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Letter to the Editor

Evolutionary Constraints and the Neutral Theory[1]

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Summary. The neutral theory of molecular evolution postulates that nucleotide substitutions inherently take place in DNA as a result of point mutations followed by random genetic drift. In the absence of selective constraints, the substitution rate reaches the maximum value set by the mutation rate. The rate in globin pseudogenes is about 5×10^{-9} substitutions per site per year in mammals. Rates slower than this indicate the presence of constraints imposed by negative (natural) selection, which rejects and discards deleterious mutations.

Key words: Neutral theory — Codon usage — Synonymous codons — Pseudogenes

The neutral theory of molecular evolution is based on an inherent evolutionary property of DNA, and on the nature of properties of species of living organisms. During evolution, mutations involving nucleotide changes take place, some of which become fixed by random genetic drift. This means that such changes resulting from point mutations spread through the species by a chance process.

The opposing ("selectionist") view states that mutational changes accumulate in a species exclusively by the action of positive Darwinian selection; that is, there has to be some selective advantage for the mutants to become fixed.

The neutralist–selectionist controversy has continued for over 15 years, but we believe that the neutral theory has gained much strength by recent developments in molecular genetics. (See [1] for a comprehensive review.) Note that both sides accept that negative selection is common, and that deleterious mutations are eliminated from the population. What is at issue is whether those mutational changes that spread through the species are fixed by Darwinian selection acting on advantageous mutations (the "selectionist" conclusion), or instead by random genetic drift acting on selectively neutral (selectively equivalent) or nearly neutral mutations (the "neutralist" conclusion). The two views are diametrically opposed. For a discussion of some of the misunderstandings on the meaning of the neutral theory, see Kimura [1].

According to the neutral theory, the rate of fixation is at a maximum when there are no adaptive constraints on the DNA sequences involved. This is because the rate of evolution is equal to the mutation rate for selectively neutral mutations [2], and is at a maximum when all the mutations are neutral, i.e., when none of them are selected against. Constraints result from negative feedback that eliminates deleterious changes by natural selection. A familiar example of such constraints is the case of histone proteins 3 and 4 in cows and peas; these proteins have functions that are so essential and so similar in widely differing species that there are only 6 differences in a total of 237 amino acid residues. But many silent nucleotide substitutions occurring in codon third positions of histone genes are not so constrained, and hence are not discarded. In a comparison of 187 codons in sea urchin H3 and H4 histone genes, 63 silent nucleotide substitutions occurred simultaneously with only 2 amino-acid-al-

[1] We wish to dedicate this paper to the memory of Professor Jack Lester King

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tering nucleotide substitutions (replacement substitutions [3]). Even more striking is a comparison of two nonallelic gene sets for H3 and H4 in yeast that code identical H3 and H4 histone proteins [4]. The two pairs of genes are translated in the same cell. There were 28 silent nucleotide substitutions in the 135 codons of the H3 gene and 4 in 102 codons of the H4 gene, without a single replacement substitution in either gene [4].

The fixation of neutral nucleotide substitutions in mammalian evolution, as shown by comparisons of globin pseudogenes, occurs at the rate of about 5×10^{-9} per nucleotide per year [5]. Fixation rates of amino acid replacements are usually much lower than this because of constraints. Fibrinopeptides show high rates of amino acid replacements and are a favorite example of neutral changes [5], but in our opinion their sequences contain too many gaps to enable truly accurate comparisons, as pointed out previously [6]. Also, some positions in fibrinopeptide A are notably less changeable than others [6]. Generally speaking, synonymous nucleotide substitutions show very similar rates among proteins that have widely different amino acid substitution rates [5].

Various authors have proffered evidence of evolutionary constraints as disproof of the neutral theory. Some examples are the nonrandom use of synonymous codons; the high evolutionary rate at early stages of evolution, which contradicts the "molecular evolutionary clock"; and conservation of helical regions in mRNA molecules.

Blaisdell [7] concluded that "bias in the choice of the occupant of site 3 in codons makes it unlikely that mutations in site 3 are selectively neutral." This would apply only to site 3 positions for which bias in the choice has been shown. If the magnitude of selective advantage or disadvantage of a mutant allele is very small, its behavior in the population is essentially the same as that of a strictly neutral allele. More precisely, if the absolute value of the selection coefficient (s) of the mutant allele is much smaller than the reciprocal of $2N_e$, where N_e is the "effective" (or breeding) size of the population [i.e., if $|s| \ll 1/(2N_e)$], then the behavior of the allele is mainly controlled by random genetic drift rather than by natural selection. Therefore, there is no need for mutations to be selectively neutral in the strict sense to validate the neutralist position. It is only necessary that $|s| \ll 1/(2N_e)$.

Returning to the problem of nonrandom usage of synonymous codons, the real issue from our standpoint is whether the observed nonrandomness is caused by positive selection or by negative selection. The selectionist interpretation is that among synonymous codons produced by mutation, those better fitted to the environment are predominantly chosen to replace those less fit, thus leading to a nonrandom pattern. Neutralists, on the other hand, assume that such nonrandomness reflects the fact that not all synonymous changes are selectively equivalent and therefore not all of them have equal probability of becoming fixed by random drift.

Kimura [5] pointed out that nonrandom use of synonymous codons occurs because of differences in the availabilities of synonymous tRNA species in the cell resulting from stabilizing selection [5, 8]. It was long ago suggested that differences in the amounts of synonymous tRNAs that paired with different codons might affect translation rates in protein synthesis [9, 10]. Experimental evidence showing that differences in the levels of various tRNAs for the same amino acid actually occur, and that differences exist in the usage of synonymous codons, was not then available, but has appeared subsequently, as discussed in [5]. Zuckerkandl and Pauling [10] cited an earlier expression of the same idea by Itano [11]. They wrote that "one can therefore not say, without resorting to an auxiliary hypothesis, that the apparent slower rate of HbS synthesis as compared to HbA synthesis in HbA/HbS heterozygotes is perhaps due to the appearance of a codon whose corresponding transfer-RNA is present in limiting amounts" [10].

This seemed to say that in the case of HbS and HbA, tRNA differences would not affect translation rates, unless an auxiliary hypothesis was valid. This hypothesis was that the identity of the degenerate (third) base of the codon might influence the rate of polypeptide synthesis. The authors then noted that, after all, the rate of synthesis of HbS might be not lower than that of HbA, but they also said that the possibility of "isosemantic substitutions as a significant factor in the regulation of polypeptide synthesis is not ruled out."

The conclusion by Kimura [5] cited above is based on experimental results, especially those of Ikemura [12–14], who measured the relative abundances of many tRNAs in *Escherichia coli* and *Saccharomyces cerevisiae*. He correlated these measurements with codon usage in these organisms and reviewed earlier work by other investigators. Ikemura found that codon choices in yeast genes "were constrained by a combination of tRNA availability and nature of its codon recognition [13]." In *E. coli*, the correlation between tRNA abundance and codon frequency was strongest for genes that coded the most abundant proteins. This is the reverse of what one would expect from the "selectionist" argument that if nonrandom usage is the result of positive Darwinian selection, then increased usage should cause more rapid evolution. By positively selecting among mutational changes, those changes that best fit a specific tRNA species will speed up evolution as

compared with a situation in which there is no particular choice. (This latter case would occur when all mutational changes are equally accepted and become fixed by random drift, without positive selection.) But the observation is in the opposite direction: A stronger bias slows evolutionary change, and, as Miyata [15] has stated, "the evolutionary rate of synonymous substitution is negatively correlated with the degree of bias in codon utilization."

The mathematical theory of Kimura [8], which shows that Ikemura's finding can be incorporated into the framework of the neutral theory, makes use of the concept of random drift under stabilizing or centripetal selection. Actually, this theory is more general, and shows that under stabilizing phenotypic selection, extensive neutral evolution can occur at the molecular level. Milkman [16], who arrived at essentially the same idea independently, called this "a unified selection theory." More recently, Milkman [17] has written that, with Kimura's theory [8], the neutralist–selectionist conflict has finally been resolved (see pp 328–334 of [17]).

The proposal that an evolution was particularly rapid at its early stages is based on errors of geologic dating [5], and in any case, perturbations of rate do not vitiate the molecular evolutionary clock [3]. The third proposed piece of evidence against the neutral theory is conservation of helical regions in mRNA molecules. This is an example of a constraint that slows the substitution rate.

To reiterate, the existence of bias in the choice of synonymous site 3 nucleotides in certain codons would be evidence for a selective constraint rather than a contradiction of the neutral theory. The neutral theory is concerned with the mechanism by which a mutated change spreads through the species. It claims that the majority of evolutionary change is caused by random genetic drift in the species, under continued mutational pressure. It does not claim that all the mutational changes at the time of occurrence in individuals are selectively neutral.

In sum, the neutral theory of molecular evolution has been substantiated and strengthened by numerous recent observations based on DNA sequencing. These show that the rate of nucleotide substitution in evolution tends toward a maximum rate in the absence of constraints, and that diminution below the maximum rate is evidence for the existence of constraints (negative selection). The maximum rate so far found in mammals, as observed in evolutionary nucleotide substitution of globin pseudogenes, appears to be about 5×10^{-9} substitutions per nucleotide site per year.

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