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Uniformity in the Nonsynonymous Substitution Rates of Embryonic β-Globin Genes of Several Vertebrate Species

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Summary. The nucleotide substitution rate in structural portions of the embryonic β-globin genes of placental mammals is lower than that for the adult β-globin genes. This difference occurs entirely within the class of substitutions that result in nonsynonymous (replacement) differences between these genes, and therefore represents a constraint on the structure of the mammalian embryonic β-globin proteins relative to the adult proteins (Shapiro et al. 1983; Hardison 1984). A similar effect has also been observed in marsupial mammals (Koop and Goodman 1988). In an effort to determine whether the observed rates are evidence of a uniform degree of selective constraint on the embryonic β-globin genes, analyses were performed that compared replacement substitution rates. The analyses reveal that embryonic β-globin genes appear to have been fixing replacement substitutions at nearly the same average rate not only in placental and marsupial mammals but in avian and amphibian species as well. In contrast, the adult β-globin genes from these organisms appear to have a more variable rate of replacement substitution with an especially low rate for birds. In the chicken (*Gallus gallus*), the adult β-globin gene replacement substitution rate appears to be lower than the embryonic replacement substitution rate.

Key words: Molecular evolution — Nucleotide substitution rates — Molecular clocks — Embryonic β-globin genes

Introduction

The β-globin gene superfamily, because of the large number of gene sequences available, has been widely examined for the purpose of understanding the evolution of DNA sequence. Complete sequences have been determined for some or all of the β-globin genes from numerous vertebrates including human, mouse, goat, rabbit, opossum, chicken, and frog (see notes to Table 1 for scientific binomens). The β-globin genes of each of these organisms reside in closely linked clusters, the organizations of which provide evidence for numerous and varied occurrences of gene duplication, deletion, and correction (see Collins and Weissman 1984 for review, and additional references herein).

In addition, each of the organisms listed above represents a taxonomic branch in which distinct embryonic and adult forms of β-globin have evolved. Where it has been investigated, namely in the placental and marsupial mammals, indications are that within each of these lineages, the proteins expressed in the embryonic developmental compartment are evolving more slowly than those expressed in the adult, as inferred from the lower rate of replacement substitution for the embryonic β-globin genes (Shapiro et al. 1983; Hardison 1984; Koop and Goodman 1988). Based upon this, the suggestion was made early on that embryonic and adult β-globin genes would have to be treated separately for the purpose of establishing such things as molecular evolutionary clocks (Shapiro et al. 1983; Hardison 1984). Information that is obviously relevant to such purposes is the relationship between the evolutionary rates of β-globin genes expressed at the same developmental stage in different vertebrate lineages. This report describes the results of analyses comparing replacement substitution rates among the embryonic and adult β-globin genes of several vertebrate classes which diverged from each other as long as 380 million years (Myr) ago.

Materials and Methods

Analysis of Divergence. The analyses of divergence for pairs of sequences were performed by a program written by F. Fuller, which was based on the procedures described by Perler et al. (1980). Nucleotide diversity (average divergence) within a population of species, average divergence between populations, and associated standard errors were calculated using a program provided by M. Nei and L. Jin using algorithms described in Nei and Jin (1989).

Hypothesis tests concerning these averages were calculated using the commercially available SAE (Statistical Analysis for Engineers) package (Barnes 1988).

Figure 1 was produced using Sigmaplot version 3.1.

Gene Sequences. Gene sequences were obtained from the sources given in the notes to Tables 1 and 2. When appropriate, one or two codons were deleted from near the beginning of the protein coding sequence of these genes to achieve alignment with *Xenopus* β or goat β^{A}, respectively, for the divergence analysis.

Results

Divergence at Nonsynonymous Sites between β-Globin Genes Expressed at the Same Developmental Stage

The corrected percentage of divergence at replacement sites between several vertebrate embryonic β-globin genes and between the adult β-globin genes of the same organisms is shown in Table 1. The pairwise divergence values at each taxonomic divergence point were used to calculate an average divergence and standard error of the average divergence by the method of Nei and Jin (1989). Perler corrected divergence values were entered into the Nei and Jin program so they would not be further modified (as Jukes–Cantor values) and because nearly identical results for standard errors were obtained using either the unweighted pair-group method using arithmetic averages (UPGMA) or neighbor-joining (NJ) tree-making method options, the NJ results were used to avoid making the assumption of constant rates of nucleotide substitution.

The average corrected percentage divergence at replacement sites obtained above was plotted against the species divergence time determined from the fossil record in Fig. 1. For plotting nucleotide divergences over a range of 380 Myr, several approximations and estimates were made. All orders of placental mammals were assumed to have diverged from each other at the same time based on the estimate of the time of the mammalian radiation (Romero-Herrera et al. 1973). Divergence times for the other taxonomic groups were the centers of the ranges provided by several references (Wilson et al. 1977; Wernke and Lingrel 1986; Koop and Goodman 1988). These times represent the currently prevailing interpretation of the fossil record for these groups (see Discussion). Two plots are shown, one for embryonically expressed β-globin genes, and one for the adult expressed β-globin genes from the same species for comparison. The embryonic values essentially describe a straight line. The correlation coefficient (r) for the best line (linear regression by the method of least squares) drawn between the average nucleotide divergences is 0.988 with a coefficient of determination ($r^2 \times 100$) indicating that 97.6% of the relationship is explained by linearity. The error bars, drawn at one standard error, show no overlap at this level between taxonomic divergence times. The best line has a y-intercept 1.3%, very close to the origin. The unit evolutionary period (UEP) is equal to 11.6 Myr/% (0.86×10^{-9} substitutions per site per year). These analyses indicate that the replacement substitution rate for the embryonic β-globin genes of these species, averaged over time, is nearly the same.

In contrast, the adult plot is less linear. It has an r value of 0.887 and a coefficient of determination of 78.7%. The best line has a y-intercept of 6.0%. The calculated UEP = 10.1 Myr/% (0.99×10^{-9} substitutions per site per year). The bird/mammal divergence average falls well off the best line, and, in fact, the nucleotide divergence values at the bird/mammal divergence are not significantly different from those at the metatherian/eutherian divergence (at $\alpha = 0.05$). These data suggest that the chicken adult β-globin replacement site substitution frequency is different and substantially lower than that of the other adult β-globin genes.

Nonsynonymous Substitution Rates in the Chicken β-Globin Genes

Sometime after the divergence of birds and mammals, distinct genes arose in each of these lineages from the orthologous ancestral β-globin gene and eventually evolved into the modern embryonic and adult β-globin genes (Czelusniak et al. 1982). In bird β-globin evolution, represented by the chicken, additional developmentally specific β-globin genes also evolved, resulting in the modern β-globin locus, 5′-ρ-β^{H}-β-ϵ-3′. In this locus, β encodes the adult β-globin, β^{H} encodes a transiently expressed hatching β-type globin, and ρ and ϵ encode the predominant and lesser embryonic β-type globins, respectively (Dolan et al. 1981, 1983; Roninson and Ingram 1981, 1982). Molecular evidence supporting the independent evolution of embryonic and adult β-globin genes in birds is strong. Replacement site divergence between chicken embryonic and adult β-globin genes is less than 10% (Roninson and Ingram 1982), whereas replacement site divergences between mammalian and avian β-globin genes for both embryonic and adult are between 20 and 30% (see Ta-

Table 1. Corrected percentage divergence at replacement and silent sites for vertebrate β-globin genes

Embryonic		Rβ4	Hε	Gε^{1}	Mεy2	Oε	Cρ	Xtβ
			67.2 ± 7.7[a]			69.7 ± 8.9[a]	103.2 ± 14.2[a]	135.4 ± 19.5[a]
Rβ4		—	58	74	57	68	108	161
Hε		8	—	59	61	60	98	110
Gε^{1}	8.4 ± 1.2[b]	9	6	—	95	63	84	126
Mεy2		11	9	8	—	88	123	145
Oε	12.9 ± 1.8[b]	13	13	12	14	—	103	122
Cρ	23.8 ± 2.7[b]	25	25	24	24	21	—	149
Xtβ	35.8 ± 3.5[b]	37	35	33	31	38	40	—

Adult		Rβ1	Hβ	Gβ^{A}	Mβ^{maj}	Oβ	Cβ	Xβ
			47.8 ± 5.9[a]			86.4 ± 12.4[a]	83.0 ± 10.8[a]	139.5 ± 21.7[a]
Rβ1		—	42	34	64	95	81	121
Hβ		5	—	37	54	91	67	158
Gβ^{A}	12.0 ± 1.4[b]	11	12	—	56	84	81	113
Mβ^{maj}		13	14	18	—	76	76	161
Oβ	23.7 ± 2.7[b]	21	21	26	27	—	110	142
Cβ	25.5 ± 2.6[b]	24	24	28	27	24	—	143
Xβ	49.2 ± 4.4[b]	48	50	47	56	49	45	—

Percentage divergences for pairs of sequences were determined by the method of Perler et al. (1980). Replacement and silent site divergences are given in the lower left and upper right portions, respectively, for both the embryonic and adult parts of the table. Nucleotide diversities (average divergences) within populations of species (shown for the placental mammals) and average divergences between populations were determined. Standard errors were computed as described in Nei and Jin (1989) using their Eq. (3) for obtaining the standard error for diversity (average divergence) within a population and their Eq. (19) for obtaining the standard error for average divergence between populations. The numbers of potential replacement and silent sites were assumed to be 335 and 106 nucleotides, respectively, in a typical β-globin gene sequence. Error values obtained from computations using the neighbor-joining (NJ) tree-making method are shown. The sources of the sequences are as follows: Rβ4, Hardison (1983); Hε, Baralle et al. (1980); Gε^{1}, Shapiro et al. (1983); Mεy2, Hansen et al. (1982); Oε and Oβ, Koop and Goodman (1988); Cρ, Roninson and Ingram (1981); Xtβ, Banville et al. (1983); Rβ1, Hardison et al. (1979); Hβ, Lawn et al. (1980); Gβ^{A}, Schon et al. (1981); Mβ^{maj}, Konkel et al. (1979); Cβ, Dolan et al. (1983); Xβ, Patient et al. (1983). R, H, G, M, O, C, and X stand for rabbit (*Oryctolagus cuniculus*), human (*Homo sapiens*), goat (*Capra hircus*), mouse (*Mus musculus*), opossum (*Didelphis virginiana*), chicken (*Gallus gallus*), and frog (*Xenopus laevis*), respectively

[a] Silent average divergence ± SE

[b] Replacement average divergence ± SE

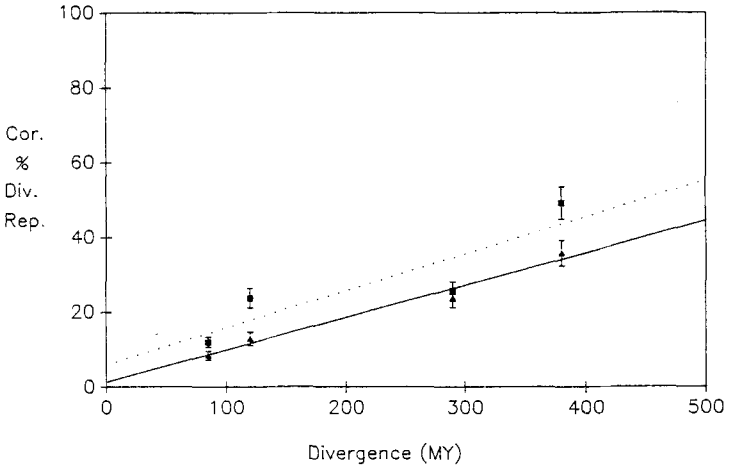

Fig. 1. Relationship between corrected percent divergence at replacement sites and time of divergence based on the fossil record for embryonic and adult vertebrate β-globin genes. Average divergences (±SE) from Table 1 are plotted against millions of years of divergence (see text for references). Divergence times: mammalian radiation, 85 Myr; marsupial vs placental mammals, 120 Myr; avians vs mammals, 290 Myr; amphibians vs avians/mammals, 380 Myr. ▲, embryonic average; ■, adult average. Solid line, embryonic regression line; dotted line, adult regression line.

ble 1). A similar relationship exists for silent site divergences.

The average nucleotide divergence values at each taxonomic divergence point in Fig. 1 are significantly higher for the adult β-globin genes than for the embryonic in all cases except for the bird/mammal divergence (by t-test with $\alpha = 0.05$). Because the chicken adult sequence is being compared to other adult sequences known to be diverging faster than the embryonic genes in marsupials and placental mammals, one may infer that the adult nonsynonymous substitution rate in chickens may be even lower than the rate for their embryonic β-globin genes.

In order to further investigate the relationship between the embryonic and adult β-globin gene nonsynonymous substitution rates in birds, replacement divergences were determined between the chicken β, ρ, and ε genes and the functional embryonic, fetal, and adult β-globin genes of four mammals, human, rabbit, goat, and mouse. Because the chicken β-globin genes all arose from a single β-type ancestor after the divergence of the bird lineage from the mammalian lineage, differences in the substitution rates between chicken β and the chicken embryonic β-type genes ought to be apparent when these genes are compared to any mammalian β-globin gene. The analysis is independent of the precise divergence time of birds and mammals. The results are shown in Table 2. Compared to ρ and ε the chick β gene was the least divergent in comparisons to 10 of the 12 mammalian genes, substantiating the conclusion that the chicken adult β-globin gene replacement site substitution rate is actually lower than the embryonic rate.

The values in Table 2 also corroborate the previous observation concerning the lower rate of nonsynonymous substitution among mammalian embryonic β-globin genes compared to adult β-globin genes (Shapiro et al. 1983; Hardison 1984). The situation that permits the comparison of chicken adult to chicken embryonic genes in their divergence from mammalian genes also permits the reverse, i.e., the comparison of mammalian β-globin gene substitution rates by measuring divergence from any chicken gene. In mammals representing four different orders, rodents, lagomorphs, artiodactyls, and primates, the embryonic β-globin genes have lower replacement substitution rates than the adult genes when compared to any chicken gene ρ, ε, or β. As previously observed, the lower embryonic substitution rate is limited to the replacement sites. In 11 out of the 12 comparisons of silent site divergence the adult mammalian gene has a lower value than any other stage.

Table 2. Corrected percentage divergences between chicken and mammalian β-globin genes at replacement (silent) sites

		Chicken		
		ρ	ε	β
Goat	ϵ^{I}	23.6 (84.3)	21.9 (85.1)	19.7 (82.7)
	ϵ^{II}	26.0 (91.1)	28.2 (82.1)	25.1 (90.1)
	β^{A}	30.4 (73.2)	29.8 (79.5)	28.1 (80.8)
Human	ε	25.0 (97.8)	21.8 (86.3)	20.5 (69.4)
	$^{G}\gamma$	29.1 (99.6)	25.8 (101.4)	24.0 (77.5)
	β	27.3 (69.8)	25.5 (70.7)	24.3 (66.9)
Rabbit	β4	25.1 (108.2)	24.7 (99.1)	22.6 (108.5)
	β3	22.1 (145.7)	22.7 (139.8)	22.2 (129.8)
	β1	25.7 (106.5)	25.0 (103.6)	23.7 (81.3)
Mouse	εy2	24.2 (122.9)	23.1 (124.6)	22.4 (86.1)
	βh1	22.3 (107.9)	21.7 (110.1)	22.1 (104.2)
	β^{maj}	30.0 (84.6)	31.4 (95.5)	27.4 (75.9)

Corrected percentage divergences were determined by the method of Perler et al. (1980) and rounded off to the nearest tenth. Silent site divergences are shown in parentheses following the replacement site divergence values. Sources of the sequences are as in Table 1 with the addition of chicken ε (lesser embryonic), Roninson and Ingram (1982); goat ϵ^{II} (expression period undetermined), Shapiro et al. (1983); human $^{G}\gamma$ (fetal), Slightom et al. (1980); rabbit β3 (early embryonic; human γ-related), Hardison (1981); mouse βh1 (early embryonic; human γ-related), Hill et al. (1984)

Discussion

Concomitant with the observation that mammalian embryonic β-globin genes were accumulating replacement substitutions more slowly than mammalian adult β-globin genes, the suggestion was made that this difference would need to be accounted for when making molecular clocks based on globin gene substitutions (Shapiro et al. 1983; Hardison 1984). A sufficient number of embryonic β-globin gene sequences of widely divergent vertebrate species have now been obtained to make a meaningful analysis of their evolutionary rates. The data presented in Fig. 1 demonstrate that for the embryonic genes, the average rate of nonsynonymous substitution in each independent taxonomic branch was about the same as that in the others, and this average rate did not change significantly over the course of the last 380 Myr. The accumulation of nonsynonymous substitutions in the embryonic β-globin genes appears to be clocklike. The rate obtained in all of the lineages examined is the same as that in mammals, suggesting that all of the embryonic sequences may have been constrained to the same degree by the negative or purifying selection on their encoded proteins previously described for the mammals. Of course the inferred rate, UEP = 11.6 Myr/% (0.86×10^{-9} substitutions per site per year), is an average and does not imply that that rate was continuous. The actual occurrence of the substitutions may have been at a continuous rate or may have occurred in an episodic manner (as in Gillespie 1986), but over the long time frame under investigation here, presents an average that does not provide information to distinguish between the two possibilities. The result does suggest however that the embryonic environments in placental mammals, marsupial mammals, and egg-laying vertebrates may impose a similar constraint on the structure and function of the embryonic β-globin protein.

Molecular characters evolving at constant rates are sought after for the purpose of constructing molecular clocks. Molecular clocks based on replacement site divergence of globin genes have been given by Perler et al. (1980). When compared to silent site

clocks, replacement clocks show superior characteristics over long periods of time. For clocks operating on globin genes over periods in excess of about 100 Myr, the rapidly substituting, probably neutral silent sites saturate and the silent site clock apparently enters a second phase in which the remaining sites show evidence of selective pressure. Scatter during this second phase is generally substantially higher than that for replacement site divergence for the same time interval (Perler et al. 1980; see Table 1, this report). Indeed, even for periods under 85 Myr, the globin silent site clock quickly evidenced too much scatter at each taxonomic divergence point after the addition of more globin sequences to the establishing data (Schon 1982). Thus clocks based on replacement sites or protein sequence, with their much slower substitution rates, appear to be better prospects for usefulness over long periods of time (Perler et al. 1980; Zuckerkandl 1987).

Replacement site substitution rates among globin genes, however, display considerable variation. This has been shown for both different genes in the same line of descent and the same gene in different lines of descent (Shapiro et al. 1983; Hardison 1984; Harris et al. 1986; Koop and Goodman 1988; this report). In contrast, the findings reported here for embryonic β-globin genes indicate that these genes do not appear to display significant differences in replacement site substitution rates in several vertebrate classes and therefore may generate a very accurate clock.

It should be emphasized that the embryonic genes used for comparison in Fig. 1 were demonstrated orthologues in the case of the mammalian species (all embryonically expressed descendents of proto ϵ), but the genes were chosen only on the basis of function from all other branches. Thus, for example, even though bird β is more similar to mammalian embryonic genes than the bird embryonics are, the chicken ρ gene was used for the embryonic comparison because it encodes the predominant embryonic β-globin protein. Because orthology is often defined based upon maximum similarity, in instances such as the above, the compared genes may not be orthologous by this definition. However, the compared genes always arose via speciation from a common ancestral gene and are orthologous by this definition. It should also be pointed out here that β-globin genes that are expressed exclusively during fetal development were not analyzed for substitution rates over this long time frame because their existence is limited to only some of the eutherian orders. Furthermore, the fetal genes arose from different mammalian β-globin precursor genes in these orders, are thus not orthologous, and do not lend themselves to this analysis.

The data in Fig. 1 also imply that when only one β-globin gene exists in these organisms, and therefore the product of that gene has to function in developmental compartments which include the embryonic one, then the gene is constrained like an embryonic gene. This situation applies in amphibians immediately after divergence, in the line that goes to birds and mammals prior to their divergence from each other, and in the newly diverged bird and mammal ancestors. This requirement is inferred from the linearity of the embryonic plot in Fig. 1. Once gene duplication results in distinct embryonic and adult genes, then evolution of the adult gene (and the embryonic gene) proceeds at its distinct rate.

The low apparent rate of replacement substitution in the chicken adult β-globin gene has been previously observed (Dickerson and Geis 1983; Nei 1987). Factors that might contribute to this effect include an actual lower replacement substitution rate for chicken β, and the possibility of an error in interpretation of the fossil record placing the bird/mammal divergence too early. The latter possibility has some support from both molecular data from other chicken genes and morphological evidence that may group chickens with mammals rather than reptiles, thus permitting a later divergence of birds and mammals (Gardiner 1982; Dickerson and Geis 1983; Lovtrup 1985; Nei 1987). The two possibilities are not mutually exclusive nor would a change in the position of birds necessarily create a problem for the embryonic β-globin clock.

If one were to assume that all of the deviation of the bird/mammal adult replacement site divergence average from the regression line is due to error in the divergence time, one could restore linearity to the greatest extent by displacing the bird/mammal divergence to 171 Myr ago (compared to the 290 Myr ago used in Fig. 1). This extreme position, if correct, would worsen the linearity of the embryonic points substantially. However, there is compelling reason to believe that, in fact, the chicken adult β-globin replacement substitution rate is lower than that of other vertebrates and this factor at least contributes to the low bird/mammal divergence values. Roninson and Ingram (1982) determined, by the identical method used in this study, that the replacement site divergence between the adult βs of chickens and ducks is very low at 1.1% (duck embryonic β-globin gene sequence is not available). These bird orders are believed to have diverged some 70–100 Myr ago (Wilson et al. 1977) probably shortly after the evolution of the distinct bird adult β-globin gene. Therefore, the replacement substitution rates of the adult β-globin genes from both of these birds are very likely quite low and this may account for a large part of the effect observed. A minor ad-

justment of the bird/mammal divergence to a more recent time for whatever reason would actually improve the linearity of the embryonic β-globin replacement divergence points with optimum linearity occurring at 247 Myr ago.

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