Differences in DNA Methylation Patterns and Expression of the CCRK Gene in Human and Nonhuman Primate Cortices

Ruxandra Farcas,* Eberhard Schneider,* Katrin Frauenknecht,† Ivanela Kondova,‡ Ronald Bontrop,‡ Jürgen Bohl,† Bianca Navarro,§ Markus Metzler,|| Hans Zischler,¶ Ulrich Zechner,* Angelika Daser,* and Thomas Haaf**

*Institute for Human Genetics, Johannes Gutenberg University, Mainz, Germany; †Department of Neuropathology, Johannes Gutenberg University, Mainz, Germany; ‡Department of Comparative Genetics and Refinement, Biomedical Primate Research Center, Rijswijk, The Netherlands; §Institute of Legal Medicine, Johannes Gutenberg University, Mainz, Germany; ||Department of Pediatrics, University of Erlangen-Nuremberg, Erlangen, Germany; and ¶Institute for Anthropology, Johannes Gutenberg University, Mainz, Germany

Changes in DNA methylation patterns during embryo development and differentiation processes are linked to the transcriptional plasticity of our genome. However, little is known about the evolutionary conservation of DNA methylation patterns and the evolutionary impact of epigenetic differences between closely related species. Here we compared the methylation patterns of CpG islands (CGIs) in the promoter regions of seven genes in humans and chimpanzees. We identified a block of CpGs in the cell cycle–related kinase (CCRK) gene that is more methylated in the adult human cortex than in the chimpanzee cortex and, in addition, it exhibits considerable intraspecific variation both in humans and chimpanzees. The species-specifically methylated region (SMR) lies between the almost completely methylated 5′ region and the completely demethylated 3′ region of the presumed CCRK CGI promoter. It is part of an Alu-Sg1 repeat that has been integrated into the promoter region in a common ancestor of humans and New World monkeys. This SMR is relatively hypomethylated in the rhesus monkey cortex and more or less completely methylated in the baboon cortex, indicating extraordinary methylation dynamics during primate evolution. The mRNA expression level of CCRK has also changed during the course of primate evolution. CCRK is expressed at much higher levels in human and baboon cortices, which display an average SMR methylation of 70% and 100%, respectively, than in chimpanzee and rhesus macaque cortices with an average SMR methylation of 35% and 40%, respectively. The observed evolutionary dynamics suggests a possibility that CCRK has been important for evolution of the primate brain.

Introduction

Our closest extant evolutionary relatives, the chimpanzees, diverged from the human lineage only 5–8 Ma (Kumar and Hedges 1998; Haile-Selassie 2001; Enard and Paabo 2004; Benton and Donoghue 2007), and their genomic sequence is very similar to that of humans. The genetic differences between humans and chimpanzees consist of approximately 1% fixed single-nucleotide substitutions and 3% euchromatic divergence due to insertion and deletion events (Chimpanzee Sequencing and Analysis Consortium 2005; Varki and Altheide 2005). Thus, the striking species differences, that is, in cognitive abilities, must be due to changes in gene regulation rather than structural changes in the gene products. Indeed, comparative transcription analyses revealed substantial expression differences between humans and chimpanzees, in particular in their respective brain tissues. A subset of genes showed elevated expression in the human brain after the split from the chimpanzee lineage (Caceres et al. 2003; Gu and Gu 2003; Khaitovich et al. 2006).

Methylation of critical CpG dinucleotides in the cis-regulatory regions of promoters is generally thought to act as an epigenetic signal that regulates the appropriate gene expression patterns. It establishes and/or maintains an inactive chromatin structure through posttranslational histone modifications (Wolffe and Matzke 1999; Jaenisch and Bird 2003). In the “critical site” model, the methylation of specific cytosines in transcription-factor binding sites reduces binding affinity and thus, transcription. According to the “methylation density” model, the proportion of methylated cytosines across a region controls chromatin conformation and transcriptional potential.

In humans and nonhuman primates, DNA methylation is restricted to CpG dinucleotides, which are largely depleted from the genome because of their inherent mutability (deamination of methylated cytosines causing C to T transitions) (Shen et al. 1994). The most prominent compartments enriched with CpGs are repetitive DNA elements (Yoder et al. 1997), in particular Alu transposons, and CpG islands (CGIs, Ioshikhes and Zhang 2000). CGIs are 500–2,000 bp long and associated with cis-regulatory sequences in most mammalian genes. In contrast to the highly methylated CpGs in repeats, most CGIs in promoters and first exons are protected from methylation in somatic tissues (Rollins et al. 2006). Based on CpG frequency, three different classes can be distinguished. High CpG promoters (HCPs) are largely unmethylated, even when inactive. Low and intermediate CpG promoters (LCPs and ICPs) are predisposed to de novo methylation during development and differentiation (Weber et al. 2007). The somatically methylated CGIs may help to prevent ectopic gene expression.

It is tempting to speculate that changes in the methylation patterns of key genes during evolution may have preceded or dictated functional changes involving chromatin organization and/or transcription. Comparative bisulfite DNA sequencing revealed a remarkable conservation of methylation profiles between human and mouse orthologous genes in four different tissues (skin, liver, skeletal and heart muscles). Less than 5% of the analyzed loci were differentially methylated between these two evolutionarily distant species (Eckhardt et al. 2006). An array-based comparison of 145 CpG sites from 36 genes identified 18 CpGs (12 genes) that have differences in methylation between...
humans and chimpanzees (Enard et al. 2004). The observation that many more CpGs were differentially methylated in the brain of the two species than in liver or lymphocytes promotes the idea that a change in methylation patterns has contributed to the evolution of the human brain. Enlargement of the neocortex during hominoid evolution was important for the development of specific cognitive abilities in humans. Genes that have changed their regulation during evolution of the human brain may also contribute to the intraspecific variation and pathology of cognitive abilities among humans. In this light, differences in DNA methylation patterns between humans and chimpanzees can be viewed as an epigenetic footprint of genes that are crucial for human brain development and function. Here, we have compared CGI promoter methylation of seven genes in human and chimpanzee cortices. One gene, cell cycle–related kinase (CCRK), which was differentially methylated in humans and chimpanzees, was studied further in Old World monkeys.

Materials and Methods

Tissue Samples

Brain samples were obtained between 1 and 2 days postmortem from 12 humans (Homo sapiens, HSA), three chimpanzees (Pan troglodytes, PTR), one rhesus macaque (Macaca mulata, MMU), and three baboons (Papio hamadryas, PHA) (table 1). Frontal cortex tissue (area A10) was dissected by experienced neuropathologists and immediately frozen and stored at −80 °C. Human brain autopsy samples (excess materials) were from the Institute of Legal Medicine, the Department of Neuropathology, and the Department of Child Pathology at the Mainz University Medical Center. Primate samples were obtained from the Biomedical Primate Research Center, Rijswijk, Netherlands and the Primate Center, Göttingen, Germany. Genomic DNA was extracted from the cortex samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Total RNA was isolated using TRIzol Reagent (Invitrogen, Karlsruhe, Germany).

Sequence Analysis

CGISearcher and EMBOSS (http://www.ebi.ac.uk) were used to identify CGIs in putative cis-regulatory regions 500 bp downstream to 10 kb upstream of the transcription start site (TSS) of the genes of interest. When a gene contained two or more CpG rich segments, we usually analyzed the CGI nearest to the TSS. The observed CpG/expected CpG ratio was calculated as follows: (number of CpGs × number of bp)/(number of Cs × number of Gs). HCPs have a GC content of 55% and a CpG ratio of 0.75. LCPs do not contain 500-bp stretches with a CpG ratio of >0.48. ICPs are neither LCPs nor HCPs (Weber et al. 2007). Human and primate gene sequences were retrieved from the Ensembl (http://www.ensembl.org; release 44–45) and NCBI (http://www.ncbi.nlm.nih.gov; build 36) databases. Orthologous sequences were aligned with ClustalW (BioEdit-version 7.0.5.2,[6/5/05]), PCR amplified and sequenced with a Beckman Coulter CEQ 8000 Genetic Analysis System using the CEQ DTCS Quick Start Kit (Beckman Coulter, Krefeld, Germany). PCR and sequencing primers (table 2) were selected from evolutionarily conserved regions that did not exhibit nucleotide changes among humans and nonhuman primates.

Bisulfite Sequencing

Aliquots of 500 ng of genomic DNA were treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen). Sodium bisulfite converts unmethylated cytosines to uracils, which are then PCR amplified as thymines, whereas methylated cytosines remain cytosines (Frommer et al. 1992). PCR products from bisulfite-treated DNA were cloned into the pGEM-T vector (Promega, Mannheim, Germany). Plasmid DNAs were extracted and the inserts

Table 1

Cortex Samples

<table>
<thead>
<tr>
<th>Individual</th>
<th>Sex</th>
<th>Age</th>
<th>Time Postmortem</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA1</td>
<td>Female</td>
<td>66 years</td>
<td>1 day</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>HSA2</td>
<td>Female</td>
<td>80 years</td>
<td>2 days</td>
<td>Acute heart failure</td>
</tr>
<tr>
<td>HSA3</td>
<td>Female</td>
<td>83 years</td>
<td>1 day</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>HSA4</td>
<td>Female</td>
<td>82 years</td>
<td>1–2 days</td>
<td>Chronic heart insufficiency</td>
</tr>
<tr>
<td>HSA5</td>
<td>Female</td>
<td>81 years</td>
<td>1–2 days</td>
<td>Multiple organ failure</td>
</tr>
<tr>
<td>HSA6</td>
<td>Male</td>
<td>84 years</td>
<td>1 day</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>HSA7</td>
<td>Male</td>
<td>54 years</td>
<td>2 days</td>
<td>Traffic accident</td>
</tr>
<tr>
<td>HSA8</td>
<td>Male</td>
<td>31 years</td>
<td>1 day</td>
<td>Suicide</td>
</tr>
<tr>
<td>HSA9</td>
<td>Male</td>
<td>45 years</td>
<td>1–2 days</td>
<td>Tractor accident</td>
</tr>
<tr>
<td>HSA10</td>
<td>Male</td>
<td>59 years</td>
<td>3 days</td>
<td>Acute heart failure</td>
</tr>
<tr>
<td>HSA11</td>
<td>Male</td>
<td>40 years</td>
<td>1–2 days</td>
<td>Motocycle accident</td>
</tr>
<tr>
<td>HSA12</td>
<td>Female</td>
<td>17 gestat. weeks</td>
<td>1–2 days</td>
<td>Chorion amnionitis</td>
</tr>
<tr>
<td>PTR1</td>
<td>Male</td>
<td>14 years</td>
<td>12 h</td>
<td>Hemolytic anemia</td>
</tr>
<tr>
<td>PTR2</td>
<td>Male</td>
<td>7 years</td>
<td>12 h</td>
<td>Anesthesia accident</td>
</tr>
<tr>
<td>PTR3</td>
<td>Female</td>
<td>40 years</td>
<td>12 h</td>
<td>Drown in pond</td>
</tr>
<tr>
<td>MMU1</td>
<td>Female</td>
<td>24 years</td>
<td>6 h</td>
<td>Septicemia after injury</td>
</tr>
<tr>
<td>PHA1</td>
<td>Male</td>
<td>30 years</td>
<td>12 h</td>
<td>Terminated for experimental brain surgery</td>
</tr>
<tr>
<td>PHA2</td>
<td>Male</td>
<td>9 years</td>
<td>1 day</td>
<td>Terminated</td>
</tr>
<tr>
<td>PHA3</td>
<td>Male</td>
<td>9 years</td>
<td>12 h</td>
<td>Terminated</td>
</tr>
</tbody>
</table>
were sequenced with a Beckman Coulter CEQ 8000 Analysis System, using vector primers and the CEQ DTCS Quick Start Kit. One potential problem of classic bisulfite sequencing is an amplification bias in the PCR reaction that leads to preferential amplification of only a few DNA molecules from the heavily degraded bisulfite-treated starting sample, and consequently to an overrepresentation of certain alleles in the PCR product. To avoid such an amplification bias, the bisulfite-treated DNA was diluted 1:50 and 5–10 independent PCR products were generated from different aliquots of this dilution. Five to ten plasmid clones from different PCR replicates were sequenced to obtain a representative view on the methylation status of the analyzed CGI promoter. As a control, we evaluated not only the critical CpG sites, but also the conversion of non-CpG cytosines in each plasmid. This allowed us to distinguish the critical CpG sites, but also the conversion of non-CpG cytosines in each plasmid. This allowed us to distinguish different DNA molecules.

Quantitative Real-Time PCR

Aliquots of 2 μg total cortex RNA were reversely transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR analyses of CCRK were performed on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Because a commercially available QuantiTect Primer Assay did not work for all primates, we had to design our own RT-PCR primers in evolutionarily conserved regions. The forward primer (5'-AGAAAGTGGGCC-TAAGGCGGTGGG-3') is localized in CCRK exon 2 and the reverse primer (5'-GCTACCTGCAAGATGCTGCT-CAAG-3') in exon 3, spanning 2,825 bp of intronic sequence. All reactions were performed in triplicates. Each 25-μl reaction volume contained 25 ng cDNA template, 2.5 μl 10× QuantiTect Primer Assay, 12.5 μl 2× QuantiTect SYBR Green I PCR Master Mix, and RNase-free PCR water. PCR was performed with one denaturation step of 95 °C for 15 min, and 40 amplification cycles of 94 °C for 15 s, 62 °C for 30 s for CCRK or 55 °C for 30 s for B2M, respectively, and 72 °C for 40 s. Relative quantification was carried out with the delta–delta-CT method (Applied Biosystems 7500 Fast System SDS Software Version 1.3). The beta-2-microglobulin (B2M) gene (QT 00088935; Qiagen) was chosen as endogenous control, because it showed rather constant expression levels in human and nonhuman primate cortices.

Results

Comparison of CGI Promoter Methylation in Human and Chimpanzee Cortices

To get a general view on how DNA methylation patterns change during primate evolution, we compared the CGI promoters of seven genes in human and chimpanzee cortices, using classic bisulfite sequencing. The cortex was chosen, because in a previous microarray-based study, the DNA methylation differences between humans and chimpanzees were more pronounced in the brain than in other tissues (Enard et al. 2004). Although array-based approaches allow one to evaluate a large number of CpGs simultaneously and bisulfite pyrosequencing allows one to accurately quantify the methylation percentages of CpGs in short stretches (30–50 bp) of DNA, bisulfite sequencing of cloned plasmids remains the gold standard for high-resolution methylation profiling. The main advantage of classic bisulfite sequencing is that it provides information on all CpGs in 300–600 bp long CGIs. Here, we compared the methylation status of 269 CpGs from 8 CGIs (table 3). The aldehyde dehydrogenase 1 family member B1 (ALDH1B1), CCRK, insulin-like growth factor binding protein-like1 (IGFBL1), neurotrophic tyrosine kinase receptor type 2 (NTRK2), Src homology 2 domain containing transforming protein 3 (SHC3), and zinc finger protein 519 (ZNF519) genes were selected, because they are located in close proximity (<2 Mb) of evolutionary inversion breakpoints (Kehrer-Sawatzki and Cooper 2008); also, according to published microarray expression data (Enard et al. 2002; Khaitovich et al. 2006), these genes are likely to be differentially expressed between humans and chimpanzees. The O-6-methylguanine-DNA methyltransferase (MGMT) gene
was selected, because of its methylation-dependent regulation and role in brain tumorigenesis (Hegi et al. 2008).

The human–chimpanzee divergence of the analyzed cis-regulatory regions varied from 0.55% to 1.3%, which corresponds to the average sequence difference between the human and the chimpanzee genomes (Chimpanzee Sequencing and Analysis Consortium 2005). The CpG ratio (of observed vs. expected CpG dinucleotides) varied from 0.64 to 1.03 (table 3), representing intermediate (ALDH1B1, CCRK, SHC3, and ZNF519) and high CpG (IGFBPL1, MGMT, and NTRK2) promoters (Weber et al. 2007). The transcription start sites of ALDH1B1, IGFBPL1, MGMT, NTRK2, and SHC3 lie within the analyzed CGIs, and those of CCRK and ZNF519 in close proximity (<200 bp). According to the Transcriptional Regulatory Element Database (http://rulai.cshl.edu), all known and predicted transcription-factor binding sites in the analyzed regions are highly similar in humans and chimpanzees.

When we compared the methylation profiles between one human (HSA1) and one chimpanzee (PTR1) cortex, six of the eight analyzed CGIs were completely or almost completely demethylated. Only CCRK and SHC3 CGI1 were partially methylated in both species (fig. 1). Two of 32 analyzed CpG sites in ALDH1B1, 9 of 33 CpGs in CCRK, 5 of 48 in IGFBPL1, 3 of 64 in MGMT, 1 of 36 in NTRK2, 3 of 30 in SHC3, and none of 26 in ZNF519 were differentially methylated in human and chimpanzee. With the notable

Table 3
Genes Examined in This Study and Their CpG Islands

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length of Entire CGI</th>
<th>% GC</th>
<th>CpG Ratioa</th>
<th>Length of Analyzed CGI</th>
<th>% GC</th>
<th>CpG Ratioa</th>
<th>Chromosomal Position of Analyzed CGIb</th>
<th>Position of TSSb (Distance of CGI to TSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH1B1</td>
<td>656 bp</td>
<td>59.5</td>
<td>0.66</td>
<td>330 bp</td>
<td>72.7</td>
<td>0.75</td>
<td>Chr.9: 38,382,506–38,382,836 bp</td>
<td>38,382,660 bp</td>
</tr>
<tr>
<td>CCRK</td>
<td>895 bp</td>
<td>60</td>
<td>0.73</td>
<td>602 bp</td>
<td>56.3</td>
<td>0.69</td>
<td>Chr.9: 89,779,503–89,780,105 bp</td>
<td>89,779,444 bp</td>
</tr>
<tr>
<td>IGFBPL1</td>
<td>528 bp</td>
<td>56.2</td>
<td>1.03</td>
<td>378 bp</td>
<td>72.2</td>
<td>0.97</td>
<td>Chr.9: 38,414,350–38,414,728 bp</td>
<td>38,414,444 bp</td>
</tr>
<tr>
<td>MGMT</td>
<td>732 bp</td>
<td>73.8</td>
<td>0.88</td>
<td>455 bp</td>
<td>76.4</td>
<td>1.00</td>
<td>Chr.10: 131,155,195–131,155,650 bp</td>
<td>131,155,480 bp</td>
</tr>
<tr>
<td>NTRK2</td>
<td>595 bp</td>
<td>63.2</td>
<td>1.01</td>
<td>370 bp</td>
<td>65.4</td>
<td>0.99</td>
<td>Chr.9: 86,472,937–86,473,307 bp</td>
<td>86,473,285 bp</td>
</tr>
<tr>
<td>SHC3 CGI1</td>
<td>600 bp</td>
<td>55</td>
<td>0.65</td>
<td>383 bp</td>
<td>41.2</td>
<td>0.64</td>
<td>Chr.9: 90,983,522–90,983,905 bp</td>
<td>90,983,502 bp</td>
</tr>
<tr>
<td>SHC3 CGI2</td>
<td>780 bp</td>
<td>72.5</td>
<td>0.88</td>
<td>267 bp</td>
<td>69.5</td>
<td>0.64</td>
<td>Chr.9: 90,983,203–90,983,470 bp</td>
<td>90,983,502 bp (32 bp)</td>
</tr>
<tr>
<td>ZNF519</td>
<td>535 bp</td>
<td>56.1</td>
<td>0.65</td>
<td>460 bp</td>
<td>61.2</td>
<td>0.64</td>
<td>Chr.18: 14,121,809–14,122,269 bp</td>
<td>14,122,430 bp (161 bp)</td>
</tr>
</tbody>
</table>

a Ratio of observed versus expected CpG dinucleotides (for details see Materials and Methods).

b According to Ensembl release 44–45.

c The analyzed CGI contains the TSS.

FIG. 1.—Methylation patterns of orthologous CGI promoters in human (HSA1) and chimpanzee (PTR1) cortices. Five to ten different DNA molecules were sequenced for each sample. White circles represent CpG sites that were completely unmethylated and red circles CpGs that were completely methylated in all analyzed plasmids. Yellow and blue circles indicate CpGs with 60–90% methylation and 20–50% methylation, respectively. Differentially methylated CpG sites are framed.
exception of 14 of 236 individual CpG sites (6%), the overall CGI methylation patterns of 6 genes, ALDH1B1, IGFBPL1, MGMT, NTRK2, SHC3, and ZNF51, were well conserved between species.

Only one gene, CCRK, exhibited a larger cluster of species-specifically methylated CpG sites in its promoter region and, therefore, was analyzed in more detail. The analyzed human CCRK CGI promoter region contains 33 CpGs (fig. 2). Because of the human–chimpanzee sequence divergence, CpG numbers 13 and 31 are not present in the chimpanzee, whereas CpG 23A (between human CpGs 23 and 24) is only found in nonhuman primates. The 5′ region of the island (CpGs 1–16) corresponds to an Alu-Sg1 repeat. Sequence conservation of the CCRK promoter region in human, chimpanzee, rhesus monkey, baboon (fig. 2), and marmoset (Callithrix jacchus) (data not shown) indicates that insertion of this Alu-Sg1 repeat occurred before the split of humans and New World monkeys 35–40 Ma (Goodman et al. 1998; Enard and Pääbo 2004). The first CpG sites of this Alu repeat were hypermethylated in both the human (figs. 1 and 3A) and the chimpanzee cortices (figs. 1 and 4A), followed by a number of differentially methylated CpGs. The second half of the CCRK CGI (CpGs 18–33), comprising almost entirely of CpG sites after the Alu repeat, was completely demethylated in both species.

Variation of CCRK Promoter Methylation among Humans and Nonhuman Primates

To study intra- and interspecific methylation differences, we first analyzed the CCRK methylation patterns in the adult cortices of 11 unrelated human individuals and one fetal cortex (fig. 3A). CpG sites 1–5 were hypermethylated (methylation percentage greater than 75%) in all, or essentially, all analyzed adult cortices, and they displayed much less variation than CpGs 6–16 in the Alu-Sg1 repeat (fig. 3B). CpG sites 20–33 were completely demethylated in all of the analyzed cortex samples. It is noteworthy that the entire Alu repeat (CpGs 1–16) appeared to be somewhat less methylated in the fetal human cortex (fig. 3A, white bars) than in the average adult cortex (fig. 3A, gray bars).

We then performed bisulfite sequencing of the adult cortex samples from three chimpanzees, three baboons, and one rhesus macaque. Although the CCRK promoter sequence, including the Alu-Sg repeat, is well conserved in all analyzed primate species, we had to introduce six CpG positions into the CGI consensus sequence for interspecific comparisons. CpGs 9A and 16A are specific for the baboon, CpG 10A is specific for the rhesus monkey and the baboon, and CpGs 12A and 22A are specific for the rhesus monkey. CpG 23A is found in chimpanzee, baboon, and rhesus monkey, and CpGs 12A and 22A are specific for the rhesus monkey. CpG 10A is specific for the rhesus monkey and the baboon, whereas CpG 23A (between human CpGs 23 and 24) is only found in nonhuman primates. The 5′ region of the island (CpGs 1–16) corresponds to an Alu-Sg1 repeat. Sequence conservation of the CCRK promoter region in human, chimpanzee, rhesus monkey, baboon (fig. 2), and marmoset (Callithrix jacchus) (data not shown) indicates that insertion of this Alu-Sg1 repeat occurred before the split of humans and New World monkeys 35–40 Ma (Goodman et al. 1998; Enard and Pääbo 2004). The first CpG sites of this Alu repeat were hypermethylated in both the human (figs. 1 and 3A) and the chimpanzee cortices (figs. 1 and 4A), followed by a number of differentially methylated CpGs. The second half of the CCRK CGI (CpGs 18–33), comprising almost entirely of CpG sites after the Alu repeat, was completely demethylated in both species.

Expression of CCRK Gene in Human and Nonhuman Primate Cortices

In order to compare the relative CCRK mRNA levels of humans and nonhuman primates, we performed quantitative real-time RT-PCR analyses of total RNAs from four human (HSA7, 8, 10, and 11), three chimpanzee, one rhesus macaque, and three baboon cortices. The relative expression in the humans and the baboons was approximately two times higher than that in the chimpanzees and the rhesus macaque (fig. 5B). Humans and chimpanzees, which showed considerably more intraspecific variation in CCRK promoter methylation than baboons, also exhibited more variation in their gene expression levels. In this context, it is interesting to note that high CCRK expression in the humans and the baboons was associated with SMR hypermethylation, whereas low gene expression in the chimpanzees and the rhesus macaque was associated with...
hypomethylation (fig. 5A and B). When looking at each individual separately (fig. 5C), there was no evidence for a correlation between the percentage of SMR methylation and the level of CCRK expression within the human or the baboon species. In chimpanzees, the one individual (PTR1 in fig. 5C) with a hypermethylated SMR exhibited significantly lower CCRK mRNA levels than the two other analyzed individuals, who were endowed with a hypomethylated SMR.
Discussion

Comparative bisulfite sequencing revealed that most of the analyzed genes (six of seven) showed very similar CGI promoter methylation patterns in humans and chimpanzees. The SHC3 CGI1 was partially methylated, whereas CGI2 was completely demethylated in both species. With the exception of individual sites, the ALDH1B1, IGFBPL1, MGMT, NTRK2, and ZNF519 promoters were completely demethylated. The presence of a few partially or fully methylated CpGs in otherwise demethylated CGIs may represent stochastic methylation of individual sites. Consistent with an earlier study (Enard et al. 2004), the vast majority (11 of 14) of these differentially methylated individual CpGs showed an upmethylation in the human brain, compared with the chimpanzee brain. There may be a general tendency toward a slightly higher degree of DNA methylation in the human lineage. Although we cannot exclude the possibility that such methylation changes of individual CpGs contribute to species differences in brain structure and function, it is unlikely that upmethylation of a single or few sites in larger CGIs directly translates into evolutionarily fixed changes in gene regulation and expression.

One of our seven studied genes, CCRK, is endowed with a CGI that exhibited considerable intraspecific and even higher interspecific variation in the degree of methylation of a cluster of 4–6 CpGs. This SMR lies within an Alu-Sg1 repeat comprising of the first half of the CGI. Alus are the most prominent short interspersed nuclear elements, adding up to roughly 10% of the human genome (International Human Genome Sequencing Consortium 2004). More than 20 Alu subfamilies appeared and all of them have actively retrotransposed during different periods of primate evolution. Although the period(s) of rapid expansion lies in the past, Alus can still be actively retrotransposing in the human genome. It is estimated that approximately 7,000 Alus retrotransposed in the human genome after the split of the human and chimpanzee lineages (Xing et al. 2007; Goodier and Kazazian 2008). It is interesting to note that similar to CCRK, many genes contain one or several Alus in close proximity 5’ to their CGIs (International Human Genome Sequencing Consortium 2004). The neutralist or selectionist “marker” model explains this Alu enrichment by their preferential preservation in gene-flanking regions (Urrutia et al. 2008). In contrast, the “expression modifier” model assumes that Alu insertions near or in promoter regions can influence gene expression (Britten 1996). For several genes, it has been demonstrated experimentally that Alu sequences are involved in the regulation of transcription (Hamdi et al. 2000; Ludwig et al. 2005). Alus can be thought of as “centers of de novo methylation” from which methylation spreads into adjacent promoters to induce gene silencing (Santourlidis et al. 2002; Turker 2002; Levine et al. 2003; Stirzaker et al. 2004).
FIG. 4.—Variation of CCRK CGI promoter methylation among humans and nonhuman primates. CpG positions 9A and 16A are only present in the baboon sequence, 10A only in the rhesus monkey and the baboon sequence, and 12A only in the rhesus macaque sequence. (A) Methylation percentages of individual CpG sites in the adult cortices of three unrelated chimpanzees (PTR). Please note that PTR1, represented by the first bar at each site, shows much higher methylation levels at CpGs 1–17 than the two other animals. (B) Methylation percentages of individual CpG sites in the adult cortices of three unrelated baboons (PHA). (C) Methylation percentages (mean ± standard deviation) in human (n = 11), chimpanzee (n = 3), rhesus macaque (n = 1), and baboon cortices (n = 3). (D) Average methylation percentages (and standard deviations) at CpG sites 6–10A (enlargement of fig. 4C) in human, chimpanzee, rhesus macaque, and baboon cortices.
DNA methylation appears to be a major mechanism for preventing retrotransposon activity in human phylogeny and ontogeny (Yoder et al. 1997). In somatic tissues and mature germ cells, most retroelements are densely methylated and consequently transcriptionally inactive. However, genomewide methylation reprogramming during gametogenesis and early embryogenesis (Reik et al. 2001; Haaf 2006) opens a window for transcriptional activity and even retrotransposition. Following their entry in the genital ridge, the primordial germ cells undergo genomewide de-methylation (Hajkova et al. 2002); most retrotransposon-derived elements become remethylated at later stages of germ-cell development (Li 2002). In the zygote and early embryo genomewide active and passive demethylation erases most germline methylation patterns, followed by de novo methylation and establishment of somatic methylation patterns around the time of implantation (Mayer et al. 2000; Reik et al. 2001). Interestingly, Alus and some other retroelements show heterogeneous methylation patterns in somatic tissues, with a substantial fraction (10–15%) of Alus being undermethylated (Schmid 1998; Yang et al. 2004). This suggests that the silencing process is imperfect and incomplete (Whitelaw and Martin 2001). It has been shown for at least some genes that the epigenetic state of retrotransposons inserted into regulatory regions can be transmitted through the germline (transgenerational epigenetic inheritance) (Morgan et al. 1999; Rakyan et al. 2003).

Our study provides a comprehensive overview of the intra- and interspecific variability of DNA methylation of an Alu insertion in a regulatory region. In the human cortex, the methylation percentage of particular CpG sites in the CCRK promoter can vary from 0% to 100% between individuals. These dramatic epigenetic differences can have resulted from stochastic processes, endogenous mechanisms, and/or environmental perturbations. Consistent with our findings, bisulfite sequencing of Alu insertion/deletion polymorphisms revealed a significant interindividual variability in the methylation level of specific Alu elements (Sandovici et al. 2005). Interestingly, the average Alu methylation level in blood cells gradually decreased through aging (Bollati et al. 2009), whereas in the cortex, the majority of Alus showed relatively little change in methylation across the entire lifespan (Siegmund et al. 2007). Little is known about methylation differences of specific Alu repeats between humans and nonhuman primates. The average methylation percentage of the analyzed SMR in the CCRK promoter ranged from 35% in chimpanzees and 40% in the rhesus macaque to 70% in humans and 100% in baboons. Whether other loci that show intraspecies variation in Alu methylation also display such dramatic variation between species, remains to be elucidated. An Alu element in intron 6 of the p53 gene was found to be methylated in human and eight different great ape and Old World monkey species (Yang et al. 1996).
The CCRK promoter is susceptible to epigenetic modification by DNA methylation, which may be, to some extent, stochastic and result in complex patterns of transcription. The methylation differences between species were inversely correlated with gene expression. The relative CCRK mRNA levels in human and baboon cortices, where the SMR was hypermethylated, was approximately two times higher than in chimpanzees and the rhesus macaque, where the SMR was relatively hypomethylated. This is consistent with the idea that at the species level global SMR methylation abrogates a repressor activity and/or confers an enhancer-like activity. Although in general DNA hypermethylation is coupled with transcriptional repression, accumulating experimental evidence suggests that some genes can be activated by CpG methylation (Eden et al. 2001; Unoki and Nakamura 2003; Cheng et al. 2005). Within species, we did not find a clear relationship(s) between SMR methylation and gene expression. This may be largely due to the small number of individuals analyzed per species. However, in chimpanzees, one individual showed a remarkably high SMR methylation for this species and expressed the CCRK gene at very low levels. It is possible that dramatic upmethylation of normally hypomethylated sites, that is, CpGs 6 and 7, which are 100% methylated in PTR1 but completely demethylated in PTR2 and PTR3, has a different effect on the species-specific gene regulation than the evolutionary fixation of global differences in SMR methylation between species. CCRK is a member of the cyclin-dependent kinase family that is important for cell cycle control and transcriptional regulation. It appears to be indispensable for cell growth and may also act as a negative regulator of apoptosis (Liu et al. 2004; MacKeigan et al. 2005). Transcriptional upregulation of CCRK has been implicated in human glioblastoma tumorigenesis, whereas knockdown of CCRK expression significantly suppressed cell proliferation (Ng et al. 2007). It is tempting to speculate that fine-tuning of the growth-promoting and apoptosis-reducing effects of CCRK was important for human brain evolution. In contrast to baboons, both humans and chimpanzees display substantial intraspecific variations of the CCRK promoter methylation patterns, which may contribute to interindividual differences in brain development, function, and disease susceptibility. CCRK is a prime candidate gene for studying epigenetic effects (gene–environment interactions) on the human brain.

Acknowledgments

We thank Dr. Larissa Seidmann for dissecting the fetal brain sample. Cortex tissue from nonhuman primates was obtained with the support and through the European Primate Network EUPRIM-Net. This work was supported by the German Research Foundation (HA 1374/5-4).

Literature Cited


Naoko Takezaki, Associate Editor

Accepted March 9, 2009