Warm-Blooded Isochore Structure in Nile Crocodile and Turtle

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The genomes of warm-blooded vertebrates are characterized by a strong heterogeneity in base composition, with GC-rich and GC-poor isochores. The GC content of sequences, especially in third codon positions, is highly correlated with that of the isochore they are embedded in. In amphibian and fish genomes, GC-rich isochores are nearly absent. Thus, it has been proposed that the GC increase in a part of mammalian and avian genomes represents an adaptation to homeothermy. To test this selective hypothesis, we sequenced marker protein genes in two cold-blooded vertebrates, the Nile crocodile *Crocodylus niloticus* (10 genes) and the red-eared slider *Trachemys scripta elegans* (6 genes). The analysis of base composition in third codon position of this original data set shows that the Nile crocodile and the turtle also exhibit GC-rich isochores, which rules out the homeothermy hypothesis. Instead, we propose that the GC increase results from a mutational bias that took place earlier than the adaptation to homeothermy in birds and before the turtle/crocodile divergence. Surprisingly, the isochore structure appears very similar between the red-eared slider and the Nile crocodile than between the chicken and the Nile crocodile. This point questions the phylogenetic position of turtles as a basal lineage of extant reptiles. We also observed a regular molecular clock in the Archosaurus, which enables us, by using a more extended data set, to confirm Kumar and Hedges’s dating of the bird-crocodile split.

Introduction

Vertebrate genomes are heterogeneous in base composition. They can be described as a mosaic of isochores, i.e., very long DNA segments (200–1,000 kb) in which base composition is homogeneous, that belong to a small number of families characterized by different GC levels (see Bernardi [1995] for a recent review). The GC content of protein-coding genes, and, more specifically, that in third codon positions, is highly correlated with that of the isochore into which they are embedded for cold-blooded (Bernardi and Bernardi 1991) and warm-blooded (Kadi et al. 1993; Clay et al. 1996) vertebrates. Thus, the analysis of base composition in third codon positions (GC3) from orthologous coding sequences can be used to study the structure (Zoubak, Clay, and Bernardi 1996) and the evolution (Bernardi, Hughes, and Mouchiroud 1997; Robinson, Gautier, and Mouchiroud 1997; Galtier and Mouchiroud 1998) of the isochores in vertebrate genomes.

The isochore patterns appear to be very different in amphibians and fishes than in birds and mammals, as assessed by analytical DNA ultracentrifugation (Bernardi and Bernardi 1990a, 1990b) and sequence analysis (Bernardi et al. 1988; Bernardi and Bernardi 1991). The amphibian and fish genomes, more precisely, Actinopterygii, exhibit much lower compositional heterogeneity. Whereas GC-poor isochores are compositionally conserved among all vertebrates, GC-rich isochores are nearly absent in amphibian and fish genomes. These results indicate that the separation of the genomes of birds and mammals and those of amphibians and fishes resulted from a “major compositional transition” in which a sizeable proportion of the cold-blooded vertebrate genomes underwent GC increases, leading to heterogeneous warm-blooded genomes.

Debate rages on the question of isochore evolution in vertebrate genomes. The GC heterogeneity has been considered a convergently derived state that independently occurred twice, in association with the development of homeothermy in both mammalian and avian lineages (Bernardi 1993). An increase in GC content in some genomic regions could protect DNA, RNA, and proteins from degradation by heat. Sequence data available thus far have not clearly distinguished a “warm-blooded” pattern from an “amniote” pattern, since only mammal and bird sequences were representative of amniotes in the database. Thus, the isochore composition of the genomes of turtles, lizards, snakes, and crocodiles, all “cold-blooded” amniotes, is highly relevant to this question.

The purpose of the present work was to test the homeothermy hypothesis by investigating the isochore patterns in two cold-blooded reptilian groups, crocodilians and turtles. Ten protein-coding genes selected as markers of the GC transition were sequenced for *Crocodylus niloticus*, and six were sequenced for the turtle *Trachemys scripta elegans*. We compared their base compositions with those of orthologous genes of other vertebrates, cold-blooded (*Xenopus*) and warm-blooded (chicken and human). Our results demonstrate that genes with high GC contents are already present in the crocodilian and turtle genomes, indicating the presence of some GC-rich isochores and, consequently, refuting the homeothermy hypothesis.

Materials and Methods

Marker Sequences

Marker genes were chosen according to the following criteria: (1) orthologous sequences known in a mam-
Table 1
GC Content in Third Codon Positions of Aligned Sites for the 10 Genes Sequenced for Crocodylus niloticus and for Trachemys scripta elegans Compared with Human, Chicken, and Xenopus Orthologous Genes

<table>
<thead>
<tr>
<th>GENE NAME</th>
<th>%GC3</th>
<th>LENGTH² (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-ski proteinα (c-ski)</td>
<td>80.1</td>
<td>78.5</td>
</tr>
<tr>
<td>Hepatocyte growth factorα (hgf)</td>
<td>79.0</td>
<td>75.8</td>
</tr>
<tr>
<td>Pyruvate kinaseα (pk)</td>
<td>47.9</td>
<td>54.9</td>
</tr>
<tr>
<td>PPAR Alphaα (ppara)</td>
<td>41.9</td>
<td>68.8</td>
</tr>
<tr>
<td>PPAR Betaα (pparb)</td>
<td>80.5</td>
<td>76.5</td>
</tr>
<tr>
<td>PPAR Gammaα (pparg)</td>
<td>40.4</td>
<td>40.4</td>
</tr>
<tr>
<td>Retinol binding proteinα (rpl)</td>
<td>48.8</td>
<td>57.1</td>
</tr>
<tr>
<td>Ribosomal protein S6α (rps6)</td>
<td>56.9</td>
<td>45.6</td>
</tr>
<tr>
<td>SPARC proteinα (sparc)</td>
<td>56.9</td>
<td>92.9</td>
</tr>
<tr>
<td>Vitamine D receptorα (vdr)</td>
<td>34.3</td>
<td>70.1</td>
</tr>
</tbody>
</table>

* Length of orthologous sequences between the four or five species after alignment and gap removal.

Genes selected with high GC differences between humans and the two others species, Xenopus and chickens.

Genes selected with high GC differences between the two warm-blooded vertebrates and Xenopus.

Genes selected in collaboration with another group. The sequences of the chicken PPAR alpha, beta, and gamma were obtained from I. Takada and K. Umesono (personal communication).

Genes selected with low GC differences between the three species.

mal (Homo sapiens), a bird (Gallus gallus), and an amphibian (Xenopus laevis); (2) very large GC differences in third codon positions (ΔGC3) between at least two of these orthologous genes (table 1) (We also sequenced a GC-poor gene [rps6] having similar GC contents in humans, chickens, and Xenopus); (3) sufficient base conservation for the design of multiple PCR primers (see below) and a resulting fragment size that is not too small compared to complete gene size; and (4) some knowledge of tissue expression for reverse transcription–polymerase chain reaction (RT-PCR) experiments (see below).

For the first criterion, orthologous genes were selected using the HOVERGEN software (release 31; Duret, Mouchiroud, and Gouy 1994), which allowed us to check the orthology status by analyzing phylogenetic trees. Multigenic families were disregarded in most cases to avoid mistakes. After this step, genes were extracted from GenBank with QUERY_WIN (Perriere, Gouy, and Gojobori 1994) and aligned using CLUSTAL W (Thompson, Higgins, and Gibson 1994). Two pairs of 20mer-degenerated primers (sequences available on request) were constructed for each gene based on the most conserved zones according to Escriva et al. (1997).

On the other hand, three genes of the peroxisome proliferator-activated receptor family (ppar) were sequenced in collaboration with another group (I. Takada, K. Umesono, and V. Laudet) and introduced in our data set. Other crocodilian or turtle genes, already available in GenBank but not markers of the transition, were added in the sequence analysis. These sequences are available on request. The GenBank/EMBL accession numbers for the 16 genes sequenced are AJ011391–AJ011397, AJ011515, AJ2433131–AJ2433136, AJ2433138, and AJ2433139.

RT-PCR Method

Biological material used in this study was taken from an adult female Nile crocodile (C. niloticus) from the Crocodile Farm of Pierrelatte (France) and from an adult female red-eared slider (T. s. elegans). Different tissue samples are conserved in liquid nitrogen or ethanol at the Centre d’Analyse Moléculaire de la Biodiversité (CAMB), Lyon, France.

Total RNAs were extracted from frozen liver tissues with Qiagen RNeasy kit. For each gene, a first touchdown RT-PCR was performed under various conditions with the Perkin-Elmer AmpliTaq Gold Taq polymerase, followed by a second run with nested primers to enhance specificity of the products, according to Escriva et al. (1997). Resulting fragments were purified (Qiagen Qiaquick system), ligated into pUC18 (Amersham SureClone ligation kit) and cloned into Escherichia coli strain DH5-α. Fluorescence sequencing (Amersham kit) of some clones was performed using a Pharmacia automatic sequencer ALF express.

Sequence Analysis

Sequences of C. niloticus and T. s. elegans were aligned by hand with the already-known vertebrate sequences using SEAVIEW (Galtier, Gouy, and Galtier 1996). Phylogenetic reconstructions were made with PHYLO_WIN (Galtier, Gouy, and Galtier 1996) by neighbor joining applied to nonsynonymous evolutionary distances (Kd) (Li 1993). Kd distances between crocodiles (Kd(ch)) or chickens (Kd(ch)) and their most recent

$T = d1 + d2 + d3$
common ancestor (fig. 1) were calculated for each gene as follows, with humans as outgroup:

$$K_{a(ch)} = \frac{1}{2}(K_{a(ch-hu)} - K_{a(ch-cr)} + K_{a(ch-hu)})$$

$$K_{a(cr)} = K_{a(ch-cr)} - K_{a(ch)}$$

The relative-rate tests to compare substitution rates between two lineages or two groups of sequences were done using the program RRTree (Robinson et al. 1998). For dating the bird-crocodile divergence time, we applied the lineage-specific method (fig. 1) developed by Kumar and Hedges (1998). This method is not biased by high or low evolutionary rate in the outgroup lineage.

Results
GC Content in Crocodilian and Chelonian Coding Genes

The RT-PCR method used allowed only partial sequencing of coding genes. Thus, fragments of various sizes (ranging from 252 to 981 bp) were obtained, representing 7,182 bp of the nuclear genome of *C. niloticus* and 3,741 bp of that of *T. s. elegans* (table 1). The GC levels in third codon positions in orthologous sequences from humans, chickens, and *Xenopus* and in gene fragments from crocodiles and turtles are given in table 1. The GC3 differences in orthologous genes between *Xenopus* and the other species are plotted in figure 2. Globally, GC3 levels in the crocodile and turtle coding sequences appear much more similar to those of humans and/or chickens than to those of *Xenopus* (table 1 and fig. 2). Indeed, the GC3 levels of nearly all coding sequences are statistically different between the two cold-blooded reptiles and the amphibian (fig. 2C and D, white points). Six genes (sparc, vdr, pparg, hgf, ppar, and ppara) show more than 20% difference in GC3 between *Xenopus* and the Nile crocodile (fig. 3). As expected, the low GC3 level of *rps6* in the three known species also stands in the Nile crocodile and turtle genes. For with large GC3% differences between humans and chickens (c-ski, rbp, ppara, pparg, and rps6), the GC3 level of homologous crocodilian genes is more similar to that in chicken sequences (Student’s t-test on paired data; $P = 0.98$) than to that in human sequences ($P = 0.0387$). In addition, data in table 1 indicate that the
GC3 levels of the six turtle coding genes are similar to those in crocodiles ($P = 0.6028$).

Increasing our data set with previously known sequences in GenBank enabled us to corroborate the above conclusion with a strong positive correlation between GC3 values of orthologous genes from chickens and crocodiles ($r = 0.85, P < 0.0001$) (fig. 2A) or from chickens and turtles ($r = 0.77, P = 0.003$) (fig. 2B). No such correlations were observed for comparison between crocodile and *Xenopus* ($r = 0.07, P = 0.8061$) (fig. 2C) or between turtle and *Xenopus* GC3 values ($r = 0.35, P = 0.3360$) (fig. 2D). The most surprising result is the extremely high correlation coefficient obtained for the turtle/crocodile comparison ($r = 0.97, P < 0.0001$) (fig. 4), although it was supported by a small gene sample. The original evolution of base composition in *ppara* and *pparg* genes, which show higher GC levels in *Xenopus*, could be explained by a secondary translocation of these genes (Bernardi, Hughes, and Mouchiroud 1997).

**Divergence Time Between Birds and Crocodilians**

Before dating the bird/crocodile split, we tested potential evolutionary-rate differences between the avian and crocodilian lineages. The relative-rate tests were computed on nonsynonymous substitution rates ($K_a$) using human sequences as outgroup (fig. 1). For 13 of the 16 genes tested, we did not detect any significant difference between chicken and crocodilian nonsynonymous rates. Three genes presented significant rate differences: *c-ski*, *eno*, and *znf*. Paralogy or functional changes could explain the significant $K_a$ differences for these three genes. Thus, these genes were disregarded for divergence time estimation.

The divergence of birds and mammals (310 MYA; Benton 1990) provides a reliable point at which to anchor the molecular clock in amniotes. For each gene, the divergence time between the bird and crocodilian lineages was estimated by the lineage method (Kumar and Hedges 1998), with humans as outgroup. The bird/crocodilian divergence times range from 80 Myr for the *pparg* gene to 280 Myr for the *rhd* gene (table 2), with an average of $180 \pm 62$ Myr. This value increases to $196 \pm 50$ Myr if the two genes with the shortest divergence times (*pparb* and *pparg*) are disregarded. Moreover, the divergence time obtained with a combined analysis of the 12 protein-coding genes, 200 Myr, increases to 215 Myr without the two *ppar* genes.

**Discussion**

**Crocodile/Bird Divergence**

As expected, phylogenetic analysis of the 10 presently sequenced genes from the Nile crocodile strongly

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**Table 2**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>No. of Sites Used</th>
<th>$K_{a,CH}$ (%)</th>
<th>$K_{a,CR}$ (%)</th>
<th>Relative-Rate Test</th>
<th>Divergence Time (Myr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-ski</td>
<td>504</td>
<td>0.19</td>
<td>1.59</td>
<td>$-1.40 \pm 0.6^*$</td>
<td>—</td>
</tr>
<tr>
<td>hgd</td>
<td>655</td>
<td>8.97</td>
<td>6.47</td>
<td>$2.50 \pm 1.9$</td>
<td>216</td>
</tr>
<tr>
<td>pk</td>
<td>432</td>
<td>2.14</td>
<td>2.08</td>
<td>$0.06 \pm 1.0$</td>
<td>226</td>
</tr>
<tr>
<td>ppara</td>
<td>642</td>
<td>0.53</td>
<td>1.43</td>
<td>$-0.90 \pm 0.6$</td>
<td>151</td>
</tr>
<tr>
<td>pparb</td>
<td>601</td>
<td>0.55</td>
<td>0.56</td>
<td>$-0.01 \pm 0.4$</td>
<td>90</td>
</tr>
<tr>
<td>prc</td>
<td>749</td>
<td>0.23</td>
<td>0.28</td>
<td>$-0.05 \pm 0.3$</td>
<td>80</td>
</tr>
<tr>
<td>rbp</td>
<td>224</td>
<td>2.32</td>
<td>3.12</td>
<td>$-0.80 \pm 1.6$</td>
<td>147</td>
</tr>
<tr>
<td>rps6</td>
<td>339</td>
<td>0.22</td>
<td>0.30</td>
<td>$-0.08 \pm 0.4$</td>
<td>141</td>
</tr>
<tr>
<td>sparc</td>
<td>403</td>
<td>1.29</td>
<td>1.15</td>
<td>$0.14 \pm 0.8$</td>
<td>162</td>
</tr>
<tr>
<td>vdr</td>
<td>436</td>
<td>6.23</td>
<td>6.24</td>
<td>$-0.01 \pm 1.9$</td>
<td>271</td>
</tr>
<tr>
<td>c-mos</td>
<td>242</td>
<td>7.46</td>
<td>7.63</td>
<td>$-0.17 \pm 3.0$</td>
<td>152</td>
</tr>
<tr>
<td>endh</td>
<td>808</td>
<td>0.80</td>
<td>2.40</td>
<td>$-1.60 \pm 0.6^*$</td>
<td>—</td>
</tr>
<tr>
<td>rhd</td>
<td>707</td>
<td>2.94</td>
<td>2.43</td>
<td>$0.51 \pm 0.9$</td>
<td>280</td>
</tr>
<tr>
<td>wtp</td>
<td>503</td>
<td>1.20</td>
<td>1.15</td>
<td>$0.05 \pm 0.7$</td>
<td>221</td>
</tr>
<tr>
<td>prc</td>
<td>705</td>
<td>1.87</td>
<td>2.23</td>
<td>$-0.36 \pm 0.8$</td>
<td>194</td>
</tr>
<tr>
<td>znf</td>
<td>343</td>
<td>0.56</td>
<td>2.65</td>
<td>$-2.09 \pm 0.9^*$</td>
<td>—</td>
</tr>
</tbody>
</table>

* Genes sequenced for *Crocodylus niloticus* and known for humans, chickens, and *Xenopus*.  
  $^b$ Genes previously known for humans, chickens, *Xenopus*, and another crocodilian species, *c-mos = c-mos proto-oncogene (Crocodylus porosus); eno = alpha enolase (Alligator mississippiensis); rhd = rhodopsin (A. mississippiensis); wtp = Wilm’s tumor (A. mississippiensis).  
  $^c$ Genes previously known only for humans, chickens, and another crocodilian species, *pr = progesterone receptor (Crocodylus siamensis); znf = ZFY-related zinc finger gene (A. mississippiensis).  
  $^d$ Significant difference ($P < 0.05$).
supports the Archosauromorpha hypothesis, which groups birds and crocodilians in a sister group with bootstrap values higher than 85%, except for vdr (59%) and pk (71%). As a whole, these data definitively rule out the "Haemothermia" hypothesis, which groups birds and mammals in a sister group (Hedges, Moberg, and Maxon 1990).

For the divergence time between birds and crocodilians, a recent work has proposed a mean value of 222 ± 40 Myr (Kumar and Hedges 1998), in agreement with dilians, a recent work has proposed a mean value of 222 ± 40 Myr (Kumar and Hedges 1998), in agreement with the 240-Myr value suggested by fossil analyses (Benton 1990). The new divergence time (215 Myr) that we estimated on 10 concatenated nuclear coding genes (i.e., 2.5 more genes than in previous studies) is in accordance with Kumar's findings. Analysis of the avian/crocodilian split, performed on the complete mitochondrial genome of *Alligator mississippiensis*, gives a divergence time of 254 Myr (Janke and Aronson 1997), close to our estimate for the nuclear genome.

The Phylogenetic Position of the Turtle Among Vertebrates

Among reptiles, the phylogenetic position of turtles is presently questioned, since recent works disproved that turtles were first reptiles (Rieppel and DeBraga 1996; Zardoya and Meyer 1998). Our results are presently too scarce to provide valuable information on this question. Nevertheless, the extremely high correlation coefficient observed between GC3 levels of orthologous genes from crocodiles and turtles (fig. 4) suggests that turtles and crocodiles might be near parents, as proposed by Hedges and Poling (1999). In order to test this hypothesis, complete sequencing of marker coding genes studied here is in progress for a squamate.

GC Variability in Crocodilian and Turtle Genomes

In light of results obtained with Nile crocodile coding genes and confirmed with those of the turtle, it appears that the GC increase observed for warm-blooded vertebrates cannot be explained by homeothermy, considering that crocodiles and turtles are ectotherms.

The discrepancy between the GC3 heterogeneity observed here for *C. niloticus* and *T. s. elegans* coding genes and the compositional homogeneity obtained through analytical ultracentrifugation (Bernardi and Bernardi 1990a, 1990b) could result from the sensitivity of the latter approach. One might expect such a result if the isochore structure in the reptilian genome were intermediate between isochore structures of other cold-blooded and warm-blooded vertebrates. In fact, many molecular characters place reptilian genomes as intermediate between cold-blooded and warm-blooded vertebrates. Indeed, genome sizes are smaller and more uniform in reptilian genomes than in amphibian and fish genomes (Olmo 1991). The methylation level in reptiles is roughly only half that in fishes and amphibia (Jabbari et al. 1997) but the same as that in mammals and birds. R-banding patterns corresponding to GC-rich DNA were demonstrated in the chromosomes of turtles, snakes, and some lizards but were absent in fishes and amphibia (Schmid and Guttenbach 1988).

However, our data do not allow us to claim that the GC variability in the Nile crocodile is as high as that in birds. A larger gene sample is necessary to confirm a birdlike isochore structure in cold-blooded reptilian genomes. Nevertheless, the high correlations observed between GC3 values for chickens and crocodilians (fig. 2A) and between those for chickens and chelonians (fig. 2B) argue in favor of such a hypothesis.

Isochore Evolution in Vertebrates

The present results suggest that the GC increase observed in part of avian genomes certainly occurred before the crocodile/turtle divergence. Furthermore, although without squamate data, the GC variability in the crocodilian and chelonian genome indicates that the common reptilian ancestor of mammals and birds would already have undergone an increase in GC content.

Thus, we have some arguments for positioning the GC increase in the common ancestor of tetrapods (fig. 5). A comparison of orthologous genes from *Xenopus*, chickens, and humans has shown that the majority of increasingly GC-rich genes were the same in the two warm-blooded vertebrates and, moreover, corresponded to GC3-rich genes from *Xenopus* (Bernardi, Hughes, and Mouchiroud 1997). The finding that DNA from the most GC-rich isochores of humans hybridizes with the most GC-rich isochores of other vertebrates, including those in amphibia (Bernardi 1995), also supported this result. These observations indicate that the same genes and, consequently, the same DNA regions, are implicated in the GC increase in all tetrapods and could result from a unique event in the ancestral genome. In other respects, sequence analysis has shown that *Xenopus* and other amphibian genomes exhibit a slightly more heterogeneous genome structure than fish genomes and, notably, the Actinopterygii (data not shown). Moreover, this slight GC heterogeneity in amphibian genomes was not detected with compositional profile. This suggests that the GC increase may have taken place before the separation of amniota, perhaps in the common ancestor of tetrapods, between 450 and 350 MYA (fig. 5).

GC Increase and Mutational Pattern

Since the present results rule out the adaptive hypothesis explaining GC increase by higher stability of
DNA in homeotherm genomes, we propose another hypothesis. The origin of the regional GC increases could be explained by the variation in mutational pattern related to nonuniform gene distribution along DNA sequences (Bernardi et al. 1988; Mouchiroud et al. 1991).

Indeed, gene density is 10 to 20 times as great in the most GC-rich isochores than in the most GC-poor isochores. Moreover, the nonuniform gene distribution is likely to be common to cold-blooded vertebrates (Duret, Mouchiroud, and Gautier 1995; Andersson et al. 1996).

One argument in favor of the mutational bias hypothesis is that birds showing the strongest information compaction (McQueen, Siriaco, and Bird 1998) exhibit the highest GC increases (Kadi et al. 1993). Thus, the high gene density associated with various mutational biases linked to high recombination rate (Eyre-Walker 1993), early replication timing (Wolfe, Sharp, and Li 1989), or more efficient repair (Filipski 1990) could by itself explain the regional GC increases.

In conclusion, we propose that the establishment of GC-rich isochores, initially observed in mammalian and avian genomes, took place only once in vertebrate evolution (in the common ancestor of tetrapods) and probably resulted from a mutational bias linked to the nonuniform gene distribution. Nevertheless, if turtles and crocodiles are grouping in the same clade, the unique origin of the GC increase in amniotes cannot be advanced without squamate data. Therefore, we have undertaken the sequencing of the genes studied here for a squamate species to provide additional information about the phylogenetic position of turtles among reptiles and the origin of GC increases in some amniote genomes.

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