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Evolution

Weak male-driven molecular evolution in rodents

(Y chromosome-linked genes/X chromosome-linked genes/substitution rates/sex differences)

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ABSTRACT **In humans and rodents the male-to-female ratio of mutation rate (α_m) has been suggested to be extremely large, so that the process of nucleotide substitution is almost completely male-driven. However, our sequence data from the last intron of the X chromosome-linked (*Zfx*) and Y chromosome-linked (*Zfy*) zinc finger protein genes suggest that α_m is only $\approx$2 in rodents with a 95% confidence interval from 1 to 3. Moreover, from published data on oogenesis and spermatogenesis we estimate the male-to-female ratio of the number of germ cell divisions per generation to be $\approx$2 in rodents, confirming our estimate of α_m and suggesting that errors in DNA replication are the primary source of mutation. As the estimated α_m for rodents is only one-third of our previous estimate of $\approx$6 for higher primates, there appear to be generation-time effects—i.e., α_m decreases with decreasing generation time.**

How high is the male-to-female ratio of mutation rate (α_m) in humans and other mammals? This has been an intriguing question for human geneticists and evolutionists since Haldane (1) suggested that the rate of mutation might be much higher in males than in females because the number of germ cell divisions per generation is much larger in the male germ line than in the female germ line. Statistical analyses of hemophilia data led to an estimate of $\alpha_m = -10$ (1, 2). However, such genetic disease data presumably include deletions and insertions as well as base changes and thus cannot give an accurate estimate of α_m in terms of base changes. Miyata *et al.* (3) compared the average rate of synonymous substitution in X chromosome-linked genes with that in autosomal genes from humans and rodents and concluded that α_m is so large that the female contribution to mutation is negligible and that the process of nucleotide substitution is almost completely male-driven. The same conclusion was reached in a study of only rodent genes (4). However, as explained in *Discussion*, estimates of α_m based on the synonymous substitution rates in nonhomologous X-linked and autosomal genes may not be reliable. Indeed, recent studies of exon and intron sequences of the homologous X-linked (*ZFX*) and Y-linked (*ZFY*) zinc finger (ZF) protein genes gave estimates of $\alpha_m \leq 6$ (5–8). However, these latter estimates were based on limited data and could not reject the hypothesis that α_m is much larger than 10.

An issue that has not been addressed by previous authors is whether α_m depends on generation time. Under the assumption that errors in DNA replication during germ cell division are the primary source of mutation, α_m is approximated by the male-to-female ratio (α) of the number of germ cell divisions per generation. As explained later, α (and thus α_m) is likely to decrease with decreasing generation time, if the cell cycle time is nearly constant.

To resolve the above issues, we have sequenced the last intron of the zinc finger protein genes (*Zfx* and *Zfy*) in mice and rats. In a previous study we sequenced the homologues of these introns in humans, orangutans, baboons, and squirrel monkeys (8). These two sets of data enable us to show that α_m is not very large and that the much shorter generation time in rodents than in higher primates reduces α_m. To confirm these conclusions we obtained estimates of α in rodents and humans from published data on gametogenesis.†

MATERIALS AND METHODS

Sources of Samples. Freshly dissected liver tissues from inbred mice (strain BALB/cAncr) were gifts from B. W. McIntyre (M. D. Anderson Cancer Center at Houston) and frozen kidneys from rats (*Rattus norvegicus*, strain F334) were gifts from T. C. Douglas (University of Texas at Houston). Genomic DNA was extracted from 100 mg of tissue of each of the studied samples by the method in ref. 9.

PCR Amplification. Two 3′ PCR primers, one *Zfx* specific (corresponding to positions 1427–1403 in ref. 10) and one *Zfy* specific (corresponding to positions 1442–1414 in ref. 11), and a common 5′ PCR primer (corresponding to positions 1235–1259 in ref. 10) were designed for the amplification of the last intron of the *Zfx* and *Zfy* genes in mouse and rat. The sequences were amplified from 1 μg of genomic DNA from each of the studied individuals by a regime of 94°C (1 min), 64°C (1 min), and 72°C (2 min) for a total of 30 cycles on a Perkin–Elmer/Cetus DNA thermal cycler in a reaction mix containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 1 unit of Taq DNA polymerase, and the appropriate primers.

Cloning. The major PCR products ($\approx$1 kb for the *Zfx* introns and 1.3 kb for the *Zfy* introns) were end-filled with Klenow fragment, and the desired DNA fragments, isolated by gel electrophoresis (as noted later, the inseparable mouse *Zfy1* and *Zfy2* products were isolated together), were purified with Prep-A-Gene gene cleaning kits (Bio-Rad), treated with polynucleotide kinase, and ligated into phosphatase-treated *Sma* I–digested pBluescript SK+ vector (Stratagene). These were then transformed into competent *Escherichia coli* XL1/Blue cells and single recombinant colonies were isolated.

Sequencing. For each of the five introns (mouse *Zfx*, *Zfy1*, *Zfy2* and rat *Zfx* and *Zfy*), three clones, each derived from an independent PCR and cloning reaction, were sequenced enzymatically (12) with Sequenase version 2.0 sequencing kits (United States Biochemical) on double-stranded templates purified with Magic Miniprep kits (Promega). The sequence of one of the clones from each intron species was determined entirely on both strands; the other two were sequenced completely from only one strand. Internal se-

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Abbreviations: ZF, zinc finger; *ZFX* and *ZFY*, X-linked and Y-linked ZF genes.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X58927, mouse *Zfx*; X58928, mouse *Zfy1*; X58929, mouse *Zfy2*; X58933, rat *Zfx*; X58934, rat *Zfy*).

quencing primers were designed as sequence information accumulated, and they worked for all of the *Zfy* introns but not for the *Zfx* introns. For the *Zfx* introns, 5% Long Ranger gels (AT Biochem, Malvern, PA) were used to obtain extended sequences from the vector primers until overlapping complementary sequences were identified. Restriction maps of these preliminary sequences revealed a single *Hin*dIII site in mouse *Zfx* (position 632, Fig. 1) and a single *Hin*cII site in rat *Zfx* (position 673, Fig. 1). Each of the two introns was independently restricted at these sites into two fragments, which were cloned, and recombinant clones were isolated, purified, and sequenced as described above.

RESULTS

***Zfx* and *Zfy* Intron Sequences.** In contrast to the existence of a single Y-linked ZF gene in other placental mammals examined, including humans, rabbits, dogs, cattle, and horses, the mouse has two Y-linked ZF genes (*Zfy1* and *Zfy2*), which appear to have been derived from a duplication event during relatively recent mouse evolution (11, 13). Our PCR amplifications with the conditions specified above yielded single major products (mouse *Zfx*, rat *Zfx*, and rat *Zfy*) with the exception of male mouse genomic DNA for which two products (corresponding to *Zfy2* and *Zfy1*) were distinguishable

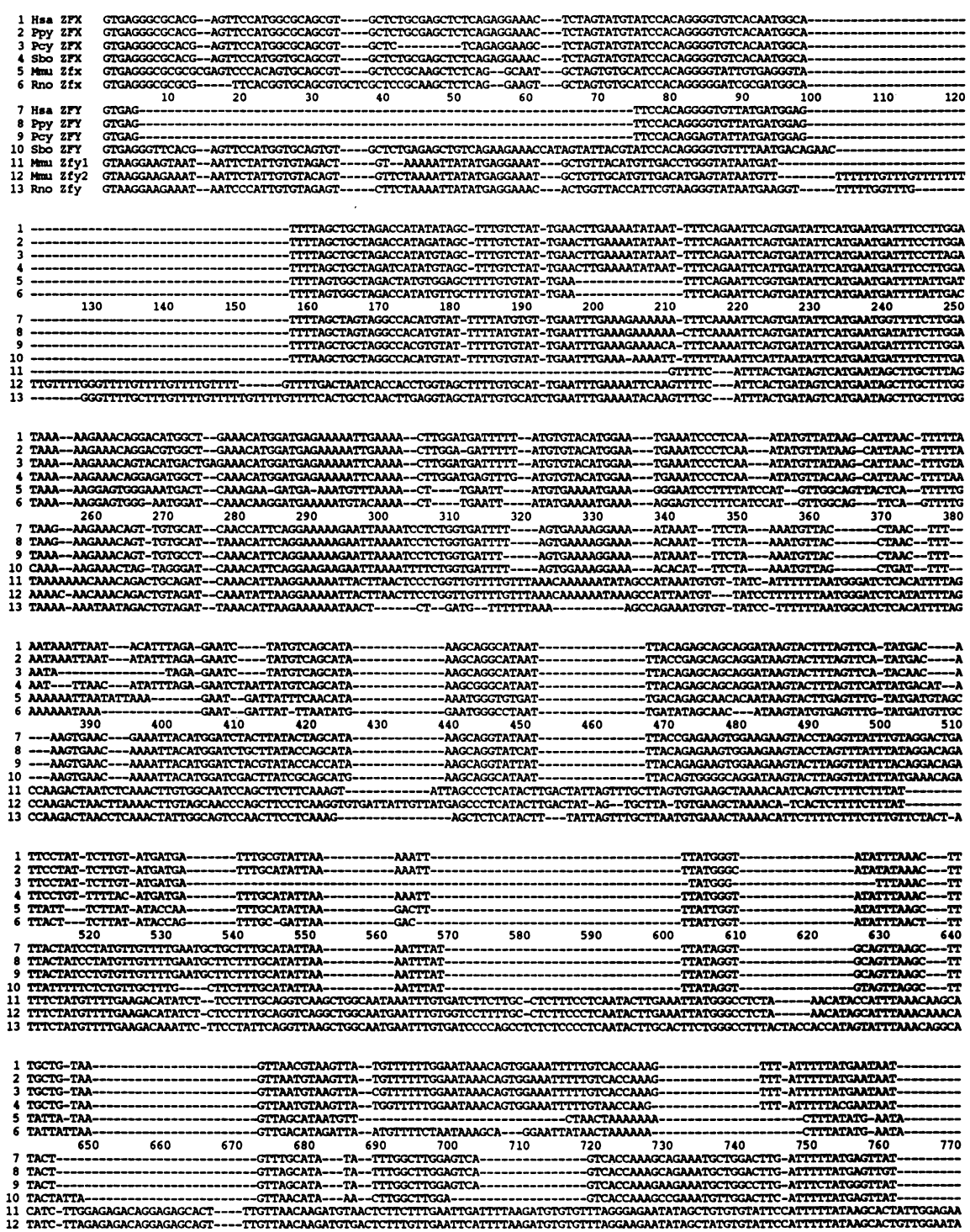

FIG. 1. (*Figure continues on the opposite page.*)

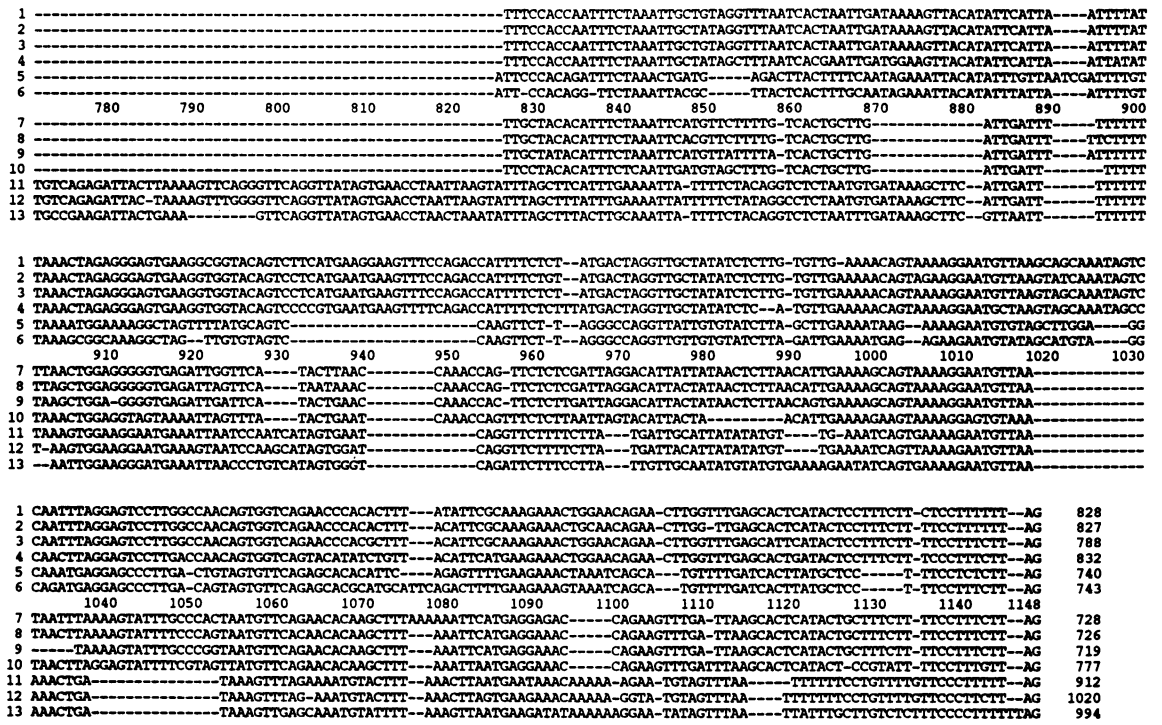

FIG. 1. Alignment of the sequences of the last intron of the X- and Y-linked ZF protein genes of primates and rodents. The X-linked introns are above and the Y-linked introns are below the line for the alignment position numbers. Species are indicated at the beginning of the alignment: Hsa, *Homo sapiens*; Ppy, *Pongo pygmaeus*; Pcy, *Papio cyanocephalus*; Sbo, *Saimiri boliviensis*; Mmu, *Mus musculus*; and Rno, *Rattus norvegicus*. The primate *ZFX* introns contain an *Alu* element (not shown) located between alignment positions 758 and 759. Total length of introns is indicated at the end of the alignment.

but inadequately separated. The latter two products were shot-gun cloned and their identities were clarified after sequencing by comparing the flanking exons included in the PCR amplification (293 bp from the *Zfx* genes and 365 bp from the *Zfy* genes) with published cDNA sequences (11, 13). All sequences completely matched the published mouse sequences (10, 11) with the exception of the mouse *Zfy1*, which contained a C residue (conserved in human *ZFX* and *ZFY* and mouse *Zfx* and *Zfy2*) at a position 7 nucleotides upstream from the 5′ splicing junction of the last intron, corresponding to the A residue at position 1317 of Ashworth *et al.* (13). The C → A transversion causes a change from glutamine to lysine. To rule out the possibility of DNA recombination during PCR amplification involving *Zfy1* and *Zfy2*, two primers were constructed to specifically amplify the potentially variant region of the *Zfy1* gene; the sequence of this product was found to be in agreement with our three independently derived sequences. This reconfirmed that the sequence of the last intron of the *Zfy1* gene determined in this study was an authentic one and the C → A transversion in Ashworth *et al.* (13) may be due to a variant or different mice strains. Illustrated in Fig. 1 is the alignment of the nucleotide sequences for the last introns of mouse and rat *Zfx* and *Zfy* genes and their primate homologues, *ZFX* and *ZFY*.

Male-to-Female Ratio of Mutation Rate. From the rodent sequences in Fig. 1 we computed the number of nucleotide substitutions per site (K) by Tajima and Nei's (14) method (Table 1). The K value between mouse *Zfy1* and *Zfy2* was significantly smaller than that between rat *Zfy* and either of mouse *Zfy1* and *Zfy2*, supporting the hypothesis that mouse *Zfy1* and *Zfy2* were derived from a relatively recent duplication (15). The K value between rat *Zfy* and mouse *Zfy2* was larger than that between rat *Zfy* and mouse *Zfy1*, but the difference was not statistically significant; the average for the two values is 0.182, which will be denoted by Y to signify that it is from Y-linked sequences. The K value between rat and mouse *Zfx* was only 0.128, which will be denoted by X. So, $Y/X = 0.182/0.128 = 1.42$. Under the assumption that errors in DNA replication during germ cell division are the primary source of mutation, $\alpha_m = \alpha$ and $Y/X = 3\alpha/(\alpha + 2)$ (ref. 3), from which we obtain $\alpha_m = 1.80$.

More rigorously, we want to estimate the ratio E(Y)/E(X), where E means taking expectation; in practice we use the observed values of X and Y to estimate the ratio and so we are actually estimating Y/X. Note that $E(Y)/E(X) \approx E(Y/X) - E(X)V(Y)/E(Y)^3$, where V(Y) is the variance of Y. From Table 1 we can assume that V(Y) is $\approx 0.016^2$, which is the smaller of the variances for the two Y values, and $E(Y) = 0.182$ and $E(X) = 0.128$. We then have $E(Y)/E(X) \approx 1.422 - 0.005 \approx 1.42$. Using the formula $V(Y/X) \approx V(Y)/E(X)^2 + E(Y)^2V(X)/E(X)^4$, we obtain a standard error of ≈ 0.21. Therefore, our estimate of E(Y)/E(X) is 1.42 ± 0.21, and the 95% confidence interval for this ratio is (1.0, 1.84), from which we find that the 95% confidence interval for α_m is approximately (1.0, 3.2); the mean of α_m is 1.80.

Evolutionary Relationships Among Mammalian *Zfx* and *Zfy* Genes. Table 2 shows the K values for all pairwise comparisons of the five rodent intron sequences obtained in this study and the eight primate intron sequences obtained in our previous study (8). Using the neighbor joining method (16), we constructed a phylogenetic tree (Fig. 2), which revealed that the rate of nucleotide substitution was faster in the Y-linked introns than in the X-linked introns, that the rodent

Table 1. Number of nucleotide substitutions per site between intron sequences

	X intron	Y intron	
Sequence	Mouse *Zfx*	Mouse *Zfy1*	Mouse *Zfy2*
Rat *Zfx*	0.128 ± 0.015		
Mouse *Zfy2*		0.071 ± 0.010	
Rat *Zfy*		0.172 ± 0.016	0.192 ± 0.017

Gaps are not included in the comparison.

Table 2. Mean (below diagonal) and standard error (above diagonal) of the number of nucleotide substitutions per 100 sites between intron sequences

		X intron						Y intron						
	Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Human X		0.7	0.8	1.5	4.1	4.1	3.0	3.0	3.0	3.1	4.9	5.1	5.7
2	Orangutan X	2.1		0.9	1.4	4.2	4.2	3.0	3.1	3.0	3.3	5.0	5.1	5.7
3	Baboon X	2.8	3.1		1.4	4.1	4.2	3.0	3.0	2.9	3.2	5.2	5.2	6.0
4	S. monkey X	7.9	7.6	7.6		4.2	4.1	3.2	3.2	3.1	3.3	5.0	5.2	5.6
5	Mouse X	37.4	39.2	38.3	39.4		2.0	4.4	4.4	4.1	4.8	6.6	6.5	7.3
6	Rat X	38.6	40.0	40.1	38.9	14.1		4.9	5.2	5.0	5.3	6.3	6.5	7.2
7	Human Y	26.2	26.2	25.9	28.6	42.2	47.8		1.1	1.2	2.1	4.6	4.6	5.6
8	Orangutan Y	26.9	27.5	26.6	29.7	43.4	51.0	4.8		1.3	2.0	4.7	4.7	5.6
9	Baboon Y	25.6	26.3	24.6	28.0	39.0	48.9	5.8	6.1		2.2	4.6	4.6	5.6
10	S. monkey Y	28.5	30.0	29.2	31.0	46.2	52.2	14.8	14.7	15.7		5.1	5.3	6.5
11	Mouse Y1	50.5	50.6	52.7	51.0	66.4	64.8	46.6	47.5	46.5	51.3		1.2	2.1
12	Mouse Y2	52.5	52.6	53.6	53.0	65.7	66.7	47.4	48.2	46.8	53.4	5.8		2.3
13	Rat Y	57.6	57.7	60.2	57.0	70.8	71.9	55.6	56.6	56.5	63.6	15.1	17.2	

Only regions that are shared by all sequences are compared—i.e., any region containing a gap in any of the sequences is excluded from comparison. For this reason, the regions compared tend to be better conserved than those excluded and the *K* values in the table tend to be smaller than those in Table 1 and in ref. 8. S. monkey, squirrel monkey.

X-linked introns evolved faster than the primate X-linked introns, and that the rodent Y-linked introns evolved faster than the primate Y-linked introns. These results are in agreement with the view that the rate of nucleotide substitution was faster in Y-linked sequences than in X-linked sequences (3, 4, 8) and that the rate of nucleotide substitution was faster in rodents than in primates (17–19).

The fact that the X- and Y-linked ZF genes are found in all eutherian species studied (11) suggests that the two genes diverged prior to the eutherian radiation. It has been proposed that the anomolously high similarity between the human *ZFX* and *ZFY* cDNA sequences resulted from gene conversion of *ZFY* by *ZFX* during primate evolution (6, 7, 20). In contrast, the sequences of the last introns of *ZFX* and *ZFY* in higher primate reveal no evidence of gene conversion (8). The additional rodent data might detect a more ancient gene conversion but the observation that the X-linked introns and the Y-linked introns belong to two separate clusters (Fig. 2) implies no gene conversion between the last introns of *ZFX* and *ZFY*. However, the branch separating these clusters is very short, and thus it remains uncertain whether the primate and the rodent *ZFY* genes share a common origin prior to or have arisen separately after the rodent–primate split.

DISCUSSION

To estimate α_m, Miyata *et al.* (3) and Wolfe and Sharp (4) obtained X/A $\approx$ 0.60, where X and A were the average synonymous substitution rates in X-linked and autosomal genes, respectively. As this ratio is even smaller than the theoretical minimum value 0.67 predicted for $\alpha_m = \infty$, it implies that α_m is infinitely large. This approach, however, has some potential problems (8, 20). (*i*) It assumes that synonymous changes are selectively neutral. If synonymous changes are subject to some, even very weak, selective constraints, then selection is more effective in reducing the rate in X-linked genes than in autosomal genes because in males an X-linked gene is present in a single copy and in females one of the two copies may be inactivated. (*ii*) The mutation rates for nonhomologous sequences may be different, because the mutation rate depends on the nucleotide composition (e.g., G and C are more mutable than A and T) and neighboring nucleotides (21, 22). The X-linked and autosomal genes they used are not homologous. The large variation in synonymous rate among genes (4, 20) might be due to either or both of the preceding factors. However, Miyata *et al.*'s (3) study included a pair of homologous Y-linked and autosomal pseudogenes. Since this pair of sequences should not have the above potential problems, it is not clear why they gave an estimate of $\alpha_m = \infty$; however, it could be due to statistical fluctuations, for the data were limited.

In this and the previous study we have tried to avoid the above problems but there may still be some potential problems. (*i*) Although the sequences we used are homologous (i.e., share a common ancestor), they are located on different chromosomes and there is the possibility that the mutation

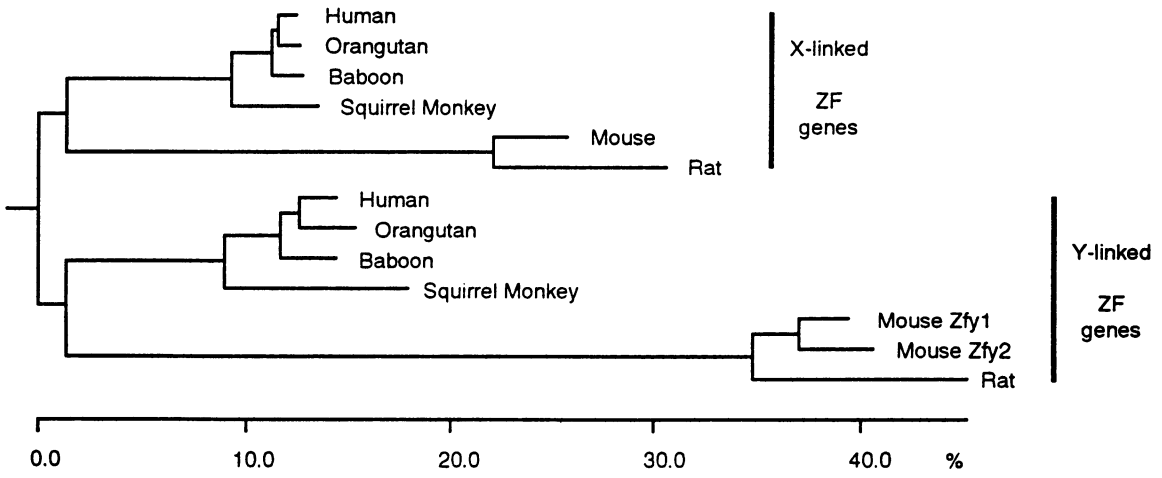

FIG. 2. Phylogenetic tree of the rodent and primate X-linked and Y-linked ZF genes.

rate per replication may not be the same for different regions of the genome (23). (*ii*) Changes in introns may not be completely free of selective constraints. In this case, the *Zfy* intron may evolve faster than the *Zfx* intron for three reasons: (*a*) Selection is likely to be less effective against the Y intron than the X intron because *Zfx* and *Zfy* are expressed in males, whereas *Zfx* alone is expressed in females or, in other words, *Zfy* might be dispensable but *Zfx* is not; (*b*) The effective population size of the Y chromosome is smaller than that of the X chromosome so that the substitution rate is expected to be faster for the Y chromosome than for the X chromosome (24); (*c*) the absence of recombination on the Y except for the pseudoautosomal region may affect the rate of substitution because of such effects as hitchhiking. However, if only a small part of the intron (e.g., signals for splicing) is subject to functional constraints, then the effect of selection would not be strong. At any rate, selection is likely to elevate the substitution rate in the Y intron and thus also the estimate of α_m, and this would only strengthen our conclusion that the difference in mutation rate between male and female rodents is much smaller than estimated previously.

Previously we obtained an estimate of $\alpha_m \approx 6$ in higher primates but the 95% confidence interval was from 2 to 84, so we could not exclude the possibility that α_m is indeed very large (8). The most significant result of the present study is that our estimate of α_m in rodents is only 1.8 with the 95% confidence interval from 1 to 3, indicating weak male-driven evolution in rodents. With the addition of the rodent data one can now be quite confident that the α_m values in primates and rodents are far smaller than suggested by Miyata *et al.* (3) and Wolfe and Sharp (4).

Limited data on the sex difference in spontaneous mutation rate are available for the mouse, as a by-product of the extensive work on the mutagenic effect of radiation (see ref. 25). The observed frequencies per locus were 7.9×10^{-6} for the males and 4.9×10^{-6} for the females, leading to a ratio of 1.6, which is close to our estimate. However, six of the seven mutations observed in females occurred in the offspring of one female and could be due to a premeiotic event. When these six mutations were counted as one, the total number of observed mutations in the females was reduced to 2, and the frequency per locus in the females became 1.4×10^{-6}, so that the male-to-female ratio increased to 5.6. This is higher than our estimate, but not significantly so because it is based on only two observed mutations.

We have made an effort to estimate the numbers of germ cell divisions per generation in females (n_f) and males (n_m) and the ratio $\alpha = n_m/n_f$ in mice, rats, and humans from published data on gametogenesis (details to be presented elsewhere). In the development of a female mouse, the number of DNA replications in the germ line (i.e., from zygote to mature egg) is $n_f \approx 28$. In the development of a male mouse, the number of germ cell divisions in the germ line from zygote up to the formation of stem spermatogonia is ≈ 30. Spermatogenesis requires 10 further DNA replications to the production of spermatids, initiating on day 6 after birth and occurring on average every 8.6 days in adult male mice (26, 27). The span of high reproductivity in laboratory mice is approximately from 2 to 8 months. If we assume that the average reproductive age of male mice in the wild is 5 months, then a stem spermatogonium would have gone through $5 \times 30/8.6 \approx 17$ divisions and the total number of DNA replications from birth to age 5 months is approximately $n_m = 30 + 10 + 17 = 57$. Therefore, $\alpha = n_m/n_f \approx 57/28 = 2.0$. If the average reproductive age is 2 or 8 months, then α becomes 1.7 and 2.4, respectively.

For the rat, $n_f \approx 29$, the number of cell divisions in the male germ line to form stem spermatogonia is ≈ 32, the spermatogenesis cycle occurs every ≈ 12.9 days (27), and the period of maximal fertility of laboratory rats occurs between 100 and 300 days after birth. If we assume that the average reproductive age of males in the wild is 7 months, then a stem spermatogonium would have gone through $7 \times 30/12.9 = 16$ divisions, the total number of DNA replications in the male germ line is $n_m \approx 32 + 10 + 16 = 58$, and thus $\alpha \approx 58/29 = 2.0$.

In humans, $n_f \approx 33$. The estimation of n_m is more difficult because data are more scanty than those for rodents. We estimate the number of cell divisions from zygote to stem spermatogonia at puberty to be ≈ 40; spermatogenesis requires five further DNA replications, and the spermatogenesis cycle occurs every ≈ 16 days or 23 cycles per year. If the average reproductive age of males is 20 years, then the number of DNA replications for stem spermatogonia from puberty (age 13) to 20 is $(20 - 13) \times 23 \approx 160$, $n_m \approx 40 + 5 + 160 = 205$, and $\alpha \approx 205/33 = 6.2$. If the average reproductive age of males is 25, then α becomes ≈ 9.7.

Although these estimates of α are rough, they are very close to the estimates of $\alpha_m \approx 2$ for rodents and $\alpha_m \approx 6$ for humans and other higher primates. This agreement suggests that errors in DNA replication during germ cell divisions are indeed the primary source of mutation and that the contribution of replication-independent factors such as oxygen radicals to mutation is considerably less important. Moreover, the 3-fold difference between our estimates of α_m for rodents and higher primates and also between our estimates of α for the two groups of organisms suggests that α_m and α decrease with decreasing generation time.

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