary rate

INTRODUCTION

Comparison of human and other hominoids is definitely necessary to understand human evolution (e.g., see Saitou 2005). Chimpanzee chromosome 22, orthologous to human chromosome 21, have been fully sequenced recently for human-chimpanzee comparative genomics (The International Chimpanzee Chromosome 22 Consortium, 2004), however, we need outgroup species to infer human specific changes. There are such gene comparison studies of human and other hominoids (Chen and Li 2001; O'hUigin et al. 2002; Wildman et al. 2003; Kitano et al. 2004). Those studies mainly used protein-coding sequence data. Some studies (Chen and Li 2001; Yi et al. 2002) compared human and other hominoid non-coding sequence data. However, data from 5' upstream non-coding regions are much less abundant than data from coding regions. 5' upstream non-coding regions of genes have important role for controlling gene expression. Accumulation of analyses of these regions between human and other hominoids is important to understand human evolution.

The nucleotide substitutional difference between the human and chimpanzee genomes was estimated to be 1.23% based on a total of 19,813,086 bp BAC end sequence comparison (Fujiyama et al. 2002), while the whole chromosome comparison between human chromosome 21 and chimpanzee chromosome 22 showed somewhat higher value, 1.44% (The International Chimpanzee Chromosome 22 Consortium, 2004). Many of those differences are probably caused by mutations occurred in so-called junk DNA (Ohno 1972), and have no effect on phenotypic difference between human and chimpanzee. Some proportion of nucleotide changes, however, must be responsible for human-specific characters, such as large brain size, bipedalism, etc. King and Wilson (1975) proposed that genetic changes at the gene expression control region are more important than changes in the protein-coding region.

We thus estimated the rate of nucleotide substitution of 5' upstream gene region in hominoids in this study. We then compared it with corresponding synonymous substitution rates. These rates were also compared with those for mouse (*Mus musculus*) and rat (*Rattus norvegicus*). Finally, implication of human specific changes in the 5' upstream region is discussed.

MATERIALS AND METHODS

We determined nine 5' upstream gene regions (see Table 1) for human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), and orangutan (*Pongo pygmaeus*) in this study. Six genes (DDC, DMD,

Table 1. List of genes whose 5' upstream regions were compared

Symbol	Name	Chromosomal location
DDC	dopa decarboxylase	7p11
DMD-1	dystrophin	Xp21.2
DMD-2	dystrophin	Xp21.2
INMT	indolethylamine N-methyltransferase	7p15.3-p15.2
MAOA	monoamine oxidase A	Xp11.4-p11.3
NEF3	neurofilament 3	8p21
NGFB	nerve growth factor, beta polypeptide	1p13.1
PLP2	proteolipid protein 2	Xp11.23
POU3F2	POU domain, class 3, transcription factor 2	6q16

We used description in the NCBI database on gene symbols, names, and chromosome locations.

MAOA, NEF3, NGFB, and POU3F2) are brain and/or neuron related genes. Two alternative 5' regions were sequenced for the DMD gene. The 5' upstream regions for two non brain-neuron specific genes (INMT and PLP2) were also sequenced. INMT is known to be expressed in several human tissues such as adult thyroid, adult adrenal gland, adult lung, and fetal lung (Thompson et al. 1999), and PLP2 is expressed in colonic epithelial cell (Olivia et al. 1993).

DNA was extracted from peripheral blood sample of Japanese individuals with informed consent for human samples. DNA samples for chimpanzee, gorilla and orangutan were also used, and those samples were the same as used by Kitano et al. (2004). We sequenced two human samples and two chimpanzee samples for each DMD-1, NGFB, and POU3F2 gene, and these two sequences were identical with each other (data not shown). Hot-start PCR was applied to each selected segment (PE GeneAmp PCR system 2400 and 9700). All PCR primers were designed based on human sequences in the database. A list of primers used in this study is available from TK. PCR products were confirmed by 1.5% agarose gel electrophoresis, and purified using Micro Spin Columns (Amersham Biosciences). The purified PCR products were sequenced by using BigDye Terminator Cycle Sequencing Kit and ABI PRISM 377/310 DNA sequencer (PE Biosystems). Both strands were read using PCR primers and inner primers when long sequences were read. Overlapping two peaks on a site was regarded as heterozygous and the site was designated by following Nomenclature Committee of the International Union of Biochemistry (NC-IUB).

CLUSTAL W ver.1.8 (Thompson et al. 1994) was used for multiple alignments. Aligned regions without gaps were used for following analyses. Numbers of nucleotide substitutions per site (d) were estimated by using one-parameter method (Jukes and Cantor 1969). Numbers of synonymous substitutions per synonymous site were estimated by using Nei and Gojobori's (1986) method.

Figure 1 shows 5' upstream regions of eight genes whose sequences were determined in this study. Because the first exons of two genes (DDC and NGFB) were non-coding exons, start codons were not shown in the figure. Both alternative non-coding exons of DMD were also sequenced. DMD-1 and DMD-2 correspond to the brain type (Boyce et al. 1991) and the Purkinje cell type (Holder et al. 1996), respectively. For simplicity, we used terms 'DMD-1' and 'DMD-2' in this paper.

RESULTS

We determined nine 5' upstream regions for four hominoid species (human, chimpanzee, gorilla, and orangutan), and their DDBJ/EMBL/GenBank International Nucleotide Sequence Database accession numbers are listed in Table 2. A total of 4157 nucleotide sites could be compared, and 108 variant sites were detected. Variant nucleotide sites are shown in Table 3. Two sites (120 of NEF3 and 218 of NGFB) had three character states. Therefore, a total of 110 changes are necessary to explain these variant sites.

Because numbers of nucleotide substitutions on each branch were low, we added nucleotide substitutions occurred on all branches of Fig. 2. The total number of nucleotide substitutions among human, chimpanzee, gorilla, and orangutan (d) were estimated from the total number of nucleotide changes (m) among human, chimpanzee, gorilla, and orangutan. The total d for hominoids was 0.027 ± 0.003. Divergence times of human, chimpanzee, gorilla, and orangutan are shown in Fig. 2 following Chen and Li (2001). By adding divergence times of all branches, the total time was 34.8–46.6 MY (million years), and this value was used to estimate rates of nucleotide substitution. The total rate ranged from 0.52×10^{-9} to 0.85×10^{-9}. There were no specific rate differences between brain-neuron expressed genes (DDC, DMD-1, DMD-2, MAOA, NEF3, NGFB, and POU3F2) and the remaining genes (INMT and PLP2).
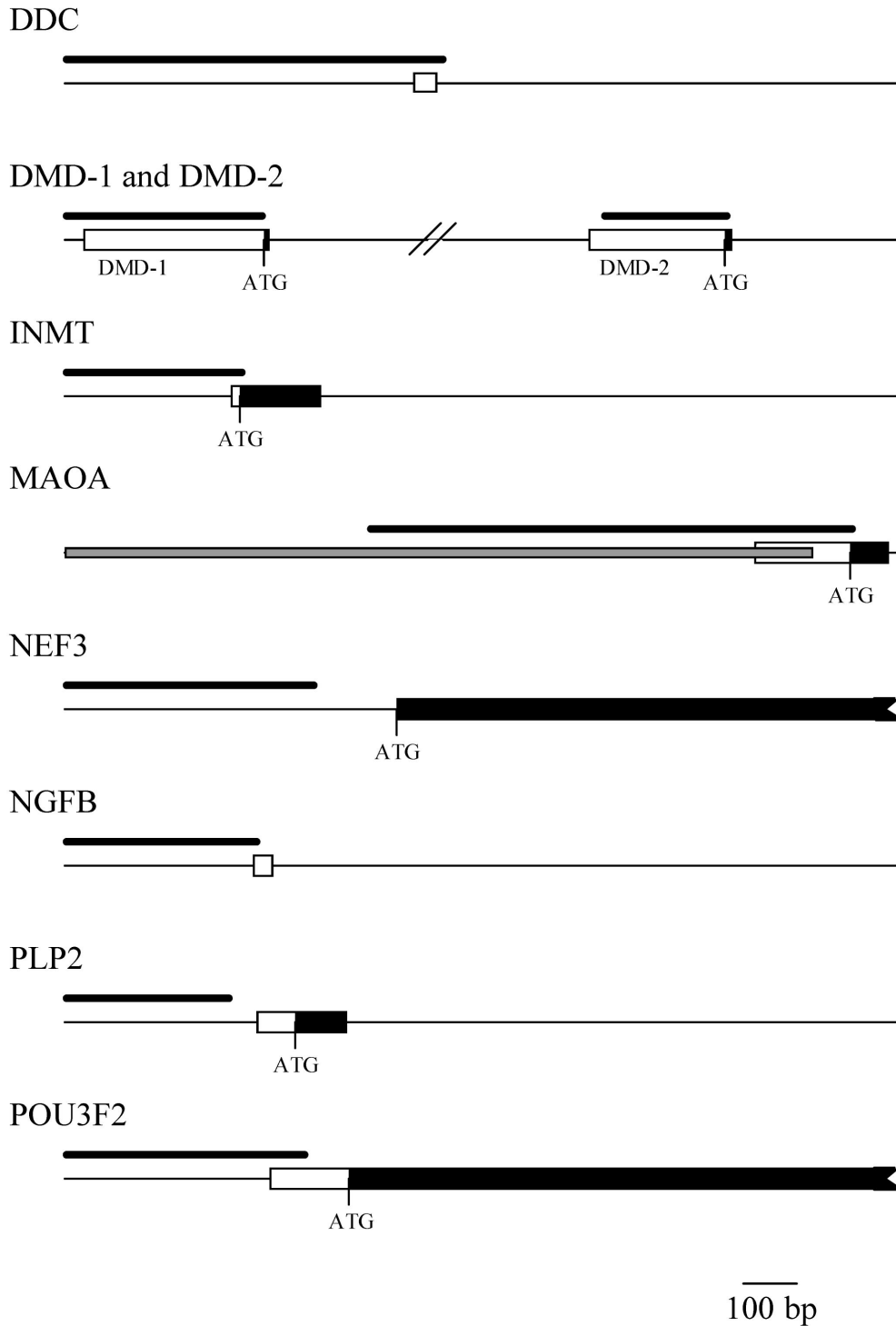
Fig. 1.   Genome structures of the nine sequenced regions.   Coding and non-coding exons are shown by close and open boxes, respectively.   Thick bars show sequenced regions.   5' upstream region of MAOA overlaps with 3' region of LOC352786 indicated by a grey box.

We used BLAST homology search system to retrieve homologous sequences from rat and mouse from the genome database.   However, we could retrieve mouse and rat counterparts only for DMD-1, DMD-2, and POU3F2. Because the regions around translation and/or transcrip- tion start sites of those six remaining sequences had homology between human and mouse-rat, we retrieved same length of sequences with hominoids from regions just upstream of these homologous regions, and used them as possible mouse and rat counterpart sequences (see

Table 2.　List of DDBJ/EMBL/GenBank accession numbers* and NCBI gene numbers whose nucleotide sequences were compared

(A) 5' upstream region

Symbol	Human, Chimpanzee, Gorilla, Orangutan, Mouse, Rat
DDC	AB037497, AB037498, AB037499, AB037500, NT_039515, NW_047430
DMD-1	AB037487, AB037489, AB037491, AB037492, NT_039709, NW_044437
DMD-2	AB037493, AB037494, AB037495, AB037496, NT_039709, NW_044437
INMT	AB041362, AB041363, AB041364, AB041365, NT_039343, NW_047693
MAOA	AB042829, AB042830, AB042831, AB042832, NT_039700, NW_048034
NEF3	AB042833, AB042834, AB042835, AB042836, NT_039606, NW_047454
NGFB	AB037481, AB037483, AB037485, AB037486, NT_039239, NW_047627
PLP2	AB041366, AB041367, AB041368, AB041369, NT_039700, NW_048035
POU3F2	AB037475, AB037477, AB037479, AB037480, M88300, NW_043830

(B) CDS region

Symbol	Human, Chimpanzee, Mouse, Rat
DDC	M76180, XM_519096, AF071068, BC087032
INMT	AF128847, XM_527713, M88694, XM_347233
MAOA	BC008064, #, BC029100, XM_343764
NEF3	–, –, X05640, Z12152
NGFB	X52599, AY665265, M17298, M36589
PLP2	L09604, AC149133, AK003522, BC061844
POU3F2	–, –, M88300, XM_345510

*DDBJ/EMBL/GenBank accession numbers starting with AB listed above were determined in this study. All of them are 5' upstream regions for hominoids.　#: Thirteen entries (AY684852, AY684860, AY685683, AY685686, AY685688, AY685690, AY685692, AY685694, AY685696, AY685698, AY685700, AY685702, and AY685704) were used for chimpanzee MAOA.　–: not analyzed.

Table 3.　Variant nucleotide sites of nine 5' upstream regions of human, chimpanzee, gorilla, and orangutan

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                DDC                      DMD-1    DMD-2         INMT
      1 1 1 1 2 2 2 2 3 3 3 3 3 4 4 4 4 4 5 6   1 2    1 1    1 1 1 1 1 1 1 1 2 2 3
      1 4 8 9 9 0 7 7 8 1 3 3 4 1 3 5 5 5 2 5 5 7 8 0 1   5 7 6 1  2 5 4 8  1 1 3 6 7 2 6 6 6 6 7 8 9 1 8 0
      3 5 8 4 6 6 0 1 7 3 4 8 5 8 2 0 1 9 8 1 6 1 3 0 2   8 7 6 3  3 1 0 2  0 2 6 1 3 7 1 3 4 5 5 3 6 9 0 3
Hum   C G C G G T A T A C C A C C C T C A G G T A A C C   C C A A  C T A T  G C C G G G G G A G C G C A C T
Chi   C G C G C T A T A C C A C C T G G G G C T A A G C   C C G A  C C A T  A T C A G G A A T G G G C G C T
Gor   C G C A G T A T G C C A C T C G G G A C C C A C G   C C A A  C T G C  G C T G G C G G A G G G C G T C
Ora   T A T G G G T C A A T G G C C G G G G C C C G C G   G T G G  T T A T  G C C G A G G G A A G C G G C C
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                MAOA                            NEF3                 NGFB    PLP2       POU3F2
      1 1 1 1 2 2 2 3 3 3 3 4 5 6 6 6 6 6 7 7 7 8 9      1 1 1 1 2 2 2 3 3 3 3 3 4    1 1 2 2    1 1 1 1 2 2 2 2    4
      2 6 2 3 4 9 5 8 9 0 0 7 8 0 4 2 5 6 9 4 6 9 4 0    3 3 6 7 9 0 2 6 6 5 6 6 2 4 5 8 8 0   3 6 0 8 1 1  3 4 5 8 8 0 0 2 7   7 4
      0 7 2 5 3 6 1 4 8 2 4 7 2 0 3 0 8 1 8 1 8 9 3 6    1 4 9 4 5 2 0 1 3 2 0 6 3 7 7 2 3 9   3 4 6 4 0 8  2 1 5 2 3 4 6 8 4   4 7
Hum   C C T T C G T A T A T C A T G G G G C G T G G C    A T T T A G G T A T G G G T C C C G   G C T G G A  C C C C T C G T C   C C
Chi   T C T T C G T A C A T C A T G A G G C G T G G C    A T T T A C G T A C G A G T C A A G   G C T G G C  C C C C T C G T C   C C
Gor   T C T C T G T T C A T C A T T A G G T G C G G C    A T T C A G C T A C G G G A G C C A   G C G G G C  C C C C T C G T C   C C
Ora   T T C C C C C T C G C T G G G A A A C A T C A A    G C C T G G A C G C C G A T C C C G   C A T C A T  T T T A G A A C A   T G
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Hum, Chi, Gor, and Ora denote human, chimpanzee, gorilla, and orangutan, respectively. Numbers indicate nucleotide positions of each DNA region.

Table 2 for list of sequence IDs used for comparison). The total d between mouse and rat was 0.100 ± 0.005 (Table 4). Since the divergence time between mouse and rat is a matter of argument (Wilson et al. 1977; Jacobs. and Pilbeam 1980), we used two kinds of divergence times between mouse ,and rat to estimate rates of nucleotide substitutions; 40.7 MYA (million years ago) (Kumar and Hedges 1998) and 30 MYA (Kitano et al. 1999). The total rate ranged from 1.17×10^{-9} to 1.76×10^{-9}. The total rate of nucleotide substitution in hominoids was lower than

that in rodents.

Figure 3 shows relationship of d between hominoids and rodents for each region. Hominoid d and rodent d are positively correlated (correlation coefficient is 0.63). This is mainly because three regions (DMD-1, DMD-2, and POU3F2; shown in gray circle in Fig. 3) where primates-rodents homology was found low d both in hominoids and mouse/rat, while the remaining six regions, in which no clear homology was found between hominoids and mouse/rat, showed higher d both in hominoids and
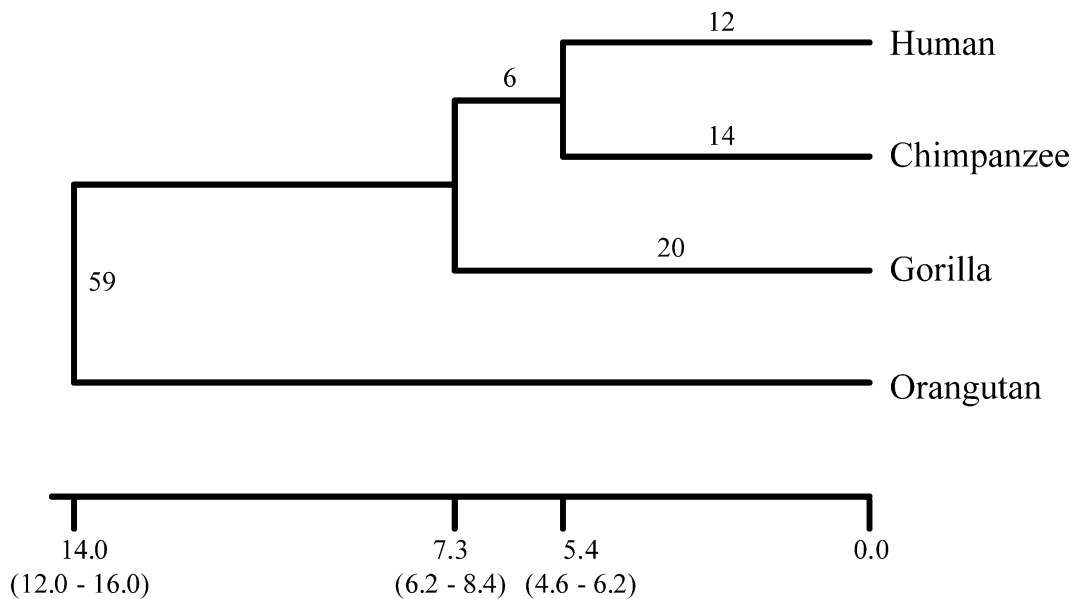

Fig. 2. A species tree of human, chimpanzee, gorilla, and orangutan. Numbers of each branch indicate nucleotide changes. Divergence times for three speciation events are shown on the time scale bar. Ranges of divergence times (MY, million years) are shown in parentheses.

Table 4. Nucleotide differences and substitution rate for 5' upstream regions of nine protein coding genes for Hominoids and Rodents

Gene	Hominoids			Rodents (mouse vs. rat)		
	m	bp	d	m	bp	d
DDC	25	718	0.036 ± 0.007	70	718	0.104 ± 0.013
DMD-1*	4	372	0.011 ± 0.005	1	238	0.004 ± 0.004
DMD-2*	4	232	0.017 ± 0.009	11	207	0.055 ± 0.017
INMT	16	321	0.052 ± 0.013	35	321	0.118 ± 0.020
MAOA	24	917	0.027 ± 0.005	125	917	0.150 ± 0.014
NEF3	19	469	0.042 ± 0.010	56	469	0.130 ± 0.018
NGFB	7	364	0.019 ± 0.007	42	364	0.125 ± 0.020
PLP2	9	311	0.030 ± 0.010	15	311	0.050 ± 0.013
POU3F2*	2	453	0.004 ± 0.003	19	437	0.045 ± 0.010
Total	110	4157	0.027 ± 0.003	374	3982	0.100 ± 0.005

m: number of nucleotide changes among human, chimpanzee, gorilla, and orangutan, bp: number of compared sites, d: number of nucleotide substitutions per site.
*Mouse and rat sequences were found to be homologous to hominoid ones by BLAST homology search (NCBI mouse and rat genome).
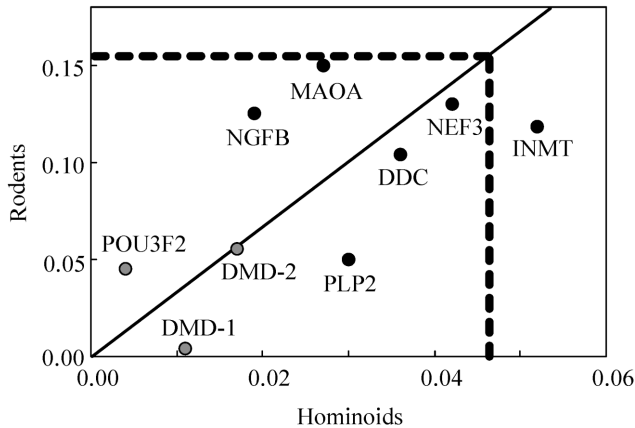
Fig. 3. Comparison of evolutionary distances for ten 5' upstream regions between rodents and hominoids, based on data shown in Table 4. Three regions designated with gray circles are those with asterisks in Table 4. Two broken lines represent evolutionary distances for rodents (horizontal) and for hominoids (vertical) under the pure neutral evolution. The genomic divergence between mouse and rat (Table 6) was used as the pure neutral evolutionary distance for rodents. The value of genomic divergence between human and chimpanzee (0.012) multiplied by 3.77 was used as the pure neutral evolutionary distance for hominoids, because the total time of all branches (34.8–46.6 MY, mean 40.7 MY) was 3.77 times higher than the time between human and chimpanzee (10.8 MY = 5.4 × 2). The diagonal line represents equal selective constraints between rodents and hominoids. This line is through the origin and the intersection of the two broken lines.

mouse/rat. This suggests that relative intensity of functional constraints had been more or less constant through the mammalian evolution. One 5' upstream region for hominoids, INMT, showed the evolutionary distance larger than that expected under pure neutral evolution

(see Fig. 3). This suggests that the 5' upstream region of this gene reduced its importance in the primate lineage.

DISCUSSION

The average rate of nucleotide substitution for human and chimpanzee non-coding DNA has been reported to be 0.99×10^{-9} (Yi et al. 2002) or 0.86×10^{-9} (Wildman et al. 2003). The total rate of the 5' upstream region of hominoids (from 0.52×10^{-9} to 0.85×10^{-9}) estimated by this study was lower than those of previous results. We estimated the rate of nucleotide substitution by using human, chimpanzee, gorilla, and orangutan data sets. When we estimate rates of nucleotide substitution by using only human and chimpanzee, the overall rate for human and chimpanzee ranged from 0.41×10^{-9} to 0.82×10^{-9} ($m = 26$, $d = 0.006 \pm 0.001$). Divergence time (4.6–6.2 MYA, see Fig. 2) between human and chimpanzee was used estimate the rate of nucleotide substitutions. The overall rate of human and chimpanzee was still lower than those of previous reports (Yi et al. 2002; Wildman et al. 2003).

Rates of nucleotide substitution of various regions were summarized as nonsynonymous (0.85×10^{-9}), synonymous (4.61×10^{-9}), 5' UTR (1.96×10^{-9}), 3' UTR (2.10×10^{-9}), introns (3.14×10^{-9}) and pseudogenes (3.52×10^{-9}) in various mammalian genes (Li and Graur 1991). It is shown that the rate of nucleotide substitution in 5' upstream regions is lower than those in other regions except for nonsynonymous sites. Studies of divergence between human and chimpanzee (Chen et al. 2001; Shi et al. 2003) also showed similar tendency. 5' upstream regions do not code gene products, but these regions probably have functional roles to gene expression. Our result showed

Table 5. Nucleotide differences and substitution rate for synonymous sites of five protein coding genes for Hominoids (human vs. chimpanzee) and seven genes for Rodents (mouse vs. rat)

CDS region	Hominoids*			Rodents**		
	s	S	dS	s	S	dS
DDC	5	307.5	0.016 ± 0.007	78.5	349.33	0.267 ± 0.032
INMT	1	112.42	0.009 ± 0.009	48	199	0.291 ± 0.045
MAOA	9	371.5	0.025 ± 0.008	41	372.58	0.119 ± 0.019
NEF3	–	–	–	93.5	547.42	0.194 ± 0.021
NGFB	5	173.83	0.029 ± 0.013	24	176.58	0.150 ± 0.031
PLP2	3	119.67	0.025 ± 0.015	16	117.33	0.151 ± 0.039
POU3F2	–	–	–	25	320.5	0.082 ± 0.017
Total	23	1084.92	0.021 ± 0.005	326	2082.74	0.176 ± 0.010

s: number of synonymous changes, S: number of synonymous sites, dS: number of synonymous substitutions per synonymous site, –: not analyzed genes.
DMD gene coding region sequences were not available neither for hominoids nor murids, so this gene was not listed in this table.
*Comparison between human and chimpanzee.   **Comparison between mouse and rat.

Table 6.   Comparison of nucleotide differences of human-chimpanzee and mouse-rat

Region	d(Human-Chimpanzee)	d(Mouse-Rat)	d(M-R)/d(H-C)
5' upstream	0.006 ± 0.001	0.100 ± 0.005	16.7
Synonymous	0.021 ± 0.005	0.176 ± 0.010	8.4
Genomic	0.012*	0.155**	12.9

*From Fujiyama et al. (2002).   **From Abe et al. (2004).

Table 7.   Differences between human and other hominoid on possible transcription factor binding sites

Gene	Site	Human sequence (factor)	Ape sequence (factor)
DDC	451	TCGCCA (F-ACT1)	TCCCCA (AP-2alphaA)
	451	GCCAGTC (CP2)	CCCAGTC (–)
INMT	219	AAGGAA (c-Ets-2)	AAGGAG (–)
	219	GGAAAG (NF-1)	GGAGAG (–)
MAOA	620	CAGCTG (XPF-1)	CAACTG (c-Myb)
NEF3	252	CTGCCT (–)	CTGCCC (Sp1)
NGFB	218	ACCACA (AML1)	ACCACY (–)
	218	CAGCCC (–)	CYGCCC (Sp1)

On a site 218 of NGFB, chimpanzee and gorilla showed 'C' and orangutan showed 'T', so 'Y' was used.

lower rate of nucleotide substitution in 5' upstream regions. This suggests that 5' upstream regions have evolved under strong functional constraints.

It has been reported that rates of coding region in hominoids are lower than those of rodents (Wu and Li 1985; Gu and Li 1992; Kitano et al. 1999). In this study, we showed that 5' upstream regions also have lower rates of substitution in hominoids than in rodents. To assess whether the lower rate of substitution in hominoids than in rodents is due to generation time effects or stronger constraint in hominoids, we estimated rate of synonymous substitutions for the same genes. For hominoid comparisons, we used distances between human and chimpanzee, because data for other hominoid species were not available. Table 5 shows the comparison of synonymous substitutions for hominoids and rodents (mouse vs. rat). We then summarized distance differences between hominoids and rodents as shown in Table 6. In all three comparisons (5' upstream region distance, synonymous distance, and genomic divergence), the evolutionary distance between mouse and rat is 8–17 times higher than that between human and chimpanzee. The ratio of the species divergence time between the two species pair, t(M-R)/t(H-C), should be at most 7 times, if there is no difference in evolutionary rate in primates and rodents. This indicates that somehow the evolutionary rate has been higher in rodents than in primates.

One good characteristic of simultaneous sequence comparison of closely related species is clear deduction of species-specific changes. We thus extracted eleven changes out of total 110 changes as human lineage specific changes (see Table 3). We searched putative transcription factor binding sites by using TESS (Transcription Element Search System) under anticipation that even one nucleotide substitution at the human lineage might caused a significant phenotypic change through change of gene expression pattern. Only five possible transcription elements differences between human and other hominoids were observed, as listed in Table 7. Those site differences may affect gene expression differences between human and other hominoids, however, we need further experiments to confirm them.

ELECTRONIC-DATABASE INFORMATION

NCBI Home Page**,** http://www.ncbi.nlm.nih.gov/
Project Silver Home Page, http://sayer.lab.nig.ac.jp/

~silver/index.html

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