J Mol Evol (1993) 36:599–601

Journal of Molecular Evolution

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A Differential Molecular Clock in Enolase Isoprotein Evolution

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Enolase (EC 4.2.1.11) catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, a step in glycolysis vital to cellular function. In mammals, there appear to be three dispersed active enolase genes. (See Craig et al. 1990.) The sequence homology between enolases from sources as diverse as *Escherichia coli, Arabidopis,* and *Homo sapiens* (e.g., Van Der Straeten et al. 1991) strongly supports the presence of an ancestral enolase gene which has diversified by speciation of the organism as well as by duplications within organisms. Here we show that despite similar total levels of divergence of enolase isoprotein codons between man, rat, and mouse, the degree of divergence varies markedly between the three different isoproteins NSE, MSE, and NNE.

Mammals display three enolases, neurone-specific enolase (NSE), muscle-specific enolase (MSE), and nonneuronal enolase (NNE). For man, rat, and mouse, a wide collection of sequences is now available. We have sequenced human-neurone-specific enolase (NSE) cDNA and human gene ENO3, which encodes muscle-specific enolase (MSE). Other groups have sequenced human non-neuronal enolase (NNE) cDNA, all of the corresponding rat (*Rattus norvegicus*) and mouse (*Mus musculus*) cDNAs, and in several instances the genes also. Authorship is identifiable from the GenBank entries, accession numbers X13120; X51956; X16504; M14328; X16287-X16290; M22349; M27833; M36768; X66610; X51957; Y00691; Y00979; X07726-X07729; M11931; X02610; X52379; X52380; and X62669. The analysis presented here depends on the veracity of identification of small numbers of protein substitutions causing a given isoprotein to differ in sequence between human and rat: however, the combination of data from independent groups and clones, now available for relevant enolase proteins, cDNAs, and genes, effectively excludes the problem of sequencing errors confounding the identification of natural variation. A cDNA frameshift of 20 nucleotides (real or artefactual) affecting deduced rat MSE sequence aligning with yeast enolase positions 177–183 (Chin et al. 1981) was discounted from the point mutation analyses presented here; human NNE and NSE positions aligning with yeast enolase position 397 were arbitrarily taken to be threonine; and human NSE position 3 was confirmed to be glutamic acid following review of our own data: the agreement of data was otherwise high.

Perfect alignment between all mammalian enolases is possible. In addition a near-perfect alignment with yeast enolase-1 is possible and has the additional advantage that the sequences are then referable to the crystal structure known for the latter (Lebioda et al. 1989). The numbering system of the latter is therefore used here: sites equivalent with yeast sites 139, 140, and 267 are absent in the rat and human isoproteins.

For paralogous comparisons in human, the total amino acid differences are: for NNE/MSE,74; MSE/NSE,72; and NNE/NSE,74; in rat, NNE/MSE,76; MSE/NSE,74; and NNE/NSE,76; and in

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Table 1. A. Total silent nucleotide and amino-acid replacement substitutions for NNE, MSE, and NSE coding sequences observed between man, rat, and mouse

	Silent substitutions		Replacement substitutions	
	Observed	Expected	Observed	Expected
NNE	149	135.3	33	19
MSE	123	135.3	14	19
NSE	134	135.3	10	19
	$\chi^2 = 2.52$ (2 *df*) $p > 0.1$		$\chi^2 = 15.80$ (2 *df*) $p < 0.001$	

Table 1. B. Total amino-acid replacement substitutions for enolase isoprotein orthologues when man (*Hs*), rat (*Rn*), and mouse (*Mm*) are considered pairwise

Amino-acid substitutions	NNE	MSE	NSE	
Hs vs *Rn*	26	13	8	$\chi^2 = 11.02$ $p < 0.01$
Rn vs *Mm*	17	7	7	$\chi^2 = 6.45$
Hs vs *Mm*	24	11	6	$\chi^2 = 12.63$ $p < 0.01$

mouse, NNE/MSE,70; MSE/NSE,69; and NNE/NSE, 73. The total length of each protein, excluding the initiator methionine, is 432 amino acids. The approximately equidistant divergences average 17% and suggest that a "burst" event took place in evolution to create all three isogenes: saturation divergence appears unlikely since it is not the same 70 or so sites involved in each pairwise comparison and more remote species such as yeast (Chin et al. 1981) display many more differences.

By contrast, there are fewer differences in orthologous comparisons, presumably because the isoprotein divergence (deducible to be perhaps 300 Myr from Segil et al. 1988; Clark-Rosenberg and Marangos 1980; Britten 1986) predated rat/human speciation (75–110 Myr: Britten 1986). Table 1 shows the total number of silent and replacement substitutions between man, rat, and mouse for each gene. Chi-squared test indicates that there is no significant difference in silent-site divergence but that there is a highly significant ($p < 1/1000$) difference in amino-acid replacement substitutions between the three genes. Gene-specific amino-acid substitution rates have been observed previously (Langley and Fitch 1974) and in the case of globin proteins have been shown to be attributable to isogene-specific rates of fixation of codon changes which cause amino-acid substitution, rather than to isogene-specific rates of nucleotide substitution (Harris et al. 1986). However, the underlying mechanisms remain obscure. Amongst enolases, isoprotein NNE seems to be evolving faster. Both complete multiple alignments and modeling based on yeast enolase-1 C_a positions (not shown) indicate that the amino-acid substitutions are quite widely scattered over the surface of the protein.

We have considered the following possibilities to explain our finding:

1. That inhomogeneities of nucleotide content for the three isogenes (e.g., between codon positions 1, 2, and 3) are causing similar silent nucleotide substitutional rates (mostly at position 3) but different coding substitutional rates (mostly at positions 1 and 2). We have not been able to demonstrate such a pattern.
2. That the chi-squared test is rendered inappropriate by interdependence (rather than the assumed independence) of amino acids in isoproteins; e.g., once one substitution occurs, others occur as an obligatory or favorable cascade. It is not obvious from the distribution of substitutions on the model structure that this might have occurred for NNE, but it could not be excluded without extensive phylogenetic data enabling the history of the series of substitutions to be tracked.
3. That a true chance event ($1/1{,}000$–$1/10{,}000$) has created the picture described here. Sequences of cDNAs for NNE, MSE, and NSE in other mammals would be of interest in this respect. However, it should be noted that the faster amino-acid divergence rate in NNE is already apparent across two independent speciation events (Table 1B).
4. That interactions of the tissue-specific isoproteins, NSE and MSE, with other cellular components (Brady and Lasek 1981; Batke et al. 1988), impose selective constraints to a greater extent than with NNE.

The differential substitutional clock described suggests differential selective pressures for the different isoproteins. It would be of interest to test whether this observation extends to more remote species, which might also help to discriminate between the possible mechanisms enumerated here.

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Received August 25, 1992/Revised January 20, 1993