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10. Intact guard cells observed under fluorescence microscopy show small, red-fluorescing organelles that are probably chloroplasts. Onion guard cells are known to have small, poorly developed yet active chloroplasts [M. Shaw and G. A. Maclachlan, *Can. J. Bot.* **32**, 784 (1954)]. They are also distinct because they lack starch [O. V. S. Heath, *New Phytol.* **48**, 186 (1949)].
11. The light source was a 12-volt tungsten halogen lamp (Sylvania) in a lamp housing and filter holder [Reichert (Vienna, Austria) Zetopan] used with a constant voltage transformer (± 3 percent) (Sola), and a stepwise 12 volt transformer (Reichert). A heat filter (Reichert 2KG 2/g) and a 2-cm ice-cold water filter were used in all treatments. Filter combinations (all from Reichert, except when noted) and light intensity at the microchamber level (ergs per square centimeter per second) were: low-intensity blue, blue filter BG 12/h, 11,540; high-intensity blue, blue filter BG 12/h, 34,630; low-intensity red, neutral density filter NG3, red filter RG610, Wratten filter Kodak 301, 55,800; high-intensity red, red filter RG610, Wratten filter Kodak 301, 317,460; green, green filter VG 9/h and green interference filter λ = 546 mm, 19,240; white, neutral density filter NG 3, 50,500. Identical filter combinations gave different light intensities when the distance between the source and the chamber was changed. An infrared filter (Corning CS7-56), added to all filter combinations, gave infrared and heat readings which allowed us to obtain corrected values for visible light intensity. The temperature inside the microchambers, monitored with a thermocouple pair and a laboratory thermometer (Bayley Bat-4) was 22° to 24°C throughout the light exposure.
12. Protoplasts under high-intensity red light showed a 7 percent increase in volume beyond dark controls, as compared with a 38 percent increase under low-intensity blue light. The blue-red quantum ratio was 1 : 44.
13. Preparations that either include or are largely free of mesophyll-cell protoplasts can be obtained by adequate manipulation of digestion times and washing procedures (*9*).
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15. A blue-light photoreceptor in the guard cells has been postulated (*5*, *14*).
16. Epidermal peels and paradermal slices of young onion cotyledons (*9*) were mounted in the microchambers in 0.15 and 0.23*M* mannitol, respectively. After 2 to 3 hours in the dark, 50 m*M* KCl (epidermal peels) and 30 m*M* KCl (paradermal slices) were introduced; the pore size of 30 to 50 stomatal complexes per chamber were measured with a split-image eyepiece (Vickers) under green light. A 90-minute light treatment (*11*) was given and a second pore size determination made. The results (pore size in micrometers at the beginning and end of treatment ± standard error) were, epidermal peels: blue light 6.3 ± 1.0, 7.2 ± 1.2; blue light without K^+ 6.8 ± 0.9, 6.5 ± 1.0; red light 6.5 ± 1.0, 6.4 ± 1.1; same preparation to blue light 7.1 ± 1.1; dark 6.2 ± 0.6, 6.4 ± 0.8; same preparation to blue light 7.0 ± 0.9; paradermal slices: blue light 5.6 ± 0.7, 7.5 ± 1.1; red light 4.6 ± 0.9, 5.9 ± 1.0; red without K^+ 6.2 ± 1.1, 6.4 ± 1.0; dark 4.5 ± 0.6, 4.7 ± 0.9; same preparation to blue light 7.2 ± 1.1. Each experiment was repeated once, with similar results. The results with paradermal slices agree with previous work done with onion leaves [H. Meidner, *J. Exp. Bot.* **19**, 146 (1968)]. The results with epidermal peels are small in magnitude, yet reproducible. No further attempts to increase the response were made.
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21. The argument is based on the chemiosmotic theory [P. Mitchell, *Biol. Rev.* **41**, 445 (1966)]. The fact that the *p*H of guard cells increases when stomata open [K. Raschke and G. D. Humble, *Planta* **115**, 47 (1973); M. G. Penny and D. J. Bowling, *ibid.* **122**, 209 (1975)] is well established, although it has been commonly interpreted as a consequence of the exchange of K^+ for H^+ during K^+ uptake. The electrical potential inside guard cells is little understood. C. K. Pallaghy [*Planta* **80**, 147 (1968)] and M. G. Penny and D. J. Bowling [*ibid.* **119**, 17 (1974)] were unable to correlate stomatal opening with changes in membrane potentials. Recently I. I. Gunar, I. F. Zlotnikova and L. A. Panichkin [*Sov. Plant Physiol.* **22**, 704 (1975)] reported an increase in the negative electrical potentials inside the guard cells of *Tradescantia* within 5 minutes of their illumination.
22. We thank Dr. W. Briggs and S. Britz of the Carnegie Institution of Washington for many helpful discussions and the use of equipment. We also thank S. Westrate of Scientific Instrument Corporation for generous assistance with equipment. Supported by NSF grant BMS 74-15256 to P.K.H.

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Newly Evolved Repeated DNA Sequences in Primates

Abstract. *Repeated DNA sequences in primates having identical or nearly identical members and exhibiting unusual phylogenetic specificity were analyzed. They appeared in repeated DNA sequences in each group of primates, probably within the last 10 to 15 million years, and are conserved to the same extent as unique DNA sequences. The finding allows a new approach to the construction of evolutionary trees.*

The DNA genomes of mammals and other metazoans consist of nucleotide sequences present once per haploid genome (single-copy or unique sequences) and sets of sequences that are reduplicated many times, called families of repeated sequences (*1*). Most of the DNA sequences that are in the repeated class are repeated 1000 times or more; only a small fraction of repeated DNA consists of sequences repeated less than 1000 times.

It was proposed that repeated sequences arose by the amplification of sequences previously present one or only a few times per haploid genome (*2*) and that sequences amplified in the distant evolutionary past now consist of repeated families whose members are only distantly related to one another, while sequences amplified recently consist of repeated sets whose members are mutually closely related or identical (*1*, *2*). Since repeated sequences are at a higher concentration in DNA than single-copy sequences they reanneal more rapidly (that is, at low C_0t, where C_0t is the concentration of nucleotides in moles per liter and

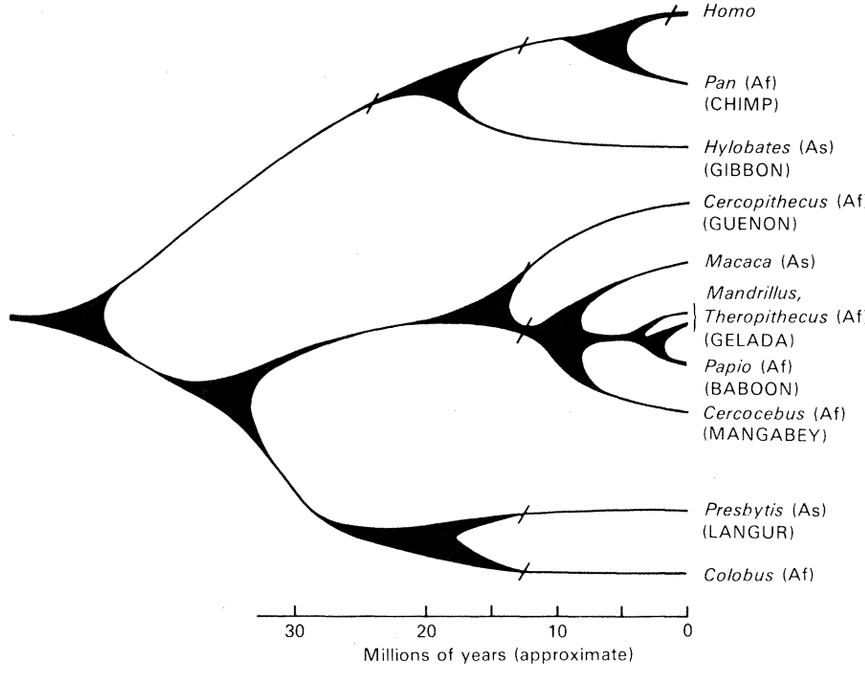

Fig. 1. Time of appearance of newly repeated DNA sequences in primates. The slash marks on the phylogenetic tree indicate the time of appearance of newly evolved repeated DNA sequences in primates, on the basis of the hybridization results of Table 1 and the unpublished experiments cited in the text.

t is the time in seconds) when denatured DNA is incubated under reannealing conditions (*1*). Moreover, since most families of repeated DNA sequences consist of members that are related but are different from one another in nucleotide sequence, cross-hybridization between related members of an ancestral repeated DNA family gives rise to reannealed complexes of relatively low thermal stability (*1*).

Members of a newly amplified family of repeated DNA sequences should reanneal to form DNA · DNA of high thermal stability (*3*). Furthermore, if the sequences were amplified after two animals diverged from a common ancestor, the newly evolved repeated family would exist in one of these species but would occur as single-copy sequences in the other and would not participate in low C_0t hybridizations (*3*).

Using this rationale, I purified putative newly evolved repeated DNA sequences from DNA of Old World primates. Native DNA was sheared, freed of RNA by incubation with NaOH, labeled to 10^8 cpm/μg with ^{125}I, denatured, reannealed to a C_0t of about 5, and DNA · DNA of high t_m (melting temperature) was purified from the reannealed mixture. Empirically, it was found that 70°C was the optimum reannealing temperature in 0.12*M* phosphate buffer for the isolation of fast-repeat high t_m sequences (FRHT), and that duplexes eluting from hydroxyapatite (HAP) between 80° and 100°C were most enriched for phylogenetically specific sequences.

Sequences of ^{125}I-labeled DNA that had been purified in this fashion were hybridized to an excess of unfractionated, unlabeled DNA from several primates. The hybridization assay was carried out at 80°C to a C_0t of 4 to select for interactions between nearly perfectly complementary DNA sequences. The results of such tests are presented in Table 1, and several features of the system are apparent.

1) If a given preparation of FRHT ^{125}I-labeled DNA is used and unfractionated DNA from Old World primates is hybridized to it, only two hybridization responses are evident: the percentage of the ^{125}I-labeled DNA that can be hybridized to unfractionated DNA from the same monkey and seen with some related monkeys or a lower "baseline" hybridization obtained with unfractionated DNA from other Old World primates. The results take an "all-or-none" form expected of newly evolved repeated DNA sequences as opposed to a phylogenetic gradation expected of ancestral DNA sequences.

2) Grouping animals that share common FRHT sequences yields a solution that is consistent with phylogenetic analysis. Thus, the baboons, macaques, and mangabeys share common FRHT sequences; two guenons share different FRHT sequences; the one species of colobus examined has distinct FRHT sequences; and the FRHT sequences of the monkeys mentioned above are not found in langurs or apes.

3) The FRHT fraction consists of at

Table 1. Hybridization of ^{125}I-labeled FRHT DNA sequences to total DNA from primates. Five micrograms of ^{125}I-labeled DNA in 1 ml of 0.12*M* phosphate buffer, *p*H 6.8, was denatured and reannealed at 70°C for 18 hours. Reannealed DNA was attached to HAP in 0.14*M* phosphate buffer, *p*H 6.8, imperfect duplexes were eluted by washing the DNA-HAP at 80°C, and the FRHT fraction was collected by elution at 100°C. Occasionally (Table 1, columns 6 and 7), FRHT was eluted from HAP at 80°C in 0.48*M* phosphate buffer, reattached to HAP in 0.14*M* phosphate buffer, then reeluted between 80° and 100°C. For hybridization, FRHT DNA (approximately 5000 count/min) was mixed with 25 μg of unlabeled, unfractionated DNA in 100 μl of 0.12*M* phosphate buffer. Nucleic acids were denatured at 100°C, then reannealed for 15 minutes at 80°C. Unreacted DNA was removed by washing the HAP at 80°C three times with 20 volumes of 0.14*M* phosphate buffer. Hybridized DNA was eluted at 100°C. Values are presented as the percentage of the ^{125}I hybridized; values obtained in experiments done without the unlabeled DNA (2 to 4 percent of the ^{125}I) were subtracted.

Source of unlabeled DNA		Source of ^{125}I-labeled FRHT						
Latin name	Common name	(1) *P. cynocephalus*	(2) *C. atys.*	(3) *M. sylvana*	(4) *M. mulatta*	(5) *C. neglectus*	(6) *C. aethiops*	(7) *C. badius*
Papio cynocephalus	Yellow baboon	20	14	21	23	4	1	7
Papio anubis	Dogface baboon	20	12	20			0	
Papio hamadryas	Royal baboon	21	12	23			1	
Papio papio	Western baboon	18	14	20			1	
Theropithecus gelada	Gelada baboon	19	13	23	24	3	1	
Mandrillus Sphinx	Mandrill baboon	19	13	20	23	2	1	
Cercocebus torquatus	Red crowned mangabey	18	12	18			−1	
Cercocebus atys.	Sooty mangabey	19	13	17	25	3	2	6
Macaca sylvana	Barbary ape	19	13	20			2	
Macaca mulatta	Rhesus monkey	19	13	18	24	3	0	8
Macaca cyclopis	Rock macaque	22	12	18			1	
Macaca iris	Crab-eating macaque	19	12	22				
Macaca speciosa	Stump-tail macaque	21	15	18	22	1		
Macaca fascicularis	Sumatran macaque	20	12	21			−1	
Cynopithecus niger	Celebes ape	18	15	21				
Cercopithecus neglectus	DeBrazza guenon	6	2	3	5	27	38	7
Cercopithecus aethiops	Grivet; vervet	6	4	3	5	27	41	3
Colobus badius	Red colobus	6	3	6	5	6	2	38
Presbytis obscurus	Spectacled langur	5	3	3	4	6	1	5
Hylobates lar	White-handed gibbon	4	3	3	2	3	0	−1
Hylobates hoolock	Hoolock gibbon		3	3	1		−2	
Pan troglodytes	Chimpanzee	3	0	3	3		0	
Homo sapiens	Human	3	3	3	0	3	0	
Ateles spp.	Spider monkey		−1	0			0	
Alouatta spp.	Howler monkey		−1	0			0	
Saimiri spp.	Squirrel monkey		1	2			0	
Cebus spp.	Capuchin		1	−2			0	
Logothrix spp.	Woolly monkey		0	0			−3	
	Lemur	−2	−2					
	Slow loris	−2	−2					

Table 2. Hybridization of recycled ^{125}I-labeled FRHT sequences from human DNA to DNA from apes. The FRHT sequences were prepared from DNA of normal human blood leukocytes (Table 1, legend). The percentage of this fraction that hybridized to DNA for primates in the standard assay (Table 1, legend) was as follows: human DNA was 45 percent, chimpanzee DNA was 40 percent, gibbon DNA was 38 percent, baboon DNA was 32 percent. To remove conserved sequences, ^{125}I-labeled FRHT (12×10^6 count/min) fraction was hybridized to 50 μg of unfractionated, unlabeled DNA from *P. cynocephalus* at 80°C to a C_0t of 20, then *unhybridized* DNA was collected by HAP chromatography (85 percent of the input). The unhybridized DNA was then annealed to unfractionated DNA from selected primates (Table 1). The experiment shown represents the best result obtained of experiments attempted with three different preparations of ^{125}I-labeled human DNA. The patterns of the results in the remaining two experiments were similar, although the recycled DNA hybridized less well to human DNA (12 and 7 percent). Of the primates tested, humans have been the most difficult source of newly evolved DNA sequences. The reason for this is not known.

Source of DNA	^{125}I hybridized (%)	Δt_m (°C)
Human	20.8	0
Chimpanzee	10.9	0
Gibbon	7.8	−2
Baboon	3.8	
No DNA	2.1	

least three sequence components. One component is the putative newly evolved repeated DNA sequences, identified by its specific hybridization behavior at 80°C and low C_0t. A second component is conserved among the Old World primates (excepting prosimians). This component can be removed by preparative hybridization (Table 2) or minimized by purifying FRHT sequences by two cycles of HAP chromatography (Table 1, legend). A third component appears to consist of contaminating ancestral repeated DNA sequences. This component is inactive in the hybridization assay at 80°C but does react ($C_0t_{\frac{1}{2}} = 0.1$) at 60°C and shows a phylogenetic gradation expected of ancestral sequences (not shown).

The newly evolved repeated DNA sequences of the baboons, macaques, and mangabeys are totally shared; none of these have repeated sequences lacking in the remaining two genera. Thus, when baboon ^{125}I-labeled FRHT sequences are annealed to DNA from macaques and the common sequences are removed, no sequences specific to the baboons remain (not shown). By the same test, no genera-specific sequences were detected in macaques or mangabeys.

It is conceivable that the newly evolved repeated DNA sequences of the different Old World primates were generated at a particular evolutionary time (Fig. 1). The time of appearance of the sequences of the baboon-macaque-mangabey group can be pinpointed rather accurately to the time before these animals diverged from a common ancestor but after that ancestor split from the guenons. The time of appearance of the newly evolved repeated DNA sequences of other Old World monkeys cannot yet be pinpointed as closely. Newly evolved repeated sequences in humans may have been generated more than once, since there appears to be one set not shared with chimpanzees, a second set shared with chimpanzees but not found in gibbons, and a third set common to the apes but missing in the repeated DNA sequences of other Old World monkeys (Table 2).

The newly evolved repeated DNA sequences of baboons, macaques, and mangabeys evolve at about the same rate as unique sequence DNA and much more slowly than satellite DNA sequences, as judged by the t_m of intergeneric hybrids (Table 3). The t_m of the intergeneric hybrids is 1° to 2°C lower than that of the homologous system, indicating a nucleotide sequence difference of about 1 to 3 percent from one genus to the other (*4, 5*). The newly evolved repeated DNA sequences in these animals must have originated 10 to 15 million years ago; hence the rate of nucleotide change is about 0.2 percent per 10^6 years, in excellent agreement with measurements on rate of change of nonrepeated DNA sequences (*5*).

Table 3. Thermal stability of hybrids involving ^{125}I-labeled FRHT sequences. Hybrids were formed as described in the legend to Table 1. Unreacted DNA was washed away at 80°C with 0.14*M* phosphate buffer. The DNA-HAP was successively washed with buffer that was 2°C warmer than the preceding buffer and the fraction of duplex DNA removed at each washing was determined. The t_m of homologous hybridizations was 86.5°C (tested with all of the FRHT preparations reported in Tables 1 and 3), and the t_m values were reliable within 0.5°C. Results were expressed as the t_m difference in degrees Celsius (Δt_m) between the intergeneric or interspecific hybridization and the homologous hybridization. Numbers in parentheses indicate the number of species tested from each genus. The range of Δt_m values reflects the spread obtained with DNA from the different species. All t_m's were done at least in duplicate.

Source of unlabeled DNA	Δt_m(± 0.3°)C with ^{125}I-labeled FRHT from:	
	Papio cynocephalus	*Macaca mulatta*
Papio (4)	0 to −0.3	−1.0 to −1.5
Theropithecus (1)	−1.0	−1.0
Mandrillus (1)	−1.1	−1.5
Macaca (6)	−1.0 to −1.6	0 to −0.5
Cercocebus (2)	−1.9 to −2.3	−0.9 to −1.2

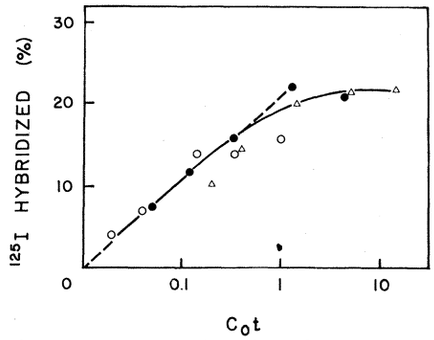

Fig. 2. Rate of hybridization of ^{125}I-labeled FRHT to an excess of unfractionated DNA. Hybridization mixtures were constructed as described (Table 1), except that 0, 0.5, 1.0, 3.0, 10.0, or 37.5 μg of unlabeled DNA per 100 μl of hybridization mixture was used. Mixtures with these amounts of DNA, made in replicate, were hybridized for 5, 15, or 60 minutes at 80°C in 0.12*M* phosphate buffer, *p*H 6.8. Values are expressed as the percent of the ^{125}I hybridized compared to the product of DNA concentration × time (C_0t) (*1*), corrected to 60°C conditions. ○, 5 minutes hybridization; ●, 15 minutes hybridization; △, 60 minutes hybridization. Values obtained with no unlabeled DNA were subtracted (2 percent).

About 30,000 copies of FRHT sequences exist in DNA from baboons as judged by the $C_0t_{\frac{1}{2}}$ of the annealing of ^{125}I-labeled FRHT baboon DNA sequences to an excess of unfractionated baboon DNA. The $C_0t_{\frac{1}{2}}$ is about 0.1, corrected to 60°C (Fig. 2). Since about 1 percent of the DNA consists of newly evolved repeated DNA sequences by the hybridization test of Table 1, there would be per haploid genome about one such family with members 1000 nucleotides long, about ten different newly evolved, repeated sequence families with members 100 nucleotides long, and so on.

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