cDNA sequence analysis, gene expression and protein localisation of the inhibin α -subunit of Australian brushtail possum (*Trichosurus vulpecula*)

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ABSTRACT

An inhibin α -subunit cDNA sequence from the Australian brushtail possum (*Trichosurus vulpecula*) has been identified and analysed. The cDNA includes an open reading frame encoding a predicted precursor protein of 361 amino acids. The predicted protein sequence includes four possible proteolytic cleavage sites, 12 evolutionarily conserved cysteine residues and three potential N-linked glycosylation sites. The mature α -subunit is the carboxyl terminal fragment (α C) consisting of 131 amino acids. The full-length precursor protein shows a mean identity with eutherian homologues of 69.8%. The homology is not evenly distributed, with the putative α C fragment showing the highest level (79.7%). Using Northern hybridisation, an α -subunit transcript of approximately 1.6 kb was detected in adult possum ovary. Using *in situ*

hybridisation and immunocytochemistry, inhibin α -subunit was localised exclusively to the granulosa cell layers of follicles. Hybridisation and immunostaining for the inhibin α -subunit were first observed in granulosa cells of primary follicles and the expression continued throughout all stages of follicular growth. Inhibin α -subunit mRNA and protein were also detected in cells of the corpus luteum. In summary, results indicate considerable conservation of the structure and possible function of the inhibin α -subunit protein since the divergence of the marsupial and eutherian mammalian lineages. The expression data suggest that, in the adult possum, inhibin may have a role in ovarian follicular growth from the primary stage of development.

Journal of Molecular Endocrinology (1998) **21**, 141–152

INTRODUCTION

The mammalian reproductive cycle is regulated through the actions and interactions of hormones secreted by the hypothalamus, pituitary and gonads (Woodruff *et al.* 1987). Follicular development and spermatogenesis are dependent primarily on the actions of follicle-stimulating hormone (FSH) and luteinising hormone (Richards *et al.* 1986, Steinberger 1971). Inhibin, a protein hormone originally purified from ovarian follicular fluid (Ling *et al.* 1985, Miyamoto *et al.* 1985, Robertson *et al.* 1985), has been shown to exert a negative feedback on FSH secretion (Steinberger & Steinberger 1976, Schwartz & Channing 1977). In

addition to regulating gonadotrophin secretion, inhibin exerts paracrine and autocrine effects within the gonads (Findlay 1993).

Inhibin is a heterodimeric glycoprotein consisting of an α -subunit and a β -subunit, joined by disulphide bridges. Two forms of the inhibin β -subunit, termed β A and β B, have been isolated (Mason *et al.* 1985); in combination with an α -subunit, they form inhibin-A and inhibin-B respectively. In addition, homodimers of the β -subunits form activin, which enhances pituitary FSH production (Ling *et al.* 1986). Inhibin is primarily produced by ovarian granulosa cells (Erickson & Hsueh 1978) and by Sertoli cells of the testis (Steinberger & Steinberger 1976). Inhibin

α -subunit precursor cDNA sequences for six eutherian mammalian species have been published: porcine (Mason *et al.* 1985), human (Mayo *et al.* 1986), bovine (Forage *et al.* 1986), rat (Esch *et al.* 1987, Woodruff *et al.* 1987), murine (Albano *et al.* 1993) and equine (Yamanouchi *et al.* 1995). However, comparatively little is known about the structure, function or localisation of inhibins in marsupials.

In the present paper, a cDNA sequence, the gene expression and the protein localisation of the brushtail possum inhibin α -subunit are reported.

MATERIALS AND METHODS

All experiments were performed with approval granted by the Animal Ethics Committee at Wallaceville Animal Research Centre and in accordance with the 1987 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand.

Reverse transcriptase-PCR

Adult possums were killed and their ovaries were collected and stored at -70°C . Total RNA was extracted using TRIzol Reagent (Gibco BRL, Grand Island, NY, USA) and first-strand cDNA was synthesised using Superscript II reverse transcriptase (Superscript Preamplification System, Gibco BRL). Target cDNA sequences were amplified by PCR. Five microlitres of first-strand cDNA were amplified using 5 U Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany) in a total volume of 50 μl containing 20 pmol of each primer and the manufacturer's recommended buffer. PCR was conducted in a thermal cycler (Perkin Elmer Cetus Corporation, Norwalk, CT, USA) with one cycle (94°C for 1 min), followed by 35 cycles (94°C for 30 s; 60°C for 1 min; 72°C for 2 min) and a final extension at 72°C for 7 min. The PCR primers were designed from conserved nucleotide sequences identified by comparison of eutherian inhibin α -subunit cDNA sequences (human (Mayo *et al.* 1986), mouse (Albano *et al.* 1993), rat (Woodruff *et al.* 1987), bovine (Forage *et al.* 1986), porcine (Mason *et al.* 1985), equine (Yamanouchi *et al.* 1995)): forward primer 5'-CAGGGAGCTGGTGCTGGCCAA-3' (150–170); reverse primer 5'-AGCCAAGCTCTGGAAAGAGA-3' (866–886). The positions of the primers are indicated between brackets and correspond to the respective bases of the cDNA sequence (Fig. 1). A single amplification product of the predicted size (737 bp) was produced and

corresponded to bases 150–886 of the cDNA sequence (Fig. 1).

To obtain the full-length cDNA sequence, gene specific primers (GSPs) were designed for both 3' and 5' RACE (rapid amplification of cDNA ends) (Frohman *et al.* 1988). 3' RACE GSPs: GSP1 5'-TGGTGACACTGCTGGCGATGTC-3' (512–533); GSP2 5'-GCTTGCTGCAGCGTCCATCA-3' (800–819). 5' RACE GSPs: GSP1 5'-CCAGCAGTGTCAC-3' (514–526); GSP2 5'-GTGGACTTCGGCACCCACCTTG-3' (474–495); GSP3 5'-GAGGCCAGTGTGGAACCACAG-3' (451–471). The RACE reaction conditions were as specified by the manufacturer (Gibco BRL).

Cloning and sequencing

PCR amplification products were electrophoresed through 1% (w/v) low melting point agarose gels (SeaPlaque, FMC BioProducts, Rockland, ME, USA), extracted (Qiaquick Gel Extraction Kit, Qiagen, Hilden, Germany), ligated into pGEMTeasy (Promega, Madison, WI, USA) and transformed into competent *Escherichia coli* DH5 α cells (Hanahan 1985). Plasmids having inserts were identified by restriction enzyme digestion and the insert sequences determined using an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). All enzymes were used according to the manufacturer's specifications (Boehringer Mannheim). The full-length cDNA sequence was derived from the original 737-bp fragment and the different 3' and 5' RACE pieces.

Northern hybridisation

Total RNA was extracted from adult possum ovary and liver using TRIzol (Gibco BRL) and 5 μg electrophoresed through a 1% (w/v) agarose gel in denaturing electrophoresis buffer (7.0% (v/v) formaldehyde, $1 \times$ MOPs (20 mM 4-morpholinopropanesulphonic acid pH 7.0, 4.8 mM sodium acetate, 1.0 mM EDTA). RNA transcript size was estimated by comparison with a 0.24–9.5-kb RNA ladder (Gibco BRL) and the positions of 18S and 28S ribosomal RNA bands. Gels were equilibrated in $20 \times$ SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) and RNA was transferred by capillary action to Hybond N membranes (Amersham International plc, Little Chalfont, Bucks, UK). A possum inhibin α -subunit cDNA clone was radiolabelled ($[\alpha\text{-}^{32}\text{P}]\text{dCTP}$) using the Rediprime DNA labelling system (Amersham). The probes and membranes were hybridised in hybridisation buffer (0.5 M Na_2HPO_4 –0.5 M NaH_2PO_4 , pH 7.2, 7% (w/v) SDS, 1 mM EDTA, 10% (w/v)

1	GACAGAGGTC			CAGAAGCTGA			GGGCAACAGG			AAGCTCAGGC			GAGGCGATCC			TATGCAACCC			60		
61	CTG	GCC	ATG	CTG	CCT	CTG	CTG	CTC	CCC	CTG	CAG	CTT	CTG	CTG	CTG	ATG	GTT	ATG	AAG	GGC	120
		Met	Leu	Pro	Leu	Leu	Leu	Pro	Leu	Gln	Leu	Leu	Leu	Leu	Met	Val	Met	Lys	Gly		18
121	GGG	CAT	GGA	TGC	CAG	GGT	CCA	GAA	CTA	GAC	AGG	GAG	CTG	GTG	CTG	GCC	AAA	GTA	AGG	GCA	180
19	Gly	His	Gly	Cys	Gln	Gly	Pro	Glu	Leu	Asp	Arg	Glu	Leu	Val	Leu	Ala	Lys	Val	Arg	Ala	38
			▲	•																	
181	CTG	GTG	CTG	GAT	GCT	TTG	GGC	CCT	CCC	AAT	GCC	TCC	AAG	GAT	GGT	GGC	AAG	CCT	GTG	GCC	240
39	Leu	Val	Leu	Asp	Ala	Leu	Gly	Pro	Pro	Asn	Ala	Ser	Lys	Asp	Gly	Gly	Lys	Pro	Val	Ala	58
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241	CAG	CGC	CTG	ACC	CGC	AGA	CAT	GCC	CAC	ACT	GGG	GGG	TCC	ACA	CGC	AGA	AGC	ATG	GAA	AAT	300
59	Gln	Arg	Leu	Thr	Arg	Arg	His	Ala	His	Thr	Gly	Gly	Ser	Thr	Arg	Arg	Ser	Met	Glu	Asn	78
						▲										▲					
301	GAA	GAT	GAG	GAC	CTT	TCA	CAA	GTC	ATC	CTT	TTC	CCA	ACC	ACA	GGT	CCT	GGC	TGT	GAG	GAT	360
79	Glu	Asp	Glu	Asp	Leu	Ser	Gln	Val	Ile	Leu	Phe	Pro	Thr	Thr	Gly	Pro	Gly	Cys	Glu	Asp	98
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361	GAA	CCA	GAG	GCC	AGA	GCG	GCT	GAG	GGC	CTG	TTT	ACA	TAC	ACA	TTC	AGG	CCT	TCC	CTG	CAT	420
99	Glu	Pro	Glu	Ala	Arg	Ala	Ala	Glu	Gly	Leu	Phe	Thr	Tyr	Thr	Phe	Arg	Pro	Ser	Leu	His	118
421	ACT	CGA	AGT	CGC	CAG	GTG	ACA	GCT	GCC	CAG	CTG	TGG	TTC	CAC	ACT	GGC	CTC	GAC	AAG	GTG	480
119	Thr	Arg	Ser	Arg	Gln	Val	Thr	Ala	Ala	Gln	Leu	Trp	Phe	His	Thr	Gly	Leu	Asp	Lys	Val	138
481	GGT	GCC	GAA	GTC	CAC	AAT	GAC	TCA	GGC	CCC	ATG	GTG	ACA	CTG	CTG	GCG	ATG	TCT	TCG	GGG	540
139	Gly	Ala	Glu	Val	His	Asn	Asp	Ser	Gly	Pro	Met	Val	Thr	Leu	Leu	Ala	Met	Ser	Ser	Gly	158
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541	GGT	CCC	ATG	GCA	GTG	CCA	ATA	TTA	CTG	GGC	CCA	GCC	CCG	CCT	CAC	TGG	GCT	GTG	CTT	CAC	600
159	Gly	Pro	Met	Ala	Val	Pro	Ile	Leu	Leu	Gly	Pro	Ala	Pro	Pro	His	Trp	Ala	Val	Leu	His	178
601	CTG	GCA	GCC	CCT	GCA	TTC	ACA	CTG	CTG	ACC	CGT	CCC	CTC	CTT	GTG	CTG	CTG	CTT	CGC	TGC	660
179	Leu	Ala	Ala	Pro	Ala	Phe	Thr	Leu	Leu	Thr	Arg	Pro	Leu	Leu	Val	Leu	Leu	Leu	Arg	Cys	198
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661	CCA	AAC	TGC	CCC	TGC	CCA	GCC	CAG	CTC	GAT	GCC	ACC	CCC	TTC	CTG	GTG	GCA	CAC	ACC	AGG	720
199	Pro	Asn	Cys	Pro	Cys	Pro	Ala	Gln	Leu	Asp	Ala	Thr	Pro	Phe	Leu	Val	Ala	His	Thr	Arg	218
		•			•																
721	GCC	CGC	CCA	CCC	AGT	GTT	GGG	GAG	AGG	GCT	CGT	CGC	TCC	CCT	CTT	CCA	CCT	CCC	TGG	CCC	780
219	Ala	Arg	Pro	Pro	Ser	Val	Gly	Glu	Arg	Ala	Arg	Arg	<u>Ser</u>	<u>Pro</u>	<u>Leu</u>	<u>Pro</u>	<u>Pro</u>	<u>Pro</u>	<u>Trp</u>	<u>Pro</u>	238
													▲								
781	TGG	TCT	CCT	GCT	GCT	CTG	CGC	TTG	CTG	CAG	CGT	CCA	TCA	GAG	GAT	CCA	GCT	GCC	CAT	GCT	840
239	Trp	Ser	Pro	Ala	Ala	Leu	Arg	Leu	Leu	Gln	Arg	Pro	Ser	Glu	Asp	Pro	Ala	Ala	His	Ala	258
841	GAC	TGC	CAT	CGG	GCT	GCC	CTC	AAC	ATC	TCT	TTC	CAG	GAG	CTT	GGC	TGG	GAC	CAG	TGG	ATC	900
259	<u>Asp</u>	<u>Cys</u>	<u>His</u>	<u>Arg</u>	<u>Ala</u>	<u>Ala</u>	<u>Leu</u>	<u>Asn</u>	<u>Ile</u>	<u>Ser</u>	<u>Phe</u>	<u>Gln</u>	<u>Glu</u>	<u>Leu</u>	<u>Gly</u>	<u>Trp</u>	<u>Asp</u>	<u>Gln</u>	<u>Trp</u>	<u>Ile</u>	278
	•							*													
901	GTT	CAC	CCA	CCT	AGC	TTC	ATC	TTC	CAT	TAC	TGC	CAT	GGG	GGC	TGT	GGG	CTG	GTC	CCC	TCC	960
279	<u>Val</u>	<u>His</u>	<u>Pro</u>	<u>Pro</u>	<u>Ser</u>	<u>Phe</u>	<u>Ile</u>	<u>Phe</u>	<u>His</u>	<u>Tyr</u>	<u>Cys</u>	<u>His</u>	<u>Gly</u>	<u>Gly</u>	<u>Cys</u>	<u>Gly</u>	<u>Leu</u>	<u>Val</u>	<u>Pro</u>	<u>Ser</u>	298
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961	CCA	GTC	CTG	TCT	CCT	GGG	GCT	GCC	CTC	ACG	CCT	TCT	CAG	CCA	CTG	CCC	CTG	GGC	CCA	GGC	1020
299	<u>Pro</u>	<u>Val</u>	<u>Leu</u>	<u>Ser</u>	<u>Pro</u>	<u>Gly</u>	<u>Ala</u>	<u>Ala</u>	<u>Leu</u>	<u>Thr</u>	<u>Pro</u>	<u>Ser</u>	<u>Gln</u>	<u>Pro</u>	<u>Leu</u>	<u>Pro</u>	<u>Leu</u>	<u>Gly</u>	<u>Pro</u>	<u>Gly</u>	318
1021	TCC	CGG	CCT	TGT	TGT	GCT	GCT	ATG	CCT	AGC	ACC	ATG	AGA	CCC	CTT	CGA	GTC	CGT	ACC	ACC	1080
319	<u>Ser</u>	<u>Arg</u>	<u>Pro</u>	<u>Cys</u>	<u>Cys</u>	<u>Ala</u>	<u>Ala</u>	<u>Met</u>	<u>Pro</u>	<u>Ser</u>	<u>Thr</u>	<u>Met</u>	<u>Arg</u>	<u>Pro</u>	<u>Leu</u>	<u>Arg</u>	<u>Val</u>	<u>Arg</u>	<u>Thr</u>	<u>Thr</u>	338
				•																	
1081	TCA	GAC	GGA	GGC	TAT	TCC	TTC	AAG	TAT	GAG	ATA	GTG	CCC	AAC	CTC	CTC	ACT	CAA	CAC	TGT	1140
339	<u>Ser</u>	<u>Asp</u>	<u>Gly</u>	<u>Gly</u>	<u>Tyr</u>	<u>Ser</u>	<u>Phe</u>	<u>Lys</u>	<u>Tyr</u>	<u>Glu</u>	<u>Ile</u>	<u>Val</u>	<u>Pro</u>	<u>Asn</u>	<u>Leu</u>	<u>Leu</u>	<u>Thr</u>	<u>Gln</u>	<u>His</u>	<u>Cys</u>	358
																			•		
1141	GCC	TGC	ATA	TAG	GGAGGACC			ACCCTCTCTC			CAAGTAATGG			CCTAACATCC			ATTAATGCTG				1200
359	<u>Ala</u>	<u>Cys</u>	<u>Ile</u>	***	361																
	•																				
1201	GTGTTGGCCA				GAATGAAAGC			CAGATAACCA			CAGAGAGGAG			ACAAGGTCAG			AGGACAGATC				1260
1261	CCCATCTCCT				AGTACTGGGT			CCTTCCTGTC			CAAGGTCACC			CTCATCGATT			GTAAACCGAT				1320
1321	AAATAAACAG				TTCAGCATAA			CTAAAAAA			AAAAAA			AAAAAA			AAAAAA				1377

FIGURE 1. Nucleotide (cDNA) and deduced amino acid sequences of possum inhibin α -subunit gene. The mature inhibin α -subunit (α C) sequence and the consensus polyadenylation signal (AATAAA) are underlined. The positions of potential N-linked glycosylation sites (*), cysteine residues (●) and potential proteolytic cleavage sites (▲) are indicated, as is the termination codon (***) (Genbank Accession Number: AF033340).

dextran sulphate) (Church & Gilbert 1984) for 16–20 h at 55 °C. The hybridised membranes were sequentially washed in $2 \times \text{SSC}-0.1\%$ SDS (10 min at room temperature), in $2 \times \text{SSC}-0.1\%$ SDS (30 min at 65 °C) and finally in $0.1 \times \text{SSC}-0.5\%$ SDS (15 min at 55 °C) before autoradiography at -70 °C with an intensifying screen.

***In situ* hybridisation**

The *in situ* hybridisation methodology has been described previously (Tisdall *et al.* 1994). Radio-labelled ($[\alpha\text{-}^{32}\text{P}]\text{UTP}$) RNA probes were synthesised from a 737-bp α -inhibin cDNA cloned into pGEMTeasy (Riboprobe Gemini system, Promega).

Ovaries from seven adult possums were fixed in 4% (w/v) phosphate-buffered paraformaldehyde and embedded in paraffin. Sections 5 μm thick were cut and transferred to Superfrost-Plus glass slides (Erie Scientific, Portsmouth, NH, USA), deparaffinised in xylene and hydrated through an alcohol series to water. Sections were immersed in 0.2 M HCl (20 min, room temperature) and washed in $2 \times \text{SSC}$ for 30 min. The sections were digested with proteinase K (2 $\mu\text{g}/\text{ml}$, Boehringer Mannheim) at 37 °C for 5 min, immersed in 0.1 M triethanolamine pH 8.0, 0.25% (v/v) acetic anhydride (twice for 5 min, room temperature) and then washed in $2 \times \text{SSC}$ for 5 min at room temperature before dehydration through an alcohol series to absolute ethanol.

Hybridisation was performed in 50% (v/v) formamide, 0.3 M NaCl, 10 mM Tris-HCl pH 6.8, 10 mM sodium phosphate pH 6.8, 5 mM EDTA pH 8.0, $1 \times$ Denhardt's solution (0.02% (w/v) each of BSA, Ficoll and polyvinyl pyrrolidone), 10% (w/v) dextran sulphate, 50 mM dithiothreitol, 1 mg/ml tRNA and approximately 2×10^5 c.p.m./ μl ^{32}P -labelled sense or antisense riboprobe at 55 °C for 18 h. After hybridisation, the slides were washed sequentially in $5 \times \text{SSC}$ (50 °C for 15 min, twice); $2 \times \text{SSC}/50\%$ (v/v) formamide (65 °C for 15 min, twice) and $2 \times \text{SSC}$ (37 °C for 5 min, four times). The sections were incubated with RNase A (Boehringer Mannheim) (20 $\mu\text{g}/\text{ml}$ in 0.4 M NaCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA) at 37 °C for 30 min and then sequentially washed in $2 \times \text{SSC}/50\%$ (v/v) formamide (65 °C for 30 min), $2 \times \text{SSC}$ (37 °C for 15 min) and $0.2 \times \text{SSC}$ (37 °C for 15 min). The slides were dehydrated through an alcohol series, air dried, coated with autoradiographic emulsion (LM-1 emulsion; Amersham) and exposed at 4 °C for ten days. The slides were developed (D19 developer; Kodak, Rochester, NY, USA) and fixed photographically to produce visible silver grains over the sites of hybridisation. The

tissue sections were stained with haematoxylin and photographed under both light- and dark-field illumination on an Olympus BH-2 microscope. Hybridisation of duplicate sections with a ^{32}P -labelled sense riboprobe was used as a negative control.

Immunohistochemistry

Paraffin-embedded sections were deparaffinised with xylene and rehydrated through an alcohol series to water. Endogenous peroxidase activity was quenched with exposure to 3% (v/v) hydrogen peroxide in methanol for 30 min. After antigen retrieval by heating the sections in a pressure cooker containing 0.01 M citrate buffer (pH 6.0) for 1 min, the sections were washed in Tris-HCl buffer (TBS; 0.5 M Tris(hydroxymethyl)methylamine, 0.9% (w/v) NaCl; pH 7.6) three times for 10 min. Sections were then incubated with non-immune rabbit serum (diluted 1 : 5 in TBS) for 30 min to reduce non-specific immunostaining. Mouse anti-inhibin α -subunit (Serotec, Oxford, UK; clone R1; diluted 1 : 100 in TBS+0.1% (w/v) BSA) was applied to the sections and incubated overnight at 4 °C in a humid chamber. All other incubations and washes were performed at room temperature. The sections were then washed three times for 10 min in TBS and incubated for 30 min with the secondary antibody, biotinylated rabbit anti-mouse IgG (Dakopatts, Dako Corporation, Carpinteria, CA, USA) diluted 1 : 500 in TBS containing 0.1% (w/v) BSA and 1% (v/v) non-immune possum serum.

After the incubation with the primary antibody, all sections were washed three times for 10 min in TBS and incubated with an avidin-biotinylated horseradish peroxidase complex (Dakopatts), following the manufacturer's instructions, for 30 min. Subsequently, the sections were given three 10-min washes in TBS and the signal was enhanced by use of a tyramide signal amplification kit (NEN Life Science Products, Boston, MA, USA), following the manufacturer's instructions. The immunoperoxidase activity was visualised with 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) in TBS+0.03% (v/v) hydrogen peroxide in which the sections were incubated for 5 min. Following a final wash of 5 min in TBS, the sections were counterstained in Gill's haematoxylin, dehydrated through graded alcohols and xylene and finally mounted under coverslips using DePeX mounting medium.

The absence of non-specific staining was determined by the replacement of clone R1 with non-immune mouse ascites fluid.

FIGURE 2. Schematic presentation of the possum inhibin α -subunit precursor. Coding sequences are boxed, and untranslated sequences are represented by a line. The dotted box represents the coding region for the signal peptide. The numbers refer to the amino acid residues. The position of cysteine residues (S) and potential N-linked glycosylation sites (*) are indicated. α C, C-terminal fragment of α -inhibin; α N, N-terminal fragment of α -inhibin; pro, pro-fragment of α -inhibin.

RESULTS

Sequence analysis

The possum inhibin α -subunit cDNA nucleotide and corresponding predicted amino acid sequences are shown in Fig. 1. The cDNA sequence, including the polyA tail, is 1377 nucleotides in length and includes a 1086-nucleotide open reading frame (nucleotides 67–1152) encoding a 361-amino acid precursor protein with a predicted molecular mass of 38.9 kDa. The cDNA sequence contains a 3' untranslated region of 225 bases, which includes a typical polyadenylation signal (5'-AATAAA-3') located 15 bases before the polyA tail.

Using homologies with eutherian inhibin α -subunits, the proposed site of signal peptide cleavage was assigned between Gly²¹ and Cys²² (Forage *et al.* 1986, Mayo *et al.* 1986, Yamanouchi *et al.* 1995) and three evolutionary conserved proteolytic processing sites were identified at Arg⁶³–Arg⁶⁴, Arg⁷³–Arg⁷⁴ and Arg²²⁹–Arg²³⁰ (Figs 1, 3). By analogy with the processing of eutherian homologues, the α N– α C fragment (amino acids 65–361) is predicted to be derived from the full-length precursor by cleavage at the C-terminal site to the dibasic motif, Arg⁶³–Arg⁶⁴ (Fig. 2) and further proteolytic processing at Arg²²⁹–Arg²³⁰ generates two fragments of 166 (α N; 65–230) and 131 (α C; 231–361) amino acids (Fig. 2). The mature α -chain is the 131-amino acid α C fragment, leading to a calculated molecular mass of 14.2 kDa. Three potential N-linked glycosylation sites (Asn–X–Thr/Ser; Pless & Lennarz 1977) were found in the predicted α -inhibin protein sequence (Asn⁴⁸, Asn¹⁴⁴ and Asn²⁶⁶). There are 12 evolutionarily conserved cysteine residues in the precursor α -subunit: one at Cys²², four in the α N fragment (96, 198, 201, 203) and seven in the α C fragment (260, 289, 293, 322, 323, 358, 360) (Figs 1, 3).

The deduced amino acid sequence of the possum inhibin α -subunit precursor protein was aligned with six eutherian homologous sequences (Fig. 3). Quantitative comparisons between the sequences revealed mean identities of 71.8% (coding region nucleotide sequence), 69.8% (deduced amino acid sequence of full-length precursor protein), 70.5% (deduced amino acid sequence of 'pro' fragment), 65.8% (deduced amino acid sequence of α N fragment) and 79.7% (deduced amino acid sequence of α C fragment).

Gene expression in adult ovary

Northern hybridisation using a possum inhibin α -subunit cDNA probe detected a transcript of approximately 1.6 kb in possum ovarian RNA with no hybridising transcript detected in possum liver RNA (negative control) (Fig. 4).

The cell-type distribution of α -inhibin gene expression within the ovary was investigated by RNA *in situ* hybridisation. The inhibin α -subunit RNA probe hybridised specifically to the granulosa cell layers of follicles, whereas no expression was detected in the theca cell layers or in the ovarian stroma (Fig. 5B, D, F). The α -inhibin gene expression was examined in the granulosa cells of follicles at different stages of development. No α -inhibin mRNA was detected in primordial follicles containing only one layer of flattened granulosa cells (Fig. 5B, D). Expression of α -inhibin was observed in primary follicles (those in which one to two layers of cuboidal granulosa cells were forming; Fig. 5D) and in all subsequent stages of follicular development (Fig. 5B). In large antral follicles, expression was evident in the cumulus granulosa cells surrounding the oocyte, but not in the oocyte itself (Fig. 5F). Finally, α -inhibin gene expression was detected in corpus luteum (Fig. 5H).

	1	●	★50
possum	MLPLLLLPLQL LLLMVMKGGH GCQGPELDRE LVLAKVRALV LDALGPPNAS		
equine	MVPP...L. ...L.PQ... ...S.... I.....FAVT		
human	MV.H.L. F..LTPQ... S...L.A..FAVT		
bovine	MW.Q.-. ...LAPQ... ..H.L.....FPVT		
porcine	MWPQ.-. ...LAPRS.....FAVT		
mouse	MVSQRS.L. ...LTLRDVD S.....V..K..FAMD		
rat	MVIQPS.L. ...LTLQDQVD S.....V..K..FAMD		
	51	▼	● 100
possum	KDGGKPVAQR LTRRHAHTGG STRRSMENED EDLSQVILFP TTGPGCEDEP		
equine	GE..D.GVR. .P....-L.. FA..GS.P.E ..V..A.... AS.SR.....		
human	RE..D.GVR. .P....LG.F TH.G.EPE.E ..V..A.... A.DAS...KS		
bovine	GE..D.GVR. .H....-V.. FM..GS.P.. Q.V..A.... AA.AS.G...		
porcine	GE..D.GVR. .P....-V.. FM..GS.P.E ..V..A.... A..AR.G...		
mouse	GE..D.GIR. .P....-V.. FMH.TS.P.E ..V..A.... A..AT...Q.		
rat	GE..G.GIR. .P....-L.. FMH.TS.P.E ..V..A.... A..AT...QA		
	101		★
possum	EAR-----AA EGLFTYTRFP SLHTRSQRVT AAQLWFHTGL DKVGAIEVHND		
equine	A.GELAQE.E Q.....M... .Q.M..... .S.H..... .RQ.TAAS.S		
human	A..GLAQE.ER.M... .Q.....S..... .RQ.TAAS.S		
bovine	D.GE----.EV.Q. .Q.....S..... .RQETAAS.S		
porcine	A.GELARE.EV... .Q..H.....S..... .RQ.MAAAS.S		
mouse	A..GLAQE.EV... .Q.I..H....S..... GRKSTAAAS.S		
rat	A.GGLAQEPEV... .Q.I..H....S..... .RKSTAAAS.S		
	151		200
possum	SGPMVTLLAM SSGGPMAPVI LLGPAPPHWA VHLAAPAFT LLTRPLLVLL		
equine	.E.LLG...LV T..Q...C...S.LP ...H.V....		
human	.E.LLG...L .P...V..M S..H..... .TS.LS ...H.V....		
bovine	.E.LLG..VL T.....P..M S..Q...R... .TS..P ...H.V.A...		
porcine	..LLD...L .R..V..M S..Q...R... ..S.LP ...H.V....		
mouse	.A.LLD..VLV S.VQG..R... ..S..P ...H.I....		
rat	.R.LLD..VLV S..QS..R... ..S..P ...H.I....		
	● ● ●	▼	250
possum	LRCPCPCPA QLDATPFLVA HTRARPPSVG ERARRSPLPP PWPWSPAALR		
equine	...L.S.S. RPE..... .G...T...TP.LS...		
human	...L.T.S. RPE..... .T...G...TPLM S...S...		
bovine	...L.S.ST RPE..... .K...G...TP.LS...		
porcine	...L.S.S. RPE..... .G...TA.LS...		
mouse	...L.S.SG RPET..... .A..A...TPSVS...		
rat	...L.S.SG RPET..... .A..A...APSMS...		
	251	● ★	● ●
possum	LLQRPSEDPA AHADCHRAAL NISFQELGWD QWIVHPPSFI FHYCHGGCGL		
equine	P.E.. ...N..... R...R... ..S...		
human	P.E.. ...N...V... ..E R...Y... ..S...		
bovine	P.E.. R.....Y.....		
porcine	P.E.. V.....S. R.....S...		
mouse	P.E.. ...F..... R.....S..M		
rat	P.E.S ...F..... R.....S..M		
	301	● ●	350
possum	VPSPVLS--- PGAALTSPSQ LPLGPGSRPC CAAMPSTMRP LRVRTTSDGG		
equine	SAP.D.PLPV .EVPP..I.. .S.V..AQ.. ...L.G... .H.....		
human	HIP.N..LPV ..PP..A.. YS.L..AQ.. ...L.G... .H.....		
bovine	S.PQD.PLPV .VPP..V.. .S.V..AQ.. ...L.G... .H.....		
porcine	PTL.N.PLSV ..PP..V.. .L.V..AQ.. ...L.G...S ..S...		
mouse	PT.DL-PLPV ..VPP..V.. .F.V..AK.. ...L.GS.TS ..S...		
rat	PT.DL-PLPV ..PP..A.. .F.V..AK.. ...L.GS..S ..S...		
	351	● ●	
possum	YSFKYIEIVN LLTQHCACI	361	
equine	T...	367	
human	T...	366	
bovine	M...	360	
porcine	T...	364	
mouse	M... .I.....	366	
rat	M... .I.....	366	

FIGURE 3. Comparison of the deduced amino acid sequence of possum inhibin α -subunit precursor protein with six eutherian mammal homologues (equine (Yamanouchi *et al.* 1995), human (Mayo *et al.* 1986), bovine (Forage *et al.* 1986), porcine (Mason *et al.* 1985), mouse (Albano *et al.* 1993) and rat (Woodruff *et al.* 1987)). The sequences are aligned to show maximum homology. Positions at which sequences are identical with that of the possum are indicated by dots. Space insertions are indicated by -. The positions of conserved cysteine residues (●), potential N-linked glycosylation sites (*) and proteolytic cleavage sites (▼) are indicated. The length for each protein sequence is given at the end of each sequence.

FIGURE 4. Northern blot analysis of total RNA extracted from possum liver (lane 1) and ovary (lane 2) hybridised with a possum inhibin α -subunit probe. The positions of molecular weight standards are indicated. Kb=kilobase.

No hybridisation above random background was observed using the sense control probe (data not shown).

Protein localisation in adult ovary

The immunocytochemical localisation of inhibin α -subunit in adult possum is shown in Fig. 6. Inhibin α -subunit protein was localised to granulosa cells of developing follicles, whereas no immunostaining was detected in the theca cell layer or in the ovarian stroma (Fig. 6A). Immunostaining for the inhibin α -subunit was first observed in granulosa cells of primary follicles and this staining was evident throughout folliculogenesis (Fig. 6A, B). α -Inhibin protein was also detected in cells of the corpus luteum (Fig. 6C).

DISCUSSION

This paper reports the cDNA sequence and pattern of expression of the inhibin α -subunit gene of the brushtail possum. Comparison of the predicted amino acid sequence with those of six eutherian homologues gives a mean sequence identity for the mature protein of 79.7%, which is lower than the mean homology observed between the eutherian α -inhibin sequences (87.5%). This is not unexpected, given the 130 million years separating the evolution of the marsupial and eutherian lineages (Janke *et al.* 1997).

It seems that the major functional groups of the amino acid sequence have been conserved during evolution: the positions of proteolytic cleavage processing sites, N-linked glycosylation sites and the number and positions of cysteine residues. It is known that, in eutherians, inhibin α -subunit is synthesized as a precursor protein that is processed by sequential proteolysis, first removing the 20 amino acids signal sequence (Forage *et al.* 1986, Mayo *et al.* 1986), and then cleavage at three dibasic sequences producing various forms of different lengths, of which the C-terminal fragment (α C) is considered the mature α -subunit (Mason *et al.* 1985, Mayo *et al.* 1986, Forage *et al.* 1986, Woodruff *et al.* 1987, Albano *et al.* 1993, Yamanouchi *et al.* 1995). In the present study, the cDNA sequence for possum predicts a molecular mass of 14.2 kDa for the α C fragment, similar to the calculated molecular mass of the mature α -subunit in porcine (Mason *et al.* 1985), bovine (Forage *et al.* 1986) and equine (Yamanouchi *et al.* 1995) species. In possums, there are no data available with respect to the actual molecular masses of the different forms of the inhibin α -subunit resulting from proteolytic processing. However, the presence of potential N-linked glycosylation sites indicates that it is likely to be larger than that predicted from the amino acid sequence (Forage *et al.* 1986). Such glycosylation accounts for the difference in size between that calculated from the amino acid sequence and the empirically measured values of porcine (Mason *et al.* 1985, Mayo *et al.* 1986) and bovine α -inhibins (Forage *et al.* 1986). The potential N-linked glycosylation sites are preserved at Asn¹⁴⁴ and Asn²⁶⁶ across the different species (Fig. 3). There is, however, an additional potential N-linked glycosylation site in the possum α -inhibin sequence at Asn⁴⁸. The number and distribution of cysteine residues in the amino acid sequences are also evolutionarily conserved between possum and eutherian species. There are four cysteine residues in the α N fragment amino acid sequence, suggesting possible intrachain disulphide bonds that enable the separation of the α N fragment from the α C fragment (Wang & Johnson 1993). The odd number (seven) of cysteine residues in the α C fragment suggests that at least one may be free to form a disulphide bond with the inhibin β -subunit during heterodimer formation (Wang & Johnson 1993).

The α C fragment of possum α -inhibin shows 79.7% amino acid identity with the six eutherian α -inhibin sequences reported. This is greater than the identity for the full-length precursor protein (69.8%) and for the α N- α C fragment (71.2%). The comparatively high level of sequence conservation

FIGURE 5. Localisation of α -inhibin mRNA in adult possum ovary using *in situ* hybridisation. Left, bright-field photomicrographs stained with haematoxylin; right, corresponding dark-field photomicrographs. (A, B) Hybridisation of the α -inhibin antisense riboprobe to the granulosa cells of preantral and antral follicles. No hybridisation is evident in the granulosa cells of primordial follicles or in the stroma. (C, D) Hybridisation of the α -inhibin antisense riboprobe to the primary follicle with one layer of granulosa cells (asterisk). No hybridisation is evident in the granulosa cells of primordial follicles. (E, F) Hybridisation of the α -inhibin antisense riboprobe to the granulosa cells and cells of the cumulus oophorus but not to the thecal cells and oocyte of an antral follicle. (G, H) Hybridisation of the α -inhibin antisense riboprobe to cells of the corpus luteum. a, antral follicle; CL, corpus luteum; G, granulosa cells; O, oocyte; p, primordial follicle; pa, preantral follicle; S, stroma; T, thecal cells. Scale bars represent 100 μ m.

FIGURE 6. Photomicrographs showing immunocytochemical localisation of inhibin α -subunit in adult possum ovary. (A) Immunostaining in the granulosa cells of preantral (arrow) and antral follicles. No immunostaining is evident in the granulosa cells of primordial follicles or in the stroma. (B) Immunostaining in the granulosa cells of primary follicles (asterisk). (C) Immunostaining in cells of the corpus luteum. a, antral follicle; p, primordial follicle; S, stroma. Scale bars represent 100 μ m.

of the α C fragment might indicate that it is the dominant form of the different cleavage products, which forms in combination with a β -subunit biologically active inhibin. Furthermore, the greater conservation of the 'pro' fragment (70.5%) compared with the α N fragment (65.8%) might be of importance, as it forms, in combination with the α C fragment, the protein, pro- α C, the latter being an α -subunit product that has been isolated from follicular fluid and serum (Knight *et al.* 1989, Schneyer *et al.* 1990). Recently, Groome *et al.* (1995) raised the possibility that pro- α C and other precursor forms of the α -subunit may themselves be functional.

Northern hybridisation revealed that the possum inhibin α -subunit gene is expressed in ovary as a transcript of approximately 1.6 kb which is similar to that reported for the inhibin α -subunit of pig (Mason *et al.* 1985), rat (Woodruff *et al.* 1987), sheep (Fleming *et al.* 1992) and horse (Yamanouchi *et al.* 1995).

In situ hybridisation and immunocytochemistry localised inhibin α -subunit to the granulosa cells of the developing follicle. These data are in accordance with the findings of previous immunohistochemical (Merchenthaler *et al.* 1987), *in situ* hybridisation (Woodruff *et al.* 1988, Torney *et al.* 1989, Jih *et al.* 1993, Tisdall *et al.* 1994, Engelhardt *et al.* 1995, Braw-Tal 1994), *in vitro* (Erickson & Hsueh 1978, Henderson & Franchimont 1981) and physiological (McNatty *et al.* 1993) studies in eutherian mammals. In addition to the expression of α -inhibin in granulosa cells, there have been reports of α -inhibin expression in the thecal cell layer in cattle (Torney *et al.* 1989) and in thecal and stromal cells in the rat ovary (Meunier *et al.* 1988, Jih *et al.* 1993). In this study, no evidence of α -inhibin mRNA or protein was found in the thecal or stromal cells of the possum ovary. Hybridisation and immunostaining for the inhibin α -subunit were first observed in granulosa cells of primary follicles and also in granulosa cells at subsequent stages of follicular growth. It appears that α -inhibin expression is switched on in follicles after the transition from flattened to cuboidal granulosa cells. To our knowledge, this is the first time that α -inhibin expression has been reported at such an early stage of folliculogenesis. In previous studies of eutherian mammals, α -inhibin expression was first observed in follicles in the late preantral stage (Woodruff *et al.* 1987, Meunier *et al.* 1988, Braw-Tal 1994) or the antral stage (Woodruff *et al.* 1988, Torney *et al.* 1989, Tisdall *et al.* 1994). The increased levels of α -inhibin mRNA and protein (shown by the increasing number of silver grains and the intensity of immunostaining) with increasing follicular

diameter in the adult possum support previous correlations between follicle size or granulosa cell number and follicular α -inhibin expression (Torney *et al.* 1989, Guthrie *et al.* 1992, Li *et al.* 1997) or inhibin content (Tsonis *et al.* 1983, Hillier *et al.* 1989, Campbell *et al.* 1991, McNatty *et al.* 1993) in eutherian mammals. As expression is associated with antrum formation and the highest level of gene expression is found in estrogenic follicles selected to ovulate, the main role may be to limit the number of less mature follicles that ovulate (Guthrie *et al.* 1992). The present data suggest that, in marsupials, inhibin may, besides limiting the maturation of ovulatory follicles, have an additional role in the early stages of ovarian follicle development.

Inhibin α -subunit mRNA and protein were also detected in the corpus luteum. This is in accordance with the findings of previous studies in rat (Davis *et al.* 1987, Woodruff *et al.* 1987, Cuevas *et al.* 1987, Merchenthaler *et al.* 1987) and human (Davis *et al.* 1986) luteal cells. In contrast, Woodruff *et al.* (1988) failed to detect any α -inhibin subunit mRNA in rat luteal cells. Furthermore, there was no evidence of α -inhibin subunit expression in the corpus luteum in sheep (Fleming *et al.* 1992, Tisdall *et al.* 1994, Rodgers *et al.* 1989) and cattle (Rodgers *et al.* 1989, Torney *et al.* 1989). Collectively, these data indicate that inhibin production by the corpus luteum may be species specific.

In eutherians, there is a more widespread distribution of inhibin α -subunit than of inhibin β A-subunit (Torney *et al.* 1989). It has been reported in different species that all follicles expressing inhibin β A-subunit also expressed inhibin α -subunit, but that there were follicles which expressed inhibin α -subunit but in which inhibin β A was not found (Torney *et al.* 1989, Jih *et al.* 1993, Braw-Tal 1994, Engelhardt *et al.* 1995). Braw-Tal (1994) concluded that these smaller follicles are producing a considerable amount of free inhibin α -subunit, which is not able to suppress FSH release, but may have an important paracrine/autocrine role in follicular development. The presence of α -inhibin mRNA and protein in follicles from the primary stage of follicular growth in the adult possum ovary may point to a similar paracrine/autocrine role for inhibin α -subunit in small follicles during folliculogenesis in marsupials.

In conclusion, these results indicate considerable conservation of the structure and possible function of the inhibin α -subunit protein since the divergence of the marsupial and eutherian mammalian lineages. The expression data suggest that, in the

adult possum, inhibin may have a role in ovarian follicular growth from the primary stage of development.

ACKNOWLEDGEMENTS

The authors thank Dr D Eckery and L Colbourne for tissue collection. Thanks to A Barkus for assistance with figure preparation and to L Still and L O'Donovan for the preparation of histological sections. This work was financially supported by the New Zealand Ministry of Agriculture (Policy Division).

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RECEIVED 2 March 1998