Nuclear and Mitochondrial DNA Comparisons Reveal Extreme Rate Variation in the Molecular Clock

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The discovery that the rate of evolution of vertebrate mitochondrial DNA is rapid, compared to the rate for vertebrate nuclear DNA, has resulted in its widespread use in evolutionary studies. Comparison of mitochondrial and nuclear DNA divergences among echinoid and vertebrate taxa of similar ages indicates that the rapid rate of vertebrate mitochondrial DNA evolution is, in part, an artifact of a widely divergent rate of nuclear DNA evolution. This disparity in relative rates of mitochondrial and nuclear DNA divergence suggests that the controls and constraints under which the mitochondrial and nuclear genomes operate are evolving independently, and provides evidence that is independent of fossil dating for a robust rejection of a generalized molecular clock hypothesis of DNA evolution.

ANIMAL MITOCHONDRIAL DNA (mtDNA) is widely employed in evolutionary studies (*1–3*) because it is small, maternally transmitted, easily purified, and consists mostly of coding sequences. No variation is known in its gene content, and gene order differs only among major taxonomic groups. Despite these conservative traits, vertebrate mtDNA evolves at a rate five to ten times that of vertebrate single copy nuclear DNA (scnDNA) (*4–6*). Whether this extreme rate difference is constant can be used to test the validity of the hypothetical clock like behavior of DNA. A constant relative evolutionary rate difference between mtDNA and scnDNA is necessary, though not sufficient, for a clock hypothesis; however, nonconstancy in relative rates is a sufficient criterion for rejecting such a hypothesis (*7*). In previous studies [summarized in (*8*)] it was necessary to rely on paleontological estimates of divergence times, which are often subject to considerable uncertainty (*9*). In contrast, we now compare relative sequence divergences of the genomes of different subcellular compartments within organisms; thus, the conclusions are independent of divergence time estimates.

To test the generality of the evolutionary rate difference found in vertebrate mtDNA and scnDNA, estimates of mtDNA divergence obtained from restriction endonuclease cleavage map comparisons among the sea urchins *Strongylocentrotus purpuratus, S. franciscanus*, and *S. droebachiensis* were compared with published scnDNA divergence estimates for these taxa (*10, 11*). The amounts of within-species mtDNA sequence polymorphism were also assessed, both as a measure of the validity of the interspecific comparisons and because exceptionally high levels of sequence polymorphism have been reported for the scnDNA's (*12*).

Individual mtDNA samples were prepared (*13*) from 24 *S. purpuratus* (16 from Corona del Mar, California; 8 from Vancouver, British Columbia), 11 *S. franciscanus* (8 from Corona del Mar; 3 from Vancouver), and 22 *S. droebachiensis* (9 from Vancouver; 13 from Woods Hole, Massachusetts). After restriction endonuclease digestion, DNA fragments were end-labeled and assayed by electrophoresis in both 1.2% agarose and 3.5 to 6% polyacrylamide gels (*14*). In a few cases, cloned *S. franciscanus* mtDNA was used to confirm the locations of cleavage sites. Interspecific sequence divergences were estimated from comparisons of the cleavage maps (Fig. 1 and Table 1) (*15*) and intraspecific divergences from fragment mobility comparisons (*16*).

Comparisons of *S. droebachiensis* and *S. franciscanus* with *S. purpuratus* yield respective divergence estimates of 6 and 11% for mtDNA and 7 and 19% for scnDNA (*11, 17*) (Table 1). In contrast, primate taxa with roughly comparable mtDNA divergences (8.8 and 17.2%) have scnDNA divergences of only 1.8 and 3.7% (Table 1). Clearly, a clock hypothesis calibrated from the primate data predicts sea urchin scnDNA divergences of only a few percent. The evolutionary rates of echinoid mtDNA and scnDNA thus appear to be approximately equal, in contrast to the five- to tenfold greater rate observed for vertebrate mtDNA (Table 1).

When paleontologically constrained divergence times are taken into account, the observed rate variation appears to be due to rate changes in scnDNA rather than in mtDNA. Paleontological estimates of the minimum and maximum possible divergence times for these sea urchin species are 5.3 and 23.7 million years (*18*). Over comparable time ranges, amounts of mtDNA and scnDNA sequence divergence among these sea urchins are similar to mtDNA

Table 1. Comparisons of mtDNA and scnDNA divergences. Divergence time ranges reflect uncertainties in or disagreements about interpretation of the fossil record. Sea urchin mtDNA divergences (±1 SE) were calculated according to the method of Nei and Tajima (*15*). Ava I sites that were also cleaved by Xho I were not included in the calculations, and the *r* value for Ava I was adjusted accordingly. The site forming the unmapped 39-bp Hind III fragment (legend to Fig. 1) was assumed to be homologous between *S. purpuratus* and *S. droebachiensis*. MtDNA sequence divergences were estimated from restriction site maps for the sea urchins and from DNA sequence data for the primates. All scnDNA divergences are from DNA hybridization experiments. The use of these three techniques is reasonable, since restriction site comparisons have been shown to be accurate estimators of DNA sequence divergences (*26*); thus, all mtDNA and all scnDNA divergences were calculated from comparable estimators. The use of primate DNA comparisons to represent vertebrates appears justified; though the data are less extensive, similar rate differences between mtDNA and scnDNA have been reported for amphibians and rodents (*6*).

Species compared	Sequence divergence (%)		Time since divergence (million years)
	mtDNA	scnDNA	
S. purpuratus–*S. droebachiensis*	6.3 ± 1.4* 6.0 ± 1.4†	7 (*11*)	5.3–23.7‡
S. purpuratus–*S. franciscanus*	11.3 ± 2.2* 11.1 ± 2.2†	19 (*11*)	5.3–23.7‡
S. droebachiensis–*S. franciscanus*	8.9 ± 1.8* 9.3 ± 1.8†		5.3–23.7‡
Chimpanzee–human	8.8 (*5*)	1.8 ± 0.05 (*22*)	4–6 (*23*)
Chimpanzee–orangutan	17.2 (*5*)	3.7 ± 0.1 (*22*)	10–20 (*23*)
Human–orangutan	16.1 (*5*)	3.6 ± 0.1 (*22*)	10–20 (*23*)
Human–Old World monkey	23.2 (*20*)	7.5–8.0 (*24*)	20–25 (*25*)
Human–New World monkey		13.0–13.3 (*24*)	35–40 (*25*)
Human–prosimian		23.7–24.5 (*24*)	55–60 (*25*)

*Divergence calculated excluding polymorphic sites. †Divergence calculated including polymorphic sites.
‡This divergence time range is most conservative, and represents the maximum possible (that is, the earliest fossils assignable to the family Strongylocentrotidae) and the minimum possible (that is, the earliest date by which all three species were present as fossils) divergence time estimates. There are no known fossil data that allow one to estimate the individual species divergences within this range (*18*).

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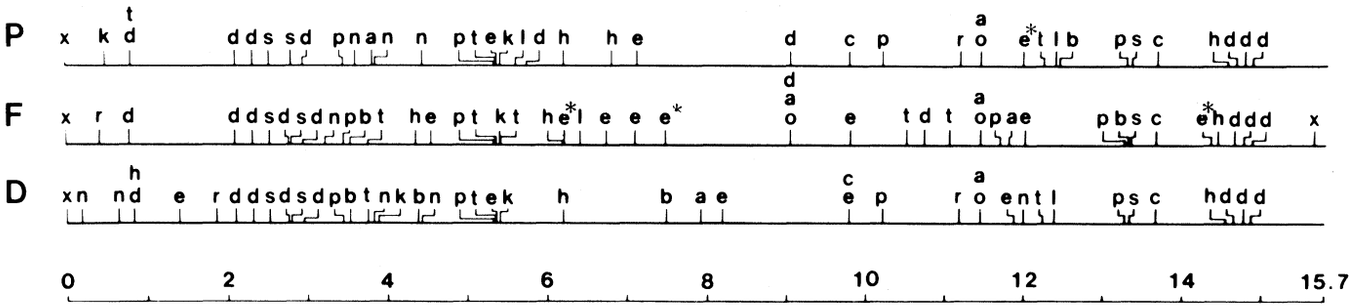

Fig. 1. Restriction endonuclease cleavage maps of *S. purpuratus* (P), *S. franciscanus* (F), and *S. droebachiensis* (D) mtDNA's. The scale is in kilobases, with the common Xba I site designated as zero. Sites are indicated as follows: a, Ava I; b, Bam HI; c, Cla I; d, Hind III; e, Eco RI; h, Hpa I; k, Kpn I; l, Sal I; n, Nci I; o, Xho I; p, Pst I; r, Eco RV; s, Sst II; t, Bst EII; and x, Xba I. Asterisks indicate polymorphic sites. No Sma I sites were present. The maps were aligned by their common Xba I, Xho I, and Cla I sites. Criteria used to infer site homology have been described (*4*). The Hind III sites at positions 0.8 and 14.8 and the Eco RI sites at 12.0 differed by 25 to 50 bp among the species. Because this variation could be due either to cleavage site changes or to deletions or additions external to the sites (*21*), divergence estimates based on both possibilities were calculated (Table 1). A Hind III fragment of 39 bp is present in *S. purpuratus* and *S. droebachiensis*, but was not mapped. MtDNA cleavage maps for *S. droebachiensis* collected from the eastern and western North American coasts were identical for the 16 enzymes employed. The number of Xba I sites and the number and positions of Eco RI and Hind III sites in the *S. franciscanus* map differ from a published map of cloned *S. franciscanus* mtDNA (*27*). Although some of these differences are probably due to sequence polymorphism and to different levels of resolution in the two studies, these explanations seem insufficient to explain why the region between the Eco RI sites at 4.6 and 7.5 kb in the above map for *S. franciscanus* and the corresponding region between the Eco RI sites at 8.3 and 11.1 kb in the *S. franciscanus* map in figure 4 of (*27*) appear to be inverted relative to one another.

divergences observed among vertebrates (Table 1). In contrast, the amount of time required to accumulate corresponding amounts of vertebrate scnDNA sequence divergence is 25 to 50 million years (Table 1), a considerably greater period of time. Thus, in the context of the paleontological data, the simplest interpretation is that the relative divergence rate between mt- and scnDNA can vary five- to tenfold, and that this fluctuation appears to be mostly in the scnDNA rate.

In support of these conclusions, Britten (*8*) has recently estimated the scnDNA divergence rate of sea urchins to be approximately five times that of vertebrates, and comparisons among continental *Drosophila* have indicated approximately equal divergence rates for mtDNA's and scnDNA's (*3, 19*). This fluctuation in the relative rates of mtDNA and scnDNA evolution is clear evidence that the genomes of distinct subcellular compartments within an organism are under different controls and evolutionary constraints, which are not uniform and evolve differently in different taxa. These observations do not invalidate the cautious use of rate constancy assumptions to estimate relative divergence times among closely related taxa (where the controls and constraints on the genomes are likely to be similar) or the use of molecular data to obtain branching orders among even less closely related taxa; however, they clearly demonstrate the necessity of establishing that appropriate boundary conditions exist and provide one empirical means of testing for this. Of particular interest is that this change in evolutionary rate of nuclear DNA occurred between vertebrates and a member of a putative sister taxon.

It was also of interest to investigate the intraspecific levels of mtDNA sequence polymorphism because elevated levels of sequence polymorphism have been observed in scnDNA's of these urchins (*12*). Digestion of mtDNA with the restriction endonucleases Mbo I, Msp I, Hinf I, Hae III, and Taq I produced, on average, a total of about 130 fragments per individual. The electrophoretic mobilities of these fragments among 16 *S. purpuratus* from Corona del Mar and Vancouver populations were compared, and a mean intraspecific sequence divergence of 0.99% (SE 0.55%, range 0.13 to 2.23%) was calculated (*16*). Preliminary estimates of <1% sequence polymorphism were calculated among ten *S. franciscanus* and among eight *S. droebachiensis*. This level of variation is well within the range found among vertebrate species (*2*), and is much less than the divergence levels observed between the sea urchin species (Table 1). In addition to cleavage site polymorphism, variations in fragment mobility corresponding to size differences of 1 to 5 bp were observed. These occur more frequently among the sea urchin mtDNA's than human mtDNA's (*20*) and are probably due to small deletions or insertions, as reported for human and lizard mtDNA's (*21*).

Our results indicate that the mitochondrial and nuclear DNA's of these sea urchins evolve at approximately the same rate, in contrast with vertebrates, in which the mitochondrial DNA rate is five to ten times faster than the nuclear DNA rate, and thus invalidate the hypothesis of a single molecular clock. These results are robust to inaccuracies in paleontological estimates of divergence times. If these time estimates are accurate, however, this rate variation can be attributed mainly to fluctuations in the rate of evolution of nuclear DNA, rather than to a change in the rate of mitochondrial DNA evolution.

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Physiological Variation in α-Adrenoceptor-Mediated Arterial Sensitivity: Relation to Agonist Affinity

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Vascular smooth muscle from different arteries of the rabbit varies in sensitivity to norepinephrine, even when factors known to contribute to this variation are excluded. Sensitivity to norepinephrine mediated through the α-adrenoceptor is linearly related to the agonist dissociation constant, but is not significantly related to receptor reserve. These results suggest that agonist affinity is the primary determinant of sensitivity to norepinephrine, at least in these arteries, and that this is a locally regulated characteristic which may account for regional sensitivity changes.

ARTERIAL SMOOTH MUSCLE EXHIBits both species and regional variation in its reactivity to norepinephrine (NE) (*1*). Even when factors known to contribute to this variation (for example, more than one type of receptor and local systems for sequestering or metabolizing NE) are excluded, remarkable variation of unknown basis remains (*2*). Differences in agonist affinity and receptor number (density) have been proposed as possible causes of variation in tissue sensitivity to drugs, but experimental evidence is lacking. In the rat vas deferens (*3*) and rabbit ovarian artery (*4*), the ability of various α-adrenoceptor agonists to evoke contraction was found to be related to the dissociation constants or affinity of these substances for the α-adrenoceptor. However, it is not known whether the varied sensitivities of different arteries to the same agonist are related to the affinity of that agonist for its receptors in these vessels. This possibility can be tested in the rabbit since the sensitivity to NE in a number of its arteries varies by more than two orders of magnitude.

We now report that the variation in the sensitivity to NE of 12 arteries of the rabbit from differing vascular regions can be correlated with the dissociation constant of NE for the α-adrenoceptor in these arteries. In all of these vessels the maximum response to NE was equal to the capacity of the tissue to contract, and the contractions that were used to determine NE sensitivities and dissociation constants were due to the action of NE on α_1-adrenoceptors (see below).

Arterial ring segments (3 to 3.5 mm) were mounted in vitro in Krebs solution, at 37°C, equilibrated with 95% O_2 and 5% CO_2. Standard pharmacological procedures were used for measurement of isometric changes in wall tension (*5*). Segments were stretched to their optimum rest tension, which had been determined in preliminary experiments with each vessel type. Desmethylimipramine ($1 \times 10^{-7} M$), deoxycorticosterone acetate ($1 \times 10^{-5} M$), and propranolol ($1 \times 10^{-6} M$) were added to the bath solution to block neuronal and extraneuronal uptake of NE and to block β-adrenoceptors, respectively. These are recognized factors that influence the response of the blood vessel to NE (*6*). Norepinephrine was added cumulatively to generate data for a dose-response curve from which the agonist sensitivity ($pD_2 = -\log EC_{50}$, where EC_{50} is the median effective concentration) was obtained. After the maximum contraction in response to NE had been recorded, the addition of serotonin ($1 \times 10^{-3} M$) or histamine ($1 \times 10^{-3} M$) did not cause further contraction of any artery. EC_{50}'s were obtained from contractile responses between 20% and 80% of maximum by linear regression analysis of probit response–log concentration data. One determination was made from each artery. A minimum of five of each type of artery was studied, each one from a different rabbit. Some experiments with each type of artery were carried out after removal of the endothelium by rubbing. The effectiveness of this procedure was confirmed by an absence of dilation in response to acetylcholine ($1 \times 10^{-8} M$ to $1 \times 10^{-6} M$) and by microscopic examination after $AgNO_3$ processing and en face examination (*7*). The agonist dissociation constant (K_A) was determined according to the method of Furchgott and Bursztyn (*8*). After control responses were obtained, the tissues were treated with dibenamine ($3 \times 10^{-7} M$ to $1 \times 10^{-6} M$) for 15 minutes and washed for another 30 minutes before NE was added again. Equieffective concentrations of NE before (A) and after (A') dibenamine treatment were obtained. The slope and y-intercept of the regression line of $1/A$ against $1/A'$ were used to calculate K_A (slope − 1/intercept). The contractions in

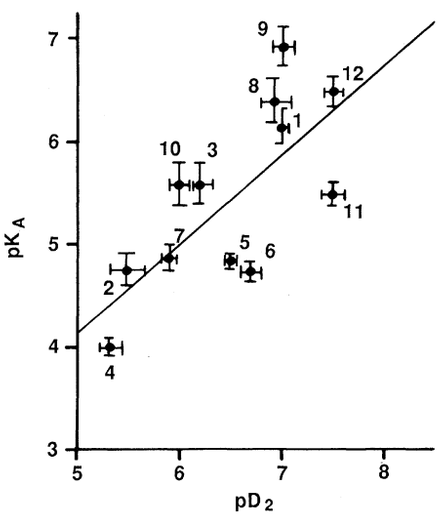

Fig. 1. Relation between the norepinephrine dissociation constant for the α-adrenoceptor (pK_A) and sensitivity (pD_2) of 12 arteries from the rabbit. The equation of the regression line is $y = 0.88x - 0.23$ and the SEM of the slope is 0.26 ($P < 0.01$). Brackets show the standard errors of means of at least five determinations, each one on an artery from a different rabbit: 1, abdominal aorta; 2, superior mesenteric artery; 3, renal artery; 4, ovarian artery; 5, common iliac artery; 6, external iliac artery; 7, internal iliac artery; 8, large pulmonary artery; 9, medium pulmonary artery; 10, basilar artery; 11, ear artery, and 12, thoracic aorta.

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