The Evolution of Mammalian Olfactory Receptor Genes

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ABSTRACT

We performed a comparative study of four subfamilies of olfactory receptor genes first identified in the dog to assess changes in the gene family during mammalian evolution, and to begin linking the dog genetic map to that of humans. The human subfamilies were localized to chromosomes 7, 11, and 19. The two subfamilies that were tightly linked in the dog genome were also tightly linked in the human genome. The four subfamilies were compared in human (primate), horse (perissodactyl), and a variety of artiodactyls and carnivores. Some changes in gene number were detected, but overall subfamily size appeared to have been established before the divergence of these mammals 60–100 million years ago.

THE mammalian olfactory receptor gene family is made up of hundreds of genes that encode an immense variety of membrane-bound G protein-coupled receptors (BUCK and AXEL 1991; NEF *et al.* 1992; PARMENTIER *et al.* 1992; RAMING *et al.* 1993; SCHURMANS *et al.* 1993; BEN-ARIE *et al.* 1994; GAT *et al.* 1994; SULLIVAN *et al.* 1996). These receptors share several hallmark sequence motifs, yet are quite variable in the region of the protein thought to be responsible for binding odorants (BUCK and AXEL 1991). The size and diversity of the gene family make it possible for mammals to recognize and discriminate thousands of different odorants. This size and diversity, together with the importance of olfaction in the natural history of mammals, make this group of genes a particularly interesting subject for comparative analysis.

Olfaction is an important part of the mammalian lifestyle, playing a role in social behavior, acquisition of food, and evaluation of the environment. Mammals differ in their level of reliance on the sense of smell, and in their olfactory sensitivity and discrimination (MOULTON 1960, 1967; STODDART 1980). Differences in olfactory acuity could be due to many different factors. One possible factor is the anatomy (*i.e.*, size and position) of the olfactory neuroepithelium in the nose. Although there is no simple relationship between surface area of olfactory epithelium and sensitivity to odorants, animals that do not rely heavily on their sense of smell often have a relatively small olfactory neuroepithelium (MOULTON 1967). Among mammals the olfactory apparatus is particularly well developed in most carnivores, perissodactyls, artiodactlys, rodents, and bats. Significant reduction of the olfactory apparatus is seen primarily in primates and aquatic mammals (MOULTON 1967). Dogs, which rely heavily on their sense of smell and are therefore considered macrosmatic, have an olfactory sensitivity up to 100 times greater for the detection of certain compounds than microsmatic humans, who rely more heavily on other senses such as vision (MOULTON 1960). Humans have $\sim$10 cm^2 of olfactory epithelium, whereas German Shepherd dogs have 95–169 cm^2, Boxers have $\sim$120 cm^2, and even tiny Pekinese have 30 cm^2 (LAURUSCHKUS 1942; MULLER 1955; MOULTON and BEIDLER 1967). Differences also exist in the fraction of the brain devoted to the sense of smell in macrosmatic and microsmatic mammals (MOULTON 1967). In addition to this anatomical variation, mammals may differ with respect to the size and diversity of the family of olfactory receptor genes in their genomes. Some of these genetic differences may result from natural selection based on olfactory ability. Olfactory receptor genes are often organized in clusters of closely related genes (REED 1992; BEN-ARIE *et al.* 1994; ISSEL-TARVER and RINE 1996; SULLIVAN *et al.* 1996). Therefore unequal crossing over between family members could lead to an increase or decrease in the number of genes in the gene family, as observed for the α globin genes in primates (ZIMMER *et al.* 1980). It is possible, for example, that macrosmatic mammals may have a larger or more diverse array of olfactory receptor genes than microsmatic mammals.

Mammalian olfactory receptor genes have been cloned from rat, mouse, human, and dog (BUCK and AXEL 1991; NEF *et al.* 1992; PARMENTIER *et al.* 1992; RAMING *et al.* 1993; SCHURMANS *et al.* 1993; BEN-ARIE *et al.* 1994; GAT *et al.* 1994; ISSEL-TARVER and RINE 1996; SULLIVAN *et al.* 1996). Our interests in olfactory receptors include a comparison of the sequence, number, and map positions of orthologous genes in different mammals. (Orthologues are homologues from different species.) To carry out such a comparison, we have studied four subfamilies of olfactory receptor genes that we recently identified in dogs (ISSEL-TARVER and RINE

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1996). These subfamilies are defined by hybridization in Southern blots of genomic DNA; members of a subfamily hybridize with one another, but do not cross-hybridize with members of other subfamilies. We compared the four subfamilies in macrosmatic dogs with those in microsmatic humans. We mapped the orthologous human genes, sequenced members of two subfamilies for comparison with their canine counterparts, and characterized subfamily size and genomic organization in the two mammals. To better understand what changes have occurred in these subfamilies over the course of mammalian evolution, and to learn whether these changes reflect the phylogeny or the ecology of different mammals, we evaluated the same four subfamilies in a variety of species.

As described recently (ISSEL-TARVER and RINE 1996), the dog genes CfOLF1, CfOLF2, CfOLF3, and CfOLF4, the starting points for this comparative analysis, share the hallmark sequence motifs common to olfactory receptors. Each of these four genes defines a subfamily of olfactory receptor genes. The subfamilies represented by the four genes range in size from two to 20 genes. All four genes (and other members of the subfamilies that they represent) are expressed in canine olfactory epithelium. They are not detectably expressed in canine lung, liver, ovary, spleen, testis, or tongue. Members of a given subfamily are clustered in the genome, with even the largest subfamily, represented by CfOLF4, arranged into a small number of clusters. The CfOLF1 and CfOLF2 subfamilies are tightly linked in the dog genome. No differences were detected in the number of genes per subfamily among the 26 breeds of dog tested.

MATERIALS AND METHODS

Southern blot hybridizations: Genomic DNA (10 μg per lane) was digested with restriction enzymes and electrophoretically separated on 0.8% agarose gels. The DNA was transferred to nitrocellulose or Genescreen nylon membranes (DuPont) and hybridized in 0.5 M $NaHPO_4$ pH 7.2, 7% SDS, 1 mM EDTA, and 100 μg/ml salmon sperm DNA at 50–55° (AUSUBEL *et al.* 1987). Washes were done at 50–55° (lower stringency) or 55–60° (higher stringency) in 40 mM NaHPO4 pH 7.2, 1% SDS, 1 mM EDTA. Probes specific to each of the four dog olfactory receptor genes designated CfOLF1–4 were generated by PCR using the cloned genes as 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 with ^{32}P-dCTP by random priming with the Multiprime DNA Labelling System (Amersham).

Mapping genes using a somatic cell hybrid panel: The human subfamilies detected with the dog CfOLF1–4 probes were mapped to human chromosomes through the use of a commercially available Southern blot of *Pst*I-digested DNA from human/rodent hybrid cell lines (Oncor, Gaithersburg, MD). Hybridizations were carried out at 42° in 50% formamide, 6× SSPE, 5× Denhardt's reagent, 1% SDS, and 100 μg/ml salmon sperm DNA. Washes were done at 52° in 0.16× SSPE and 0.1% SDS.

Screening human genomic libraries: Human chromosome-specific Charon 21A libraries were obtained from the American Type Culture Collection. The chromosome 11 library, designated LL11NS01, was made from a complete *Hin*dIII digest (DEAVEN *et al.* 1986). The chromosome 7 library, designated LA07NS01, was made from a complete *Eco*RI digest (DEAVEN *et al.* 1986). Using standard procedures, the libraries were screened with the appropriate dog gene probe at medium stringency (55°) and washed at 55° in 0.2× SSC, 0.1% SDS (SAMBROOK *et al.* 1989).

Sequencing of the genomic clones: Restriction fragments of the phage containing the human genes of interest were identified by Southern blot analysis using the appropriate dog gene as a probe, and the fragments were subcloned into the vector pBluescript (Stratagene). Sequences of the candidate olfactory receptor genes were obtained using the Sequenase 2.0 kit (USB) or with the Auto Read kit and the ALF Sequencer (Pharmacia). The two genes sequenced were designated HsOLF1 and HsOLF3 for *Homo sapiens* olfactory receptor genes 1 and 3.

Yeast artificial chromosome (YAC) library screens: YAC libraries were screened by PCR using primers specific to the human genes HsOLF1 and HsOLF3. The primers used to screen for HsOLF1 were H1-L (5′-CTACACGTTGGTCACTGAG-3′) and H1-R (5′-GGTAGATCGTCACTGAAGTC-3′). The primers used to screen for HsOLF3 were H3-L (5′-CCTGTTTGTCCTGTTCTTGGTC-3′) and H3-R (5′-AACCACTGTGAGGTGAGAGG-3′). YACs positive by PCR for the presence of the genes were subsequently screened by Southern hybridization, as described above.

Mammalian DNA samples: Most mammalian DNA samples were extracted from tissues obtained from the Museum of Vertebrate Zoology frozen tissue collection (University of California, Berkeley). Other tissues and DNA samples were gifts from ROBERT WAYNE (University of California, Los Angeles), ELAINE OSTRANDER (FHCRC, Seattle), DANIKA METALLINOS (University of California, Davis), STEPHEN GLICKMAN (University of California, Berkeley), MELLISSA DEMILLE (University of California, Berkeley), and CHARLES ISSEL (University of Kentucky). Human genomic DNA was a gift from ANNA DI RIENZO (University of California, Berkeley). High molecular-weight genomic DNA was extracted from tissues according to standard procedures (SAMBROOK *et al.* 1989).

RESULTS

Comparison of dog and human olfactory receptor gene subfamilies: Southern hybridization experiments allow comparisons of the number of genes per gene family in different mammals (IRWIN *et al.* 1989). We previously characterized four canine olfactory receptor genes (CfOLF1–4) and the four subfamilies that they represent (ISSEL-TARVER and RINE 1996). To compare the orthologous subfamilies in humans, hybridization experiments were performed using the four dog olfactory receptor genes as probes on Southern blots of human and dog genomic DNA (Figure 1). Hybridization experiments with the CfOLF1–4 gene probes on Southern blots of dog genomic DNA did not show significant

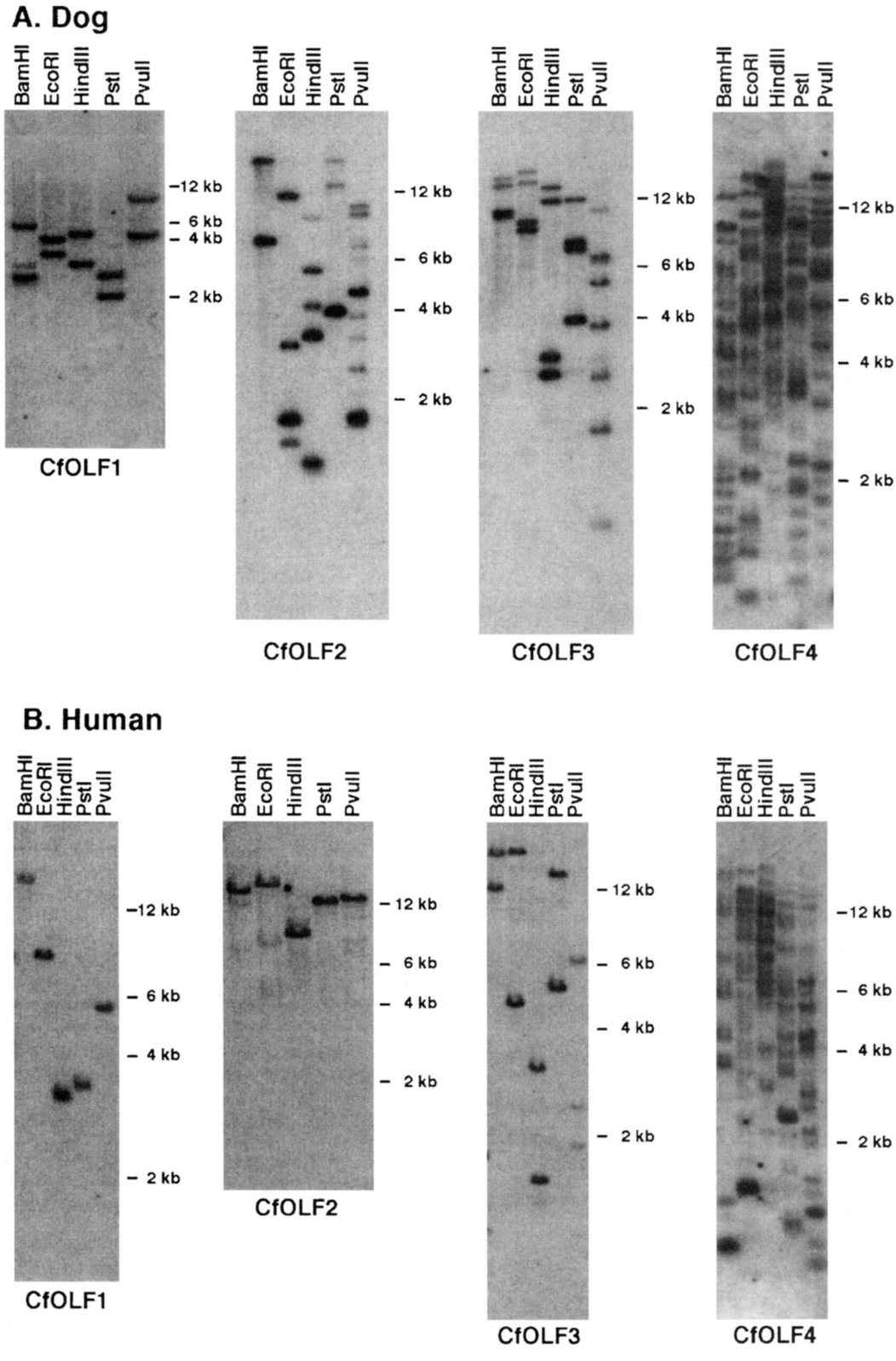

FIGURE 1.—Southern blots hybridized with canine olfactory receptor genes CfOLF1, CfOLF2, CfOLF3, and CfOLF4. Dog (A) and human (B) genomic DNA were digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Pvu*II and electrophoretically separated on 0.8% agarose gels. The DNA was transferred to nylon membranes and hybridized with ^{32}P-labeled probes corresponding to the four dog genes. Blots were washed at 55–60°. A is reproduced from ISSEL-TARVER and RINE (1996).

differences in banding patterns between conditions of high and moderate stringency. Conditions for these Southern blots were thus set at moderate stringency, such that the background was low, but comparisons among these distantly related mammals were possible. By definition, members of a subfamily hybridize with one another, but do not cross-hybridize with members of other subfamilies. Members of so-called hybridization subfamilies share at least 80% identity (LANCET and BEN-ARIE 1993). Because olfactory receptor genes are small and lack introns, one band on a Southern blot generally corresponds to one gene.

The smallest of the four olfactory receptor gene subfamilies in dogs is represented by the CfOLF1 gene. A

Southern hybridization experiment using a probe that encompassed 909 bp of the CfOLF1 open reading frame revealed two cross-hybridizing bands in dog genomic DNA digested with each restriction enzyme except *Bam*HI, which showed a faint third band due to a *Bam*HI site near the end of the CfOLF1 open reading frame. In contrast, the CfOLF1 probe revealed just one cross-hybridizing band in human genomic DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, or *Pvu*II. Thus, as defined by these hybridization conditions, there appeared to be two members of this subfamily in the dog genome and only one member of this subfamily in the human genome.

A probe encompassing 840 bp of the canine CfOLF2 open reading frame revealed two to five cross-hybridizing bands in the lanes containing dog genomic DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I. There was a *Pvu*II site in the CfOLF2 open reading frame that was apparently shared by the other members of this canine subfamily, resulting in nine or 10 bands in the *Pvu*II digest. Thus the CfOLF2 subfamily had two to five members in the dog genome. The CfOLF2 probe revealed one strong cross-hybridizing band and two to four very light bands in each of the five restriction digests of human DNA, indicating that this human subfamily had one member with strong similarity to its dog counterparts, and two to four members with less similarity.

A probe that encompassed 912 bp of the CfOLF3 open reading frame revealed four strong cross-hybridizing bands in all but one of the dog genomic DNA digests. There was a faint fifth band in the *Eco*RI and *Hin*dIII digests, perhaps due to an internal restriction site in a subfamily member, or to a more distantly related olfactory receptor gene. There was a *Pvu*II site in the CfOLF3 open reading frame that was shared by the other members of this canine subfamily, resulting in eight bands in the *Pvu*II digest. The CfOLF3 probe revealed two cross-hybridizing bands in *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I digests of human DNA. As in dog, the *Eco*RI and *Hin*dIII digests showed another faint band. The *Pvu*II digest showed four light bands, indicating that the *Pvu*II site near the middle of the canine CfOLF3 gene was also present in the human orthologues. Thus, under these hybridization conditions, the dog CfOLF3 subfamily had four members, and the human subfamily had two members.

The CfOLF4 probe, encompassing 868 bp of the open reading frame, revealed the largest of the four subfamilies in both dogs and humans. The pattern in the Southern hybridization of human DNA looked similar to that of the dog, with 15–25 cross-hybridizing bands per lane. Thus there appeared to be ~20 member genes in both species.

Taken together, these data suggested a similar size of subfamilies between dog and human, with a tendency toward larger family sizes in dog relative to human.

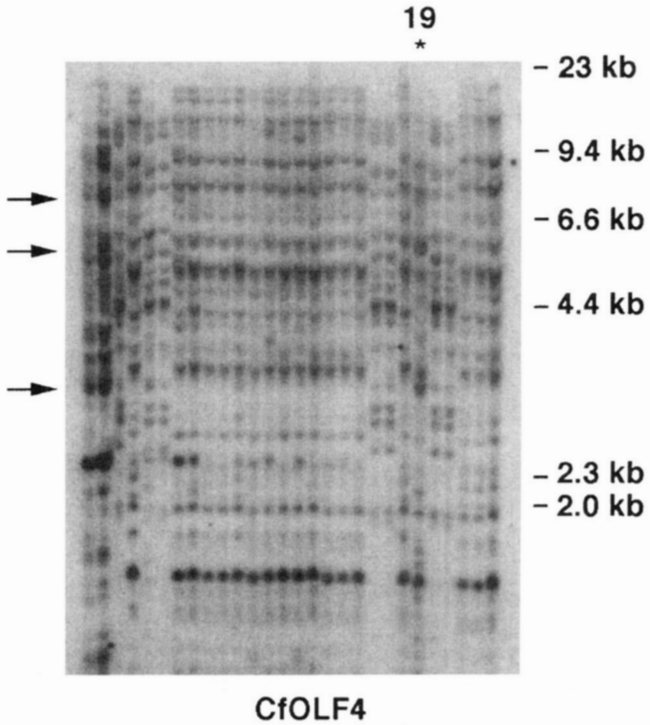

FIGURE 2.—Southern blot of a somatic cell hybrid panel, hybridized with canine olfactory receptor gene CfOLF4. A Southern blot of *Pst*I-digested genomic DNA from human male (lane 1), human female (lane 2), mouse (lane 3), hamster (lane 4), and 24 monochromosomal hybrid cell lines representing human chromosomes 1–22 and X and Y was hybridized with the CfOLF4 probe. Some human-specific bands that hybridized to the probe (marked with arrows) mapped to human chromosome 19.

Chromosomal location of the human olfactory receptor gene subfamilies: The four dog olfactory receptor gene subfamilies map to at least three different canine chromosomes (ISSEL-TARVER and RINE 1996; E. OSTRANDER and L. ISSEL-TARVER, unpublished results). To determine the chromosomal position of the four human olfactory receptor gene subfamilies, the four dog gene probes were individually hybridized to a Southern blot of genomic DNA from 24 somatic cell hybrids, each of which contained a single human chromosome in a rodent cell background.

The human orthologue of the CfOLF1 subfamily mapped to human chromosome 11. The human gene with strongest similarity to the CfOLF2 gene also mapped to chromosome 11. Both members of the human subfamily that hybridized to CfOLF3 were present on chromosome 7 (data not shown.) It was difficult to determine to which chromosome or chromosomes the human genes that hybridized to the CfOLF4 probe mapped. This subfamily was large in mouse and hamster as well as human, so the rodent background largely obscured the human cross-hybridizing bands. It was possible, however, to discern some human-specific bands in the lane corresponding to human chromosome 19 (Figure 2).

Isolation and characterization of phage containing human orthologues of CfOLF1 and CfOLF3: Although

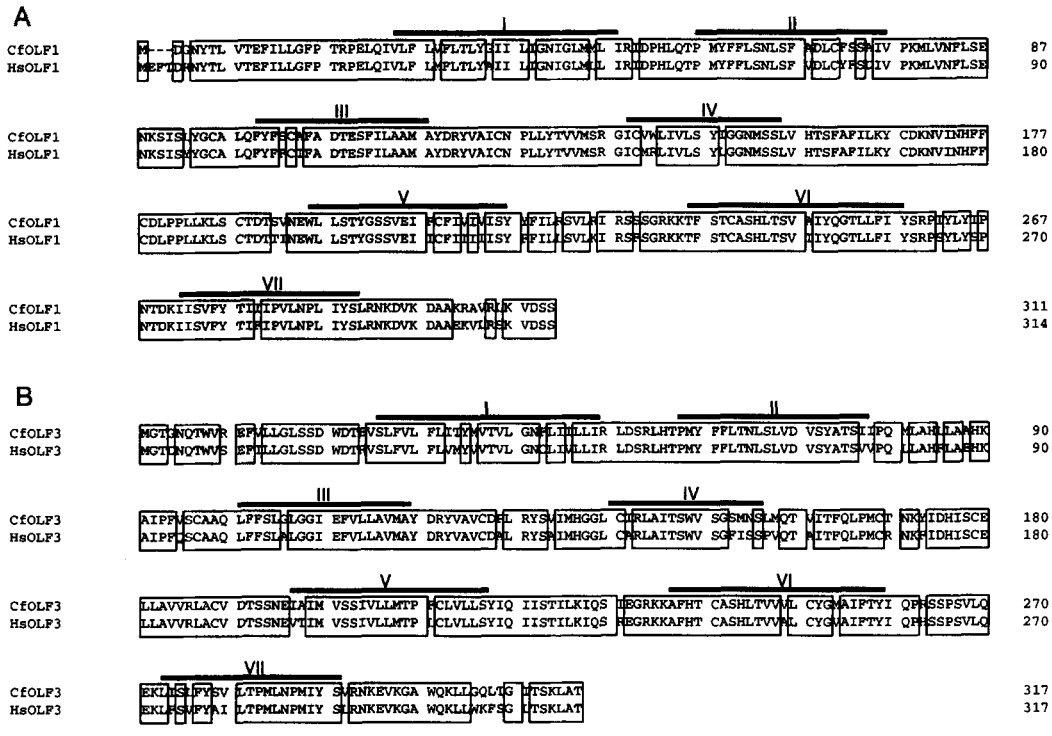

FIGURE 3.—Protein sequences encoded by olfactory receptor genes CfOLF1, CfOLF3, HsOLF1, and HsOLF3. The deduced amino acid sequences of CfOLF1 and HsOLF1 (A), and CfOLF3 and HsOLF3 (B) are shown with residues identical in the orthologous genes boxed. The predicted positions of the seven hydrophobic domains (I–VII) are indicated. The proteins encoded by CfOLF1 and HsOLF1 are 88% identical, and the proteins encoded by CfOLF3 and HsOLF3 are 86% identical. Human sequences have been submitted to GenBank (accession numbers U56420 and U56421).

a number of olfactory receptor genes have been cloned from a variety of animals, rarely have a gene and its orthologue from another species been definitively identified as a pair and both members sequenced. Comparisons between such genes can provide information about the extent of conservation between orthologous olfactory receptor genes, and about the evolution of the olfactory receptor gene family in general.

To identify the human orthologue of the canine olfactory receptor CfOLF1 subfamily, a probe encompassing most of the open reading frame of CfOLF1 was used to screen a human chromosome 11-specific Charon 21A genomic library. One phage was isolated that hybridized strongly to the dog probe. A restriction enzyme digest of the clone revealed a fragment of the expected size for the human orthologue of the CfOLF1 subfamily. The gene, referred to as HsOLF1, was subcloned and sequenced. The predicted protein encoded by the HsOLF1 gene was 88% identical to CfOLF1 (Figure 3).

To identify one of the human orthologues of the canine olfactory receptor CfOLF3 subfamily, a probe encompassing the majority of the open reading frame of CfOLF3 was used to screen a human chromosome 7-specific Charon 21A genomic library. A clone was isolated that hybridized strongly to the probe. A restriction enzyme digest of this human clone revealed a fragment of the expected size for one of the two members of this human subfamily. This human gene, designated HsOLF3, was subcloned and sequenced. The predicted protein encoded by this human gene was 86% identical to CfOLF3 (Figure 3).

Both HsOLF1 and HsOLF3 contained all the residues and motifs common to known olfactory receptors (BUCK and AXEL 1991; NEF *et al.* 1992; PARMENTIER *et al.* 1992; NGAI *et al.* 1993; RAMING *et al.* 1993; RESSLER *et al.* 1993; SCHURMANS *et al.* 1993; BEN-ARIE *et al.* 1994; SULLIVAN *et al.* 1996). Both predicted proteins had hydrophobicity plots consistent with a structure containing seven transmembrane segments. In common with other members of the olfactory receptor family, each had a potential N-linked glycosylation site in the extracellular N-terminal domain of the protein and conserved cysteines in the first and second extracellular loops, which may form a disulfide bridge. Both had the conserved SY in the fifth transmembrane domain and a conserved serine in the third cytoplasmic loop. Close matches to the PMY(L/F)FL and MAYDRYVAIC motifs common to olfactory receptors were found in both protein sequences, as were potential phosphorylation sites in the C-terminal domain.

Reciprocal Southern blot hybridizations: Southern blot hybridizations described above using the dog CfOLF1 and CfOLF3 probes on human genomic DNA revealed orthologous human subfamilies with one and two member genes, respectively. To test whether this gene count was artificially low due to the use of a probe from a different species, four replicate Southern blots of dog and human genomic DNA were hybridized separately with probes corresponding to CfOLF1, CfOLF3, HsOLF1, and HsOLF3 (Figure 4). Except for differences in intensity, the CfOLF3 and HsOLF3 probes revealed identical patterns in the Southern blot hybridizations. The CfOLF1 and HsOLF1 probes revealed nearly identical patterns of two bands in dog genomic DNA and one band in human genomic DNA, with the HsOLF1 probe revealing a couple of very faint extra bands in the dog genomic DNA. Thus these two subfam-

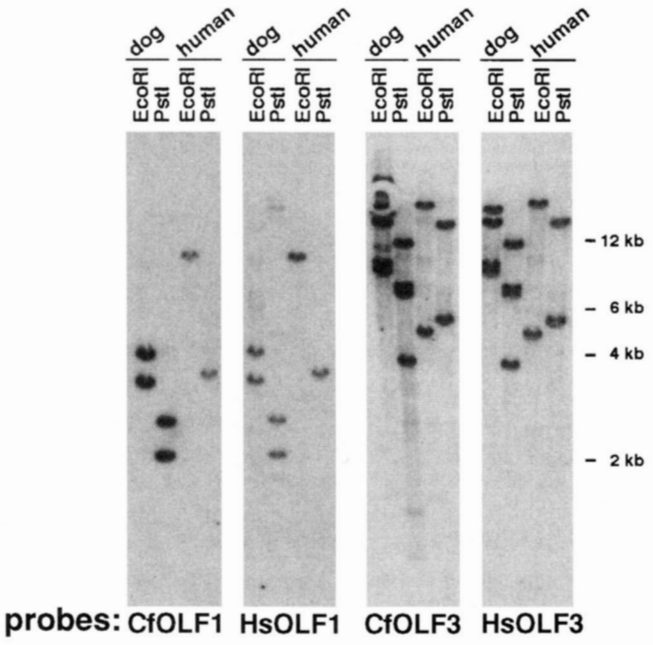

FIGURE 4.—Southern blots of dog and human genomic DNA hybridized with olfactory receptor genes CfOLF1, HsOLF1, CfOLF3, and HsOLF3. Four replicate Southern blots of dog and human genomic DNA digested with *Eco*RI and *Pst*I were hybridized with CfOLF1, HsOLF1, CfOLF3, and HsOLF3. The patterns seen in the hybridizations with the dog probes and their human orthologues were almost identical. Blots were washed at 55°.

ilies, as defined by hybridization, were indeed smaller in humans than in dogs.

Refined localization of the human olfactory receptor genes: To map the human HsOLF1 and HsOLF3 genes more precisely, PCR primers specific to each gene were used to screen human chromosome 11 (G. EVANS, University of Texas Southwestern Medical Center) and chromosome 7 (E. GREEN, NCHGR) YAC libraries.

Two chromosome 11 YACs, designated yMY832g7 and yMY774e1, were identified in the screen with HsOLF1-specific primers. Southern blot hybridizations confirmed that the HsOLF1 gene was present in both YACs (Figure 5A). A lighter band that hybridized to the CfOLF1 probe was also apparent, possibly signalling the presence of another gene with weaker homology to CfOLF1 on this YAC. These overlapping YACs map to human chromosome 11q11 and both contain STS marker D11S1313 (G. EVANS, personal communication).

In dogs, the CfOLF1 and CfOLF2 subfamilies are within 45 kb of one another (ISSEL-TARVER and RINE 1996). To determine whether the two subfamilies are also closely linked in the human genome, the CfOLF2 gene probe was hybridized to Southern blots of restriction digests of the two YACs that carried the HsOLF1 gene (Figure 5A). These hybridization experiments indicated that the human gene that hybridized strongly to the CfOLF2 probe was present on both of these YACs. Thus the linkage of these two subfamilies in dogs was conserved in humans.

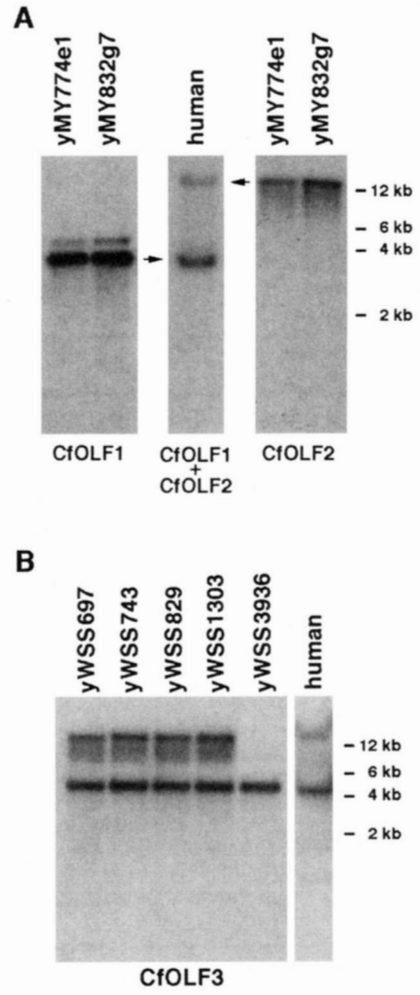

FIGURE 5.—Southern blots of YACs carrying human olfactory receptor genes. (A) Replicate Southern blots of *Pst*I-digested genomic DNA from yeast strains carrying human chromosome 11 YACs yMY774e1 and yMY832g7 were hybridized with either the CfOLF1 or CfOLF2 gene probe. For comparison, a Southern blot of *Pst*I-digested human genomic DNA was hybridized with a mixed CfOLF1 and CfOLF2 probe. Both of these overlapping YACs carry the human orthologues of the CfOLF1 and CfOLF2 subfamilies. (B) A Southern blot of *Eco*RI-digested human genomic DNA and genomic DNA from yeast strains carrying human chromosome 7 YACs yWSS697, yWSS743, yWSS829, yWSS1303, and yWSS3936 was hybridized with the CfOLF3 gene probe. Four of the five YACs carried both members of the human subfamily corresponding to CfOLF3; yWSS3936 carried only one of the two subfamily members.

Five overlapping human chromosome 7 YACs (GREEN *et al.* 1995) were identified by PCR screening with the HsOLF3 primers. Southern hybridization experiments indicated that YACs yWSS697, yWSS743, yWSS829, and yWSS1303 carried both members of the HsOLF3 subfamily, whereas yWSS3936 carried only one of the subfamily members (Figure 5B). All five YACs mapped to the 7q35 region, and the four YACs that contained both genes shared a region of overlap with one another that was not shared in common with yWSS3936. The genetic marker AFM277zf5 (sWSS1322) is present on some of the four YACs containing both

TABLE 1

Species used in analysis

Common name	Scientific name	Order	Family	Abbreviation
Human	*Homo sapiens*	Primates	Hominidae	HUMAN
Dog	*Canis familiaris*	Carnivora	Canidae	DOG
Dingo	*Canis dingo*	Carnivora	Canidae	DINGO
Wolf	*Canis lupus*	Carnivora	Canidae	WOLF
Coyote	*Canis latrans*	Carnivora	Canidae	COYOTE
Fennec fox	*Fennecus zerda*	Carnivora	Canidae	FNCFOX
Grey fox	*Urocyon cinereoargenteus*	Carnivora	Canidae	GRYFOX
Island fox	*Urocyon littoralis*	Carnivora	Canidae	ISLFOX
Raccoon dog	*Nyctereutes procyonoides*	Carnivora	Canidae	RACDOG
Cat	*Felis catus*	Carnivora	Felidae	CAT
Bobcat	*Lynx rufus*	Carnivora	Felidae	BOBCAT
Lion	*Panthera leo*	Carnivora	Felidae	LION
Hyena	*Crocuta crocuta*	Carnivora	Hyaenidae	HYENA
Olingo	*Bassaricyon alleni*	Carnivora	Procyonidae	OLINGO
River otter	*Lutra longicaudis*	Carnivora	Mustelidae	RIVOTR
Sea otter	*Enhydra lutris*	Carnivora	Mustelidae	SEAOTR
California sea lion	*Zalophus californicus*	Carnivora	Otariidae	CSLION
Steller sea lion	*Eumetopias jubata*	Carnivora	Otariidae	SSLION
Axis deer	*Axis axis*	Artiodactyla	Cervidae	AXDEER
Fallow deer	*Dama dama*	Artiodactyla	Cervidae	FLDEER
Goat	*Capra hircus*	Artiodactyla	Bovidae	GOAT
Sheep	*Ovis aries*	Artiodactyla	Bovidae	SHEEP
Peccary	*Tayassu tajacu*	Artiodactyla	Tayassuidae	PECARY
Horse	*Equus caballus*	Perissodactyla	Equidae	HORSE

members of the HsOLF3 subfamily (E. GREEN, unpublished data). Thus, like their dog counterparts, the members of this human subfamily were clustered together.

Some members of the large human subfamily with homology to the CfOLF4 gene in dogs appeared to be located on chromosome 19. A chromosome 19-specific cosmid library was screened by hybridization with the CfOLF4 gene probe, and clones that hybridized strongly to the probe even at high stringency were localized to 19p13.1 and p13.2 (A. OLSEN, personal communication). These clones accounted for a small fraction of the homologous human bands.

A survey of orthologous olfactory receptor gene subfamilies: The agreement between the reciprocal hybridizations of the dog CfOLF genes to human genomic DNA and of the human HsOLF genes to dog genomic DNA established that cross-species hybridization provided a reliable means of evaluating the size of olfactory receptor gene subfamilies among mammals. To determine the extent of the changes in size of the four olfactory receptor gene subfamilies in different mammals, the dog probes corresponding to CfOLF1–4 were hybridized at slightly lower stringency to Southern blots of *Eco*RI digests of genomic DNA from a variety of mammalian species. The species used in this comparative analysis are listed in Table 1. There is considerable controversy concerning the relationships among the mammalian orders (NOVACEK 1989, 1992; LI *et al.* 1990; GRAUR 1993), but a phylogenetic tree based on mitochondrial DNA sequences is shown in Figure 6 (KRETTEK *et al.* 1995).

The CfOLF1 subfamily was small in all species compared (Figure 7A). Under these hybridization conditions, there appeared to be one subfamily member in human, Steller sea lion, California sea lion, sea otter, river otter, olingo, cat, bobcat, lion, and hyena. (The faint second band present in the *Eco*RI digests of bobcat and lion DNA was not present in digests with other restriction enzymes, and was thus likely due to an *Eco*RI

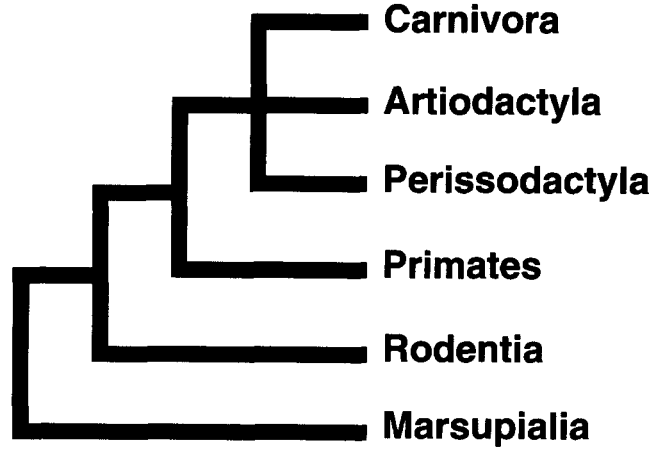

FIGURE 6.—Phylogenetic relationships among six mammalian orders. This arrangement is based on a comparative analysis of mitochondrial protein-coding genes (KRETTEK *et al.* 1995). The relationship among carnivores, artiodactyls and perissodactyls is unresolved.

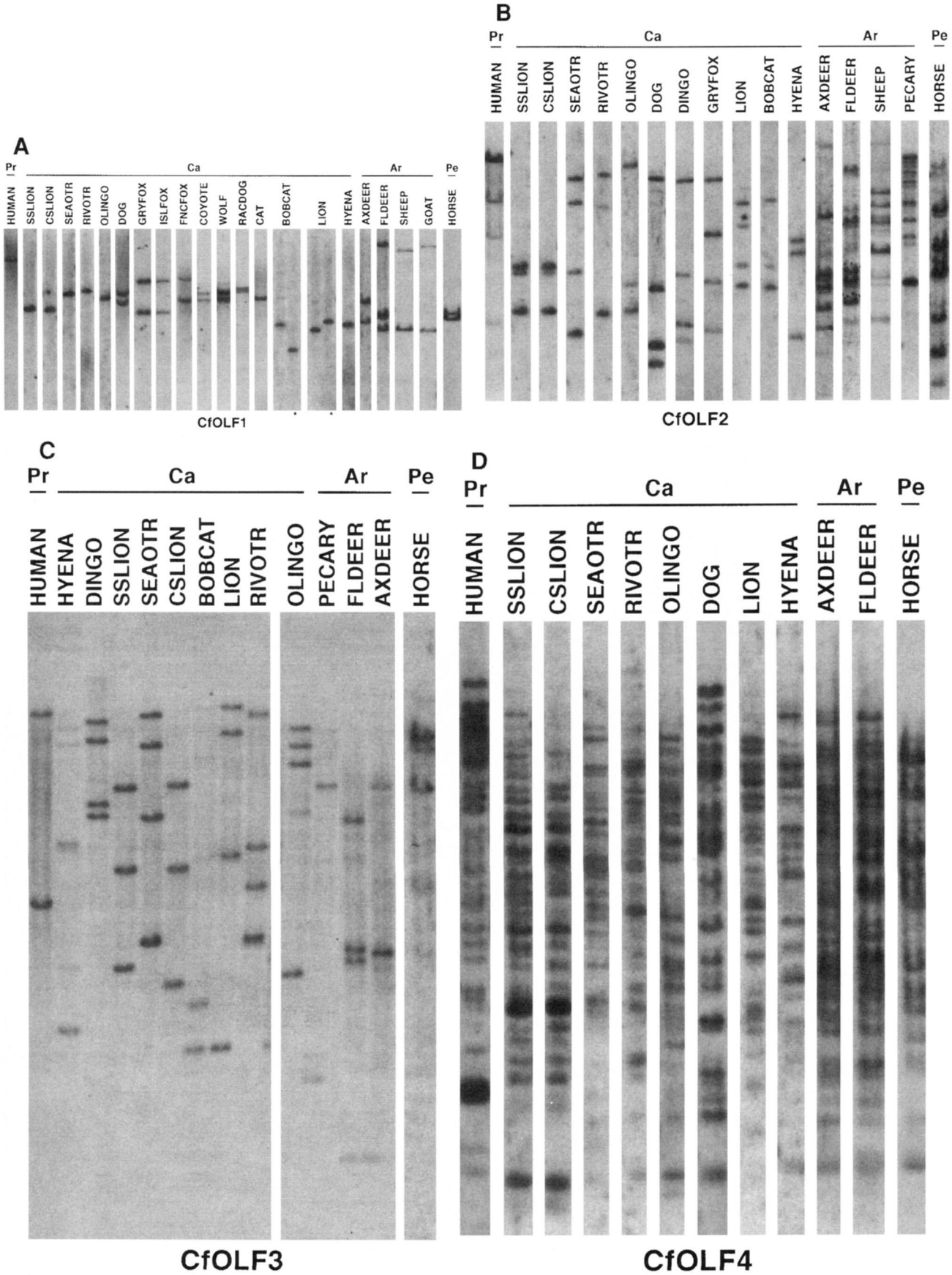

FIGURE 7.—Comparisons of the CfOLF1, CfOLF2, CfOLF3, and CfOLF4 subfamilies in a variety of mammals. Southern blots of *Eco*RI-digested genomic DNA from mammals from the orders Primates (Pr), Carnivora (Ca), Artiodactyla (Ar) and Perissodactyla (Pe) were hybridized with CfOLF1 (A), CfOLF2 (B), CfOLF3 (C), and CfOLF4 (D) gene probes. All blots were washed at 55°. The lanes marked with an asterisk in A are *Hin*dIII-digested DNA and show the absence of the faint second band.

site within the gene.) There were two to four subfamily members in dog, grey fox, island fox, fennec fox, coyote, wolf, raccoon dog, axis deer, fallow deer, sheep, goat, and horse. A comparison of the CfOLF1 subfamily in dog and cat genomic DNA digested with five restriction enzymes illustrates that the differences among mammals seen in Southern blots of *Eco*RI-digested DNA were confirmed in Southern blots using other enzymes (Figure 8A). Thus all dog-like carnivores (canids), all artiodactyls, and the perissodactyl had more than one member of the subfamily. All noncanid carnivores and the one primate appeared to have just one CfOLF1 subfamily member.

The CfOLF2 subfamily (Figure 7B) was relatively small (two to six members) in human, Steller sea lion, California sea lion, sea otter, river otter, olingo, dog, dingo, grey fox, lion, bobcat, and hyena. In axis deer, fallow deer, sheep, peccary, and horse the subfamily consisted of 10–15 members. This difference is more clearly illustrated in a side-by-side comparison of the CfOLF2 subfamilies in Steller sea lion and axis deer (Figure 8B). Thus this subfamily was smaller in carnivores and human, and larger in artiodactyls and the perissodactyl. These differences could be explained by a decrease in subfamily membership on the branches leading to humans and carnivores, or by increases on the branches leading to artiodactyls and perissodactyls.

The CfOLF3 subfamily was of moderate size, four to seven genes, in all mammals tested except human, which had a subfamily with two member genes (Figure 7C). The CfOLF4 subfamily appeared to be similarly stable in size, although it had a larger membership, ~20 genes, in all mammals tested (Figure 7D). The large size of this subfamily precluded detection of small changes in gene number.

DISCUSSION

The mammalian olfactory receptor gene family is an interesting subject for comparative analysis. This gene family is critical to one of the five sensory windows mammals have into the world around them, and as such can be subject to strong selection. The family is made up of hundreds of genes that are similar but not the same, and that are likely to experience different selective pressures due to their different odorant specificities. A previous study found evidence of positive selection for diversity among members of the same subfamily in catfish (Ngai *et al.* 1993). We were interested in a comparison of individual subfamilies across species borders. The four subfamilies revealed by canine olfactory receptor genes CfOLF1–4 provided four opportunities for us to study the evolution of olfactory receptor gene subfamilies.

Comparison of the genomic organization of olfactory receptor subfamilies in dog and human revealed many similarities (this work and Issel-Tarver and Rine 1996). In both dog and human, the subfamilies identified by the CfOLF1 and CfOLF2 genes were tightly linked. This reflected conservation of the genomic organization and provided additional evidence that we were indeed studying orthologous subfamilies in the dog and human genomes. In both dog and human, the members of the subfamily revealed by the CfOLF3 probe were clustered in a single array. In both species at least some members of the subfamily identified by CfOLF4 were clustered together. The linkage of sub-

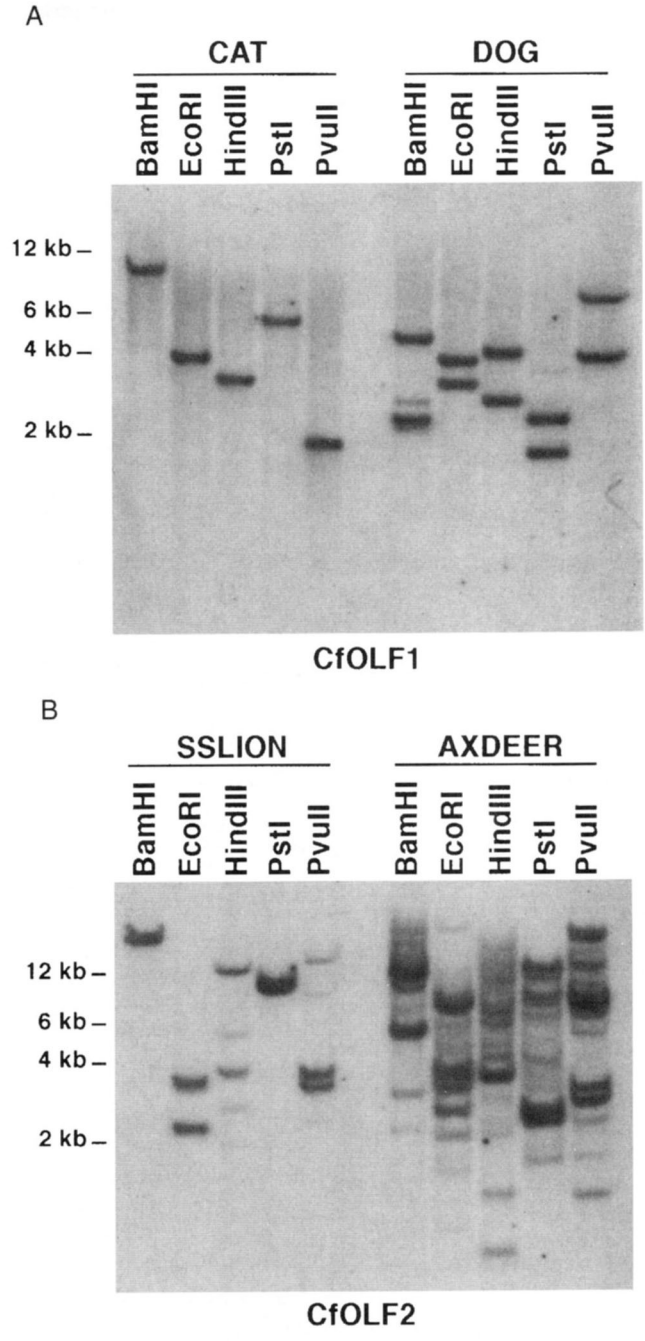

FIGURE 8.—Comparisons of the CfOLF1 and CfOLF2 subfamilies in selected mammals. Southern blots of genomic DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Pvu*II were hybridized with CfOLF1 (A) and CfOLF2 (B) gene probes. (A) The comparison between the CfOLF1 subfamilies in cat and dog; this blot was washed at 60°. (B) The comparison between the CfOLF2 subfamilies in Steller sea lion and axis deer; this blot was washed at 55°.

family members seen here has also been observed in other olfactory receptor gene subfamilies (REED 1992; NGAI *et al.* 1993; SCHURMANS *et al.* 1993; BEN-ARIE *et al.* 1994; SULLIVAN *et al.* 1996), and is common for gene families whose numbers have increased through unequal crossing-over (ZIMMER *et al.* 1980; MAEDA and SMITHIES 1986).

The human subfamilies identified by the CfOLF1 and CfOLF2 gene probes in Southern hybridization experiments both mapped to chromosome 11q11. The subfamily revealed by the CfOLF3 gene probe mapped to human chromosome 7q35. Some members of the subfamily with similarity to CfOLF4 were located on human chromosome 19p13. These data, combined with the locations of other human olfactory receptor genes on chromosome 17p13-p12 (SCHURMANS *et al.* 1993; BEN-ARIE *et al.* 1994) and chromosome 19 (REED 1992) provide evidence that members of the olfactory receptor gene family are widely scattered throughout human chromosomes. Similarly, members of the mouse olfactory receptor gene family were recently found to be present on at least seven mouse chromosomes (SULLIVAN *et al.* 1996).

The dog CfOLF1 gene and its human HsOLF1 orthologue encoded proteins that were 88% identical. The dog CfOLF3 gene and the HsOLF3 gene from the orthologous human subfamily encoded proteins that were 86% identical. This level of conservation between the dog and human genes is on par with that seen, for example, between the tyrosinase genes in dogs and humans (GIEBEL *et al.* 1991; TANG *et al.* 1996). There was no evidence of gene conversion of a region of the genes by a member of another subfamily (BUCK and AXEL 1991).

When subfamilies orthologous to the canine CfOLF1 subfamily were examined by comparative Southern blot hybridizations, some changes in gene number were apparent. There were clear differences in subfamily size even among carnivores, with canids having two members of the subfamily and all other carnivores having only one. With the exception of human, most other mammals tested had two to four members of the CfOLF1 subfamily. These changes in gene number could have come about in a number of ways. One possibility is that there was a duplication in the CfOLF1 subfamily in the common ancestor of carnivores, artiodactyls, and perissodactyls, and subsequent loss of the duplicate copy within the carnivore lineage. [if the Felidae branched first within carnivores (VRANA *et al.* 1994), then two losses must have occurred.] A second possibility is that there were separate expansions on the canid line and on the lines leading to artiodactyls and perissodactyls. These gains and/or losses could be produced by unequal crossing over. Alternatively, gene conversion between these genes and members of other olfactory receptor gene subfamilies could lead to the appearance of more or fewer cross-hybridizing bands. A Southern blot of the YACs carrying the HsOLF1 gene showed a second band that hybridized to the CfOLF1 probe, albeit with five- to 10-fold less intensity than the HsOLF1 gene itself, indicating that there may be other genes related to CfOLF1 nearby in the genome of human (and other mammals). These genes would not have been identified as subfamily members in the genomic Southern blots described here (requiring >80% identity), but may be members of the subfamily as defined by sequence homology (>60% identity) (LANCET and BEN-ARIE 1993).

The CfOLF2 subfamily seemed to come in two sizes, with carnivores and human having a moderate number of member genes (two to six), and artiodactyls and the perissodactyl having a larger number (10–15). One might expect that, because of their tight linkage, the CfOLF1 and CfOLF2 subfamilies would experience parallel increases or decreases in membership, due to duplications or deletions in their shared domain. In artiodactyls and perissodactyls both subfamilies are larger than they are in humans and some carnivores, perhaps due to expansion of shared regions. In canids, on the other hand, the CfOLF1 subfamily is larger than it is in other carnivores, but the CfOLF2 subfamily is the same size.

The CfOLF3 subfamily has not changed much in size over the course of mammalian evolution, although humans had the smallest CfOLF3 subfamily membership in this analysis. The CfOLF4 subfamily was large in all mammals in this comparison, and no significant changes in gene number were detected.

Changes in gene number as determined by Southern blot analysis could have resulted from unequal crossing over between homologous chromosomes or sister chromatids, mediated by members of this gene family or other repetitive elements. Alternatively, rapid sequence evolution, possibly due to gene conversion by a member of another subfamily, could result in gain or loss of cross-hybridization with the probe. The hybridization experiments that we performed did not allow us to evaluate the frequency of pseudogenes in these subfamilies, but our sequencing data from dog and human along with expression data from the dog were consistent with a large fraction of the cross-hybridizing bands representing functional genes (this work and ISSEL-TARVER and RINE 1996). Overall, with the exception of the CfOLF2 subfamily, it seemed that the number of genes per subfamily was more or less established before the divergence of these mammals. A subfamily that was small in one mammal (*e.g.*, the CfOLF1 subfamily) tended to be small in other mammals, and a subfamily that was large in one mammal (*e.g.*, the CfOLF4 subfamily) tended to be large in other mammals. There was a slightly smaller repertoire of olfactory receptor genes in these subfamilies in the microsmatic human relative to macrosmatic mammals. However, the numbers of genes in mammals such as the dog and cat were about the same as in sea lion, which has a relatively undeveloped olfactory apparatus. It would be interesting to ex-

amine the subfamily sizes in other mammals with less complex noses, such as other pinnipeds, nonhuman primates, and whales, to further investigate the link between size of the gene family and olfactory ability (MOULTON 1967; STODDART 1980). Thus far we are more impressed by the similarities in gene number of each subfamily than by the differences.

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