The vomeronasal organ (VNO), a sensory organ that detects nonvolatile pheromones, is found in most terrestrial vertebrates but may be absent in humans (1, 2). In most species, the paired cigar shaped organ is present at the rostral end of the nasal cavity and sensory neurons within the VNO send projections to the accessory olfactory bulb. In humans a small pit is retained in the nasal septum, but cells within the presumptive VNO do not express many of the markers of mature sensory neurons (1, 2). The accessory olfactory bulb is not evident in humans or in any other Old World (OW) monkey or ape (3). Nonetheless, it has been claimed that in humans some pheromones are transduced by the VNO (4). Indeed, it has been difficult to determine unequivocally whether the VNO is indeed functional or nonfunctional in humans and higher primates (1).

Candidate signaling components for VNO sensory transduction that are specifically expressed in the VNO have been recently identified, including several large families of receptors (V1R/V3Rs and V2Rs) (5–7) and an ion channel TRPC2 (8). TRPC2 is localized to VNO sensory microvilli, suggesting a role in sensory transduction (8). Knockout studies show that TRPC2 is essential for the functional or nonfunctional in humans and higher primates (1).

Sequence of Human TRPC2 (hTRPC2).

Coding sequence for hTRPC2 was identified on 11p15.5 (GenBank accession no. AC060812) by comparison with mouse TRPC2 (mTRPC2) or homology to the partial cDNA for hTRPC2 (12), and the position of intron/exon boundaries was assigned based on similarity to consensus motifs. DNA sequences were assembled by using VECTOR NTI suite 6 (InforMax, Bethesda). A reconstructed full-length hTRPC2 cDNA consisting of 2,667 nt was obtained by conceptually splicing exons 1, 5, and 8 identified from the genomic sequence, and the partial cDNA sequence of hTRPC2, which contains the remaining exons.

PCR Amplification and Sequencing of Primate Genomic DNA.

Genomic DNA from the following primate species was obtained from Therion (Troy, NY): squirrel monkey (Saimiri sciureus), common marmoset (Callithrix jaccus), owl monkey (Aotus azarae), and black and white ruffed lemur (Varecia variegata). Genomic DNA from the following species was obtained from O. Ryder (San Diego Zoo, San Diego): sumatran orangutan (Pongo pygmaeus), bonobo (Pan paniscus), common chimpanzee (Pan troglodytes), western lowland gorilla (Gorilla gorilla), gibbon (Hylobates syndactylus), drill (Mandrillus leucophaeus), titi monkey (Alouatta seniculus), and ring-tailed lemur (Lemur catta). Genomic DNA from rhesus macaque (Macaca mulatta) and human was from CLONTECH. Note that by using chimp or bonobo DNA as template we were able to obtain amplification in only two of the five reactions; therefore, we do not include these data in our analyses.

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PCR primers and conditions for this and other experiments in which primate DNA was amplified were as follows. PCR primer pairs: (i) AGCCCTAAGCCTGAGATCTGGAAC vs. GCTGCACCTGAGAGCGGCGACAGG; (ii) GTACAACCTCCA-TCTGCCAGCAAG vs. CCAGTGACCAAGGCGAGGAGGAGGG; (iii) either GCTGGGGCTGCCACATGCATGCGC vs. GGCCAGGGCGGTAGAAGCTGAGCATGC (most primates) or GCCACTGGCCGCTCCCTCCTCGTGGGCTT vs. GAAAGAGACCTTCAGCCCAAGAACTTTGGG (human, macaque, langur); (iv) GGATGATGTCGATCGTTGAGTGA vs. GAAAGAGATGGATAGGGATGTCGGAATGTC; and (v) GACCATACCTGACTGCAAGGAGG vs. GCCAGACTCTCG-CCGAAAGCCAGAGTCC. Conditions were as follows: 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, 200 mM NTPs, 4 mM each primer, 0.05 units/μl Taq polymerase (Fisher), and 4 ng/μl template. PCR products were purified (QiAquick, Qiagen, Valencia, CA) and directly sequenced off of both strands by using the original PCR primers (Laragen, Los Angeles). Note that direct sequencing of the PCR products is less prone to artifacts than sequencing subcloned PCR products. Only sequence that was confirmed on both strands was used in the

Methods

The paper was submitted directly (Track II) to the PNAS office.

Abbreviations: VNO, vomeronasal organ; OW, Old World; NW, New World; hTRPC2, human TRPC2.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos.AY227676–AY227690, AY231370–AY231395, AY231613–AY231626).

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For 14 species, we were able to obtain 745–770 bp of sequence. Because of failure of one or more PCRs, common marmoset, squirrel monkey, and orangutan gave 605, 645, and 597 bp of sequence, respectively.

**Analysis of Sequence Data.** Sequences were aligned and the ratio of nonsynonymous substitutions per nonsynonymous site vs. synonymous substitutions per synonymous sites, $K_{\text{a}}/K_{\text{s}}$, was determined by ODEN (19). The numbers of nonsynonymous and
synonymous nucleotide differences ($d_s$ and $d_i$) and the numbers of nonsynonymous and synonymous sites ($l_s$ and $l_i$) were obtained for all possible pairs of sequences. $K_s$ and $K_a$ were estimated from $d_s$, $d_a$, $l_s$, and $l_i$, with Jukes and Cantor's correction (20). To test the significance of the deviation of $K_s/K_a$ from one, a two-tailed Fisher's exact test was conducted with the null hypothesis $d_s/d_a = l_s/l_i$. To examine the compatibility of two values of $K_s/K_a$, we conducted $2 \times 2$ Fisher's exact test with the null hypothesis that $d_s/d_a$ is constant. To test the heterogeneity in the rate of molecular evolution (measured by $d = d_s + d_a$), a relative rate test was carried out (21).

Results

Identification of the Full-Length hTRPC2 Gene. Based on sequence obtained from a partial cDNA clone, it was previously noted that hTRPC2 was a pseudogene (12). To obtain the full-length coding sequence of hTRPC2, we queried the human genome database with mouse and rat TRPC2 cDNA sequences. Only one region of the genome (11p15.5) showed a high degree of homology to rodent TRPC2 cDNAs (85% nucleotide identity). The coding region of hTRPC2 is contained on 13 exons (Fig. 1A). Within the deduced cDNA for hTRPC2, we have identified six deleterious mutations that generate stop codons (Fig. 1B); two of these are indels (insertion/deletions) and four are missense mutations.

Deleterious Mutations in the TRPC2 Gene of Primates. To ascertain when in primate evolution TRPC2 became a pseudogene, we determined when each of the deleterious mutations found in the human sequence occurred. Genomic DNA from 15 extant primate species was used in PCRs to obtain sequence of the TRPC2 gene. In the sequences of nonhuman primates, we identified three additional frameshift or non-sense mutations that were not found in the human sequence. Of the nine mutations, the earliest two mutations arose in the common ancestor of OW monkeys and apes (Fig. 2). These non-sense mutations both lead to a truncation of the cytoplasmic C terminus, a region that is likely to be important but may not be essential for the function of the protein (22). Occurring more recently were several mutations that would clearly generate a nonfunctional protein. (i) In the common ancestor of gorillas and humans, two frameshift mutations arose at the N terminus of the protein and a non-sense mutation arose after the sixth transmembrane domain. (ii) In the common ancestor of extant OW monkeys, there was a 1-bp insertion that is predicted to generate a stop codon between the third and fourth transmembrane domains (mutation 7 in Fig. 2). We did not detect any frameshift or non-sense mutations in sequences of TRPC2 from prosimians or New World (NW) monkeys. Thus, it appears that deleterious mutations in the TRPC2 gene began to accumulate in the common ancestor of OW monkeys and apes.

Selective Pressure on the TRPC2 Gene During Primate Phylogeny. To confirm the preceding analysis, and to gain more insight into the selective pressure on the TRPC2 gene across primate phylogeny, we measured rates of synonymous and nonsynonymous substitutions among TRPC2 sequences from a range of primate species. Synonymous substitutions are nucleotide substitutions that do not alter the amino acid sequence. In the absence of selective pressure and under neutral evolution, synonymous substitutions per synonymous site (denoted $K_s$) occur at a rate equal to nonsynonymous substitutions per nonsynonymous site (denoted $K_a$); i.e., $K_s/K_a = 1$. Any significant deviation from this prediction can be interpreted as indicating that the gene is under selective pressure and thus is likely to be functional. In the case of receptor proteins that interact with ligands in the environment, selection may favor adaptive changes in amino acid sequences, leading to an elevated value of $K_s/K_a$ over part of the amino acid sequence. This is the case for mammalian odorant and vomeronasal receptors (23, 24). On the other hand, in the case of molecules that interact with intracellular messengers, such as TRPC2 and related ion channels, it might be expected that selection would be purifying, corresponding to a low value of $K_s/K_a$.

Because of the presence of a large number of introns, we generated sequence for analysis (745–770 bp for most species) by concatenating coding sequence from four exons. We eliminated the possibility that this analysis introduced a bias in two ways: (i) to eliminate ascertainment bias, before the analysis we removed codons for which the human had a non-sense mutation and we corrected frameshift mutations; and (ii) we confirmed that for the mouse and rat genes, comparison of the partial sequence gave a similar result, as a comparison of the full sequence (in both cases $K_s/K_a = 0.08$).

$K_s/K_a$ values were then obtained for all pairwise comparisons of TRPC2 sequence data from 15 primate species and two rodent species (Fig. 3). In addition, we reconstructed the most likely ancestral sequence at each node of the phylogenetic tree by using maximum parsimony phylogeny analysis (PHYLP; ref. 25) and used these sequences to calculate $K_s/K_a$ on each branch of the tree (Fig. 3; ref. 26). Parsimony analysis yielded three trees that differed only in branches leading from the common ancestor of NW monkeys to extant NW monkeys, and there was no significant difference in the evaluation of $K_s/K_a$, by using these trees.

Fig. 2. Inferred time of occurrence of deleterious mutations in TRPC2 during primate phylogeny. (A) Mutations are numbered 1–9. Mutations 1–6 are found in human at the positions indicated in Fig. 1. Mutation 6 was found in all OW monkeys and hominoids but not in NW monkeys or prosimians; therefore, we infer that it occurred in the common ancestor of OW monkeys and apes. Mutation 9 is a missense mutation that was found in all OW monkeys and in gibbon but not in other hominoids, suggesting that either that a reversion event occurred more recently in evolution (indicated by a black circle with a white 9 on the tree) or that the mutation arose independently more than once. Two frameshift mutations (7 and 8) were found only in OW monkeys. Mutation 7 is a 1-bp insertion at a position equivalent to mouse D483 and mutation 8 is a 13-bp deletion spanning the same position. The deletion event therefore restores the reading frame. (B) A schematic representation of the TRPC2 ion channel indicating the position of each mutation. Black bars represent the transmembrane domains.
The $K_a/K_s$ value when comparing the two rodent sequences was significantly different from the neutral expectation ($K_a/K_s = 0.08 \pm 0.0001$, Fisher’s exact test) and was somewhat lower than the average value of 0.14 obtained when a large number of orthologous mouse and rat genes were examined (27). Thus, the TRPC2 gene exhibits a high degree of purifying selection in the rodent lineage, consistent with its known functional significance (9, 10).

The prosimians are considered the most primitive primates and are thought to have retained many of the characteristics of the common ancestor of modern primates. We find a high degree of purifying selection on the TRPC2 gene ($K_a/K_s = 0.10 \pm 0.0001$) in comparing sequence from two strepsirrhine prosimians (black and white ruffed lemur and ring-tailed lemur; Fig. 3). In addition, we find a high degree of purifying selection on the branch of the phylogenetic tree leading to the common ancestor of these two species ($K_a/K_s = 0.07 \pm 0.0001$). These data imply that the TRPC2 gene has been retained in prosimians.

NW monkeys comprise a diverse group of species, most of which have retained a VNO and an anatomically distinct accessory olfactory bulb (3). In pairwise comparisons of sequences of TRPC2 from NW monkeys, only one comparison yielded a $K_a/K_s$ value that was significantly different from unity (owl monkey vs. titi: $K_a/K_s = 0.33 \pm 0.0001, P < 0.02$). We found a significant relaxation of purifying selection on the TRPC2 gene in NW monkeys as evidenced by the significantly higher $K_a/K_s$ values obtained from pairwise comparisons of NW monkey sequences (range, 0.33–0.94) as compared to the $K_a/K_s$ value obtained in a comparison of rat and mouse TRPC2 [$P < 0.03$ for all (15/15) comparisons; $2 \times 2$ Fisher’s exact test]. An examination of $K_a/K_s$ values along the branches leading to the NW lineage shows a high degree of purifying selection up to a common ancestor of all NW monkeys (Fig. 3). A relaxation of selective pressure occurred more recently in evolution, suggesting that in some species of the NW monkeys the VNO may be vestigial or redundant. The recent relaxation of selective pressure may not have allowed sufficient time for deleterious mutations to accumulate in the TRPC2 gene.

Pairwise comparisons of sequence data among OW monkeys
and apes shows no evidence for selective pressure on the TRPC2 gene (mean $K_s/K_a = 0.86$, range = 0.55–1.97; $P > 0.05$ for 20/21 comparisons). In the lineage leading to humans, we find that selective pressure on the TRPC2 gene was maintained in the anthropoid ancestor (the common ancestor of NW monkeys, OW monkeys, and apes) and was thereafter relaxed in a common ancestor of OW monkeys and apes ($\sim$25–40 million years ago; Fig. 3). Following this time point, $K_s/K_a$ values along branches leading to OW primates and apes are not significantly different from unity. Note that in one branch (leading from the common ancestor of apes to the gibbon), the $K_s/K_a$ differs from unity at $P < 0.05$. However, the Bonferroni correction, which adjusts significance criteria in cases in which multiple significance tests are performed, requires a value of $P < 0.002$ (assuming 22 comparisons). The finding that selective pressure was relaxed on the TRPC2 gene in the common ancestor of OW monkeys and apes is consistent with our preceding analysis, which showed that at this same point in evolution deleterious mutations began to accumulate in the TRPC2 gene.

Genes that are under relaxed selective pressure will accumulate base substitutions at a higher rate than genes that are under purifying selection (28). To determine whether the rates of nucleotide substitutions within the TRPC2 gene varied among branches of the phylogenetic tree, we performed the relative rates test (21) on all pairwise comparisons of primates species, by using both rat and mouse as outgroups. The relative rates test confirms that mutations have accumulated in the OW monkey and ape lineage at a rate significantly higher than in the prosimians, consistent with the contention that in the OW monkey and ape lineage the TRPC2 gene is not under selective pressure (23/24 comparisons were significant at $P < 0.05$; one tailed relative rates test; 24 comparisons were from two prosimians compared to six OW monkeys and apes with two outgroups). Note that the test is very conservative, because the generation time for prosimians is shorter than for OW monkeys and apes, and therefore it would be expected that neutral substitutions would occur at a higher rate in prosimians.

**Discussion**

Our data show that among present-day primates there has been strong selective pressure on the TRPC2 gene only in a strepsirhine prosimian, the lemur. These data are consistent with literature indicating that lemurs have retained a functioning VNO (29). Surprisingly, we find that selective pressure on the TRPC2 gene, although high in a common ancestor of NW monkeys, is relaxed in several species of extant NW monkeys. It is interesting to note that, although NW monkeys show prominent scent marking behavior, it has not been demonstrated that this form of chemical communication acts through the VNO or that it serves a unique function. Notably, in the marmoset, in which ovulation is suppressed among subordinate females in a social group, visual as well as chemical cues appear to play a role in suppressing ovulation (30). Interestingly, a recent study of V1R pheromone receptor genes in the marmoset has revealed the presence of only pseudogenes (31). This may be because of either extreme sequence divergence of functional V1R sequences (24), as the authors suggest, or a lack of functional significance of the VNO in this species. Our data suggest that, in NW monkeys, signaling through the VNO may, if present, be redundant, leading to a relaxation of selection on all signaling components within the VNO.

Within the lineage leading to humans, our evidence suggests that selective pressure on the TRPC2 gene was relaxed in a common ancestor of OW monkeys and apes. In present-day OW monkeys and apes, we find no evidence for selective pressure on TRPC2. These data correspond with evidence indicating that OW monkeys and apes have not retained a distinct accessory olfactory bulb and that many species of OW monkeys and apes lack a VNO (3). They are also consistent with the observed paucity of intact V1R receptor genes in the human genome; in a comprehensive study, only five V1R receptors with intact ORFs were identified, as compared with ~200 V1R pseudogenes (18). We presume that, at a certain point in human evolution, ancestral species may have relied more on visual and auditory signals, rather than on chemical signals, for communicating social and reproductive status. Remarkably, it is at the time in evolution when selective pressure on TRPC2 is relaxed that the common ancestor of OW monkeys and apes developed trichromatic color vision through a gene duplication of the green/red opsin gene (32, 33). Indeed, many species of OW monkeys signal sexual and social status via colorful skin pigmentation of the face or genitalia. Thus, it is interesting to speculate that, in the evolution of OW monkeys and apes, an enhanced reliance on vision may have led to a reduced reliance on chemical signaling in mediating social interactions.

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