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Evolution

Evidence for higher rates of nucleotide substitution in rodents than in man

(molecular clock/generation-time effect/synonymous substitution/nonsynonymous substitution/neutral theory)

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ABSTRACT **When the coding regions of 11 genes from rodents (mouse or rat) and man are compared with those from another mammalian species (usually bovine), it is found that rodents evolve significantly faster than man. The ratio of the number of nucleotide substitutions in the rodent lineage to that in the human lineage since their divergence is 2.0 for synonymous substitutions and 1.3 for nonsynonymous substitutions. Rodents also evolve faster in the 5′ and 3′ untranslated regions of five different mRNAs; the ratios are 2.6 and 3.1, respectively. The numbers of nucleotide substitutions between members of the β-globin gene family that were duplicated before the man–mouse split are also higher in mouse than in man. The difference is, again, greater for synonymous substitutions than for nonsynonymous substitutions. This tendency is more consistent with the neutralist view of molecular evolution than with the selectionist view. A simple explanation for the higher rates in rodents is that rodents have shorter generation times and, thus, higher mutation rates. The implication of our findings for the study of molecular phylogeny is discussed.**

The molecular clock or rate-constancy hypothesis (1) has been very controversial (2, 3) since its proposal. A very important aspect of the controversy is whether generation time has any significant effect on the rate of molecular evolution. The resolution of this question is highly relevant to the neutralist–selectionist controversy. The rate constancy *per year* has been supported by many comparative studies of protein sequences and immunological distances (2, 3), and it often has been argued that such observations support the neutral-mutation hypothesis (3). A criticism against this argument is that, if the hypothesis is true, the rate should be constant *per generation* rather than *per year* as the rate of mutation is sometimes believed to be (4). The resolution is also highly relevant to the study of molecular phylogeny because the rate constancy *per year* has often been assumed in phylogenetic reconstruction and in estimation of divergence times between species or genes (5). In view of these important bearings, we shall reevaluate the above issue by comparing the rates of nucleotide substitution in man and rodents. We shall use only tests that do not require knowledge of species divergence times, particularly the relative rate test (6). The availability of a large number of DNA sequences makes such tests feasible.

An advantage of using nucleotide sequences is that we can distinguish nucleotide changes that cause amino acid replacements (nonsynonymous changes) from those that do not (including synonymous changes in protein coding regions and changes in noncoding regions). Such a distinction is important because many DNA–DNA hybridization studies showed a generation time effect (2, 7, 8), whereas studies of amino acid changes often suggested the contrary (2). It is possible that the generation time effect is not so strong on the rate of nonsynonymous substitution as on the rates of synonymous substitution and nucleotide substitution in noncoding regions. We shall test the generation time effect, considering these different types of nucleotide substitution separately.

METHODS

Calculation of Numbers of Nucleotide Substitutions. The method (9) used here enables one to calculate the number of synonymous nucleotide substitutions and that of nonsynonymous substitutions separately. Briefly, we classify nucleotide sites as 4-fold degenerate, 2-fold degenerate, or nondegenerate. At a 4-fold degenerate site, all nucleotide changes are synonymous, whereas at a nondegenerate site every change is either nonsynonymous or nonsense. At a 2-fold degenerate site, a change is synonymous if it is transitional (C ↔ T or A ↔ G) and is nonsynonymous (or nonsense) if it is transversional. One exception to this rule in the nuclear genetic code is the first positions of four of the six arginine codons. Also, the third positions of the three isoleucine codons are actually 3-fold degenerate. We resolve these two problems by making some minor adjustments (9). (These problems do not occur in the mammalian mitochondrial genetic code.)

For a site of i-fold degeneracy (i = 0, 2, or 4), we can estimate the number of transitional substitutions, $A(i)$, and that of transversional substitutions, $B(i)$, by using Kimura's (10) formulas:

$$A(i) = (1/2)\ln\{1/[1 - 2P(i) - Q(i)]\} - (1/4)\ln\{1/[1 - 2Q(i)]\}, \quad [1]$$

$$B(i) = (1/2)\ln\{1/[1 - 2Q(i)]\}, \quad [2]$$

where $P(i)$ and $Q(i)$ are the proportions of transitional and transversional differences, among i-fold degenerate sites, between the two nucleotide sequences compared. The total number of substitutions per i-fold degenerate site is $K(i) = A(i) + B(i)$. We note that $A(2)$ and $B(2)$ denote the numbers of synonymous and nonsynonymous substitutions per 2-fold degenerate site, respectively; $K(4) = A(4) + B(4)$, the number of synonymous substitutions per 4-fold degenerate site; and $K(0) = A(0) + B(0)$, the number of nonsynonymous substitutions per nondegenerate site. The number of (synonymous) substitutions per synonymous site (K_S) and the number of (nonsynonymous) substitutions per nonsynonymous site (K_A) are given by

$$K_S = [L(2)A(2) + L(4)A(4)]/[(1/3)L(2) + L(4)] \quad [3]$$

$$K_A = [L(0)K(0) + L(2)B(2)]/[(2/3)L(2) + L(0)] \quad [4]$$

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Abbreviation: UT, untranslated region.

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where $L(i)$ is the number of i-fold degenerate sites. Here we follow the convention and count each 4-fold degenerate site a synonymous site, each nondegenerate site a nonsynonymous site, and one-third of a 2-fold degenerate site as synonymous and two-thirds as nonsynonymous.

Relative Rate Test. To test if the nucleotide substitution rates are the same in two different lineages, we use the scheme in Fig. 1, which is known as the "relative rate test" (6). In such a test, one compares the evolutionary distance between species 1 and a reference species with that between species 2 and the reference species. The advantage of this test is that it requires no knowledge of divergence times between species.

For synonymous rates, we want to test if $K_{13} = K_{23}$ for 4-fold degenerate sites and if $A_{13} = A_{23}$ for 2-fold degenerate sites (K_{ij} and A_{ij} are, respectively, the total number of nucleotide substitutions and the number of transitional substitutions between species i and j). For nonsynonymous rates, we want to test if $K_{13} = K_{23}$ for nondegenerate sites and if $B_{13} = B_{23}$ for 2-fold degenerate sites. These differences can be obtained from Eqs. **1** and **2**. We have to determine the variances of $A_{13} - A_{23}$, $B_{13} - B_{23}$, and $K_{13} - K_{23}$ for all three types of sites. The variance of $A_{13} - A_{23}$, for example, is equal to $\mathrm{Var}(A_{13}) + \mathrm{Var}(A_{23}) - 2\mathrm{Cov}(A_{13}, A_{23})$. The approximate variances of A, B, and K were given by Kimura (10) as follows (for convenience, we drop the subscript ij and do not specify the type of site in question):

$$\mathrm{Var}(A) = [a^2P + c^2Q - (aP + cQ)^2]/L \quad [5]$$

$$\mathrm{Var}(B) = b^2Q(1 - Q)/L \quad [6]$$

$$\mathrm{Var}(K) = [a^2\mathrm{P} + d^2Q - (aP + dQ)^2]/L, \quad [7]$$

where $a = 1/(1 - 2P - Q)$, $b = 1/(1 - 2Q)$, $c = (a - b)/2$, $d = (a + b)/2$, and L is the number of sites for that particular class. The corresponding covariance terms are given by

$$\begin{aligned}\mathrm{Cov}(A_{13}, A_{23}) = {} & a_{13}a_{23}\,\mathrm{Cov}(P_{13}, P_{23}) + a_{13}c_{23}\,\mathrm{Cov}(P_{13}, Q_{23}) \\ & + c_{13}a_{23}\,\mathrm{Cov}(Q_{13}, P_{23}) \\ & + c_{13}c_{23}\,\mathrm{Cov}(Q_{13}, Q_{23}), \quad [8]\end{aligned}$$

where a_{ij}, c_{ij}, P_{ij}, and Q_{ij} are as defined above. $\mathrm{Cov}(B_{13}, B_{23})$ and $\mathrm{Cov}(K_{13}, K_{23})$ take similar forms and are not presented. All the terms in Eq. **8** can be computed from sequence comparisons, but it is somewhat tedious. A simpler approach is to use the relation (M. Nei, personal communication): $\mathrm{Cov}(A_{13}, A_{23}) = \mathrm{Cov}[(A_{10} + A_{03}), (A_{20} + A_{03})] = \mathrm{Var}(A_{03})$ because A_{10}, A_{20}, and A_{03} are mutually independent. To obtain $\mathrm{Var}(A_{03})$, we need to know P_{03} and Q_{03} and then use Eq. **5**. From Eqs. **1** and **2**, we can obtain the approximate solutions:

$$Q_{03} = [1 - \exp(-2B_{03})]/2 \quad [9]$$

$$\mathrm{P}_{03} = [1 - Q_{03} - \exp(-2A_{03} - B_{03})]/2, \quad [10]$$

where $B_{03} = (B_{13} + B_{23} - B_{12})/2$ and $A_{03} = (A_{13} + A_{23} - A_{12})/2$. Knowing P_{03} and Q_{03}, we also can compute, from Eqs. **6** and **7**, $\mathrm{Var}(B_{03})$ [$= \mathrm{Cov}(B_{13}, B_{23})$] and $\mathrm{Var}(K_{03})$ [$= \mathrm{Cov}(K_{13}, K_{23})$].

Our simulation study has shown that the variances obtained by both methods agree well with the simulated values within the degree of sequence divergence to be considered below. In the analyses of actual data, the values obtained by the two methods are usually close, and we present only those obtained by the second method. We assume that nucleotide substitution follows a Poisson process. Numerical computations have indicated that the difference between two identical, independent Poisson variables approaches a normal distribution when the sum of their means exceeds 20. Therefore, we may determine the level of significance according to the procedure for the standardized normal test, if the total number of substitutions between the two sequences compared is larger than 20 for the type of site under consideration.

RESULTS

Relative Rate Test: Coding Regions. Table 1 shows the differences in substitution numbers between the rodent (rat or mouse) and human lineages, with dog, rabbit, cow, pig, or goat as a reference. Although some of the reference species may not be equally related to man and rodents, this should introduce no serious error because mammalian orders probably diverged at about the same time (38). Actually, in order to minimize errors in the estimation of K_{13} and K_{23}, the reference species should be chosen so that the points 0 and 0′ in Fig. 1 are as close as possible.

For synonymous substitution, the evidence for higher substitution rates in rodent than in man is rather strong. In only 3 out of 24 comparisons is the synonymous rate higher in the human lineage; in all three cases, the difference is not significant. In the remaining 21 comparisons, the synonymous rate is higher in the rodent lineage; the difference is significant in 8 comparisons. When all 11 genes are combined together, the synonymous rates at 2-fold degenerate sites and 4-fold degenerate sites are very significantly higher in the rodent lineage than in the human lineage ($P < 0.0001$ for both 2-fold and 4-fold degenerate sites). From Table 1 it is straightforward to calculate the number of substitutions from the time of human–rodent divergence to the present along each lineage. With all genes combined, there have been 0.099 and 0.223 synonymous substitutions per 2-fold degenerate site along the human and rodent lineages, respectively. The corresponding numbers are 0.232 and 0.427 for 4-fold degenerate sites. In other words, the synonymous rate in the rodent lineage has been about twice as high as that in the human lineage since the time of their divergence.

For nonsynonymous substitution, the rates also appear to be faster in the rodent lineage, although the differences are not so dramatic as in the case of synonymous substitution. In 16 of the 24 comparisons, the rodent lineage evolves faster; among them 4 (from four different genes) are significant. By contrast, the human lineage evolves faster in only five comparisons; among them three (from two different genes) are significant. With all 11 genes combined, the difference in rate between rodents and man is significant for nondegenerate sites ($P < 0.015$) and marginally significant for 2-fold degenerate sites ($P = 0.05$). A simple calculation will show that the rodent lineage has a nonsynonymous rate about 30% higher than that in the human lineage since the time of their divergence (0.090 vs. 0.069 substitutions per nonsynonymous site by Eq. **4**).

Our statistical test is probably somewhat too stringent because it is based on the assumption that nucleotide substitution follows a Poisson process. However, the final conclusion remains the same if we test the null hypothesis of equal rates by computing the binomial probability of having the number of negative signs observed in each type of nucleotide substitution.

Relative Rate Test: Untranslated Regions. In Table 2, we compare the 5′ untranslated region (5′UT) and 3′ untranslated region (3′UT) of mRNAs of five different genes from man, rodent, and either bovine or goat. The number (K) of nucleotide substitutions per site is calculated from Eqs. **1** and **2** as the sum of A and B (no need for classification of sites). The human lineage has a higher substitution rate in

Table 1. Differences in the number of substitutions per site between the human (species 1) and rodent (species 2) lineages in various genes

	No. of nonsynonymous substitutions per site								No. of synonymous substitutions per site							
	Nondegenerate sites				2-fold degenerate sites				2-fold degenerate sites				4-fold degenerate sites			
Gene	K_{12}	K_{13}	K_{23}	$K_{13}-K_{23}$	B_{12}	B_{13}	B_{23}	$B_{13}-B_{23}$	A_{12}	A_{13}	A_{23}	$A_{13}-A_{23}$	K_{12}	K_{13}	K_{23}	$K_{13}-K_{23}$
Proinsulin	0.07	0.06	0.09	−0.03 (0.02)	0.05	0.03	0.09	−0.06 (0.04)	0.34	0.32	0.28	0.04 (0.13)	0.77	0.36	1.00	−0.65* (0.33)
Actin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.26	0.10	0.20	−0.10 (0.06)	0.42	0.28	0.50	−0.22* (0.10)
GH	0.21	0.21	0.07	0.14† (0.03)	0.09	0.09	0.03	0.06* (0.03)	0.39	0.33	0.34	−0.02 (0.10)	0.61	0.57	0.36	0.21 (0.12)
GPH-A	0.17	0.17	0.03	0.14† (0.03)	0.01	0.01	0.02	−0.00 (0.02)	0.40	0.19	0.35	−0.17 (0.13)	0.68	1.07	0.71	0.36 (0.53)
Prolactin	0.23	0.15	0.27	−0.12† (0.03)	0.13	0.09	0.12	−0.03 (0.04)	0.33	0.27	0.28	−0.01 (0.08)	1.11	0.55	1.05	−0.50* (0.25)
Relaxin	0.37	0.32	0.39	−0.07 (0.05)	0.09	0.08	0.11	−0.04 (0.03)	0.37	0.22	0.38	−0.16 (0.10)	0.90	0.52	0.88	−0.37 (0.23)
Four signal peptides	0.27	0.23	0.31	−0.08 (0.05)	0.11	0.05	0.15	−0.10 (0.06)	0.40	0.17	0.29	−0.12 (0.13)	0.71	0.32	0.71	−0.40* (0.18)
POMC	0.12	0.06	0.12	−0.06† (0.02)	0.08	0.02	0.05	−0.04 (0.03)	0.18	0.15	0.22	−0.07 (0.05)	0.65	0.35	0.56	−0.21* (0.10)
IgK	0.32	0.42	0.43	−0.01 (0.06)	0.15	0.27	0.26	0.01 (0.08)	0.46	0.19	0.61	−0.43 (0.24)	0.74	0.48	0.72	−0.24 (0.20)
IgG	0.23	0.22	0.29	−0.07* (0.03)	0.13	0.11	0.11	0.0 (0.04)	0.31	0.11	0.32	−0.21* (0.08)	0.83	0.37	0.65	−0.28* (0.14)
α-Globin	0.09	0.09	0.10	−0.02 (0.02)	0.04	0.05	0.04	0.01 (0.03)	0.42	0.09	0.45	−0.35† (0.13)	0.70	0.41	0.50	−0.10 (0.13)
β-Globin	0.14	0.13	0.16	−0.03 (0.03)	0.04	0.06	0.14	−0.09† (0.03)	0.27	0.17	0.30	−0.13 (0.09)	0.39	0.30	0.46	−0.15 (0.10)
Total	0.17	0.15	0.17	−0.02* (0.01)	0.08	0.06	0.08	−0.02 (0.01)	0.32	0.18	0.31	−0.12† (0.03)	0.66	0.41	0.60	−0.19† (0.04)
No. of sites	3602.0				1094.3				1094.3				895.7			

A_{ij}, B_{ij}, and K_{ij} are, respectively, the number of transitional substitutions, the number of transversional substitutions, and the total number of substitutions between species (sp.) i and sp. j. The numbers in the parentheses are the standard errors. The sources of DNA sequences are given below; the reference for sp. 1 (human) is given immediately after the name of the gene. In proinsulin (11), sp. 2 = rat (12) and sp. 3 = dog (13); in actin (14), sp. 2 = rat (15) and sp. 3 = rabbit (16); in growth hormone (GH) (17), in glycoprotein hormone α subunit (GPH-A) (18), and in prolactin (17), sp. 2 = rat (17, 19) and sp. 3 = bovine (17, 20); in relaxin (21), sp. 2 = rat (22) and sp. 3 = porcine (23); in pro-opiomelanocortin (POMC) (24, 25), sp. 2 = mouse (26) and sp. 3 = bovine (26); in immunoglobulin constant region kappa chain (IgK) (27) and gamma chain (IgG) (28), sp. 2 = mouse (28, 29) and sp. 3 = rabbit (30, 31); and in the two globins (32, 33), sp. 2 = mouse (34, 35) and sp. 3 = goat (36, 37). The four signal peptides are from prolactin, relaxin, GH, and GPH-A.

*Significant at 5% level.

†Significant at 1% level.

only one comparison. In the remaining nine comparisons, the rodent lineage evolves faster; the difference is more than twice the standard error in seven cases and more than 3 times the standard error in one case. (We have not used the standardized normal test mentioned above, because the number of substitutions in many cases is less than 20.) When all of the five genes are combined, the differences in 5'UT

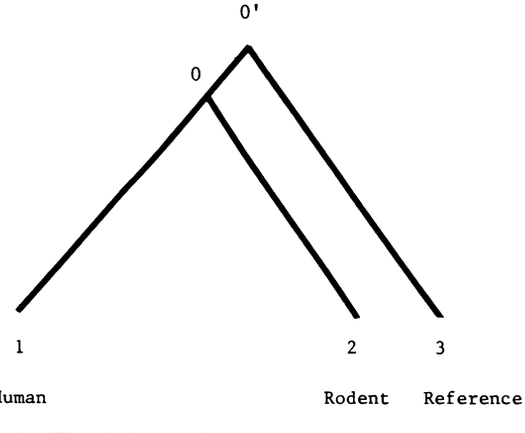

FIG. 1. The phylogenetic relationship of the three species used in the relative rate test.

Table 2. Differences ($K_{13} - K_{23}$) in numbers of nucleotide substitutions per site in the 5' and 3' untranslated regions (UT) of mRNA between the human and rodent lineages

	5'UT		3'UT	
Gene	K_{12}	$K_{13} - K_{23}$	K_{12}	$K_{13} - K_{23}$
α-Globin	0.25	−0.11 ± 0.10	0.34	−0.18* ± 0.09
β-Globin	0.21	−0.18* ± 0.08	0.45	−0.19* ± 0.09
ACTH	0.29	0.03 ± 0.06	0.37	−0.17* ± 0.08
PTH	0.35	−0.30* ± 0.12	0.42	−0.25† ± 0.07
GH	0.27	−0.32 ± 0.17	0.30	−0.16* ± 0.07
Total	0.28	−0.13† ± 0.04	0.38	−0.20† ± 0.04
	K_{13} = 0.17, K_{23} = 0.30		K_{13} = 0.23, K_{23} = 0.43	
No. of sites	266		539	

Sources of DNA sequences are the same as those in Table 1, except for corticotropin (ACTH; refs. 24, 39, and 40) and parathyroid hormone (PTH; refs. 41 and 42). Species 1 is always human, species 2 is mouse except for PTH and growth hormone (GH) (rat) genes, and species 3 is cow except in α-globin gene (goat).

*>2 standard errors.

†>3 standard errors.

and 3'UT are more than 3 times and 5 times the standard errors, respectively. Our calculation shows that the rodent line evolves 2.6 times faster in 5'UT and 3.1 times faster in 3'UT than does the human line. These figures are less reliable than those for the coding regions because the numbers of sites are smaller and also because the alignment of DNA sequences in noncoding regions is generally less reliable than the alignment of coding regions.

Comparisons Between Paralogous Genes. Another type of divergence-time independent test is to compare homologous genes that share their origin by gene duplication (paralogous genes), instead of speciation. If a gene duplication occurred prior to (but not too long before) the split of human and mouse lineages, then we can test the generation time effect by studying if there have been more substitutions between the two paralogous genes in mouse than in man.

Table 3 shows the results from the analysis of the β-globin gene family. We make all pairwise comparisons among the adult genes (denoted A; β of human and β_{maj} of mouse), the fetal genes (denoted F; $^{A}\gamma$ of human and βH1 of mouse), and the embryonic genes (denoted E; ε of human and y2 of mouse). The duplication producing the fetal and embryonic genes is believed to have preceded the mammalian radiation; this duplication, in turn, was preceded by the earlier duplication that produced the adult gene and the ancestor of the fetal and embryonic genes (48–50). These genes are, therefore, suitable for the study of the generation time effect.

In the first half of Table 3, we see that paralogous genes in mouse are always more divergent than those in human. This is true for both synonymous and nonsynonymous substitutions. Again, the difference is larger for synonymous substitutions than for nonsynonymous substitutions: the ratios of the number of substitutions in mouse to those in human are 1.13, 1.17, and 1.50 for nonsynonymous substitutions and 1.24, 1.56, and 1.71 for synonymous substitutions. These ratios are lower than those calculated from Table 1 because the rates in the two species compared are not independent; for example, comparisons of the mouse adult–fetal and human adult–fetal β-globin genes share the same history between the adult–fetal duplication and the human–mouse speciation. This may also partially explain why the ratios are highest for the comparisons between the fetal and embryonic genes: this duplication is closer to the time of speciation.

Table 3. Numbers of nucleotide substitutions per site between genes of the β-globin gene families of human and mouse

β-Globin genes	No. of substitutions per site	
	Nonsynonymous site	Synonymous site
Human (A)–human (F)	0.182	0.731
Mouse (A)–mouse (F)	0.206	0.904
Human (A)–human (E)	0.158	0.623
Mouse (A)–mouse (E)	0.184	0.974
Human (F)–human (E)	0.099	0.562
Mouse (F)–mouse (E)	0.148	0.960
Human (A)–mouse (A)	0.127	0.492
Human (F)–mouse (F)	0.138	0.708
Human (E)–mouse (E)	0.093	0.576
Human (A)–mouse (F)	0.206	1.081
Human (A)–mouse (E)	0.176	0.902
Human (F)–mouse (A)	0.184	0.674
Human (F)–mouse (E)	0.127	0.630
Human (E)–mouse (A)	0.164	0.660
Human (E)–mouse (F)	0.133	0.723

The adult genes (A) are β in man (33, 43) and β_{maj} in mouse (35); the fetal genes (F) are $^{A}\gamma$ in man (44) and βH1 in mouse (45); the embryonic genes (E) are ε in man (46) and y2 in mouse (47).

The above observation can be attributed to higher substitution rates in mouse only if we can also rule out major gene conversions between human adult, fetal and embryonic genes. (Otherwise, a smaller number of substitutions may be explained by a recent gene conversion). To do that, we note that if there has been no major gene conversion between, say, the human adult and fetal genes, one would expect the adult genes of human and mouse to be more closely related than each is to the fetal gene of the same species. On the other hand, had there been major gene conversions between the human adult and fetal genes, both genes would have been more or less equally distant to their homologues in mouse. The same rule applies to the comparisons between the adult and embryonic genes and between the fetal and embryonic genes. The second half of Table 3 indeed suggests the absence of major gene conversions between these genes: Orthologous genes (genes sharing an origin by speciation) are usually more closely related than paralogous genes.

Finally, we note that there are actually two adult genes and two fetal genes in both man and mouse. [The duplications that led to both pairs of genes in both species occurred after the duplication events mentioned earlier (48, 49).] We have made similar comparisons using these genes and reached the same conclusion as above.

DISCUSSION

In the relative rate tests, we have used pig, cow, goat, dog, and rabbit as reference species. The first three species belong to *Artiodactyla* and the fourth to *Carnivora*. These two mammalian orders are generally thought to have branched off before or around the time of the primate–rodent split (5, 38). Thus, the first four species are apparently good references. The evolutionary position of rabbit is quite uncertain (38), but this should not affect our results much because rabbit was used as a reference in only three cases. We further emphasize that, except in the case of nonsynonymous substitution, the differences in rates shown in Tables 1 and 2 are usually too large to be due to errors in the assumption of the phylogenetic relationship. In addition, the results shown in Table 3 do not involve any assumption on mammalian phylogeny. Therefore, we believe that we have provided strong evidence for higher rates of nucleotide substitution in rodents than in man. Limited evidence from α- and β-globin genes has been given earlier (51, 52).

A simple explanation for the higher nucleotide substitution rates in rodents is that rodents have shorter generation times and, thus, higher mutation rates. An immediate question is why the difference in the synonymous rate between rodent and man is only 2-fold, not so conspicuous as their difference in generation time, which is probably 100-fold. There are four possible reasons. First, mutation rate seems to depend more on the number of DNA replications or cell cycles per unit time in the germ line than on the number of generations per unit time. The sex difference in the rate of mutation to the hemophilia A condition in humans, estimated to be nearly 10 times higher in males (53), lends support to a cell-cycle effect. It has been estimated that the number of cell cycles per year is only about 7.5 times higher in mouse than in man (54). Second, our estimates of the substitution rates refer to the average rates in each of the two lineages since their divergence. Third, there may be replication-independent mutations (55). Fourth, the effective population size (N_e) in the rodent lineage has probably been larger than that in the human lineage (38). As synonymous mutations are not completely free of selective constraints (3), an increase in N_e may reduce to some extent the synonymous rate because

selection against slightly deleterious mutants is more effective in large populations.

The preceding reasoning also can explain why the generation-time effect is weaker on nonsynonymous substitution than on synonymous substitution. The reduction in substitution rate resulting from an increase in N_e would be even greater in the case of nonsynonymous substitution, for nonsynonymous mutations are generally subject to stronger selective constraints than synonymous mutations. This line of reasoning is consistent with the neutralist view (3). On the other hand, under the selectionist view, the rate of substitution is mainly governed by $N_e u_A s$, u_A being the rate of advantageous mutation and s being their average selective advantage (3). This relation would predict a difference in nonsynonymous rate considerably larger than observed because rodents appear to have had higher N_e and u_A than man.

Since our results suggest the generation-time effect to be weak on nonsynonymous substitution, it is easy to explain why many studies based on protein sequence data and immunological data did not show the generation-time effect (2) because these studies essentially examined nonsynonymous substitutions. On the other hand, DNA hybridization studies often showed the generation-time effect (7, 8), for these studies presumably examined the average of the genome, which would contain many weakly constrained regions such as synonymous sites and introns.

Our results raise cautions on the use of the molecular clock in the estimation of divergence time. The generation-time difference between rodent and man has only about a 2-fold effect on the synonymous rate because our study refers to the long-term average along each lineage. Actually, the average generation time of rodents since the time of mouse–rat divergence is probably much shorter than the average mammalian generation time. Likewise, apes and man may have acquired their long generation times only in the relatively recent past. Therefore, an application of the average mammalian rate to either group may result in substantial over- or under-estimation of species divergence time. Of course, for organisms with similar generation times, synonymous substitution may still serve as a molecular clock as suggested previously (56).

Although we propose the generation-time effect as a plausible explanation for the higher substitution rates in rodents than in man, we do not rule out other explanations, such as a less accurate DNA replication system and, thus, higher mutation rates in rodents.

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1. Zuckerkandl, E. & Pauling, L. (1965) in *Evolving Genes and Proteins*, eds. Bryson, V. & Vogel, H. J. (Academic, New York), pp. 97–116.
2. Wilson, A. C., Carlson, S. S. & White, T. J. (1977) *Annu. Rev. Biochem.* **46**, 573–639.
3. Kimura, M. (1983) *The Neutral Theory of Molecular Evolution* (Cambridge Univ. Press, London), p. 367.
4. Sturtevant, A. H. (1965) *A History of Genetics* (Harper & Row, New York).
5. Dayhoff, M. O. (1972) *Atlas of Protein Sequence and Structure* (National Biomedical Research Foundation, Silver Spring, MD), Vol. 5, p. 124.
6. Sarich, V. M. & Wilson, A. C. (1967) *Science* **158**, 1200–1203.
7. Laird, C. D., McConaughy, B. L. & McCarthy, B. J. (1969) *Nature (London)* **224**, 149–154.
8. Kohne, D. E. (1970) *Q. Rev. Biophys.* **33**, 1–48.
9. Li, W.-H., Wu, C.-I & Luo, C.-C. (1985) *Mol. Biol. Evol.*, in press.
10. Kimura, M. (1980) *J. Mol. Evol.* **16**, 111–120.
11. Bell, G. I., Pictet, R. L., Rutter, W. J., Cordell, B., Tischer, E. & Goodman, H. M. (1980) *Nature (London)* **284**, 26–32.
12. Lomedico, P., Rosenthal, N., Efstratiadis, A., Gilbert, W., Kolodner, R. & Tizard, R. (1979) *Cell* **18**, 545–558.
13. Kwok, S. C. M., Chan, S. J. & Steiner, D. F. (1983) *J. Biol. Chem.* **258**, 2357–2363.
14. Hanauer, A., Levin, M., Heilig, R., Daegelen, D., Kahn, A. & Mandel, J. L. (1983) *Nucleic Acids Res.* **11**, 3503–3516.
15. Zakut, R., Shani, M., Givol, D., Neuman, S., Yaffe, D. & Nudel, U. (1982) *Nature (London)* **298**, 857–859.
16. Putney, S. D., Herlihy, W. C. & Schimmel, P. (1983) *Nature (London)* **302**, 718–721.
17. Miller, W. L. & Eberhardt, N. L. (1983) *Endocr. Rev.* **4**, 97–130.
18. Fiddes, J. C. & Goodman, H. M. (1981) *J. Mol. Appl. Genet.* **1**, 3–18.
19. Godine, J. E., Chin, W. W. & Habener, J. F. (1982) *J. Biol. Chem.* **257**, 8368–8371.
20. Nilson, J. H., Thomason, A. R., Cserbak, M. T., Moncman, C. L. & Woychik, R. P. (1983) *J. Biol. Chem.* **258**, 4679–4682.
21. Hudson, P., Haley, J., John, M., Cronk, M., Crawford, R., Haralambidis, J., Tregear, G., Shine, J. & Niall, G. (1983) *Nature (London)* **301**, 628–631.
22. Hudson, P., Haley, J., Cronk, M., Shine, J. & Niall, H. (1981) *Nature (London)* **291**, 127–131.
23. Haley, J., Hudson, G., Scanlon, D., John, M., Cronk, M., Shine, J., Tregear, G. & Niall, H. (1982) *DNA* **1**, 155–162.
24. Takahashi, H., Hakamata, Y., Watanabe, Y., Kikuno, R., Miyata, T. & Numa, S. (1983) *Nucleic Acids Res.* **11**, 6847–6858.
25. Chang, A. C. Y., Cochet, M. & Cohen, S. N. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4890–4894.
26. Uhler, M. & Herbert, E. (1983) *J. Biol. Chem.* **258**, 257–261.
27. Ellison, J. W., Berson, B. J. & Hood, L. E. (1982) *Nucleic Acids Res.* **10**, 4071–4079.
28. Hieter, P. A., Max, E. E., Seidman, J. G., Maizel, J. V. & Leder, P. (1980) *Cell* **22**, 197–207.
29. Honjo, T., Obata, M., Yamawaki-Kataoka, Y., Kataoka, T., Kawakami, T., Takahashi, N. & Mano, Y. (1979) *Cell* **18**, 559–568.
30. Heidmann, O. & Rougeon, F. (1982) *Nucleic Acids Res.* **10**, 1535–1545.
31. Emorine, L., Dreher, K., Kindt, T. J. & Max, E. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5709–5713.
32. Liebhaber, S. A., Goosens, M. J. & Kan, Y. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7054–7058.
33. Lawn, R. M., Efstratiadis, A., O'Connell, C. & Maniatis, T. (1980) *Cell* **21**, 647–651.
34. Nishioka, Y. & Leder, P. (1979) *Cell* **18**, 875–882.
35. Konkel, D. A., Maizel, J. V. & Leder, P. (1979) *Cell* **18**, 865–873.
36. Schon, E. A., Wernke, S. M. & Lingrel, J. B. (1982) *J. Biol. Chem.* **257**, 6825–6835.
37. Schon, E. A., Cleary, M. L., Haynes, J. R. & Lingrel, J. B. (1981) *Cell* **27**, 359–369.
38. Romer, A. S. (1966) *Vertebrate Paleontology* (Univ. of Chicago Press, Chicago).
39. Notake, M., Tobimatsu, T., Watanabe, Y., Takahashi, H., Mishina, M. & Numa, S. (1983) *FEBS Lett.* **156**, 67–71.
40. Nakanishi, S., Teranishi, Y., Watanabe, Y., Notake, M., Noda, M., Kakidani, H., Jingami, H. & Numa, S. (1981) *Eur. J. Biochem.* **115**, 429–438.
41. Hendy, G. N., Kronenberg, H. M., Potts, J. T. & Rich, A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7365–7369.
42. Heinrich, G., Kronenberg, H. M., Potts, J. T. & Habener, J. F. (1984) *J. Biol. Chem.* **259**, 3320–3329.
43. Spritz, R. A., DeRiel, J. K, Forget, B. G. & Weissman, S. M. (1980) *Cell* **21**, 639–646.
44. Slightom, J., Blechl, A. E. & Smithies, O. (1980) *Cell* **21**, 627–638.
45. Hansen, J. N., Konkel, D. A. & Leder, P. (1982) *J. Biol. Chem.* **257**, 1048–1052.
46. Baralle, G. E., Shoulders, C. C. & Proudfoot, N. J. (1980) *Cell* **21**, 621–626.
47. Hill, A., Hardies, S. C., Phillips, S. J., Davis, M. G., Hutchison, C. A., III, & Edgell, M. H. (1984) *J. Biol. Chem.* **259**, 3739–3747.
48. Efstratiadis, A., *et al.* (1980) *Cell* **21**, 653–668.
49. Czelusniak, J., Goodman, M., Hewett-Emmett, D., Weiss, M. L., Venta, P. J. & Tashian, R. E. (1982) *Nature (London)* **298**, 297–300.
50. Hardison, R. C. (1984) *Mol. Biol. Evol.* **1**, 390–410.
51. Kimura, M. & Ohta, T. (1972) in *Proceedings of the 6th Berkeley Symposium*, eds. LeCam, L. M., Neyman, J. & Scott, E. L. (Univ. of California Press, Berkeley, CA), Vol. 5, pp. 43–68.
52. Fitch, W. M. (1980) *J. Mol. Evol.* **16**, 153–209.
53. Winter, R. M., Tuddenham, E. G. D., Goldman, E. & Matthews, K. B. (1983) *Hum. Genet.* **64**, 156–159.
54. Vogel, F., Kopun, M. & Rathenberg, R. (1976) in *Molecular Anthropology*, eds. Goodman, M. & Tashian, R. (Plenum, New York), pp. 13–33.
55. Drake, J. W. (1970) *The Molecular Basis of Mutation* (Holden-Day, San Francisco).
56. Miyata, T., Yasunaga, T. & Nishida, T. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7328–7332.