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Genetics

Organization and expression of canine olfactory receptor genes

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ABSTRACT **Four members of the canine olfactory receptor gene family were characterized. The predicted proteins shared 40–64% identity with previously identified olfactory receptors. The four subfamilies identified in Southern hybridization experiments had as few as 2 and as many as 20 members. All four genes were expressed exclusively in olfactory epithelium. Expression of multiple members of the larger subfamilies was detected, suggesting that most if not all of the cross-hybridizing bands in genomic Southern blots represented actively transcribed olfactory receptor genes. Analysis of large DNA fragments using Southern blots of pulsed-field gels indicated that subfamily members were clustered together, and that two of the subfamilies were closely linked in the dog genome. Analysis of the four olfactory receptor gene subfamilies in 26 breeds of dog provided evidence that the number of genes per subfamily was stable in spite of differential selection on the basis of olfactory acuity in scent hounds, sight hounds, and toy breeds.**

One of the first steps in an animal's detection of a volatile compound in its environment is the binding of the odorant to G protein-coupled receptors expressed on the surface of olfactory neurons. The pattern of receptors to which the odorant binds, and thus the pattern of neurons that transmit an action potential to the olfactory bulb in the brain, allow the animal to identify the compound and respond to its presence. The gene family that encodes these odorant receptors was recently identified (1). The family is thought to be quite large, numbering in the hundreds of genes in most mammals (1–3). Representatives of the olfactory receptor gene family have been cloned from rat, human, mouse, catfish, and dog (1–9). Most appear to be expressed in the olfactory neuroepithelium, although expression of individual receptors in the testis (2) and tongue (10) have been reported.

Olfactory receptors have seven transmembrane domains and were initially cloned on the basis of their similarity to other membrane-bound G protein-coupled receptors (1). All olfactory receptors identified to date share some characteristic sequence motifs and have a central variable region that corresponds to a putative ligand binding site (1, 11). This variability in the binding site is expected for a set of receptors that must discriminate among thousands of different odorants. The genes that encode the family of olfactory receptors are relatively small (≈1 kb in size) and lack introns within the coding region. In human and mouse the genes are often found in linked arrays, with closely related genes found close to one another (3, 4). This arrangement is expected for a gene family whose numbers have increased through the process of unequal exchange (12).

The study of olfactory receptor genes from dogs is of special interest for two reasons. First, dogs have extremely sensitive noses. Humans use dogs for hunting, tracking, drug detection, and bomb sniffing because of their olfactory acuity and trainability. Second, there is great variation in the size of the olfactory epithelium in different breeds of dog, and different levels of reliance by the breeds on olfactory cues (13, 14). Thus studying olfactory receptor genes from dogs and comparing the genes in different breeds of dog could provide insight into the evolution of this gene family in response to natural and artificial selection for enhanced olfactory ability. In this study we identified genes encoding four subfamilies of olfactory receptors. We characterized their expression pattern and organization in the genome, and tested whether unequal crossing-over during the selective breeding of dogs has contributed to the present day differences in the olfactory behavior of different breeds.

MATERIALS AND METHODS

Library Construction and Screening. High-molecular-weight genomic DNA was isolated from canine spleen (15). The DNA was briefly digested with *Sau*3A and size-selected (35–50 kb) on a sucrose gradient before cloning into the SuperCos1 cosmid vector (Stratagene). The cosmid clones were packaged with Gigapack II (Stratagene) and introduced into NM554 bacteria, producing a library of 300,000 recombinants.

To create an olfactory receptor-specific probe, degenerate oligonucleotides corresponding to protein sequence PMY(L/F)FL (primer NL61) and TC(A/G)SHL (primer NL63) were used to amplify a collection of olfactory receptor gene fragments from dog genomic DNA using the polymerase chain reaction (PCR). Primer sequences were obtained from Randall Reed (Johns Hopkins University). The primers contained *Eco*RI and *Hin*dIII restriction sites (shown in boldface type) to aid in cloning, and had the following sequences, 5′ to 3′: NL61, **CGGAATTCCC**(GATC)ATGTA(CT)(CT)T(GATC)TT(CT)CT); and NL63, **ATAAGCTTAG**(GA)TG(GATC)(GC)(TA)(GATC)(GC)C(GA)CA(GATC)GT. The resulting gene fragments were digested with *Eco*RI and *Hin*dIII, subcloned into pBluescript KS+ (Stratagene), and sequenced using a Sequenase 2.0 kit (United States Biochemical). Ten of 16 clones analyzed were sufficiently similar to olfactory receptor genes from other species to be considered presumptive canine olfactory receptor genes. These 10 clones were pooled and ^{32}P-labeled by random priming with the Multiprime DNA Labeling System (Amersham). Both the cosmid library and an EMBL3 phage library of dog genomic DNA (a gift from John Gerlach, Michigan State University) were screened with this complex probe at medium stringency (55°C) and washed in 0.2× standard saline citrate (SSC) and 0.1% SDS by using standard procedures (15).

Sequencing from the Genomic Clones. Restriction fragments of the cosmids and phage containing the genes of interest were identified by Southern blot analysis using the 10-clone pool as a probe, and the fragments were subcloned into the vector pMOB (16). Sequences of the candidate olfactory receptor genes were

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Abbreviation: CHEF, contour-clamped homogeneous field electrophoresis.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U53679–U53682).

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obtained by γδ transposon-facilitated DNA sequencing (16) using an automated laser fluorescence (ALF) Sequencer (Pharmacia). Four different genes were recovered and designated CfOLF1-4 for *Canis familiaris olf*actory receptor 1–4.

Southern Blot Hybridizations. Dog genomic DNA was digested with restriction enzymes and electrophoretically separated on 0.8% agarose gels. The DNA was transferred to GeneScreen nylon membranes (DuPont) and hybridized in 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS, and 1 mM EDTA at 60°C (17). Washes were done at 60°C in 40 mM $NaHPO_4$ (pH 7.2), 1% SDS, and 1 mM EDTA. Probes specific to each of the four genes were generated by PCR using cosmid or phage templates. The CfOLF1 probe covered nucleotides 11–920 of its open reading frame and was amplified with primers 1-L (5′-AACTACACCTTGGTGACCGAG-3′) and 1-R (5′-TTAACCTTACAGCTCTCTTAGC-3′). The CfOLF2 probe covered nucleotides 27–866 of its open reading frame and was amplified with primers 2-L (5′-GAATGAATTCCTTCTCGTGG-3′) and 2-R (5′-ATCAGAGGGTTTAGCATGG-3′). The CfOLF3 probe covered nucleotides 9–921 of its open reading frame and was amplified with primers 3-L (5′-AGGTAACCAGACTTGGGT-3′) and 3-R (5′-TTGCCCTAATAGTTTCTG-3′). The CfOLF4 probe covered nucleotides 2–870 of its open reading frame and was amplified with primers 4-L (5′-TGGAACTAGAGAATGATACACG-3′) and 4-R (5′-TCCTGAGGCTGTAGATGAAG -3′). Probes were labeled as described above.

Contour-Clamped Homogeneous Field Electrophoresis (CHEF) Gels. Agarose blocks containing 5 μg of canine genomic DNA from MDCK cells were incubated with restriction enzymes *Not*I, *Pac*I, *Pme*I, and *Sfi*I (18). Gels were cast with 1% LE agarose (FMC) and placed in 0.5× TBE in a CHEF-DR3 Apparatus (Bio-Rad). The DNA was electrophoretically separated at 6 V/cm. Run times varied from 22 to 28 h, and the gels resolved fragments between 20 and 500 kb. Lambda concatamers were used as size markers. Southern transfer and hybridizations were performed as described above.

RNA Extraction and RNase Protection Experiment. Poly(A^+) RNA was extracted from ≈1 g of quick-frozen fresh tissue using the Fast Track 2.0 system (Invitrogen). To ensure that it was intact, the RNA was inspected after electrophoresis on an agarose gel containing formaldehyde and ethidium bromide. RNase protection experiments were carried out according to standard procedures (17) using an RNase A/T1 mix (Ambion, Austin, TX). Samples consisted of 1–2 μg of poly(A^+) RNA mixed with 10 μg of yeast tRNA (Sigma) and 7×10^5 cpm of a ^{32}P-labeled antisense RNA probe. Probes were made with PCR-amplified gene fragments cloned into a pCRII vector (Invitrogen) and transcribed with SP6 RNA polymerase (Promega). The subclones were sequenced to confirm that no PCR-generated mutations were present. The CfOLF1 probe covered 190 nt (nucleotide positions 221–411 of its open reading frame), CfOLF2 covered 407 nt (positions 393–800), CfOLF3 covered 188 nt (positions 580–768), and CfOLF4 covered 155 nt (positions 684–839). Protected fragments were separated on 6% acrylamide/8 M urea gels.

RESULTS

Isolation of Cosmids and Phage Containing Candidate Olfactory Receptor Genes. Oligonucleotides corresponding to conserved regions of olfactory receptor transmembrane domains 2 and 6 (1) were used as primers for PCR amplification of dog genomic DNA. The 560-bp products of this reaction were subcloned, and 10 clones with homology to olfactory receptors of other species were identified by sequence analysis. These 10 clones were pooled, labeled by random priming, and used as a probe to screen dog genomic cosmid and phage libraries. Three cosmids and one phage hybridized strongly to this mixed probe and also gave a PCR product of the expected size when amplified with the degenerate primers described

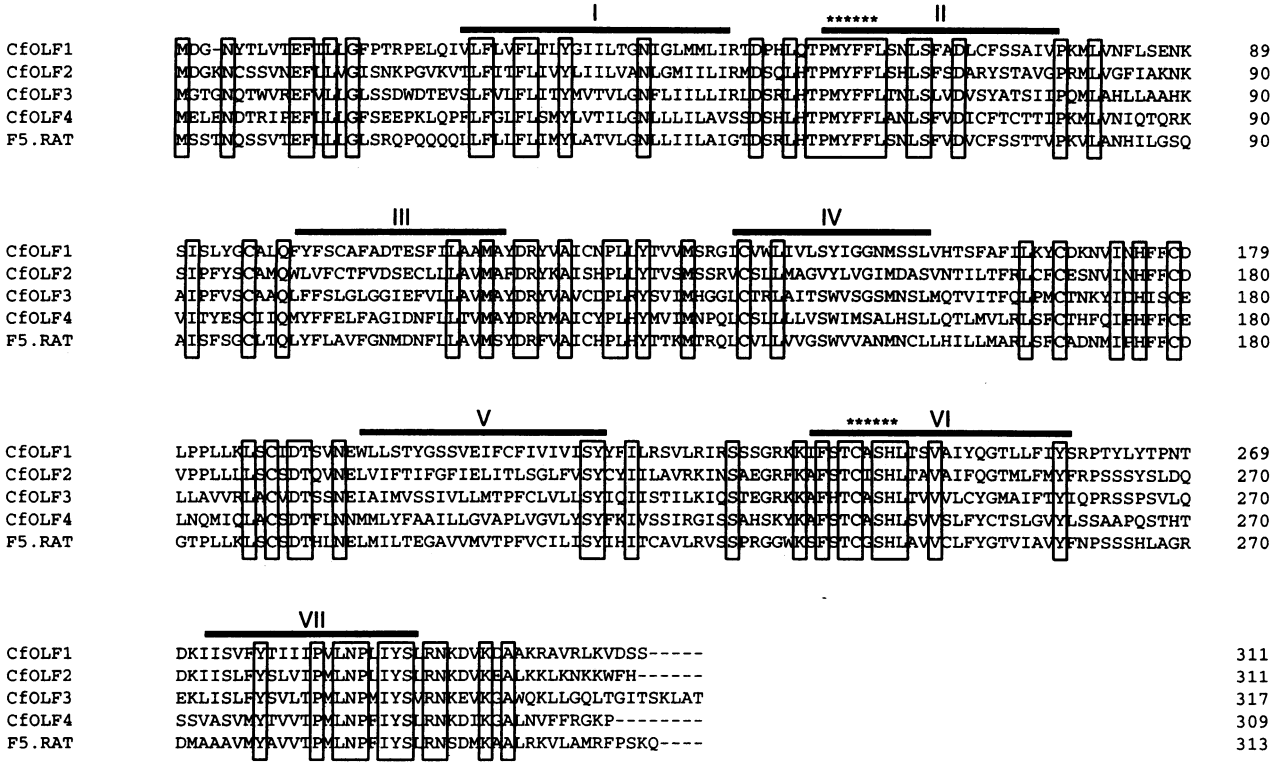

FIG. 1. Protein sequences encoded by the canine olfactory receptor genes. The deduced amino acid sequences of canine olfactory receptors CfOLF1, CfOLF2, CfOLF3, and CfOLF4 are shown aligned with rat olfactory receptor F5 (1). Residues conserved in all five proteins are shown boxed, and the predicted positions of the seven hydrophobic domains (I–VII) are indicated. Asterisks indicate the location of the conserved sequences that were used to design degenerate PCR primers NL61 and NL63.

above. Southern blot hybridizations of restriction digests of these cosmids and phage with the 10-clone pool as a probe indicated that each cosmid and phage carried a single candidate olfactory receptor gene.

The genes from these four genomic clones were subcloned and sequenced. The deduced protein sequences shared 40–64% identity with olfactory receptors from other mammalian species (1–5, 7–9) (Fig. 1). The hydrophobicity plots of all four genes were consistent with a structure containing seven transmembrane segments. In common with other members of this family, each of the four genes had a potential N-linked glycosylation site in the N-terminal domain of the protein and cysteines at positions 97 and 179, which may form a disulfide bridge. All four genes had a close match to the PMY(L/F)FL and MAYDRYVAICHPL motifs common to olfactory receptors, and also had the conserved SY at positions 217–218. The potential phosphorylation site in the C-terminal domain of almost all known olfactory receptors was also present in each of the four dog genes, as was a conserved serine in the third cytoplasmic loop. In addition the third, fourth, and fifth transmembrane domains, which constitute potential ligand binding sites, showed a variability in the four dog genes that paralleled that of other olfactory receptors. This kind of variation is expected in a gene family whose products must bind a diverse array of ligands.

Sequence Relationships Among Olfactory Receptors. According to a proposed classification system (4, 19), olfactory receptors that share at least 40% amino acid identity are considered members of the same family, and those that share at least 60% amino acid identity are considered members of the same subfamily. In comparisons of the four dog genes with one another, the two designated CfOLF1 and CfOLF2 were the most similar, sharing 52% amino acid identity. All other pairwise comparisons among the four showed amino acid identity levels of 40–43%. Thus, although all were members of the same family of genes, all were members of different subfamilies and thus provide four independent windows into the evolution of gene subfamilies.

A survey of olfactory receptors from different species revealed two rat genes, F12 and F3, with sequences very similar to that of CfOLF4 (1). Their protein sequences were 63% (F12) and 64% (F3) identical to that of CfOLF4. The sequences most similar to CfOLF1 and CfOLF2 were rat olfactory receptor OR14 (47% identical to each) and the mouse receptor fragment K17 (46% identical to CfOLF1 and 53% identical to CfOLF2 over 111 amino acids) (3, 7). The closest matches to CfOLF3 were mouse receptor OR3, which was 48% identical, and mouse receptor fragment M49, which was 49% identical over 111 amino acids (3, 5). The part of the protein covered by fragments K17 and M49 includes the highly variable region between transmembrane domains 3 and 6; therefore, the similarities of the three dog genes to the receptors represented by these fragments are probably even greater than reported.

Size of Dog Olfactory Receptor Gene Subfamilies. Southern blot hybridizations were performed to assess the complexity of the subfamilies to which the four candidate olfactory receptor genes belonged. Membership in a subfamily as defined by these Southern blots required greater sequence similarity than membership in a subfamily as defined by sequence only. Members of so-called hybridization subfamilies (19) usually share at least 80% identity, whereas members of sequence homology subfamilies need share only 60% identity.

A Southern blot of dog genomic DNA digested separately with five different restriction enzymes was hybridized to a probe that encompassed 909 bp of the CfOLF1 open reading frame. Similar hybridizations were performed with probes encompassing 840 bp of CfOLF2, 912 bp of CfOLF3, and 868 bp of CfOLF4 (Fig. 2). Because olfactory receptor genes are small and lack introns within the coding region, each band in these Southern blots

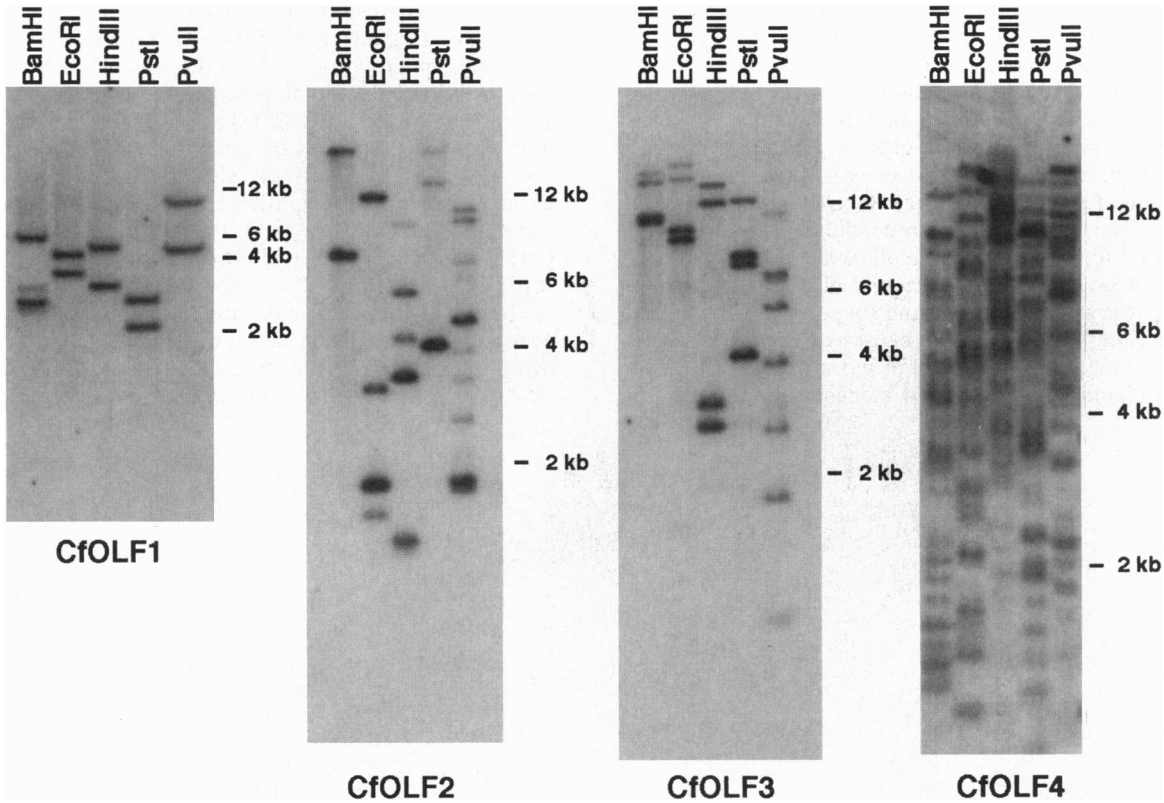

FIG. 2. Southern blots hybridized with canine olfactory receptor genes CfOLF1, CfOLF2, CfOLF3, and CfOLF4. Dog genomic DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Pvu*II and electrophoretically separated on 0.8% agarose gels. The DNA was transferred to nylon membranes and hybridized with ^{32}P-labeled probes corresponding to the four dog genes. The four subfamilies revealed by this analysis range in size from 2 (CfOLF1) to 20 (CfOLF4) genes.

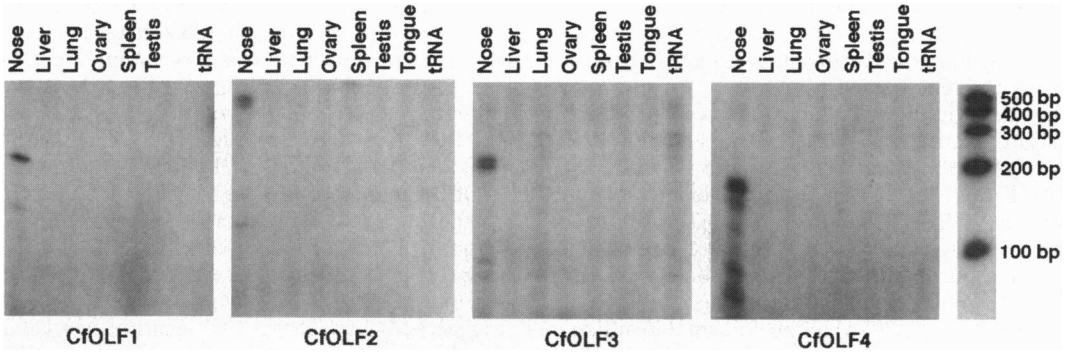

FIG. 3. RNase protection assays for the expression of canine olfactory receptor genes in diverse tissues. Subclones of CfOLF1, CfOLF2, CfOLF3, and CfOLF4 were used to make ^{32}P-labeled antisense RNA probes. Expression of the four genes appears to be restricted to the olfactory epithelium. Doublets are seen when the CfOLF2 and CfOLF3 probes are protected with nose mRNA, indicating the expression of closely related genes with differences toward the ends of the probe fragments. Multiple bands are present when the CfOLF4 probe is protected with nose mRNA, indicating the expression of multiple closely related genes. RNA markers (Ambion) were used as molecular weight standards.

generally indicated a single gene. Exceptions included cases in which the restriction enzyme either cleaved inside the open reading frame, revealed a polymorphism in the flanking DNA, or failed to separate two members of a subfamily.

All but one of the lanes in the Southern blot hybridized with the CfOLF1 probe showed two bands: *Bam*HI cut near the end of the CfOLF1 reading frame, producing a faint third band. Thus the CfOLF1 subfamily had two members.

The CfOLF2 probe revealed two to five bands in the lanes containing DNA digested with *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I. The *Pvu*II site in the CfOLF2 open reading frame was apparently shared by other members of the subfamily, resulting in eight or nine bands. Thus the CfOLF2 subfamily consisted of from two to five genes.

The first four lanes of the Southern blot hybridized with the CfOLF3 probe showed four bands. The *Pvu*II digest showed eight bands due to a *Pvu*II site near the middle of the CfOLF3 sequence, which was apparently also present in other members of the subfamily. Thus this subfamily consisted of four genes.

The largest subfamily was revealed by the CfOLF4 probe. There were between 18 and 22 bands in each lane, indicating the presence of approximately 20 genes in this subfamily.

Expression of the Dog Olfactory Receptors. To determine the expression patterns of the four candidate olfactory receptors, RNase protection experiments were performed with messenger RNA isolated from canine olfactory epithelium, liver, lung, ovary, spleen, testis, and tongue (Fig. 3).

All four genes appeared to be expressed exclusively in the olfactory epithelium. The CfOLF1 probe, representative of the smallest subfamily, was protected along its full length. The CfOLF2 probe appeared to be completely protected by one RNA species and protected along most of its length by another, suggesting the expression of at least two closely related genes with differences toward the end of the probe fragment. This same pattern of full-length and slightly less than full-length protection of the probe was also seen with CfOLF3. Each of these three genes also showed some protection of a smaller band. CfOLF4, representative of the largest subfamily, showed multiple protected species from full length to quite small, suggesting the expression of many related genes. There was a positive correlation between subfamily size as determined by genomic Southern blot and number of bands detected in these RNase protection experiments. This correlation suggested that more than one member of each subfamily was expressed in the olfactory epithelium, and that the larger subfamilies were not collections of untranscribed pseudogenes.

Genomic Organization of the Subfamilies. Many mammalian gene families are present in the genome as linked arrays, such as the α and β globin genes, immunoglobulin genes, and T-cell receptor genes (20–22). To determine whether members of the four dog olfactory receptor subfamilies were clustered, dog genomic DNA was digested with a set of restriction enzymes that have recognition sequences rare in mammalian genomes. The resulting large fragments were separated on CHEF gels, and Southern blot hybridizations were performed with each of the four gene probes (Fig. 4).

The two genes that make up the CfOLF1 subfamily appeared to be close to one another in the dog genome. Southern hybridization with a CfOLF1 probe showed a single band of ≈500 kb when the canine genomic DNA was digested with *Sfi*I,

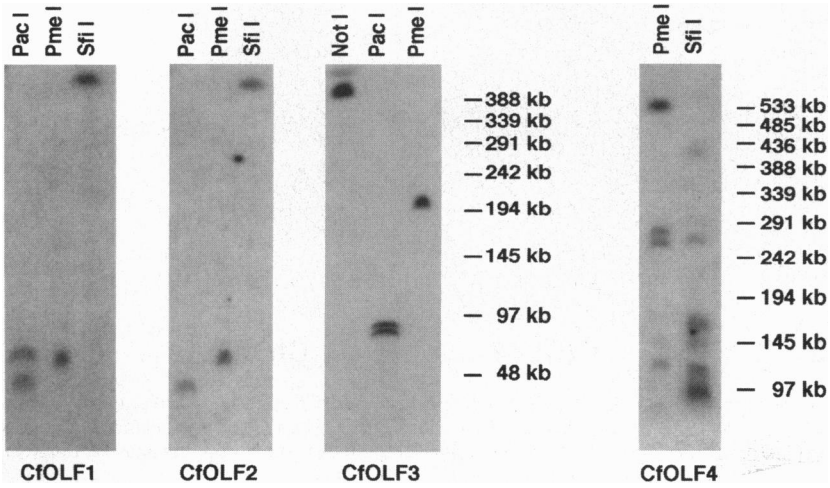

FIG. 4. CHEF gel Southern blot analysis with CfOLF1, CfOLF2, CfOLF3, and CfOLF4 probes. Canine genomic DNA from the MDCK cell line was digested with *Not*I, *Pac*I, *Pme*I, and *Sfi*I and electrophoretically separated on 1% CHEF gels. The DNA was transferred to nylon membranes and hybridized with ^{32}P-labeled probes corresponding to the four dog genes. The positions of the lambda concatamer size standards are shown.

and a single band of ≈70 kb when the DNA was digested with *Pme*I. A *Pac*I digest showed two bands of ≈45 and 70 kb in size. Thus the two members of this subfamily were most likely within 70 kb of one another with a *Pac*I site between them.

Hybridization of the same blots with the CfOLF2 probe showed a pattern strikingly similar to the one seen with CfOLF1. An *Sfi*I digest showed a single band of ≈500 kb, a *Pme*I digest showed a single band of ≈70 kb, and a *Pac*I digest showed a single band of ≈45 kb. These results indicated that members of the CfOLF2 subfamily clustered with one another, and appeared to be within 45 kb of one member of the CfOLF1 subfamily. Analysis of PCR amplification of DNA from 74 canine–rodent hybrid cell lines with primers specific to CfOLF1 and CfOLF2 was consistent with these two subfamilies being linked (E. Ostrander and L.I.-T., unpublished results).

The CfOLF3 probe hybridized to a *Pme*I digest of the DNA showed a single band of ≈200 kb. A *Not*I digest showed a single band of 400 kb, and a *Pac*I digest showed two bands of ≈80 and 90 kb. Thus the four members of the CfOLF3 subfamily were all likely located within 200 kb of one another.

Hybridizations of the CfOLF4 probe to these blots revealed a more complex pattern of bands. DNA digested with *Pme*I showed five bands ranging in size from 120 to >500 kb. An *Sfi*I digest showed six or seven bands between 90 and 500 kb. These results indicated that the members of the largest subfamily were arranged in a maximum of five clusters.

Overall it appeared that members of the same subfamily were located close to one another in the genome. In addition, the two subfamilies represented by the most similar of the four genes, CfOLF1 and CfOLF2, were tightly linked.

Breed Comparisons. Dogs were domesticated 10,000–12,000 years ago (23). Through the years humans used selective breeding to create more than 300 breeds of dog for purposes such as flock protection, water rescue, hunting, herding, tracking, and companionship. The breeds classified as scent hounds were bred for their ability to hunt and track using olfactory cues. The breeds classified as sight hounds were bred to hunt using mostly visual cues. The toy/companion breeds were bred primarily on the basis of appearance, size, and temperament.

In principle, selection of members of the scent hound breeds on the basis of enhanced olfactory sensitivity could have led to an increase in the number of olfactory receptor genes in these breeds. Because members of the olfactory receptor gene subfamilies appeared to be clustered, unequal crossing-over could lead to a change in the number of genes per subfamily. To determine whether the different selection regimens had an effect on the number of olfactory receptor genes in scent hounds relative to sight hounds or toy/companion breeds, Southern blots with genomic DNA from dogs of several different breeds were hybridized with each of the four dog gene probes. The breeds chosen for the analysis were American Foxhound, English Foxhound, Bluetick Coonhound, Bloodhound, Running Walker Hound, Beagle, Basset Hound, Dachshund, Norwegian Elkhound, and Finnish Spitz (scent hound); Borzoi, Italian Greyhound, Irish Wolfhound, Whippet, Greyhound, Rhodesian Ridgeback, Scottish Deerhound, Pharaoh Hound, Saluki, and Basenji (sight hound); and Schipperke, Pug, Shih Tsu, Lhasa Apso, Pomeranian, and Papillon (toy/companion). Genomic DNA was digested with *Eco*RI and electrophoretically separated on 0.8% agarose gels (Fig. 5).

The CfOLF1 probe showed two bands in all dogs except Beagle (scent hound), English Foxhound (scent hound), and Greyhound (sight hound); these dogs each had a third band. Additional digests with *Bam*HI, *Hin*dIII, *Pst*I, *Pvu*II, and *Xba*I indicated that the third band was the result of a small (<300 bp) duplication or insertion near one of the subfamily members and not the result of an expansion of this subfamily (data not shown). The CfOLF2 probe showed a uniform pattern of hybridization in the different breeds, with the exception of an additional band in the Norwegian Elkhound (scent hound). The CfOLF3 probe showed an invariant pattern of four bands in all breeds. The CfOLF4 probe showed a few differences in hybridization pattern, but no clear examples of a change in gene number. This analysis suggested that there have been no systematic breed-specific changes in the number of genes in the four subfamilies in spite of differential selection for olfactory ability in these groups of breeds.

DISCUSSION

The four dog genes described here appeared to be members of the olfactory receptor gene family. The predicted products of the

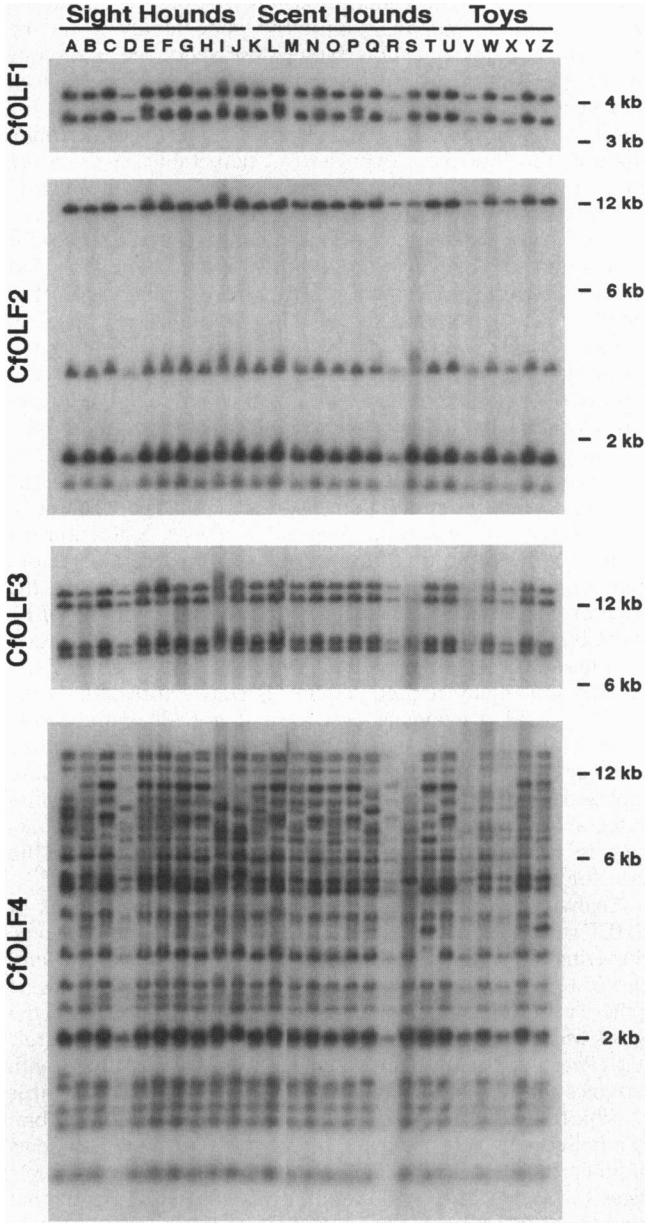

FIG. 5. Comparisons of the CfOLF1, CfOLF2, CfOLF3, and CfOLF4 subfamilies in different breeds of dog. Five micrograms of genomic DNA from 10 sight hounds (lanes: A, Borzoi; B, Italian Greyhound; C, Irish Wolfhound; D, Whippet; E, Greyhound; F, Rhodesian Ridgeback; G, Scottish Deerhound; H, Pharaoh hound; I, Saluki; and J, Basenji), 10 scent hounds (lanes: K, American Foxhound; L, English Foxhound; M, Bluetick Coonhound; N, Bloodhound; O, Running Walker Hound; P, Beagle; Q, Basset Hound; R, Dachshund; S, Norwegian Elkhound; and T, Finnish Spitz), and 6 toy/companion breeds (lanes: U, Schipperke; V, Pug; W, Shih Tsu; X, Lhasa Apso; Y, Pomeranian; and Z, Papillon) were digested with *Eco*RI and electrophoretically separated on 0.8% agarose gels. The DNA was transferred to nylon membranes and hybridized with the four ^{32}P-labeled gene probes.

genes were 40–64% identical to olfactory receptors identified in rat, mouse, and human. In addition, all the hallmark sequence motifs common to olfactory receptors were present in these genes.

The subfamilies identified by the dog gene probes in Southern hybridization experiments had as few as 2 and as many as 20 members. The ≈29 genes in the four subfamilies are only a small fraction, perhaps 5%, of the full repertoire of olfactory receptor genes thought to be present in the dog genome (2). Because hybridization experiments only reveal very closely related sequences (at least 80% identical), the subfamilies as defined by sequence homology (>60% amino acid identity) may be larger than those identified here by cross-hybridization. Further cloning and analysis of the gene family would be required to determine how large these sequence homology subfamilies are, and what fraction of the total array of olfactory receptors they represent.

All four genes were expressed in the olfactory epithelium. None of the four were expressed at detectable levels in the lung, liver, ovary, spleen, testis, or tongue, although if the level of expression were extremely low, it might have gone undetected. RNase protection experiments were useful not only for indicating the tissue in which the genes were expressed, but also for providing information about the expression of other members of the subfamilies. An RNA transcript whose sequence was identical to that of the probe would protect the probe along its full length. Mismatches between the probe and a transcript to which it had hybridized would lead to RNase-sensitive sites that are cleaved upon RNase digestion. Expression of closely related subfamily members could thus lead to the creation of different sized probe fragments. If a large number of related genes were expressed, a large number of probe fragments would be generated. RNase protection experiments indicated that in the three larger subfamilies more than one member of each subfamily was expressed in the olfactory tissue. RNase protection experiments with CfOLF2 and CfOLF3 probes each showed evidence of the expression of at least two closely related genes. In the case of CfOLF4, it seemed that many related sequences were expressed. These results provided evidence that most if not all of the cross-hybridizing bands in genomic Southern blots represented members of the olfactory receptor gene family that were expressed. The results also indicated that in these subfamilies detectable expression of all members was restricted to olfactory tissues and not found, for example, in the testis as was the case for canine receptor gene DTMT (2).

Analysis of large DNA fragments using Southern blots of CHEF gels hybridized with the four gene probes suggested some clustering of genes within subfamilies in the dog genome. Members of a subfamily were considered to be clustered in the genome if there were a smaller number of genomic fragments carrying the genes when the DNA was digested with enzymes that cut rarely (e.g. *Pac*I), compared with when the DNA was digested with enzymes that cut more frequently (e.g. *Eco*RI). Although it is possible that what appeared to be a single band on a Southern blot of a pulsed-field gel actually represented two or more fragments of similar size, the likelihood of multiple coincidences for multiple digests was small. This analysis of pulsed-field gels indicated that most likely the members of the CfOLF1 subfamily were within 70 kb of one another, the members of the CfOLF2 subfamily were within 45 kb of one another, and the members of the CfOLF3 subfamily were within 200 kb of one another. The members of the CfOLF4 subfamily appeared to be grouped into five or fewer arrays. Comparisons of the hybridization patterns of the CfOLF1 and CfOLF2 subfamilies suggested that they were also closely linked in the dog genome. This organization of family members in linked arrays is consistent with previous studies of olfactory receptor genes in human (4) and mouse (3). The clustering of the most closely related sequences (those of subfamily members) and of related subfamilies is also consistent with a mechanism of expansion of gene families by unequal crossing-over (12).

Southern blots of *Eco*RI digests of genomic DNA from 10 scent hounds, 10 sight hounds, and 6 toy/companion breeds were hybridized with the four dog gene probes to determine whether artificial selection on the basis of enhanced olfactory acuity had led to an increase in the number of olfactory receptor genes in scent hounds. Although a few size polymorphisms were found, no expansions of the subfamilies were detected. Since different breeds of dog differ as much as 16-fold in surface area of olfactory epithelium, and not simply as a function of body size (13, 24), perhaps selection on the basis of olfactory ability has primarily had an effect on total number of olfactory neurons. Such differences in neuron number could lead to global changes in sensitivity to odorants. It is also possible that selection has acted not on the number of genes per subfamily, but on the diversity of subfamilies or on levels of expression of the olfactory receptor genes. Comparisons of the arrays of olfactory receptor genes of macrosmatic mammals such as dogs with microsmatic mammals such as humans will provide a broader picture of changes in the gene family over evolutionary time.

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