

Vanishing GC-Rich Isochores in Mammalian Genomes

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ABSTRACT

To understand the origin and evolution of isochores—the peculiar spatial distribution of GC content within mammalian genomes—we analyzed the synonymous substitution pattern in coding sequences from closely related species in different mammalian orders. In primate and cetartiodactyls, GC-rich genes are undergoing a large excess of GC → AT substitutions over AT → GC substitutions: GC-rich isochores are slowly disappearing from the genome of these two mammalian orders. In rodents, our analyses suggest both a decrease in GC content of GC-rich isochores and an increase in GC-poor isochores, but more data will be necessary to assess the significance of this pattern. These observations question the conclusions of previous works that assumed that base composition was at equilibrium. Analysis of allele frequency in human polymorphism data, however, confirmed that in the GC-rich parts of the genome, GC alleles have a higher probability of fixation than AT alleles. This fixation bias appears not strong enough to overcome the large excess of GC → AT mutations. Thus, whatever the evolutionary force (neutral or selective) at the origin of GC-rich isochores, this force is no longer effective in mammals. We propose a model based on the biased gene conversion hypothesis that accounts for the origin of GC-rich isochores in the ancestral amniote genome and for their decline in present-day mammals.

THE mammalian genome is heterogeneous with respect to base composition. In human, the GC content of large genomic DNA fragments (>100 kb) ranges from 35 to 65% (NEKRUTENKO and LI 2000; LANDER *et al.* 2001). Bernardi and colleagues first discovered this pattern from cesium chloride centrifugation experiments (BERNARDI *et al.* 1985). Subsequent analyses showed that this variation of base composition affects all parts of the genomes, not only intergenic regions, but also introns and exons (and notably the third position of codons; D'ONOFRIO *et al.* 1991). Interestingly, this long-range variability of base composition is correlated with various genomic features: GC-rich regions show a lower density of LINE and a higher density of Alu repeated elements, a higher level of methylation, a higher rate of recombination, and a much higher gene density (MOUCHIROUD *et al.* 1991; EYRE-WALKER 1993; DURET *et al.* 1995; JABBARI and BERNARDI 1998; SMIT 1999; FULLERTON *et al.* 2001; LANDER *et al.* 2001). Thus, this long-range variability of GC content clearly reflects a fundamental feature of genome organization.

Bernardi and colleagues proposed a model of mammalian genomes consisting in a mosaic of compositionally homogeneous regions—the so-called isochores (see

BERNARDI 2000). The complete sequence of the human genome modified this picture a bit: in general, the GC content varies continuously (*i.e.*, there are no clear boundaries between GC-poor and GC-rich regions), and isochores (notably, the GC-rich ones) are not as homogeneous as proposed initially (NEKRUTENKO and LI 2000; LANDER *et al.* 2001). However, a highly significant spatial autocorrelation of GC content was found, with most of the structure detectable at a large (300-kb) scale (LANDER *et al.* 2001), indicating indeed a *relative* local homogeneity in base composition along mammalian chromosomes. Following EYRE-WALKER and HURST (2001), we use the term isochore to denote these large regions of relatively homogeneous base composition.

Comparison of base composition in different vertebrate species indicates that the ancestral genome of tetrapodes was probably relatively homogeneous and AT rich, and that the acquisition of GC-rich isochores occurred in the amniote lineage, after the split with amphibians, but before the divergence of mammals, birds, and reptiles, *i.e.*, ~310–350 million years ago (BERNARDI and BERNARDI 1990; BERNARDI *et al.* 1997; HUGHES *et al.* 1999). How did isochores originate? How were they maintained? Two models (one selectionist and one neutralist) have been first invoked to explain the existence of isochores: (i) selection for a high GC content in some regions of the genome (*e.g.*, BERNARDI *et al.* 1985; BERNARDI 2000) and (ii) variable mutational bias (VMB) along chromosomes (*e.g.*, WOLFE *et al.* 1989;

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FRANCINO and OCHMAN 1999). Another neutral model, called biased gene conversion (BGC), originally proposed by HOLMQUIST (1992), recently gained consideration (GALTIER *et al.* 2001; EYRE-WALKER and HURST 2001). BGC is linked to the process of recombination. During meiosis, heteroduplex DNA segments are formed from the hybridization of one maternal and one paternal DNA strand. When a heterozygous site is involved in such a heteroduplex, this results in a DNA mismatch that may be repaired by one of the cellular DNA repair systems. This repair will thus result in the loss (*i.e.*, conversion) of one of the two alleles. This process is said to be biased if the probability of conversion is not symmetric (*e.g.*, if GC alleles convert AT alleles more frequently than the reverse). Like selection, the impact of BGC on the fixation process depends on the population size (NAGYLAKI 1983). It also depends on the rate of recombination (more precisely on the rate of formation of DNA heteroduplex), the size of DNA heteroduplex, and the bias in the repair of mismatches. Several authors proposed that GC-rich isochores might result from BGC, the fixation of GC alleles being favored in genomic regions of high recombination/conversion rate (HOLMQUIST 1992; EYRE-WALKER 1993; EYRE-WALKER and HURST 2001; GALTIER *et al.* 2001).

The major selectionist hypothesis, namely adaptation to homeothermy, was dismissed after isochores were discovered in several cold-blooded species (HUGHES *et al.* 1999). It should be stressed that the particular base composition of GC-rich isochores concerns not only coding regions, but also introns and intergenic regions, and notably pseudogenes (FRANCINO and OCHMAN 1999). Therefore, if the acquisition of GC-rich isochores resulted from natural selection, this could not be due to a selective advantage at the RNA or protein level. In our opinion, neutralist models (VMB or BGC) appear more realistic.

An important progress toward the solution of this controversial issue has been brought by the analysis of polymorphism data. Indeed, the VMB model can be distinguished from the other two (selection or BGC) by studying the process of allele fixation. Under the VMB model, the probability of fixation is expected to be the same for alleles resulting from an AT \rightarrow GC mutation (*i.e.*, from A or T to G or C) as for alleles resulting from a GC \rightarrow AT mutation. Hence, the pattern of GC \leftrightarrow AT substitution is expected to be identical to the pattern of mutation. Conversely, the BGC and the selectionist models predict a fixation bias in favor of GC alleles (*i.e.*, GC alleles should have a higher probability of fixation than AT alleles). Analyses of human and mouse polymorphism data provided evidence for such a fixation bias, leading to the conclusion that GC-rich isochores result from BGC or selection, but not from mutation bias (EYRE-WALKER 1999; SMITH and EYRE-WALKER 2001). However, this conclusion was based on the assumption that the isochore structure was at equi-

librium, *i.e.*, that the GC content of a specific genomic region is not varying in time. The stationarity appeared likely because the GC content at third codon positions (GC3's) of orthologous genes taken in primates, Cetartiodactyla, Lagomorpha, and Carnivora are very close to each other (MOUCHIROUD and BERNARDI 1993), which suggests that the GC content has remained unchanged since the divergence of these different mammalian orders. Only the rodents showed a peculiar distribution of GC content (MOUCHIROUD and GAUTIER 1988; ROBINSON *et al.* 1997). An estimation of the ancestral GC3 of 27 genes sampled in various orders suggested that the ancestral mammalian isochore pattern was similar to the present-day human one (GALTIER and MOUCHIROUD 1998), consistent with the hypothesis of a stationary evolution of isochores in nonrodent mammals.

The GC content of a genomic fragment is at equilibrium when the numbers of GC \rightarrow AT and AT \rightarrow GC substitutions occurring in this fragment are equal. Interestingly, the analysis of the complete sequence of the human genome has shown that some sequences are not at equilibrium (LANDER *et al.* 2001): in defective transposons (presumably free of any selective pressure), GC \rightarrow AT substitutions are significantly more frequent than AT \rightarrow GC substitutions. This excess of GC \rightarrow AT substitutions in transposons is observed both in GC-rich and GC-poor isochores. This suggests that the GC content of the human genome might be decreasing (*i.e.*, not stationary), questioning the conclusions mentioned above. It should be noted, however, that the pattern of substitution in transposable elements might be different from that of nonrepetitive DNA, because repeated sequences can be subject to specific mutation patterns (KRICKER *et al.* 1992).

The aim of this article was therefore to directly determine whether the GC content of nonrepetitive sequences is or is not at equilibrium in mammalian genomes. For this purpose, we analyzed orthologous gene sequences in closely related species from three mammalian orders (primates, rodents, cetartiodactyls). We show that in these mammals, the GC content of genes located in GC-rich isochores has been decreasing. These results indicate that GC-rich isochores are slowly disappearing from mammalian genomes. The predictions of the three models for the origin of isochores are reevaluated in this nonequilibrium situation and compared to human sequence polymorphism data. The issue of the origin and evolution of isochores is discussed in the context of a nonstationary process.

MATERIALS AND METHODS

We selected orthologous genes from closely related species in three mammalian orders, namely primates, Rodentia, and Cetartiodactyla. In each order, a triplet of species of the form [[ingroup1, ingroup2], outgroup] was defined. In primates the ingroups were human and another Hominidae (chimpan-

TABLE 1
Average synonymous substitution rates (K_s) in the data sets of orthologous genes used in this study

Typical triplet	Order		
	Rodentia (hamster, (mouse, rat))	Cetartiodactyla (pig, (cow, sheep))	Primates (papio, (human, chimpanzee))
No. of genes	194	79	55
K_s ingroup1/ingroup2	0.19	0.08	0.02
K_s ingroup/outgroup	0.29	0.11	0.07

zee, gorilla, or orangutan), the outgroup any Catarrhini (external but as close as possible to the two ingroups, generally Papio). In Rodentia, the triplet was generally [[rat, mouse], hamster], but any triplet of species matching the [[Rattus, Mus], non-Murinae Muridae] pattern was considered acceptable. In Cetartiodactyla all comparisons involved [[Caprinae, Bovinae], Suidae], excepting one gene for which the outgroup was a Cervidae. The coding sequences of every gene available in a triplet of species as defined above were collected using the Hovergen database (release 40, May 2000; DURET *et al.* 1994), and phylogenetic trees were checked to retain only orthologous genes. Average synonymous substitution rates (computed with the method of LI 1993) in each data set are indicated in Table 1. The list of orthologous genes is available at <http://pbil.univ-lyon1.fr/datasets/Isochore2002/data.html>.

The GC content expected at equilibrium at synonymous codon position ($GC3eq$) was estimated using only the third position of fourfold degenerate codons. $GC3eq$ was computed by the ratio $u/(u + v)$, where u is the rate of AT \rightarrow GC substitutions and v the rate of GC \rightarrow AT substitutions. We measured u (and v) at the third position of fourfold degenerate codons by dividing the number of AT \rightarrow GC substitutions (GC \rightarrow AT substitutions) observed in the two ingroups by the number of AT (GC) in the ancestral sequence inferred by the maximum parsimony method (ambiguous bases were ignored).

SYNONYMOUS SUBSTITUTION PATTERNS IN MAMMALIAN GENES

Excess of GC \rightarrow AT synonymous substitutions in GC-rich genes: To determine whether the GC content of mammalian genes is at equilibrium, we analyzed orthologous coding sequences from different taxa: rodents (194 genes), cetartiodactyls (79 genes), and primates (55 genes; Table 1). The synonymous substitution process was approached using the parsimony criterion. A substitution from nucleotide X to nucleotide Y was inferred when both the outgroup and one ingroup shared state X, but the other ingroup showed state Y. We analyzed all informative substitutions: 7058 substitutions in rodents, 817 substitutions in cetartiodactyls, and 197 substitutions in primates. Genes were classified in three groups depending on their $GC3$ (<57%, 57–75%, >75%; MOUCHIROUD *et al.* 1991). The results are displayed in Table 2. A large, significant excess of GC \rightarrow AT over AT \rightarrow GC changes is found for GC-rich genes in the three orders. It has been shown previously that the GC content of GC-rich genes had been decreasing in

the rodent lineage (GALTIER and MOUCHIROUD 1998). Our results show that this erosion is also occurring in primates and cetartiodactyls.

In primates and cetartiodactyls, the present GC content is very far from the value expected at equilibrium given the observed substitution pattern (Table 2). The bias is less strong in GC-median genes, and virtually no bias is found in GC-poor genes.

Is it possible that the excess of GC \rightarrow AT substitution is due to recent translocations of GC-rich genes into a GC-poor genomic context? To test this hypothesis, we examined the synonymous substitution pattern in primates according to the GC content of the surrounding genomic region. For this purpose, we retrieved for each human gene the largest overlapping genomic sequence available in GenBank (163 kb in average). The 55 genes were split into three groups, according to the GC content of their genomic context. As shown in Table 3, the results of our analysis remain unchanged (compare with Table 2): there is a large excess of GC \rightarrow AT substitutions, especially for the genes located in regions of high GC content.

Methodological artifact? Is it possible that this result is due to a methodological artifact? The pattern [[ingroup1 = X, ingroup2 = Y], outgroup = X] is interpreted by the parsimony method as a single X \rightarrow Y substitution in the ingroup2 lineage. Parsimony can fail in case of multiple substitutions (*e.g.*, the above pattern can occur through two independent Y \rightarrow X substitutions in the ingroup1 and outgroup lineages). The method remains unbiased as long as the base composition is balanced (*i.e.*, $X\% = Y\%$): failures are equiprobable for X \rightarrow Y and Y \rightarrow X actual changes. When base composition is unbalanced, however, the maximum parsimony method tends to overestimate the proportion of substitutions from common bases to rare bases (PERNA and KOCHER 1995). In an X-rich sequence data set (*i.e.*, when the rate of Y \rightarrow X substitution is higher than the rate of X \rightarrow Y substitution), the method will overestimate the proportion of X \rightarrow Y changes. The bias can be strong even for moderate levels of divergence between sequences (EYRE-WALKER 1998; GALTIER and BOURSOT 2000). Maximum-likelihood methods (*e.g.*, GALTIER and GOUY 1998) are more robust than the

TABLE 2
Pattern of synonymous substitutions (AT \leftrightarrow GC) in mammalian genes of different GC content

Order	GC3 class (%)	All codons			Quartet codons		No CpG: GC \rightarrow AT ^c	GC3q ^d (%)	GC3eq ^e (%)
		GC \rightarrow AT ^a	AT \rightarrow GC ^a	Ratio ^b	GC \rightarrow AT ^a	AT \rightarrow GC ^a			
Rodentia	<57	934	1378	0.7	374	585	327	46	58
	57–75	2143	1923	1.1	910	904	806	64	61
	>75	431	249	1.7**	182	128	157	78	69
Cetartiodactyla	<57	155	161	1	65	65	53	44	46
	57–75	185	104	1.8**	97	45	85	64	48
	>75	162	50	3.2**	88	20	64	83	56
Primates	<57	40	25	1.6*	18	11	13	41	34
	57–75	53	32	1.7*	31	23	24	63	54
	>75	37	10	3.7**	18	4	13	80	43

Data sets were split into three groups of genes of low, medium, and high GC3 content.

^aTotal number of synonymous substitutions counted in the branches leading to the two ingroups (see Table 1 for details).

^bRatio of GC \rightarrow AT over AT \rightarrow GC substitutions. Significance was assessed by a binomial test (probability of observing that ratio or higher assuming an equal expected number of the two kinds of changes). * $P < 0.05$; ** $P < 0.01$.

^cNumber of GC \rightarrow AT substitutions at the third position of quartet codons (*i.e.*, fourfold degenerate codons), excluding all positions corresponding to a CpG dinucleotide in the ancestral sequence.

^dGC3q, average GC content at the third position of quartet codons.

^eGC3eq, GC content expected at equilibrium at the third position of quartet codons (see text). Note that given the low number of substitutions analyzed in primate GC-rich genes, this estimate might not be very accurate for that subset.

maximum-parsimony method to estimate the ancestral GC content. However, they could not be used here because at present too few genes have been sequenced in enough species for such analyses.

To assess the extent of the bias caused by the maximum-parsimony method in this analysis, we made use of a restricted data set of 20 primate genes for which an additional species was available. This fourth species was used to measure the rate of error of the maximum-parsimony method. We selected quartets of the form [[[ingroup1, ingroup2], ingroup3], outgroup] (generally [[[human, chimpanzee], gorilla or orangutan], a non-Hominidae Catarrhini]). We examined all the sub-

stitutions previously inferred from the three-species analysis, and we counted as ambiguous all the cases where the nucleotide in the ingroup³ was different from the outgroup (*i.e.*, patterns different from [[[X, Y], X], X]). Among the 78 substitutions analyzed, 9 (12%) were ambiguously oriented (*i.e.*, counted as X \rightarrow Y whereas they might correspond to Y \rightarrow X substitutions). The error rate was similar for GC \rightarrow AT (13%, 7/55) and for AT \rightarrow GC (9%, 2/23) substitutions. In GC-rich genes (*i.e.*, GC3 > 75%), there are four times as many GC \rightarrow AT as AT \rightarrow GC substitutions (12 vs. 3, a ratio very close to what was measured in the full three-species data set, see Table 2), and this ratio remains unchanged when erroneously orientated substitutions (13%, 2/15) are removed. In other words, the bias in the parsimony method is weak for this data set (presumably because evolutionary distances are very short) and cannot account for the fourfold excess of GC \rightarrow AT substitutions over AT \rightarrow GC substitutions observed in GC-rich genes from primates.

This test could not be performed in rodents and cetartiodactyls because of lack of data. We therefore analytically assessed the amount of bias of the maximum-parsimony method using EYRE-WALKER's (1998) equations. These equations give the expected number of GC \rightarrow AT and AT \rightarrow GC substitutions maximum parsimony would infer, given base composition and distances between analyzed sequences. Under the hypothesis that base composition is at equilibrium, the ratios of GC \rightarrow AT substitutions over AT \rightarrow GC substitutions that we would have expected to measure in rodent, cetartiodac-

TABLE 3

Pattern of synonymous substitutions (AT \leftrightarrow GC) in primate genes located in different isochore contexts

GC class ^a (%)	GC \rightarrow AT ^b	AT \rightarrow GC ^b	Ratio ^c
<43	60	39	1.5
43–49	40	18	2.2
>49	30	10	3.0

^aGenes were split into three groups according to the GC content of the GenBank genomic sequence containing them (average length of the genomic sequence = 163 \pm 50 kb). The limits of GC content correspond to the 33% lowest and highest GC content in the entire data set of 1892 complete human genes (see Figure 2 legend).

^bTotal number of synonymous substitutions counted in the branches leading to the two ingroups.

^cRatio of GC \rightarrow AT over AT \rightarrow GC substitutions.

tyl, and primate GC-rich genes are, respectively, 1.9, 1.4, and 1.2 (compared with 1.7, 3.2, and 3.7 observed in the data set, see Table 2). Hence, the hypothesis of equilibrium is clearly rejected in cetartiodactyls and primates (in agreement with the previous test), but not in rodents. This does not mean that base composition is at equilibrium in rodents, but simply that we cannot totally exclude that the observed excess of GC → AT substitutions is due to a methodological artifact of the maximum-parsimony method (see DISCUSSION).

Hypermutability of CpG dinucleotides: It is well known that in mammals, CpG dinucleotides are hot-spots of C → T mutations because of the deamination of methylated cytosines (COULONDRE *et al.* 1978; BIRD 1980). To determine whether these mutations could account for the excess of GC → AT substitutions, we removed from our analyses all GC → AT substitutions that occurred within a CpG dinucleotide in the reconstructed ancestral sequence. The substitutions at CpG dinucleotides correspond to 14% (rodents), 28% (primates), and 31% (cetartiodactyls) of GC → AT substitutions in GC-rich genes. But in all taxa, the number of GC → AT substitutions remained higher than the number of AT → GC substitutions after CpG's were removed (Table 2). Hence, the decrease in GC content of GC-rich genes is not due solely to the hypermutability of CpG dinucleotides.

FIXATION BIAS IN FAVOR OF GC ALLELES

An asymmetric pattern of GC/AT polymorphisms in human has been reported by EYRE-WALKER (1999; MHC sequence data) and SMITH and EYRE-WALKER [2001; single-nucleotide polymorphism (SNP) data]: a higher number of GC → AT than AT → GC polymorphic sites was found. In these analyses, it was assumed that the GC content was at equilibrium (*i.e.*, that the number of GC → AT substitutions was equal to the number of AT → GC substitutions). Hence, the difference between the mutation pattern (revealed by polymorphism data) and the substitution pattern (inferred from the hypothesis of stationarity) was interpreted as the result of a biased fixation process, either selection or BGC, favoring GC alleles (EYRE-WALKER 1999; SMITH and EYRE-WALKER 2001). However, our analysis of synonymous substitution patterns (see above) shows that the hypothesis of stationarity does not hold: the GC content is not at equilibrium. Our results, therefore, question the existence of a fixation bias favoring GC alleles—a major argument against the mutation bias hypothesis.

We reanalyzed the SNP data set (CARGILL *et al.* 1999) used by SMITH and EYRE-WALKER (2001) to check the mutation bias hypothesis further. This data set includes SNPs sampled in 114 human chromosomes and in one chimpanzee. Instead of analyzing the number of polymorphic sites of each category (either GC → AT or AT → GC), we focused on the distribution of the fre-

quencies at which these alleles occur in the sample. We chose this approach because it is not affected by the fact that the GC content is not at equilibrium: the fixation dynamics of a mutation, given that it has occurred, is independent of the stationary status of base composition. Hence, in the absence of any fixation bias, similar distributions of allele frequency are expected for GC → AT or AT → GC polymorphisms, whatever the mutation process. Conversely, if GC alleles had a higher probability of fixation than AT alleles (because of selection or BGC), the allele frequency distribution of AT → GC polymorphisms should be shifted to the right (advantageous mutations segregate at higher frequencies, on average).

Polymorphisms were oriented using the chimpanzee as an outgroup. Noncoding and synonymous polymorphisms were pooled. Nonsynonymous polymorphisms were excluded from this analysis as they undergo selection at the protein level. The total number of SNPs analyzed was 410. CARGILL *et al.* (1999) did not indicate exact allele frequencies, but classified polymorphic sites in four classes of frequency: low (<5%), median, (5–15%), high (15–50%), or very high (>50%). The distributions of allele frequencies of GC → AT and AT → GC polymorphisms in GC-rich (GC3 > 75%, 169 SNPs), GC-median (96 SNPs), and GC-poor (GC3 < 57%, 145 SNPs) genes are shown (Figure 1). A visual inspection suggests that AT → GC mutations segregate at higher frequencies than GC → AT mutations (*i.e.*, that GC alleles have a higher probability of fixation than AT alleles), especially in GC-rich genes.

A likelihood-ratio test was performed to assess the significance of this apparent discrepancy between the two observed distributions. Two multinomial models were fitted to the allele frequency data. The joint multinomial model (null hypothesis) assumes that the allelic frequencies of AT → GC and GC → AT polymorphic sites are drawn from a unique multinomial distribution (three parameters). The full multinomial model allows one free parameter for every frequency class of AT → GC and GC → AT polymorphisms (six parameters): distinct distributions are assumed for the two categories of SNPs. The likelihood was calculated under the two models according to the multinomial formula. Optimal parameter values are obvious: the expected proportions of each class of allele frequency are taken as the observed ones, separating (full multinomial model) or pooling (joint multinomial model) the distributions of AT → GC and GC → AT polymorphisms. Twice the difference in log-likelihood between the two models follows a χ^2 distribution (3 d.f.) under the null hypothesis of a common distribution. Results are displayed in Table 4. No difference between the two distributions was detected in GC-poor genes, but AT → GC polymorphisms appeared to segregate at significantly higher allele frequencies, on average, than GC → AT polymorphisms in both GC-median and GC-rich genes.

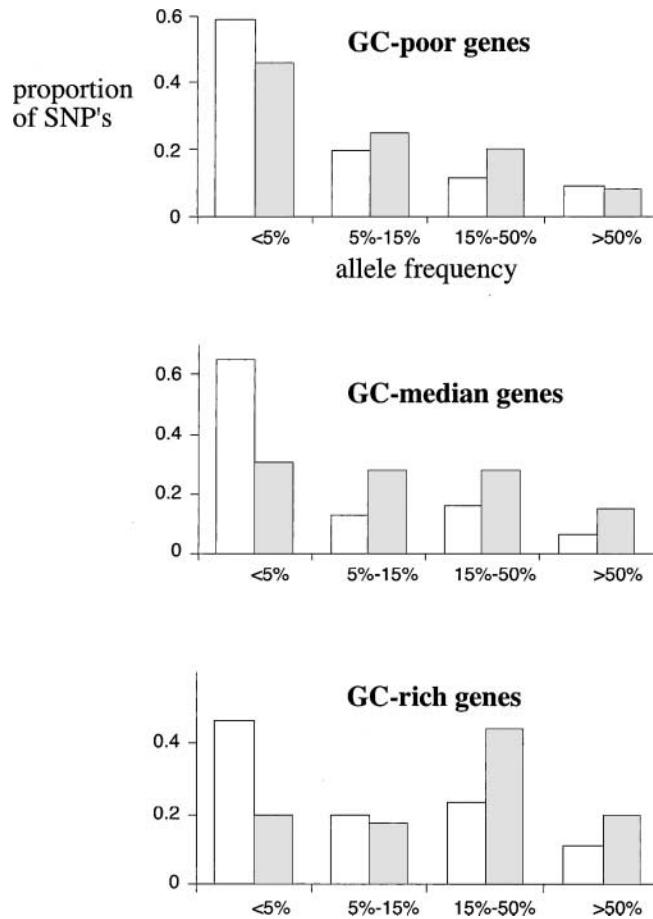


FIGURE 1.—Distribution of allele frequencies of 410 human SNPs in three classes of gene GC content. SNPs were classified in four classes depending on the frequency of the mutant allele. The proportions of allele frequency classes are represented separately for GC → AT (open bars) and AT → GC (shaded bars) polymorphic sites. Number of polymorphic sites analyzed: GC-poor genes, $n = 145$; GC-median genes, $n = 96$; GC-rich genes, $n = 169$.

A population genetics model was also fitted to the allele frequency data set, following HARTL *et al.* (1994) and AKASHI and SCHAEFFER (1997). This model assumes that a given fixation bias (BGC or selection coefficient w) favoring GC over AT alleles applies independently at every site in a panmictic population of effective size $2N$, at selection (BGC)/mutation/drift equilibrium. The expected probability density of allele frequencies under these assumptions has been derived from diffusion equations (*e.g.*, SAWYER and HARTL 1992). It depends on a single parameter, namely the $4Nw$ product. The density was numerically integrated over the relevant frequency boundaries (0–5%, 5–15%, . . .) to provide the required discrete expected distribution. Using this model, one can test the neutral hypothesis by comparing the maximum likelihood (leaving w free to vary) to the likelihood obtained by setting $w = 0$ (null, neutral hypothesis). The results were essentially consistent with the multinomial tests: no departure from neutrality was

TABLE 4

Maximum-likelihood analysis of the allele frequency distribution of GC → AT and AT → GC polymorphisms

Data set	Multinomial model	n_p^a	LRT ^c	
			$\ln(L)^b$	
GC poor	Full	6	−11.93	NS
	Joint	3	−13.65	
GC median	Full	6	−10.67	*
	Joint	3	−16.12	
GC rich	Full	6	−12.44	**
	Joint	3	−18.59	

^a Number of free parameters.

^b Maximum log-likelihood.

^c Likelihood-ratio test. NS, not significant; * $P < 0.05$; ** $P < 0.01$.

detected for the GC-poor data set, but neutrality was rejected for GC-median genes (P -value $< 1\%$). As far as the GC-rich data set was concerned, the population genetics model fitted badly to the observed distribution of allele frequency. Indeed, a comparison between this model and the full multinomial model led to rejection of the hypothesis of a panmictic population at equilibrium (twice the likelihood ratio: 13.7, 5 d.f., P -value $< 5\%$), making the test of neutrality impracticable.

This peculiar feature of GC-rich genes is a bit surprising. It can hardly be explained by a departure from demographic equilibrium or panmixy since the population history is shared by every part of the genome. We speculate that this uncommon distribution might result from a possible variation of fixation bias, w , among GC-rich regions of the genome—the above analysis assumes that a unique w applies to all the SNPs pooled in any one category. The “GC-rich gene” category might include a fraction of SNPs located in regions of the genome undergoing an exceptionally high fixation bias (see the “GC-factory” hypothesis below), the remaining SNPs of this category being located in “regular” GC-rich regions. This would explain the occurrence of many high-frequency AT → GC polymorphisms (contributed by the putative w hotspots), leading to a departure from distributions expected under the “single w ” model.

The above analyses treat SNPs as independent data. This is not the case actually: the 410 analyzed SNPs are located in the vicinity of 106 genes, so that some of them are presumably genetically linked, and their allele frequency is correlated. Please note that this should not result in a bias toward a higher frequency of some kind (*e.g.*, GC) of variants—linkage occurs independently of the AT/GC status of mutants. Linkage should essentially reduce the signal; *i.e.*, the sampling variance will be higher than that of a sample of 410 independent SNPs. Our detection of a significant fixation bias, therefore, appears conservative in this respect.

Overall, these results confirm, on a larger gene data set, a previous analysis of allele frequency distribution in human and mouse MHC genes (EYRE-WALKER 1999). Eyre-Walker's finding—a fixation bias favoring GC over AT alleles—appears valid despite the nonstationarity of the substitution process. This bias, however, appears restricted to GC-median and GC-rich regions. Note that an alternative hypothesis can be proposed to explain the difference in frequency distribution between AT and GC alleles: the excess of AT alleles segregating at low frequency could be due to a recent increase in $GC \rightarrow AT$ mutation rate. This scenario, however, seems unlikely because the putative change in mutation pattern must have occurred very recently ($< \sim 4N$ generations ago).

DISCUSSION

Erosion of GC-rich isochores: An analysis of substitution pattern in defective DNA transposons revealed an excess of $GC \rightarrow AT$ substitutions over $AT \rightarrow GC$ substitutions, suggesting that the human genome was not at equilibrium (LANDER *et al.* 2001). We show here that this pattern of substitution is observed not only in repetitive DNA but also in unique sequences: the synonymous GC content of genes located in GC-rich isochores is decreasing in the mammalian genomes, presumably as a consequence of an AT-biased mutation process.

We did not analyze noncoding sequences because there are presently not enough species for which such data are available. However, numerous works have shown that in mammals there is a strong correlation between the GC content at synonymous codon positions and the GC content of the genomic region in which a gene is located (*e.g.*, AïSSANI *et al.* 1991; CLAY *et al.* 1996). As shown in Figure 2, this correlation is confirmed here with a much larger data set. Thus, whatever the process that drives the evolution of isochores, this process acts not only in noncoding regions but also at synonymous codon positions. It should also be noted that the subset of 55 human genes that we have used to infer synonymous substitutions in primates is clearly representative of the whole data set (Figure 2). This suggests that the pattern of synonymous substitution that we measured directly reflects the evolution of isochores. This conclusion is further supported by the fact that the same pattern is observed in transposable elements that are essentially located in introns or intergenic regions (LANDER *et al.* 2001). In other words, these different results indicate that GC-rich isochores have been disappearing from the genome of these mammalian orders.

It was shown previously that the GC content of rodent genomes was less heterogeneous than that of primates (*i.e.*, GC-rich genes are less GC rich in rodents than in primates, and conversely for GC-poor genes; MOUCHIROUD and GAUTIER 1988) and that this change in GC content occurred in the rodent lineage (and hence was

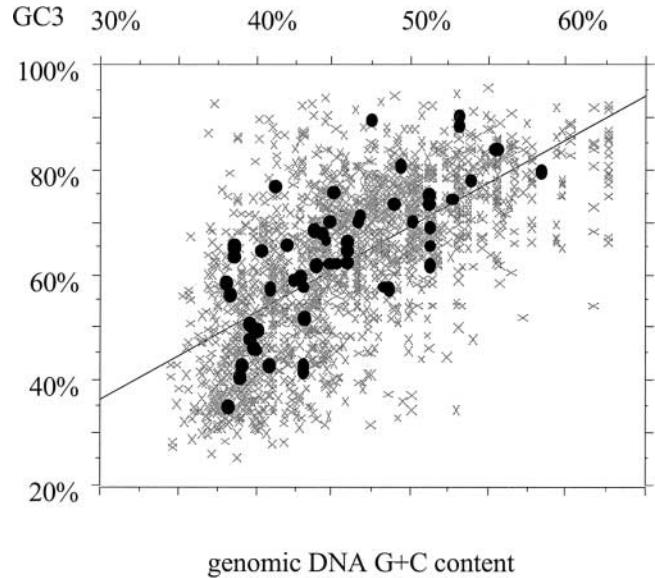


FIGURE 2.—Correlation between the GC content at third codon positions (GC3) of human protein-coding genes and the GC content of the genomic region (>100 kb) in which they are located. We have selected in GenBank (release 128, February 2002) all complete human genes (excluding those with <100 codons) annotated in genomic sequences >100 kb. This data set contains 1892 genes ($\sim 10^6$ codons), from 874 sequences (total = 137 Mb). Correlation coefficient: $R^2 = 0.43$. The subset of genes used in this work to infer synonymous substitution patterns is indicated by solid dots.

called the “murid shift”; ROBINSON *et al.* 1997; GALTIER and MOUCHIROUD 1998). However, in all other taxa analyzed (primates, Lagomorpha, Cetartiodactyla, Carnivora) the GC3's of orthologous genes are very close to each other (MOUCHIROUD and BERNARDI 1993). Hence, it was generally thought that the GC content had remained unchanged in these mammalian orders since the time of their divergence, 80–100 million years ago.

Our results show that the decrease of GC-rich isochores, previously reported in rodents, is actually occurring in primate and cetartiodactyl genomes. As far as rodents are concerned, we observed both a decrease in GC content in GC-rich isochores and an increase in GC-poor isochores (Table 2), suggesting that the murid shift might be an ongoing process. However, evolutionary distances between the rodent species we analyzed are too high for the maximum-parsimony method to be trusted. Sequence data involving more closely related species would be required for confirming the observed trend in rodents. Overall, the reports of a long-term (rodents, GALTIER and MOUCHIROUD 1998) and short-term (primates and cetartiodactyls, this study) decline of GC-rich isochores in various mammalian lineages suggest that this erosion is a general process of mammalian genome evolution. The similarity in base composition in orthologous genes from distant mammalian taxa such as primates and cetartiodactyls simply reflects the fact that they have undergone similar substitution pat-

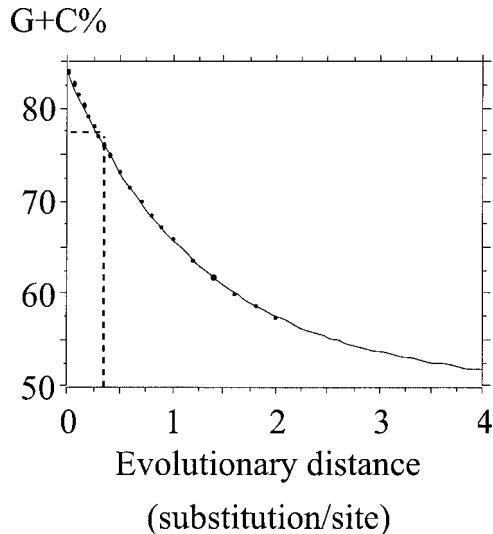


FIGURE 3.—Evolution of the GC content of a sequence (initially at 85% GC) and subject to a substitution pattern leading to an equilibrium GC content of 50% (with a transition/transversion ratio of 2).

terns and/or that there was not enough time to accumulate enough substitutions to significantly affect their GC content. Note that the time necessary to reach equilibrium can be very long. For example, consider a GC-rich sequence (say 85% GC) subject to a pattern of substitution leading to equilibrium GC content of 50% (*i.e.*, similar to the substitution pattern observed in primate or cetartiodactyl GC-rich genes). As illustrated in Figure 3, after 0.33 substitution per site (which corresponds to the average synonymous evolutionary distance between human and bovine orthologous genes), the GC content is still 77%. It is therefore possible that the decline in GC content presently observed in mammalian GC-rich isochores began a long time ago, before the divergence between the different mammalian orders.

The second major conclusion of our study, based on the analysis of allele frequency distribution, is that a directional evolutionary force is biasing the fixation process of GC/AT polymorphism toward GC in GC-rich, but not in GC-poor regions of the genome. However, this force is not strong enough to maintain GC-rich isochores, since GC \rightarrow AT substitutions are more frequent than AT \rightarrow GC substitutions. In other words, in GC-rich genes, GC alleles have a higher probability of fixation than AT alleles, but this bias is not sufficient to overcome the large excess of GC \rightarrow AT mutations.

Fixation bias favoring GC alleles: biased gene conversion: The fixation bias favoring GC alleles is likely to be a result of BGC, not selection. Indeed, different observations support the BGC model: experiments in mammalian cells have shown that the repair of DNA mismatches is biased in favor of GC; genes that frequently undergo conversions are GC rich, and there is an overall correlation between GC content and recombi-

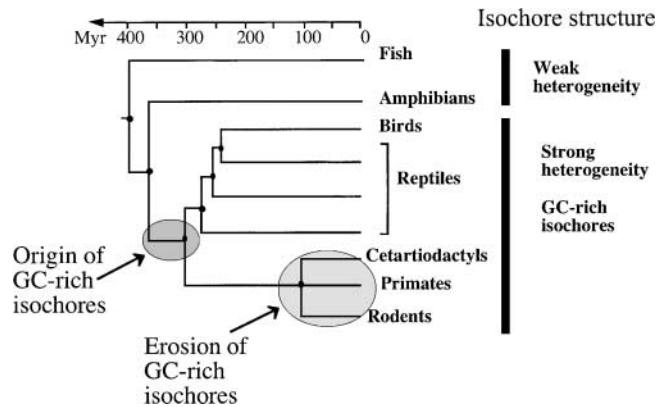


FIGURE 4.—Phylogenetic distribution of GC-rich isochores in vertebrates. Genomes of fishes and amphibians are weakly heterogeneous in base composition. Genomes of mammals, birds, and reptiles are generally highly heterogeneous and contain GC-rich isochores (HUGHES *et al.* 1999).

nation rate (reviewed in GALTIER *et al.* 2001). EYRE-WALKER and HURST (2001) also concluded that BGC was the most likely scenario for the origin of isochores. They raised, however, an important point apparently not consistent with this model: a positive correlation between substitution rate and GC content has been reported (BIELAWSKI *et al.* 2000), whereas the BGC model (like selection) predicts that the correlation should be negative. However, this prediction holds only if the GC content is at equilibrium. On the contrary, when the GC content is decreasing, one expects a positive correlation between substitution rate and GC content (PIGANEAU *et al.* 2002). In conclusion, the data are consistent with the hypothesis that the origin of GC-rich isochores is due to BGC, but that this process is no longer effective to maintain their GC content.

Origin and evolution of GC-rich isochores: The origin and evolution of isochores becomes even more mysterious in this nonequilibrium context. As mentioned in the Introduction, the acquisition of GC-rich isochores occurred in the amniote lineage, \sim 310–350 million years ago. However, the substitution process measured from the comparison of closely related species indicates that GC-rich isochores are disappearing from mammalian genomes. The fact that similar patterns are found in three different mammalian orders suggests that this erosion may have started >80 million years ago (see Figure 4).

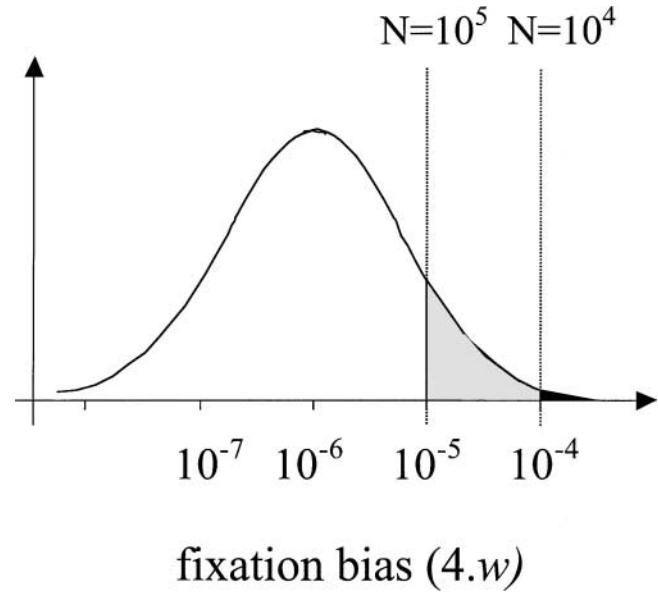
How to explain the rapid acquisition of GC-rich isochores in the amniote ancestral genome and their slow decay in mammals? A part of the answer probably lies in the asymmetry between the processes of GC increase and GC decrease. The data reported in this study are consistent with the existence of an AT-biased mutation process ($u < v$, where u is the AT \rightarrow GC mutation rate and v the GC \rightarrow AT mutation rate) *vs.* a GC-biased fixation process (w). In regions of the genome/periods of time of negligible fixation bias ($4Nw \ll 1$), the GC

content therefore approaches the mutational equilibrium through mutation/drift, at neutral rate $\theta v - (1 - \theta)u$, where θ is the current GC content. When the fixation bias is strong ($4Nw \gg 1$), however, advantaged GC mutations fix with a probability $2w$ (vs. $1/2N$ in absence of fixation bias), so that the GC content will increase at a rate close to $4(1 - \theta)uNw$, possibly much higher than the neutral rate. Therefore, short and/or localized episodes of GC increase can significantly contribute to the total amount of G and C, even if they concern a small fraction of the genome/time scale. In the light of these notions, we now propose several scenarios about the origin of GC-rich isochores, accounting for the newly reported nonequilibrium situation.

Model 1: the GC-factory hypothesis: This scenario invokes a spatial, but not a temporal heterogeneity of BGC coefficient. Imagine that a small fraction of the genome is undergoing a strong fixation bias toward GC (GC factory), while the major part of the genome is evolving neutrally (*i.e.*, without selection or BGC). Now assume that these two fractions communicate in some way (translocations or duplications). Sequences entering a GC factory would undergo a rapid increase of GC content. Such GC-rich sequences would then be released in the major component and form GC-rich isochores. Now when randomly sampling in the genome one would essentially sample genes from the major, neutrally evolving component and observe a global decrease of GC content. An example of such a process is provided by the *Fxy* gene in mouse. This gene used to undergo a rapid increase of GC content after it was translocated in the pseudoautosomal region (PERRY and ASHWORTH 1999). GC factories might also explain the uncommon frequency distribution of $AT \leftrightarrow GC$ polymorphisms assigned to the GC-rich gene category (see above).

In this hypothesis, the genome would be at a mutation-selection-migration equilibrium, where migration refers to movements of genes between regions of the genome. Although appealing, this scenario appears incompatible with the observed conservation of isochores among mammalian orders. The GC3's of orthologous genes sampled in primates, Cetartiodactyla, Carnivora, and Lagomorpha are highly correlated (MOUCHIROUD and BERNARDI 1993). Such a correlation is not expected if genes were randomly moving to/from GC factories independently in distinct lineages.

Model 2: a unique origin of GC-rich isochores: The hypothetical scenario that we propose for the origin of GC-rich isochores is the following. In the ancestral amniote, a change occurred in a DNA repair system, resulting in the preferential repair of AT:GC mismatches into GC and hence to a biased gene conversion favoring the fixation of GC alleles. As suggested by FRYXELL and ZUCKERKANDL (2000), this biased repair process might be an adaptation to the high rate of mutation of cytosine in heavily methylated genomes. As in many present-day genomes, there was probably a high variability of



fixation bias ($4.w$)

FIGURE 5.—Hypothetical distribution of the selection (or BGC) coefficient w within mammalian genomes and its implication with respect to isochore evolution. A constant-in-time, lognormal distribution of w is assumed for convenience. With a low effective population size (here, 10^4), the fraction of the genome under significant effective fixation bias ($4Nw > 1$) is minute (black). A larger ancestral effective population size (10^5) would have resulted in a GC increase of a nonnegligible part of the genome (gray).

recombination rate along the ancestral genome of amniotes. Thus, GC-rich isochores would have resulted from BGC in regions of high recombination of this ancestral amniote genome. Note that the repair of G:T mismatches in *Xenopus* is unbiased (VARLET *et al.* 1990) and, consistent with that model, *Xenopus* does not have GC-rich isochores. The enrichment in GC content in regions of high recombination rate may have continued independently in different amniote lineages, after the split among mammals, birds, and reptiles. Then, BGC ceased to be effective—at least in mammals, and the GC content of GC-rich isochores began to decrease (Figure 4).

Why did BGC cease to be effective? As mentioned previously, BGC significantly affects the fixation of GC alleles only if the product $4Nw$ is greater than one (where N is the effective population size and w the BGC coefficient). We propose two possible explanations for the decline of GC-rich isochores.

First, the evolution of GC content might be linked to variations in effective population size. For a given distribution of w among regions of the genome, the fraction of the genome undergoing significant fixation bias (and eventually becoming GC rich) increases with N , as illustrated by Figure 5. GC-rich isochores might therefore have appeared in an ancestral species larger than today's population size. After a subsequent decrease of population size, most of the genome would

be under too low a fixation bias ($4Nw$) for GC-rich isochores to be maintained.

The second possible explanation is that the evolution of GC-content might be linked to variations in recombination (and, therefore, BGC) rate. Such variations could have occurred as a result of chromosome rearrangements. The GC content of autosomal chromosomes is negatively correlated to chromosome size in human ($R^2 = 0.32$, $P = 0.006$; data from VENTER *et al.* 2001). This is probably because the per nucleotide recombination rate is higher in small chromosomes (*e.g.*, KABACK 1996; LANDER *et al.* 2001). Imagine that the ancestral amniote genome used to have some very small chromosomes. Their GC content would have increased rapidly as a consequence of their high recombination rate. These pieces of DNA would later have formed GC-rich isochores when fused with standard chromosomes. This scenario was suggested by the observation of such GC-rich microchromosomes in the genome of birds (MCQUEEN *et al.* 1998; ANDREOZZI *et al.* 2001), in which events of fusion between micro- and macrochromosomes have been reported (SHETTY *et al.* 1999). It would also account for the GC richness of telomeric regions: the random fusion of many GC-rich microchromosomes and few GC-poor macrochromosomes should result in a high proportion of GC-rich-ending fused chromosomes.

These mechanisms are possibly simplistic and not mutually exclusive. We leave them as an open hypothesis for future work on this issue. To conclude, it should be stressed that whatever the evolutionary force (neutral or selective) was at the origin of GC-rich isochores, this force is no longer effective to maintain them in mammalian genomes.

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