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High sequence similarity within *ras* exons 1 and 2 in different mammalian species and phylogenetic divergence of the *ras* gene family

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Abstract. We have determined the canine and feline *N-*, *K-*, and *H-ras* gene sequences from position +23 to +270 covering exons I and II which contain the mutational hot spot codons 12, 13, and 61. The results were used to assess the degree of similarity between *ras* gene DNA regions containing the critical domains affected in neoplastic disorders in different mammalian species. The comparative analyses performed included human, canine, feline, murine, rattine, and, whenever possible, bovine, leporine (rabbit), porcelline (guinea pig), and mesocricetine (hamster) *ras* gene sequences within the region of interest. Comparison of feline and canine nucleotide sequences with the corresponding regions in human DNA revealed a sequence similarity greater than 85% to the human sequence. Contemporaneous analysis of previously published *ras* DNA sequences from other mammalian species showed a similar degree of homology to human DNA. Most nucleotide differences observed represented synonymous changes without effect on the amino acid sequence of the respective proteins. For assessment of the phylogenetic evolution of *ras* gene family, a maximum parsimony dendrogram based on multiple sequence alignment of the common region of exons I and II in the *N-*, *K-*, and *H-ras* genes was constructed. Interestingly, a higher substitution rate among the *H-ras* genes became apparent, indicating accelerated sequence evolution within this particular clade. The most parsimonious tree clearly shows that the duplications giving rise to the three *ras* genes must have occurred before the mammalian radiation.

Introduction

Three functional *ras* genes termed *H-ras, K-ras,* and *N-ras* have been identified in humans and other mammals. The human genes vary greatly in length owing to large differences in the size of their introns, ranging from 4.5 kb for the smallest intron (*H-ras*) to 50 kb for the largest intron (*K-ras*), but they each have 4 exons with similar nucleotide sequences. The human *K-ras* gene differs from the other two in having two alternative fourth protein-coding exons, permitting synthesis of two isomorphic proteins with different C-termini (McGrath et al. 1983). Additionally, all known mammalian *ras* genes possess a 5′ non-coding exon. The promoter regions are characterized by the absence of a TATA-box, by high G-C content and multiple GC boxes, features resembling the promoters of housekeeping genes (Lowndes et al. 1989; Yamamoto and Perucho 1988; Ishii et al. 1986; Hall and Brown 1985). *Ras* genes encode closely related proteins of 189 amino acids (or 188 when the alternative exon of *K-ras* is used) with a molecular weight of 21 kDa and are thus referred to as p21. RAS proteins, which appear to be expressed in all cell types, are localized at the inner plasma membrane where they are involved in signal transduction from the extracellular environment to the cytoplasm. This mechanism is initiated by binding of guanosine triphosphate (GTP) to a specific RAS protein domain, leading to a change of conformation from inactive guanosine diphosphate (GDP)-bound to an active GTP-bound state (Satoh and Kaziro 1992). RAS proteins displaying mutations at specific GTP-binding domains either have reduced GTPase activity (if amino acids 12, 13, 59, 61 are involved) or show increased dissociation rate of guanine nucleotides (if amino acids 116, 117, 119, or 146 are affected). Mutations at these domains lead to continuous signal transduction by facilitating accumulation of constitutively active, GTP-bound RAS protein (Barbacid 1990), thus contributing to a malignant cell phenotype.

In naturally occurring (that is, non-experimental) neoplasms and experimentally induced animal tumors, the localization of *ras* mutations has been restricted to exons I and II, and most often to codons 12, 13, and 61. In this paper we present comparative data on the functionally critical *ras* sequences in different mammalian species and discuss the evolutional pattern of the *ras* genes.

Materials and methods

DNA/RNA extraction. Genomic DNA (gDNA) from peripheral blood was isolated as described previously (Miller et al. 1988). The extraction of total cellular RNA was performed according to a standard method of Chomczynski and Sacchi (1987). First-strand complementary DNA (cDNA) was prepared from total RNA with random hexamer primers and retroviral reverse transcriptase (Gibco, BRL, Gaithersburg, MD).

Polymerase chain reaction (PCR). PCR primers for the amplification of the *N-*, *K-* and *H-ras,* fragments were designed on the basis of previously published human and mammalian sequencing data. The specific oligonucleotides and the PCR conditions used to generate *N-ras* exon I and exon II fragments have been described previously (Watzinger et al. 1994).

Synthetic oligonucleotide primers used in amplification of *K-* and *H-ras* sequences were as follows: *K-ras* Ia S, 5′-gAC TgA ATA TAA ACT TgT gg-3′, and *K-ras* Ia AS, 5′-CTA TTg TTg gAT CAT ATT Cg-3′ generating a 107-bp fragment from exon I; *K-ras* IIa S, 5′-ATT CCT ACA ggA AgC AAg-3′, and *K-ras* IIa AS, 5′-CTA TAA Tgg TgA ATA TCT TC-3′ generating a 178-bp fragment from exon II; *H-ras* Ia S, 5′-gAC ggA ATA TAA TCT ggT-3′, and *H-ras* Ia AS, 5′-TCg ATg gTg ggg TCg TAC TC-3′ generating a 108-bp fragment from exon I; *H-ras* IIa S, 5′-gAC TCC TAT Cgg AAg CAA gT-3′, and *H-ras* IIa AS, 5′-CCT gTA CTg gTg gAT gTC C-3′ generating a 181-bp fragment from exon II. Conditions of amplification were as described earlier (Watzinger et al. 1994), the only modification being an adaptation of the annealing temperature for *K-ras* exons I and II and *H-ras* exon I to 50°C, and for *H-ras* exon II to 60°C.

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers U62088, U62089, U62090, U62091, U62092, U62093 and U62094.

K-RAS

	ATG	ACT	GAA	TAT	AAA	CTT	GTG	GTA	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGT	GCC	TTG	ACG
																				60
human																				
canine	nnp	ppp	ppp	ppp	ppp	ppp	ppp	p**	***	***	***	***	***	***	***	***	***	***	***	***
feline	nnp	ppp	ppp	ppp	ppp	ppp	ppp	p**	***	***	***	***	***	***	***	***	***	***	***	***
murine	***	***	**G	***	***	***	***	**G	***	***	***	***	***	***	***	***	**C	***	***	***
rattine	***	***	**G	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***

	ATA	CAG	CTA	ATT	CAG	AAT	CAT	TTT	GTG	GAC	GAA	TAT	GAT	CCA	ACA	ATA	GAG↓	GAT	TCC	TAC
																				120
human																				
canine	***	***	***	***	***	***	**C	***	***	**T	***	***	***	**T	***	***	***	***	***	***
feline	***	***	***	***	***	***	**C	***	***	**T	***	***	***	**T	***	***	***	***	***	***
murine	***	***	***	***	***	***	**C	***	***	**T	**G	**C	**C	**T	**G	***	***	**C	***	***
rattine	***	***	***	***	***	***	**C	***	***	**T	***	***	***	**T	**G	***	***↑	**C	***	***

	AGG	AAG	CAA	GTA	GTA	ATT	GAT	GGA	GAA	ACC	TGT	CTC	TTG	GAT	ATT	CTC	GAC	ACA	GCA	GGT
																				180
human																				
canine	***	**A	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
feline	***	**A	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
murine	***	**A	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
rattine	***	**A	***	***	***	***	C**	***	***	***	***	***	***	***	***	***	***	***	***	***

	CAA	GAG	GAG	TAC	AGT	GCA	ATG	AGG	GAC	CAG	TAC	ATG	AGG	ACT	GGG	GAG	GGC	TTT	CTT	TGT
																				240
human																				
canine	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
feline	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	**C
murine	***	***	***	***	***	***	***	***	***	***	***	***	**A	***	***	***	***	***	***	***
rattine	***	***	***	***	***	***	***	***	***	***	***	***	**A	***	***	***	***	***	***	***

	GTA	TTT	GCC	ATA	AAT	AAT	ACT	AAA	TCA	TTT	GAA	GAT	ATT	CAC	CAT	TAT	AG
																	290
human																	
canine	***	***	***	***	***	***	***	***	***	***	ppp	ppp	ppp	ppp	ppp	ppp	pp
feline	***	***	***	***	***	***	***	***	***	***	ppp	ppp	ppp	ppp	ppp	ppp	pp
murine	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	**
rattine	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	**

N-RAS

	ATG	ACT	GAG	TAC	AAA	CTG	GTG	GTG	GTT	GGA	GCA	GGT	GGT	GTT	GGG	AAA	AGC	GCA	CTG	ACA
																				60
human																				
canine	nnp	ppp	ppp	ppp	ppp	ppp	ppp	p**	***	***	***	***	***	***	***	***	***	***	***	***
feline	nnp	ppp	ppp	ppp	ppp	ppp	ppp	p**	***	***	***	***	***	***	***	***	***	***	***	***
murine	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	**C	***	**G
rattine	***	***	***	***	***	***	***	***	***	***	***	***	**C	***	***	***	**T	**T	T**	***
cavia porc.	***	***	***	**T	***	***	***	***	***	***	***	***	***	**C	***	***	**T	***	***	**C

	ATC	CAG	CTA	ATC	CAG	AAC	CAC	TTT	GTA	GAT	GAA	TAT	GAT	CCC	ACC	ATA	GAG↓	GAT	TCT	TAC
																				120
human																				
canine	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
feline	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
murine	***	***	***	***	***	***	***	***	**G	***	***	***	***	***	***	***	***	***	***	***
rattine	***	***	***	***	***	***	***	***	**G	***	***	***	***	***	***	***	***	***	***	***
cavia porc.	***	***	***	**T	***	***	***	***	**C	***	***	***	***	***	***	***	***↑	***	***	***

	AGA	AAA	CAA	GTG	GTT	ATA	GAT	GGT	GAA	ACC	TGT	TTG	TTG	GAC	ATA	CTG	GAT	ACA	GCT	GGA
																				180
human																				
canine	C**	***	**G	***	***	***	**C	***	***	***	***	C**	***	***a	***	***	***	***	***	**T
feline	C**	***	**G	***	***	***	**C	***	***	***	***	C**	***	***	***	***	***	***	***	**T
murine	C**	**G	***	***	**G	**T	***	***	**G	***	**C	C**	C**	***	***	***	**C	***	***	***
rattine	C**	***	***	***	**G	**T	**C	***	**G	***	***	C*A	C**	***	***	***	**C	***	***	***
cavia porc.	C**	***	**G	***	***	***	***	***	***	**T	***	C**	***	**T	**T	***	***	***	***	***

	CAA	GAA	GAG	TAC	AGT	GCC	ATG	AGA	GAC	CAA	TAC	ATG	AGG	ACA	GGC	GAA	GGC	TTC	CTC	TGT
																				240
human																				
canine	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
feline	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
murine	***	**G	***	***	***	***	***	***	***	**G	***	***	***	***	***	***	**G	***	***	***
rattine	***	**G	***	***	***	***	***	***	***	***	***	***	***	***	***	***	**G	***	***	***
cavia porc.	***	**G	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***

	GTA	TTT	GCC	ATC	AAT	AAT	AGC	AAG	TCA	TTT	GCG	GAT	ATT	AAC	CTC	TAC	AG
																	290
human																	
canine	***	***	***	***	***	***	***	**A	***	***	ppp	ppp	ppp	ppp	ppp	ppp	pp
feline	***	***	***	***	**C	***	***	**A	***	***	ppp	ppp	ppp	ppp	ppp	ppp	pp
murine	***	***	***	***	***	***	***	**A	***	***	**A	***	***	***	***	***	**
rattine	**G	***	***	***	***	***	***	**A	**C	***	**A	***	***	***	***	***	**
cavia porc.	**G	***	***	***	***	***	***	**A	***	***	**A	***	***	***	***	***	**

Fig. 1. Comparative nucleotide sequence analysis of human and other mammalian *K-*, *N-* and *H-ras* proto-oncogenes from position +23 to +270. The nucleotide sequences of feline and canine *K-*, *N-* and *H-ras* proto-oncogenes including exons I and II are shown together with the corresponding DNA regions of human and other mammalian species from which sequence information was available. Identical nucleotides at corresponding positions are indicated by asterisks; codons containing substitutions are boxed. Most of the nucleotide differences between different species represent synonymous changes without effect on the amino acid sequence of *RAS* p21, the only exceptions being bovine *H-ras* residues 86 and 87. n = non-identified nucleotides; p = primer nucleotides. Vertical arrows show the exon boundaries. (*Cavia porcellus* = guinea pig; *Mesocricetus* auratus = golden hamster). (*Fig. 1 continued on next page.*)

H-RAS

	60																			
human	ATG	ACG	GAA	TAT	AAG	CTG	GTG	GTG	GTG	GGC	GCC	GGC	GGT	GTG	GGC	AAG	AGT	GCG	CTG	ACC
canine	ppp	ppp	ppp	ppp	ppp	ppp	pp*	***	***	***	**T	**A	**C	***	**G	***	**C	**C	***	***
feline	ppp	ppp	ppp	ppp	ppp	ppp	pp*	***	***	***	**T	**A	***	***	**G	***	***	**C	***	***
murine	***	**A	***	**C	***	**T	***	***	***	***	**T	**A	**C	***	**A	***	***	**C	***	***
rattine	**A	***	**C	***	**T	***	***	***	***	***	**T	**A	**C	***	**A	***	***	**C	***	***
mesocric.	***	**A	***	**C	***	**C	***	***	***	***	**T	**A	**C	***	**A	***	***	**C	***	***
leporine	***	**G	***	***	***	***	***	***	***	***	***	***	***	**A	***	***	**C	**A	***	***
bovine	***	**G	***	***	**C	***	***	***	***	***	***	**T	**C	***	**G	***	**C	**C	***	**T

	120																			
human	ATC	CAG	CTG	ATC	CAG	AAC	CAT	TTT	GTG	GAC	GAA	TAC	GAC	CCC	ACT	ATA	GAG ↓	GAT	TCC	TAC
canine	***	***	**C	***	***	***	**C	**C	***	**T	**G	***	***	***	**C	**C	***	***	***	***
feline	***	***	**C	***	***	***	**C	**C	***	**T	**G	**T	***	***	**C	**C	***	**C	***	**T
murine	***	***	***	***	***	***	**C	***	***	***	**G	**T	**T	***	***	***	***	**C	***	***
rattine	***	***	***	***	***	***	***	***	***	***	**G	**T	**T	***	***	***	***	**C	***	***
mesocric.	***	***	***	***	***	***	**C	**C	***	***	**G	**T	***	***	**C	***	***	***	***	***
leporine	***	***	***	***	***	***	**C	**C	***	***	***	**T	***	***	**C	**C	***	**C	**G	***
bovine	***	***	**C	**T	***	**T	**C	**C	***	***	**G	***	***	***	**C	**C	***↑	**C	***	***

	180																			
human	CGG	AAG	CAG	GTG	GTC	ATT	GAT	GGG	GAG	ACG	TGC	CTG	TTG	GAC	ATC	CTG	GAT	ACC	GCC	GGC
canine	***	***	***	***	***	***	**C	***	***	***	***	T**	***	***	***	***	**C	**A	**G	***
feline	***	***	**A	***	**T	***	***	**C	***	***	***	**A	C**	***	**T	T**	**C	**G	**G	***
murine	***	**A	***	***	***	***	***	***	***	**A	**T	**A	C**	***	***	T*A	**C	**A	**A	**T
rattine	***	**A	***	**A	***	***	***	***	***	***	**T	T*A	C**	***	***	T*A	**C	**A	**A	**T
mesocric.	***	**A	***	***	***	***	***	***	***	**A	**T	***	C**	***	***	T*A	***	**A	**A	***
leporine	***	***	***	***	***	**C	**C	***	***	***	***	***	C**	***	***	***	**C	**G	**G	***
bovine	***	***	**A	***	***	**C	***	***	***	***	***	***	C**	***	***	***	**C	**G	**G	***

	240																			
human	CAG	GAG	GAG	TAC	AGC	GCC	ATG	CGG	GAC	CAG	TAC	ATG	CGC	ACC	GGG	GAG	GGC	TTC	CTG	TGT
canine	***	***	***	***	***	***	***	***	***	***	***	***	***	**G	***	***	***	**T	**C	***
feline	***	***	***	**T	***	***	***	***	***	***	***	***	***	**T	**A	**A	***	***	**C	***
murine	**A	**A	***	**T	**T	***	***	***	***	***	***	***	***	**A	***	***	***	***	**C	***
rattine	**A	**A	***	**T	**T	***	***	***	***	***	***	***	***	**A	***	***	***	***	**C	***
mesocric.	**A	***	***	***	**T	***	***	A**	***	***	***	***	***	**A	***	***	***	***	**C	***
leporine	***	***	***	***	***	***	***	***	***	***	***	***	***	**G	**C	***	***	***	**C	**C
bovine	***	***	**A	***	***	***	***	**A	***	***	***	***	***	***	***	***	***	**T	**C	**C

	290																
human	GTG	TTT	GCC	ATC	AAC	AAC	ACC	AAG	TCT	TTT	GAG	GAC	ATC	CAC	CAG	TAC	AG
canine	**A	***	***	***	***	***	***	***	***	***	**p	ppp	ppp	ppp	ppp	ppp	pp
feline	***	***	***	***	***	**T	***	***	***	***	**p	ppp	ppp	ppp	ppp	ppp	pp
murine	**A	***	***	***	***	***	***	***	**C	**C	***	***	***	**T	***	***	**
rattine	**A	***	***	***	***	***	***	***	**C	***	**A	***	***	**T	***	***	**
mesocric.	***	**C	***	***	***	***	***	***	**C	***	**A	***	***	**T	***	***	**
leporine	***	**C	***	***	***	***	***	***	**C	**C	***	***	***	***	***	***	**
bovine	***	***	**T	***	***	C**	GT*	***	**C	**C	***	***	***	***	***	***	C*

Fig. 1. *Continued.*

Direct sequencing. The PCR-amplified fragments were purified by commercial DNA purification columns (QIAquick or QIAex, QIAGEN, Hilden, FRG), and directly sequenced by the dideoxy chain-termination procedure according to the method of Casanova and coworkers (1990).

For the annealing reaction, 10 pmol of 5'end-labeled primers identical to one of the PCR primers and 0.2 pmol of PCR template were heated to 98°C for 5 min and immediately chilled on ice. Chain elongation and termination by incorporation of dideoxynucleotides were performed with a Sequenase kit (Sequenase™ Version 2.0, USB, Cleveland, OH). The sequencing products were run and displayed on 8% polyacrylamide gels, containing 7 M urea. After electrophoresis, gels were dried and autoradiographed overnight at –70°C.

Sequence data analysis. The sequences determined in the present study are registered under the following GenBank accession numbers: feline (*Felis catus*) *H-ras* (U62088), feline *K-ras* (U62089), feline *N-ras* (U62090), feline *N-ras* pseudogene (U62091); canine (*Canis familiaris*) *H-ras* (U62092), canine *K-ras* (U62094), canine *N-ras* (U62093). Additional *ras* sequences were used for sequence comparison: human (*Homo sapiens*) *H-ras* (J00277) (Capon et al. 1983), *K-ras* (M54968) (Kahn et al. 1987), *N-ras* (K00082) (Taparowsky et al. 1983); murine (*Mus musculus*) *H-ras* (Z50013) (Przybojewska and Plucienniczak 1996), *K-ras* (X02452 and X02453) (George et al. 1985), *N-ras* (X13664) (Chang et al. 1987); rattine (*Rattus norvegicus*) *H-ras* (M13011) (Ruta et al. 1986), *K-ras* (U09793) (Higinbotham et al. 1994), *N-ras* (X68394) (Van Kranen et al. 1994); canine *K-ras* (Kraegel et al. 1992), *N-ras* (Saunders et al. 1992);

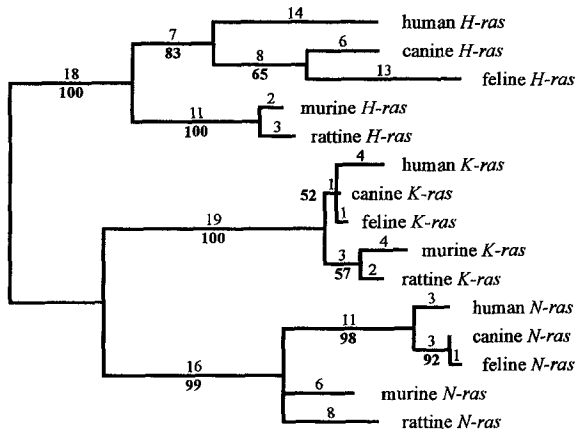

Fig. 2. Relationships among *H-*, *K-* and *N-ras* sequences from different mammals. The most parsimonious tree indicating type-specific clustering of the *H-ras, K-ras* and *N-ras* genes is based on a 248-bp section from exons I and II (tree length = 164, consistency index = 0.665). Numbers of nucleotide substitutions are indicated above the branches; bootstrap values are given below. The tree is unrooted.

A)

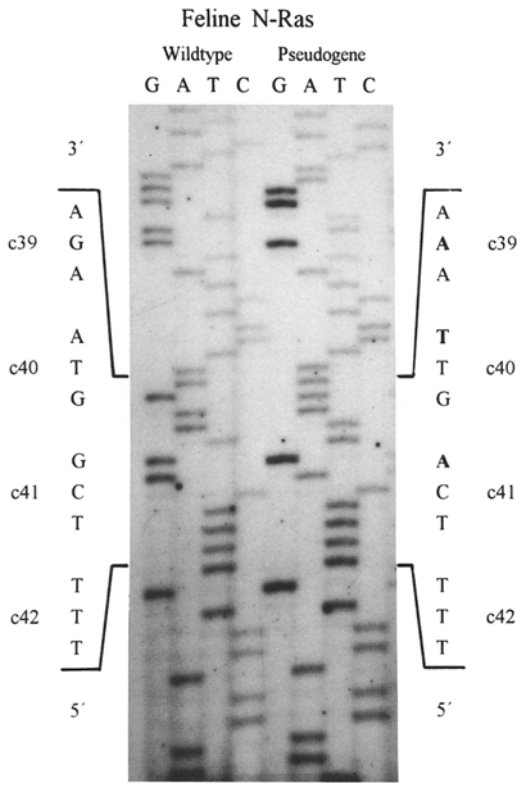

Fig. 3. Analysis of the feline *N-ras*-proto-oncogene and pseudogene. **(A)** Both feline *N-ras* wild-type gene and pseudogene were characterized by direct sequencing of antisense strands. The autoradiograph shows a comparison between antisense strand sequences of the wild-type gene and the pseudogene. Nucleotide differences from the wild-type gene are indicated in bold letters. **(B)** Comparative nucleotide sequence analysis of the feline *N-ras*-proto-oncogene and pseudogene from position +23 to +270. Homologies between the sequences are indicated by asterisks; codons displaying differences are boxed. Vertical arrows show the boundary between exons I and II of the proto-oncogene. Most of the nucleotide differences in the pseudogene represent missense mutations. Codon 41 of the pseudogene sequence represents a termination codon (shaded) owing to a C→T transition in the first codon position.

B)

***N-RAS* PSEUDOGENE**

																			54	
feline *N-ras*	nnp	ppp	ppp	ppp	ppp	ppp	ppp	pTG	GTT	GGA	GCA	GGT	GGT	GTT	GGG	AAA	AGC	GCA	
pseudogene	nnp	ppp	ppp	ppp	ppp	ppp	ppp	p**	*CA	***	***	***	***	***	***	***	***	A**	
																			108	
feline *N-ras*	CTG	ACA	ATC	CAG	CTA	ATC	CAG	AAC	CAC	TTT	GTA	GAT	GAA	TAT	GAT	CCC	ACC	ATA	
pseudogene	***	***	*C*	***	***	***	***	***	***	***	***	***	***	*G*	***	T**	*A*	***	
																			162	
feline *N-ras*	GAG↓	GAT	TCT	TAC	CGA	AAA	CAG	GTG	GTT	ATA	GAC	GGT	GAA	ACC	TGT	CTG	TTG	GAC	
pseudogene	*** ↑	***	*T*	A**	T**	***	***	***	***	*C*	**T	***	A**	***	***	G*T	C**	***	
																			216	
feline *N-ras*	ATA	CTG	GAT	ACA	GCT	GGT	CAA	GAA	GAG	TAC	AGT	GCC	ATG	AGA	GAC	CAA	TAC	ATG	
pseudogene	***	A**	***	***	***	A**	***	***	**A	***	***	***	***	***	***	***	***	***	
																			270	
feline *N-ras*	AGG	ACA	GGC	GAA	GGC	TTC	CTC	TGT	GTA	TTT	GCC	ATC	AAC	AAT	AGC	AAA	TCA	TTT	
pseudogene	***	***	**T	***	***	***	***	***	***	*G*	***	***	**T	***	***	***	***	***	
							290													
feline *N-ras*	ppp	ppp	ppp	ppp	ppp	ppp	pp													
pseudogene	ppp	ppp	ppp	ppp	ppp	ppp	pp													

hamster (*Mesocricetus auratus*) *H-ras* (M84166) (Ebert et al. 1992); bovine (*Bos taurus*) *H-ras* (X17363) (Campo et al. 1990); rabbit (*Oryctolagus cuniculus*) *H-ras* (X57123 and X57124) (Corominas et al. 1991); guinea pig (*Cavia porcellus*) *N-ras* (M15808) (Doninger et al. 1987). The program CLUSTAL V (Higgins et al. 1992) was employed for multiple sequence alignment. The program PAUP 3.1.1 (Swofford 1993) was used to construct the maximum parsimony dendrogram (heuristic search, branch-swapping algorithm: tree-bisection-reconnection). The tree is based on a 248-bp section covering nt positions 23-270. The primer binding sites were omitted from the analysis.

Results and discussion

Conservation of ras exon I and II sequences in different mammalian species. In the present study, we have determined the nucleotide sequences of exons I and II (from position +23 to +270) of canine and feline *N-*, *K-* and *H-ras* genes containing the mutational hot spot codons 12, 13, and 61 by direct sequencing of enzymatically amplified genomic DNA. Exon-intron boundaries of feline and canine sequences were determined by sequencing of complementary DNA.

The feline and canine sequences of the first two *N-*, *K-* and *H-ras* exons are shown in Fig. 1 together with the corresponding human sequences and sequences of other mammalian species. The nucleotide similarity of the feline and canine sequences to human sequences varies between 100% for *N-ras* exon I, 96% for exon II, 97% for *K-ras* exon I, 99% for exon II, 88% (feline) and 85% (canine) for *H-ras* exon I, 89% (feline) and 93% (canine) for exon II, with the majority of nucleotide differences at the third position of various codons. The few nucleotide differences to human *ras*

sequences located at other positions within individual codons included an A→C transversion at position +121 corresponding to amino acid 41, a T→C transition at position +154 corresponding to amino acid 52 of the feline and canine *N-ras* genes, and a C→T transition at position +154 corresponding to amino acid 52 of the canine *H-ras* gene. However, all differences in the canine and feline *ras* genes to the corresponding human sequences represented synonymous changes, with no effect on the amino acid sequences of the RAS proteins. Additionally, the conservation of these gene regions was confirmed by comparison of human, canine and feline nucleotide sequences of *ras* exons I and II to previously published sequences of other mammalian species (Fig. 1). The comparative sequence analysis revealed that most of the RAS-encoded proteins have identical amino acid sequences within portions of the *ras* proteins encoded by exons I and II. The only differences detected were in codons 86 and 87 in the bovine *H-ras* sequence, where asparagine and threonine are replaced by histidine and valine, and in codon 47 in the rattine *K-ras* sequence, where aspartic acid is replaced by histidine. These changes, however, do not involve any of the critical domains of p21, and are therefore unlikely to affect the function of the protein.

Phylogenetic divergence of the ras gene family. Corresponding *ras* gene segments from five mammalian species were aligned, and a maximum parsimony dendrogram was constructed to reveal the relationship among the sequences. The sequences of *H-ras, K-ras,* and *N-ras* revealed distinct clusters, each consisting of the respective gene from the five species analyzed (Fig. 2). Within each cluster, the branching pattern was in accordance with phylogenetic affinities of the species. The type-specific clustering of the *ras* sequences indicates that the differentiation among these three genes clearly precedes the radiation of mammals. Since no adequate outgroup sequence from a more distantly related organism is available, it is not possible to root the tree. Midpoint rooting can not be applied because the substitution rates on the different branches are not uniform (see below). Therefore, the succession of duplication events giving rise to the three extant *ras* genes can not be determined unequivocally on the basis of the present data. In view of the fact that the differentiation within each of the three clusters started simultaneously at the time of species divergence, the tree lengths are expected to be uniform. In contrast to this notion, we observed significant differences among the tree lengths of the three clusters: *H-ras* (64 substitutions), *K-ras* (15 subst.), *N-ras* (32 subst.). The higher substitution rate within the *H-ras* cluster is also revealed by pairwise comparison of different *ras* genes. The average nucleotide similarity within the *K-ras* and *N-ras* clusters was rather high: 97.5% for *K-ras* and 94.8% for *N-ras.* This result suggests strong selective conservation of these genes over a long evolutionary period. By contrast, the average similarity among the *H-ras* genes of the species investigated was only 89.8%. The higher degree of divergence among the *H-ras* genes is difficult to explain. Functional differentiation or relaxed selective constraints can be ruled out because all the substitutions are synonymous. It is possible, however, that the *H-ras* gene or at least the section studied represents a mutational hot spot.

N-ras pseudogene in feline DNA. In the feline *N-ras* gene, PCR amplification with primers flanking the first two exons yielded fragments of identical size when genomic DNA or complementary DNA was used as template. Sequence analysis of the amplified gDNA fragment confirmed the lack of an intron between exons I and II and revealed the presence of silent mutations, missense mutations, and a stop codon at position 41 (Fig. 3). These findings strongly suggest the presence of an *N-ras* pseudogene in the feline genome. Although the primer binding sites in the wild-type gene and the pseudogene must be very similar, we could not detect any background of pseudogene sequence by direct sequencing of gDNA fragments from feline *N-ras* exons I and II. This result can be attributed to different methodological features including more efficient primer annealing to the wild-type sequence owing to a higher degree of complementarity, and the sensitivity of the sequencing reaction, which can reveal the presence of a given sequence only if it constitutes more than 10% of the template DNA. Processed, inactivated *ras* pseudogenes were already described in the human (*Ha-ras 2* and *Ki-ras 1*) (McGrath et al. 1983; Miyoshi et al. 1984), bovine (*Ha-ras 3*) (McCaffery et al. 1989), rattine (*N-ras*) (Van Kranen et al. 1994), and murine (*N-ras*) (Chang et al. 1987) genome. To our knowledge, this is the first pseudogene in the feline genome described.

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