

# Cloning of the Cat TSH Receptor and Evidence Against an Autoimmune Etiology of Feline Hyperthyroidism

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Cats are the only nonhuman mammalian species with a high incidence of hyperthyroidism, and a better understanding of the pathogenesis of feline hyperthyroidism is of clinical relevance for veterinary medicine. The etiology of this disease in cats remains controversial. Both an intrinsic autonomy of growth and function of follicular cells as well as an autoimmune-related mechanism have been proposed. To explore the role of the autologous TSH receptor (TSHR) in the pathogenesis of hyperthyroidism in cats, we cloned the coding sequence of the feline TSHR by RT-PCR. The open reading frame consists of 2292 nucleotides and encodes a 763-amino acid protein, one amino acid less than the human TSHR. Species comparison reveals that the cat TSHR is most closely related to the canine TSHR, with 96% identity and 97% similarity in amino acid sequence. cAMP accumulation, inositol phosphate production, and TSH binding were similar in the feline TSHR,

compared with the human receptor. Analogous to the human TSHR, the cat TSHR also displays basal constitutive activity. To test the possibility that hyperthyroid cats develop antibodies that stimulate the autologous receptor, transfected cells expressing the feline TSHR were treated with sera or purified IgG obtained from 16 hyperthyroid cats. There was no increase in cAMP-dependent luciferase activity in the hyperthyroid cats, suggesting the absence of stimulatory autoantibodies. These sera were also negative for TSH-binding inhibitory Igs in an RRA. At least in the animals included in this study, there is no evidence for the presence of circulating thyroid stimulating factors as a mechanism underlying the pathogenesis of feline hyperthyroidism, and the findings support a model involving intrinsic autonomy of thyroid follicular cell growth and function. (*Endocrinology* 143: 395–402, 2002)

ASIDE FROM HUMANS, cats are the only other mammalian species in which spontaneous thyrotoxicosis occurs relatively frequently, and feline hyperthyroidism is particularly common among aging cats (1–6). The pathogenic mechanisms underlying feline hyperthyroidism remain controversial, as both an autonomous mechanism of growth and function and an autoimmune etiology have been proposed (7, 8).

In Graves' disease, the most prevalent form of hyperthyroidism in humans, excessive hormone production of the thyroid gland is caused by thyroid-stimulating antibodies that bind to the TSH receptor (TSHR) (9). Thyroid autoantibodies against the TSHR were thought to be present in sera of hyperthyroid cats, in studies using indirect immunofluorescence (10). Furthermore, purified IgG preparations from hyperthyroid cats caused a significant increase in [<sup>3</sup>H]-thymidine incorporation into DNA, as well as a strong stimulation of cell proliferation in the Fischer rat thyroid cell line FRTL-5 (11). However, none of these studies provided evidence that the tested sera or IgG directly stimulated hormone production.

In contrast to these observations suggesting a resemblance to human autoimmune hyperthyroidism, histologic and autoradiographic analyses of surgically removed feline thyroids demonstrated that the vast majority of thyrotoxic cats have multinodular goiters (3, 7). The nodules, ranging in size

from less than 1 mm to 3 cm, have an increased propensity for iodine organification and [<sup>3</sup>H]-thymidine incorporation and, in contrast to the surrounding normal parathyroid tissue, seem to be TSH-independent (3, 7, 12). In addition, xenotransplantation studies of goitrous tissue from hyperthyroid cats onto athymic (*nu/nu*) mice revealed that the cells are hyperfunctioning and proliferate autonomously, even when the endogenous TSH of the host is suppressed by concomitant administration of T<sub>4</sub> (7). Goitrous tissue from hyperthyroid cats may thus provide a useful experimental model to elucidate the pathogenesis leading to hyperplasia and hyperfunction (1, 5, 7).

In addition to its important role in thyroid follicular cell function and as a target of stimulatory autoantibodies, the TSHR can also be activated by somatic and germline mutations in the TSHR gene (13, 14). In humans, somatic TSHR mutations have been found in patients with autonomously functioning toxic adenomas (14, 15), whereas germline TSHR mutations are associated with an autosomal dominant syndrome of hereditary toxic thyroid hyperplasia or sporadic congenital hyperthyroidism (14, 16, 17). Mutational analysis of the transmembrane domain of the feline TSHR gene in cases of sporadic and familial feline thyrotoxicosis suggest that TSHR gene mutations are not involved in the pathogenesis of feline hyperthyroidism (18).

In this study, we cloned the coding sequence of the feline TSHR and investigated whether sera or purified IgG from hyperthyroid cats contains stimulatory autoantibodies against the autologous receptor.

Abbreviations: B<sub>max</sub>, Total receptor number; IC<sub>50</sub>, 50% inhibitory concentration; TBII, TSHR-binding inhibitory Igs; TSHR, TSH receptor.

## Materials and Methods

### Cloning of the coding region of the feline TSHR

Total RNA was isolated from cat thyroid tissue using the thiocyanate method. Reverse transcription was performed with 4  $\mu$ g RNA. Two overlapping fragments, A and B, were then amplified by PCR. Fragment A was generated using primers specific for the human TSHR at the 5' untranslated region (–43 to –23 bp, relative to the A of the initiation codon: 5'-GCGATTTCGGAGGATGGAGAA-3'), and a reverse primer covering nucleotides 1595–1575 of the human coding sequence (5'-CCG-GTCCAGGCGCATGGCG-3'). Fragment B was generated with a sense primer covering nucleotides 900–921 (5'-GTGTAATGACAGCAGTAT-TCGG-3') in the human coding sequence and a degenerate primer based on the alignment of the human, mouse, and rat TSHR sequences in the 3' untranslated region (5'-GGGAT/CTGGAA/GT/CGCATA/GT-TCA/TAGAAAC-3'; 2342–2368 in the human sequence). The PCR products were cloned into the TA cloning vector PCR.II (Stratagene, La Jolla, CA), and six clones of each fragment were subjected to direct DNA sequence analysis using FS AmpliTaq DNA polymerase with the ABI prism dye primer cycle sequencing kit, following the protocol of the supplier (PE Applied Biosystems, Foster City, CA). Sequencing products were analyzed using a 373A DNA sequencer (PE Applied Biosystems).

After sequence analysis of fragment A and B, the full-length coding region (–21 to +2315) of the feline TSHR was generated by PCR using *Pfu* polymerase and cloned into the *Xho*I and *Bam*HI sites of the expression vector pSVL (Pharmacia Biotech, Piscataway, NJ). The final construct was verified by direct DNA sequencing.

### Assays of TSHR function: transient expression; cAMP and luciferase assays; determination of inositol phosphates

TSA-201 cells, a clone of human embryonic kidney 293 cells (19), were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Cells were seeded into 12-well plates and transfected using the calcium-phosphate method. The wild-type feline or human TSHR cDNAs were transfected using 500 ng/well plasmids. The wild-type human TSHR cDNA in the expression vector pSVL was a gift from Prof. Gilbert Vassart (Brussels, Belgium). The empty pSVL vector was included as negative control.

For luciferase assays, a cAMP-dependent reporter plasmid (500 ng), containing 846 bp of the human glycoprotein hormone  $\alpha$ -promoter fused to the luciferase gene ( $\alpha$ -846-Luc), was cotransfected with the pSVL vector expressing wild-type feline or human TSHR, or pSVL vector only. Cells were harvested after 48 h for luciferase assays, cAMP, inositol phosphates, and TSH binding studies. Luciferase assays were performed as previously described (20, 21).

For cAMP accumulation, cells were incubated for 1 h in PBS containing 0.5 mM 3-isobutyl-1-methyl-xanthine  $\pm$  10 mIU/ml bovine TSH (Sigma, St. Louis, MO). The medium was then removed, and cAMP was extracted with ice-cold 95% ethanol (22). The cell extracts were dried under vacuum, and cAMP was determined with an enzyme immunoassay (BIOTRAK; Amersham Pharmacia Biotech, Arlington Heights, IL).

For determination of inositol phosphates, cells were incubated with 4 mCi/ml [<sup>3</sup>H]-myoinositol (American Radiolabeled Chemicals, St. Louis, MO) in DMEM supplemented with 3% FBS. The medium was removed after 24 h and replaced by PBS containing 20 mM LiCl for 30 min at 37 C. Cells were then treated with  $\pm$  100 mIU/ml bovine TSH for 30 min. After removal of the medium, ice-cold 0.4-M HClO<sub>4</sub> was added. [<sup>3</sup>H]-Labeled inositol phosphates were isolated by AG1-X8 resin chromatography (23) and measured in a scintillation counter. All experiments were performed at least four times in triplicate; groups were compared by ANOVA (Statview; Microsoft Corp., Redmond, WA).

### TSH-binding

Binding of TSH in transfected TSA cells was measured using <sup>125</sup>I-labeled bovine TSH (20, 21). Cells were incubated for 2 h at 22 C in 400  $\mu$ l assay buffer (222 mM sucrose-supplemented NaCl-free HBSS with 0.5% BSA and 20 mM HEPES, pH 7.4) containing 2–4  $\times$  10<sup>4</sup> cpm (45 mCi) <sup>125</sup>I-bovine TSH (a gift from BRAHMS Diagnostica, Berlin, Germany) in the presence of increasing amounts of unlabeled bovine TSH. Cells were washed with assay buffer and solubilized with 1 N

NaOH. Bound radioactivity was measured, and specific binding was calculated by subtracting nonsaturable binding in the presence of 10<sup>–7</sup> M unlabeled TSH. The binding studies were performed in duplicate and repeated more than six times. Data analysis and computation of the 50% inhibitory concentration (IC<sub>50</sub>) and total receptor number (B<sub>max</sub>) was performed with the Prism 2.0 software (GraphPad Software, Inc., San Diego, CA).

### cAMP-Dependent luciferase assay with serum or purified IgG from hyperthyroid cats

Serum was obtained from cats with untreated hyperthyroidism and patients with Graves' disease. IgGs were purified using a protein A column according to the protocol and with buffers provided by the supplier (Sigma). In brief, the protein column was regenerated with HEPES buffer and a regeneration buffer and then equilibrated with binding buffer at a flow rate of 1 ml/min. A total of 1–2 ml serum mixed with 2–4 ml of the binding buffer were then loaded onto the column with an approximate flow rate of 0.5 ml/min. The column was subsequently rinsed with binding buffer and attached to a desalting cartridge. Elution of the IgGs was performed by passing 5 ml elution buffer through the column and a desalting cartridge. Subsequently, the eluted proteins were dialyzed against PBS overnight, and protein concentration was determined by the Bradford assay. Cells were incubated with serum or increasing amounts of purified IgGs as indicated, for 6–24 h. Luciferase assays were performed as described above.

### Determination of TSHR antibodies by RRA

Determination of TSHR binding inhibitory Igs (TBII) was performed using a commercial RRA, following the protocol of the supplier (Kronus, San Clemente, CA). Serum from patients with Graves' disease and serum samples from the hyperthyroid cats were incubated with <sup>125</sup>I-labeled bovine TSH and solubilized porcine TSHRs and compared with a standard curve established with samples with known amounts of TBII. After incubation, receptor-bound <sup>125</sup>I-labeled TSH was separated from unbound <sup>125</sup>I-labeled TSH.

## Results

### Sequence comparison

The amino acid sequence of the feline TSHR, aligned to all known TSHR sequences, is shown in Fig. 1. The coding sequence has been deposited in GenBank (accession no. AF218264). The feline TSHR is most closely related to the canine receptor, with 96% identity and 97% similarity in amino acid sequence. The amino acid sequence of the cat TSHR is 90% identical and 92% similar to the human TSHR. The feline TSHR lacks glutamate 360, a residue that is conserved in all other species, making its overall length only 763 residues.

The feline sequence reported here differs from the published transmembrane domain sequence of Pearce *et al.* (18) at codons 487 (E487D), 624 (E624K), and 635 (I635M). This may reflect the presence of polymorphisms in the cat TSHR DNA sequence. In DNA extracted from three cell lines obtained from autonomous nodules (Gerber, H., and H. J. Peter; unpublished results), we also found a sequence variation at codon 558 (V558L) in one allele of one cell line. Functional analysis of the V558L TSHR, however, revealed that this conservative amino acid change has no apparent effect on function, and probably represents a simple polymorphism (data not shown).

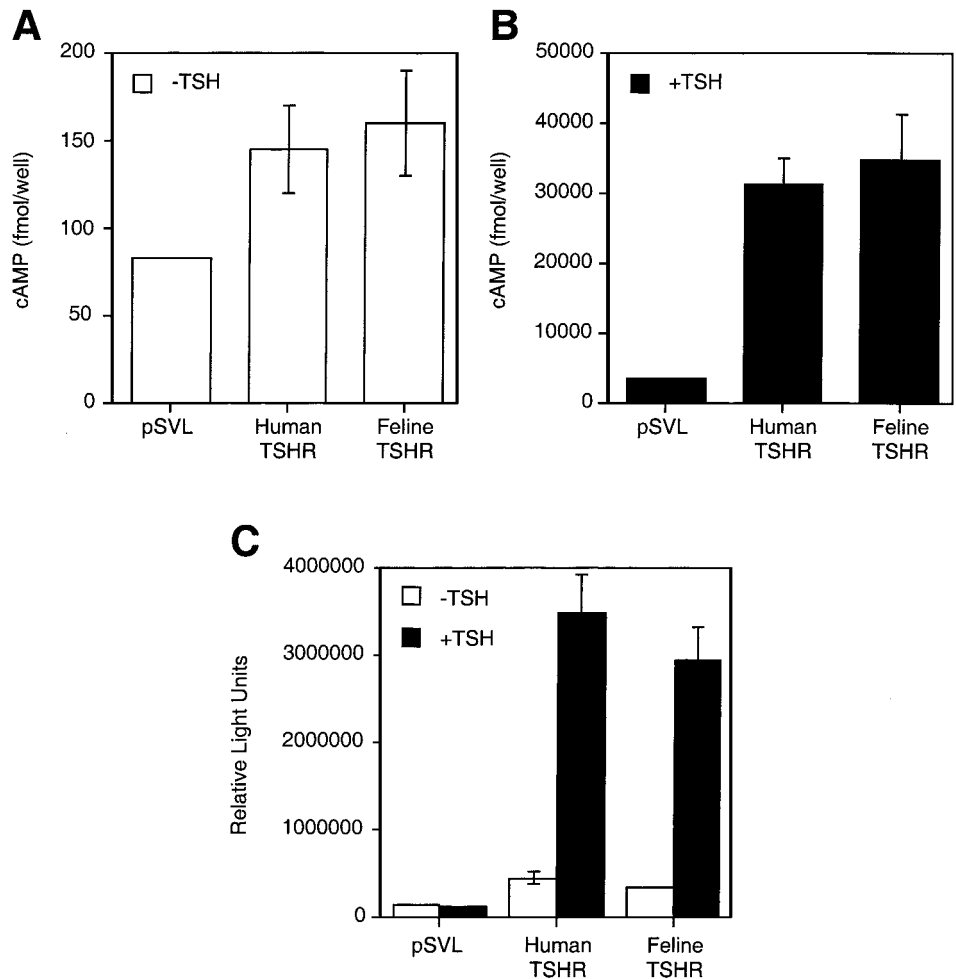
### Functional properties of the feline TSHR

Functional assays revealed that the feline TSHR has characteristics similar to those of the human TSHR (Fig. 2–4).

	10	20	30	40	50	60	70	80	90	100			
Cat	MRQTPLLQLA	LLLSLPRSLG	GKGCPSPPCE	CHQEDDFRVT	CKDIHRIPSL	PPSTQTLKFI	ETHLKTIPSR	AFSNLPLNISR	IYLSIDATLQ	RLESHSFYNI			
Dog	MRPPLLHLA	LLLALPRSLG	GKGCPSPPCE	CHQEDDFRVT	CKDIHRIPSL	PPSTQTLKFI	ETHLKTIPSR	AFSNLPLNISR	IYLSIDATLQ	RLESHSFYNI			
Human	MRPADLLQLV	LLLDLPRDLG	GKGCSSPPCE	CHQEDDFRVT	CKDIQIRPSL	PPSTQTLKLI	ETHLRTIPSH	AFSNLPLNISR	IYVSIDVTLQ	QLESHSFYNI			
Bovine	MRPTPLRLA	LFLVLPSSLG	GERCPSPPE	CRQEDDFRVT	CKDIQISPSL	PPSTQTLKFI	ETHLKTIPSR	AFSNLPLNISR	IYLSIDATLQ	QLESHSFYNI			
Sheep	MRPTPLRLA	LLLVLPSLW	GERCPSPPE	CRQEDDFRVT	CKDIQIRPSL	PPSTQTLKFI	ETHLKTIPSR	AFSNLPLNISR	IYLSIDATLQ	QLESHSFYNI			
Mouse	MRPGSLLLV	LLLALRSRLR	GKECASPPCE	CHQEDDFRVT	CKELHRIPSL	PPSTQTLKLI	ETHLKTIPSL	AFSSLPNISR	IYLSIDATLQ	RLEPHSFYNI			
Rat	MRPGSLLQLT	LLLALPRSLW	GRGCTSPPE	CHQEDDFRVT	CKELHQIPSL	PPSTQTLKLI	ETHLKTIPSL	AFSSLPNISR	IYLSIDATLQ	RLEPHSFYNI			
	110	120	130	140	150	160	170	180	190	200			
Cat	<b>SKMTHIEIRN</b>	TRSLTYIDPG	ALKELPLLKF	LGIFNTGLGV	FPDLTKVYST	DVFFILEITD	NPYMYSIPAN	AFQGLCNETL	TLKLYNNGFT	SIQGHAFNGT			
Dog	<b>SKMTHIEIRN</b>	TRSLTSDPD	ALKELPLLKF	LGIFNTGLGV	FPDVTKVYST	DVFFILEITD	NPYMASIPAN	AFQGLCNETL	TLKLYNNGFT	SIQGHAFNGT			
Human	<b>SKVTHIEIRN</b>	TRNLTVIDPD	ALKELPLLKF	LGIFNTGLKM	FPDLTKVYST	DIFFILEITD	NPYMYSIPVN	AFQGLCNETL	TLKLYNNGFT	SVQGYAFNGT			
Bovine	<b>SKVTHIEIRN</b>	TRSLTYIDSG	ALKELPLLKF	LGIFNTGLRV	FPDLTKIYST	DVFFILEITD	NPYMYSIPAN	AFQGLCNETL	TLKLYNNGFT	SIQGHAFNGT			
Sheep	<b>SKVTHIEIRN</b>	TRSLTYIDSG	ALKELPLLKF	LGIFNTGLRV	FPDLTKIYST	DVFFILEITD	NPYMYSIPAN	AFQGLCNETL	TLKLYNNGFT	SIQGHAFNGT			
Mouse	<b>SKMTHIEIRN</b>	TRSLTYIDPD	ALTELPLLKF	LGIFNTGLRI	FPDLTKIYST	DIFFILEITD	NPYMYSIPAN	AFQGLCNETL	TLKLYNNGFT	SVQGHAFNGT			
Rat	<b>SKMTHIEIRN</b>	TRSLTYIDPD	ALTELPLLKF	LGIFNTGLRI	FPDLTKIYST	DVFFILEITD	NPYMYSIPAN	AFQGLCNETL	TLKLYNNGFT	SIQGHAFNGT			
	210	220	230	240	250	260	270	280	290	300			
Cat	KLDAVYLNKN	KYLTAIDQDA	FGGVYSGPTL	LDVSYTSVTA	LPSKGLEHLK	ELIARNTWTL	KKLPLSLSFL	HLTRADLSYP	SHCCAFKNQK	KIRGILESFM			
Dog	KLDAVYLNKN	KYLSAIDKDA	FGGVYSGPTL	LDVSYTSVTA	LPSKGLEHLK	ELIARNTWTL	KKLPLSLSFL	HLTRADLSYP	SHCCAFKNQK	KIRGILESFM			
Human	KLDAVYLNKN	KYLTVIDKDA	FGGVYSGPSL	LDVSYTSVTA	LPSKGLEHLK	ELIARNTWTL	KKLPLSLSFL	HLTRADLSYP	SHCCAFKNQK	KIRGILESFM			
Bovine	KLDAVYLNKN	KYLTVIDQDA	FAGVYSGPTL	LDISYTSVTA	LPSKGLEHLK	ELIARNTWTL	KKLPLSLSFL	HLTRADLSYP	SHCCAFKNQK	KIRGILQSLM			
Sheep	KLDAVYLNKN	KYLTVIDQDA	FAGVYSGPTL	LDISYTSVTA	LPSKGLEHLK	ELIARNTWTL	KKLPLSLSFL	HLTRADLSYP	SHCCAFKNQK	NIRGILQSLM			
Mouse	KLDAVYLNKN	KYLTAIDNDA	FGGVYSGPTL	LDVSYTSVTA	LPSKGLEHLK	ELIAKNTWTL	KKLPLSLSFL	HLTRADLSYP	SHCCAFKNQK	KIRGILESFM			
Rat	KLDAVYLNKN	KYLTAIDKDA	FGGVYSGPTL	LDVSYTSVTA	LPSKGLEHLK	ELIAKNTWTL	KKLPLSLSFL	HLTRADLSYP	SHCCAFKNQK	KIRGILESFM			
	310	320	330	340	350	360	↓	370	380	390	400		
Cat	<b>CNDS</b> SIRSLR	QRKSVNALNG	PFQDEYEEYL	GDSHAGYKDN	SKFQDTRNS	HYVVFEEQ	DEILGFGQEL	KNPQETLQA	FDSDYDTC	GGNEDMVCPT			
Dog	<b>CNES</b> SIRSLR	QRKSVNTLNG	PFQDEYEEYL	GDSHAGYKDN	SQFQDTRNS	HYVVFEEQ	DEILGFGQEL	KNPQETLQA	FDSDYDTC	GGNEDMVCPT			
Human	<b>CNES</b> SMQSLR	QRKSVNALNS	PLHQEYEENL	GDSIVGYKEK	SKFQDTHNA	HYVVFEEQ	DEILGFGQEL	KNPQETLQA	FDSDYDTC	GDSEDMVCPT			
Bovine	<b>CNES</b> SIRGLR	QRKSASALNG	PFYQYEDL	GDSGAGYKEN	SKFQDTRNS	HYVVFEEQ	DEILGFGQEL	KNPQETLQA	FDSDYDTC	GGSEDMVCPT			
Sheep	<b>CNES</b> ISWGLR	QRKSASALNG	PFYQYEEEDL	GDSGAGYKEN	SKFQDTRNS	HYVVFEEQ	DEILGFGQEL	KNPQETLQA	FDSDYDTC	GGSEDMVCPT			
Mouse	<b>CNES</b> SIRNLR	QRKSVNILLR	PIYQYEEEDP	GDSNIVGYKQN	SKFQDTRNS	HYVVFEEQ	DEIVVFGQEL	KNPQETLQA	FESHYDTC	GGNEDMVCPT			
Rat	<b>CNES</b> SIRNLR	QRKSVNVMRG	PVYQYEEEDL	GDSNIVGYKQN	SKFQDTRNS	HYVVFEEQ	DEILGFGQEL	KNPQETLQA	FDSDYDTC	GGNEDMVCPT			
	410	420	<b>TM1</b>	430	440	450	<b>TM2</b>	460	470	480	490	<b>TM3</b>	500
Cat	KSDEFNPCED	IMGYKFLRIV	<b>VWFVSL</b> LALL	<b>GNVFLV</b> LILL	<b>TSHYKLT</b> VPR	<b>FLMCN</b> LAFAD	<b>FCMGM</b> YLLLI	<b>ASVDLY</b> THSE	YYNHAIDWQT	GPGCNAGGFF			
Dog	KSDEFNPCED	IMGYKFLRIV	<b>VWFVSL</b> LALL	<b>GNVFLV</b> LIVLL	<b>TSHYKLT</b> VPR	<b>FLMCN</b> LAFAD	<b>FCMGM</b> YLLLI	<b>ASVDLY</b> THSE	YYNHAIDWQT	GPGCNTAGFF			
Human	KSDEFNPCED	IMGYKFLRIV	<b>VWFVSL</b> LALL	<b>GNVFLV</b> LILL	<b>TSHYKLT</b> VPR	<b>FLMCN</b> LAFAD	<b>FCMGM</b> YLLLI	<b>ASVDLY</b> THSE	YYNHAIDWQT	GPGCNTAGFF			
Bovine	KSDEFNPCED	IMGYKFLRIV	<b>VWFVSL</b> LALL	<b>GNVFLV</b> LIVLL	<b>TSHYKLT</b> VPR	<b>FLMCN</b> LAFAD	<b>FCMGLY</b> LLLI	<b>ASVDLY</b> TQSE	YYNHAIDWQT	GPGCNTAGFF			
Sheep	KSDEFNPCED	IMGYKFLRIV	<b>VWFVSL</b> LALL	<b>GNVFLV</b> LIVLL	<b>TSHYKLT</b> VPR	<b>FLMCN</b> LAFAD	<b>FCMGLY</b> LLLI	<b>ASVDLY</b> TQSE	YYNHAIDWQT	GPGCNTAGFF			
Mouse	KSDEFNPCED	IMGYRFLRIV	<b>VWFVSL</b> LALL	<b>GNIFVLL</b> LILL	<b>TSHYKLT</b> VPR	<b>FLMCN</b> LAFAD	<b>FCMGM</b> VYLLLI	<b>ASVDLY</b> THSE	YYNHAIDWQT	GPGCNTAGFF			
Rat	KSDEFNPCED	IMGYKFLRIV	<b>VWFVSP</b> MALL	<b>GNVFLV</b> LVLL	<b>TSHYKLT</b> VPR	<b>FLMCN</b> LAFAD	<b>FCMGM</b> VYLLLI	<b>ASVDLY</b> THSE	YYNHAIDWQT	GPGCNTAGFF			
	510	520	530	540	<b>TM4</b>	550	560	570	580	<b>TM5</b>	590	600	
Cat	<b>TVFAS</b> ELSVY	<b>TLTVT</b> ILRW	YAITFAMRLD	RKIRLRHAYA	<b>IMVGG</b> WCCF	<b>LLALL</b> PLVGI	SSYAKVSICL	PMDTETPLAL	<b>AYIIL</b> VLLLN	<b>IVAFI</b> IVCSC			
Dog	<b>TVFAS</b> ELSVY	<b>TLTVT</b> ILRW	YAITFAMRLD	RKIRLRHAYA	<b>IMVGG</b> WCCF	<b>LLALL</b> PLVGI	SSYAKVSICL	PMDTETPLAL	<b>AYIIL</b> VLLLN	<b>IVAFI</b> IVCSC			
Human	<b>TVFAS</b> ELSVY	<b>TLTVT</b> ILRW	YAITFAMRLD	RKIRLRHACA	<b>IMVGG</b> WCCF	<b>LLALL</b> PLVGI	SSYAKVSICL	PMDTETPLAL	<b>AYIVF</b> VLTIN	<b>IVAFI</b> IVCSC			
Bovine	<b>TVFAS</b> ELSVY	<b>TLTVT</b> ILRW	HAITFAMRLD	RKIRLRHAYV	<b>IMVGG</b> WCCF	<b>LLALL</b> PLVGI	SSYAKVSICL	PMDTETPLAL	<b>AYIIL</b> VLLLN	<b>IVAFI</b> IVCSC			
Sheep	<b>TVFAS</b> ELSVY	<b>TLTVT</b> ILRW	YAITFAMRLD	RKIRLRHAYV	<b>IMVGG</b> WCCF	<b>LLALL</b> PLVGI	SSYAKVSICL	PMDTETPLAL	<b>AYIIL</b> VLLLN	<b>IVAFI</b> IVCSC			
Mouse	<b>TVFAS</b> ELSVY	<b>TLTVT</b> ILRW	YAITFAMRLD	RKIRLRHAYT	<b>IMAGG</b> WSCF	<b>LLALL</b> PMVGI	SSYAKVSICL	PMDTETPLAL	<b>AYIIL</b> VLLLN	<b>VVAFV</b> IVCSC			
Rat	<b>TVFAS</b> ELSVY	<b>TLTVT</b> ILRW	YAITFAMRLD	RKIRLRHAYT	<b>IMAGG</b> WSCF	<b>LLALL</b> PMVGI	SSYAKVSICL	PMDTETPLAL	<b>AYIAL</b> VLLLN	<b>VVAFV</b> IVCSC			
	610	620	630	<b>TM6</b>	640	650	660	<b>TM7</b>	670	680	690	700	
Cat	<b>YVKIY</b> ITVRN	PQYNTGDKDT	<b>KIAKR</b> MAVLI	<b>FTDFM</b> CAPI	<b>SFYALS</b> ALMN	KPLITVTNSK	<b>ILLVL</b> FYPLN	<b>SCANP</b> FLYAI	<b>FTKAF</b> QRDVF	ILLSKFGICK			
Dog	<b>YVKIY</b> ITVRN	PQYNTGDKDT	<b>KIAKR</b> MAVLI	<b>FTDFM</b> CAPI	<b>SFYALS</b> ALMN	KPLITVTNSK	<b>ILLVL</b> FYPLN	<b>SCANP</b> FLYAI	<b>FTKAF</b> QRDVF	ILLSKFGICK			
Human	<b>YVKIY</b> ITVRN	PQYNTGDKDT	<b>KIAKR</b> MAVLI	<b>FTDFM</b> CAPI	<b>SFYALS</b> ALIN	KPLITVTNSK	<b>ILLVL</b> FYPLN	<b>SCANP</b> FLYAI	<b>FTKAF</b> QRDVF	ILLSKFGICK			
Bovine	<b>YVKIY</b> ITVRN	PHYNTGDKDT	<b>KIAKR</b> MAVLI	<b>FTDFM</b> CAPI	<b>SFYALS</b> ALMN	KPLITVTNSK	<b>ILLVL</b> FYPLN	<b>SCANP</b> FLYAI	<b>FTKAF</b> QRDVF	ILLSKFGICK			
Sheep	<b>YVKIY</b> ITVRN	PHYNTGDKDT	<b>KIAKR</b> MAVLI	<b>FTDFM</b> CAPI	<b>SFYALS</b> ALMN	KPLITVTNSK	<b>ILLVL</b> FYPLN	<b>SCANP</b> FLYAI	<b>FTKAF</b> QRDVF	ILLSKFGICK			
Mouse	<b>YVKIY</b> ITVRN	PQYNTGDKDT	<b>KIAKR</b> MAVLI	<b>FTDFM</b> CAPI	<b>SFYALS</b> ALMN	KPLITVTNSK	<b>ILLVL</b> FYPLN	<b>SCANP</b> FLYAI	<b>FTKAF</b> QRDVF	ILLSKFGICK			
Rat	<b>YVKIY</b> ITVRN	PQYNTGDKDT	<b>KIAKR</b> MAVLI	<b>FTDFM</b> CAPI	<b>SFYALS</b> ALMN	KPLITVTNSG	<b>VLLVL</b> FYPLN	<b>SCANP</b> FLYAI	<b>FTKAF</b> QRDVF	ILLSKFGICK			
	710	720	730	740	750	760							
Cat	RQAQAYRGQR	VSPKNSTGIQ	VQKVTRNMRQ	SLPNMQDDYE	LLNSHLTPN	KQSHISKEYN	QTVL						
Dog	RQAQAYRGQR	VSPKNSAGIQ	IQKVTRDMRQ	SLPNMQDEYE	LLNSHLTPN	KQQQISKEYN	QTVL						
Human	RQAQAYRGQR	VPPKNSTDIQ	VQKVTHDMRQ	GLHNMEDVYE	LIENSHLTPK	KQQQISEEYM	QTVL						
Bovine	RQAQAYRGQR	VSPKNSTGIR	VQKVPPDVQR	SLPNVQDDYE	LLNSHLTPK	QQDQTSKEYK	RTVL						
Sheep	RQAQAYRGQR	VSSKNSTGIR	VQKVPPDVQR	SLPNVQDDYE	LLNSHLTPK	QQDQTSKEYK	QTVL						
Mouse	RQAQAYQGQR	VCPNNSTGIQ	IQKIPQDTRQ	SLPNMQDTYE	LLNSQLLAPK	LQQQISEEYK	QTVL						
Rat	HQAQAYQAQR	VCPNNTGTRQ	IQKIPQDTRQ	SLPNVQDTYE	PLGSSHLTPK	LGQRISEEYK	QTVL						

FIG. 1. TSHR amino acid sequences. Amino acid (aa) sequence alignment of the feline, canine, human, ovine, murine, and rat TSHRs. The feline (aa 360) and the bovine (aa 329) TSHRs are shorter, by 1 aa, and only have 763 residues. The amino acid sequence of the human TSHR is taken from Libert *et al.* (32) and differs at position 601 (Y601H) from the sequence reported by Nagayama *et al.* (34). Potential N-glycosylation sites are shown in bold and are underlined; the transmembrane domains are shown in bold. The coding sequence has been deposited in GenBank (accession no. AF218264).

FIG. 2. cAMP accumulation and cAMP-dependent luciferase assays. A and B, TSA cells were cotransfected with pSVL vector and wild-type human or feline pSVL TSHRs. cAMP was measured using an enzyme-based immunoassay, 1 h after treatment with medium (A) or medium supplemented with 10 IU/ml bovine TSH (B). Cells transfected with the wild-type human and feline TSHRs show constitutive basal activity (A) and a similar response to TSH (B). Values are the mean  $\pm$  SEM from triplicates. C, TSA cells were cotransfected with pSVL vector, wild-type human or feline pSVL TSHRs, and the  $\alpha$ -846-luc reporter. Cells were treated  $\pm$  TSH (10 mIU/ml for 6 h). Both the human and the feline TSHRs display constitutive basal activity and have a similar increase in response to TSH. Values are the mean  $\pm$  SEM from triplicates.



Basal and TSH-stimulated cAMP accumulation were similar in cells transfected with the wild-type feline TSHR, compared with cells transfected with the wild-type human TSHR (Fig. 2, A and B). Measuring cAMP accumulation after 1 h, there was detectable constitutive basal activity (Fig. 2A), but this was more readily apparent in the cAMP-dependent luciferase assay (Fig. 2C). This observation is consistent with previous studies (20, 21), and it suggests that, in addition to the differences in duration of cAMP accumulation in the two experimental systems, the luciferase assay may be more sensitive because of the integration of amplified downstream signals (24).

Basal inositol phosphate accumulation did not differ between the human and the feline TSHRs. Cells transfected with the feline TSHR displayed a significant increase in inositol phosphate accumulation in response to TSH, albeit to a slightly lesser degree than for the human TSHR (Fig. 3).

The  $IC_{50}$  value for the feline TSHR was  $1.91 \times 10^{-10}$  vs.  $3.61 \times 10^{-10}$  M for the human receptor, suggesting a slightly higher affinity of the cat TSHR for bovine TSH (Fig. 4). The  $B_{max}$  values were  $1.4 \times 10^{-9}$  mol/well for the human and  $1.2 \times 10^{-9}$  mol/well for the feline receptor, suggesting the presence of similar receptor numbers.

#### Stimulation with sera and IgG fractions from hyperthyroid cats

To determine whether hyperthyroid cats contain stimulatory antibodies to their autologous receptor, TSA cells transiently cotransfected with the feline TSHR and a cAMP-dependent luciferase reporter ( $\alpha$ -846-Luc) were treated with sera or purified IgG derived from 16 thyrotoxic cats or from patients with Graves' disease (Fig. 5A). cAMP-dependent luciferase accumulation did not increase in response to exposure of the cells to serum of hyperthyroid cats. In contrast, luciferase activity was significantly increased when cells were treated with sera from patients with Graves' disease or bovine TSH. Transfection results from cells treated with purified IgG derived from two hyperthyroid cats are shown in Fig. 5B. Increasing doses of purified IgG did not stimulate cAMP-dependent luciferase activity. However, there was a significant increase in luciferase activity when cells were treated with bovine TSH.

#### Determination of TSHR antibodies by RRA

Determination of TBII, using an RRA, did not reveal the presence of significant inhibition of  $^{125}I$ -labeled TSH binding to porcine TSHRs, and TBII values were below 13 U/liter in all cats included in the study (data not shown). In contrast,

FIG. 3. Inositol phosphate accumulation. TSA cells were transfected with pSVL vector or wild-type human and feline pSVL TSHRs. Twenty-four hours after transfection, cells were treated with 100 mIU/ml bovine TSH for 30 min. <sup>3</sup>H-labeled inositol phosphates were isolated by AG1-X8 resin chromatography and measured in a scintillation counter. Basal and TSH-stimulated inositol phosphate accumulation did not differ significantly between the human and feline wild-type TSHRs. Values are the mean ± SEM from triplicates.

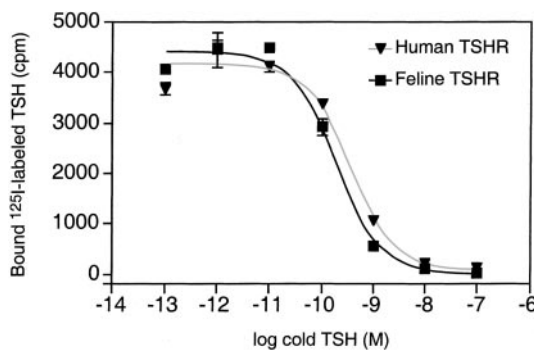
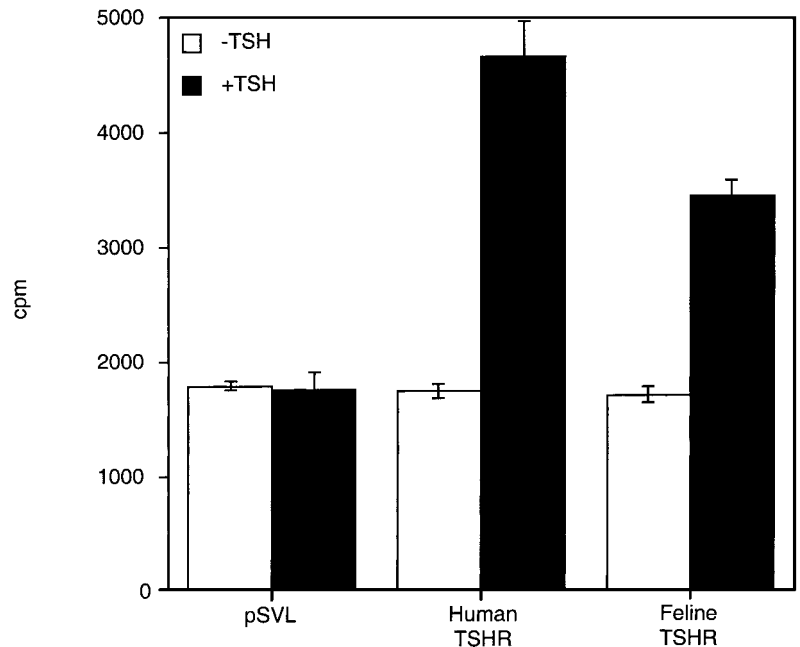


FIG. 4. TSH binding to TSHRs. Binding of TSH in transfected TSA cells was measured using <sup>125</sup>I-labeled bovine TSH and increasing amounts of unlabeled TSH. The IC<sub>50</sub> value for the feline TSHR was 1.91 × 10<sup>-10</sup> vs. 3.61 × 10<sup>-10</sup> M for the human receptor, suggesting a slightly higher affinity of the cat TSHR for bovine TSH. The B<sub>max</sub> values were 1.4 × 10<sup>-9</sup> mol/well for the human and 1.2 × 10<sup>-9</sup> mol/well for the feline receptor, suggesting the presence of similar receptor numbers. Values are the mean ± SEM from duplicates.

control sera values from two patients with Graves' disease were 50 and 80 U/liter, respectively.

### Discussion

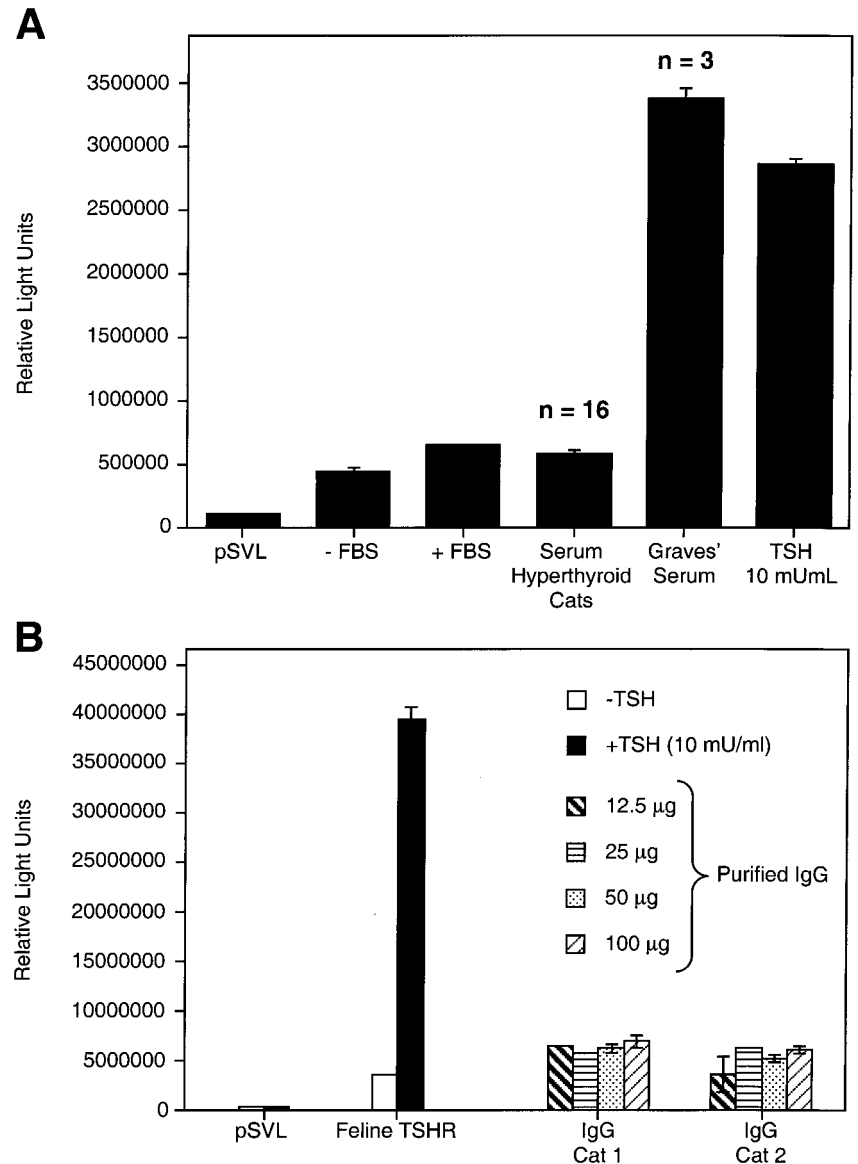
The TSHR has been the subject of intense scrutiny because of its role in thyroid follicular cell physiology and as a target of autoantibodies in human autoimmune thyroid disease (9, 25–27). More recently, it has been the focus of interest because of the detection of gain-of-function mutations that provide a molecular explanation for the development of toxic adenomas, nonautoimmune familial hyperthyroidism, and sporadic nonautoimmune congenital hyperthyroidism (13, 14). Loss-of-function mutations in this receptor are recognized to result in partial or complete resistance to TSH, with compensated or overt hypothyroidism (13, 14, 28).

The cloning of a functional cat TSHR provides an essential tool for further characterizing the molecular mechanisms

underlying feline hyperthyroidism, an important clinical entity in veterinary medicine. The availability of the feline TSHR enabled us to test the effects of sera and purified IgG from hyperthyroid cats on the autologous receptor. All previous studies addressing autoimmune mechanisms in hyperthyroid cats used either rat or porcine tissue. Therefore, it was conceivable that the negative results observed are attributable to discrete differences in epitopes among the different species (29–31). The biochemical properties of the cat TSHR are similar to those of the human TSHR. Transiently transfected mammalian cells expressing the cat TSHR failed to respond to treatments with sera or IgG purified from the hyperthyroid cats included in this study. In contrast, sera from patients with Graves' disease stimulated the feline receptor and resulted in marked increases of cAMP-dependent luciferase activity. These results suggest that the hyperthyroidism in the animals included in this study does not have an autoimmune etiology. Together with the typically multinodular goiter found in cats with hyperthyroidism, this observation is consistent with models proposing an autonomous mechanism of growth and function intrinsic to thyroid follicular cells (1, 3, 12). This observation, however, does not exclude the possibility that the pathogenesis of feline hyperthyroidism may be heterogeneous, as it is the case in humans.

Pearce *et al.* (18) isolated the cDNA of the transmembrane domain of the feline TSHR and analyzed this segment of the receptor for activating mutations that may account for sporadic and familial feline thyrotoxicosis. However, no mutations were found in this region of the TSHR, suggesting that gain-of-function mutations in the TSHR probably are not a common cause of feline hyperthyroidism. Direct sequence analysis of DNA isolated from cell lines obtained from hyperthyroid cats is congruent with the study of Pearce *et al.*, because there were no mutations in the transmembrane domain of the TSHR. The only sequence alteration we detected in DNA obtained from a cat cell line derived from an autonomous nodule, V558L, did not alter the function of the

FIG. 5. Treatment with sera and purified IgG from hyperthyroid cats. A, Cells transiently transfected with the feline TSHR were treated with medium without or with FBS (16 sera from hyperthyroid cats, 3 sera from patients with Graves' disease) or 10 mIU/ml bovine TSH. In contrast to the Graves' sera and bovine TSH, sera from hyperthyroid cats did not result in an increase of cAMP-dependent luciferase activity. The sera from 16 hyperthyroid cats and 3 patients with Graves' disease have been tested individually, and the results have then been pooled. B, Purified IgG from hyperthyroid cats were added in increasing amounts to cells transiently transfected with the feline TSHR. Increasing amounts of purified IgG did not stimulate cAMP-dependent luciferase activity. Values are the mean  $\pm$  SEM from triplicates.



receptor, a finding that is not surprising, given the conservative nature of the amino acid change.

TSHR cDNAs of various mammalian species have been cloned, including man, dog, cow, sheep, rat, and mouse (20, 32–39). Sequence comparison of the TSHRs from various species indicates a high degree of conservation (Fig. 1). A residue that was the focus of particular attention in the human TSHR is Y601, because one of the reported clones was found to have a histidine at this position (34). The other reported human cDNAs (32, 35), as well as all known TSHR sequences, identified a tyrosine at this position (Fig. 1). More recent studies indicated that substitution of tyrosine 601 by histidine results not only in loss of basal constitutive activity but also loss of coupling to Gq/11 (40, 41). Of note, the potential *N*-glycosylation sites are also highly conserved among the various TSHRs, but the human receptor has an additional *N*-glycosylation site at amino acids 113–115 (Fig. 1). The rather discrete differences in the cat TSHR do not seem to be of functional significance. Analogous to the hu-

man TSHR, the cat TSHR displays basal constitutive activity; and at higher doses of TSH, it stimulates the phospholipase C inositol phosphate pathway by coupling to Gq/11 (42).

The human TSHR is unique among the glycoprotein hormone receptors, because it is submitted to cleavage into two subunits (43–46). This intramolecular cleavage seems to involve the removal of a segment of the ectodomain (46–48). Sequence comparison of the various mammalian TSHRs reveals a high degree of conservation at cleavage site 1 (human aa 302–317) and cleavage site 2 (human aa 367–369; Fig. 1). The human receptor differs at positions 306 and 307. In the human receptor, the hydrophobic isoleucine at position 306 is replaced by methionine. At position 307, it contains the uncharged polar glutamine residue, whereas all other TSHRs contain arginine or, in the case of the ovine receptor, the nonpolar tryptophan. Whether these differences in the human receptor are of importance for the phenomenon of cleavage, and thus possibly antigenicity of the receptor, is unclear. Of note, deletion mutants encompassing site 1 shift the cleav-

age site toward the aminotermisus, suggesting that cleavage is not dependent on a specific amino acid sequence but rather occurs at a fixed distance from a protease attachment site (49).

In summary, this study demonstrates that the feline TSHR has biochemical properties similar to those of the human receptor. Sera and purified IgG from hyperthyroid cats do not stimulate the autologous receptor, and, at least in the cats included in this study, the data argue against an autoimmune etiology of their hyperthyroidism. Sequence comparisons of various mammalian TSHR sequences may provide additional insight into discrete differences that may be relevant for the structure of certain antigenic epitopes in the human TSHR and thus the pathogenesis of Graves' disease.

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