The Australian brushtail possum (*Trichosurus vulpecula*) gonadotrophin α -subunit: analysis of cDNA sequence and pattern of expression

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ABSTRACT

A cDNA sequence from the gonadotrophin α -subunit mRNA of Australian brushtail possum (*Trichosurus vulpecula*) has been determined and analysed. Comparison with seven eutherian mammalian gonadotrophin α -subunit gene sequences revealed an average of 82.6% homology between the coding region nucleotide sequences and 88.8% identity between the predicted amino acid sequences. The predicted possum gonadotrophin α -subunit protein has ten evolutionarily conserved cysteine residues, two potential *N*-linked glycosylation sites and a putative enzyme recognition sequence which it has been suggested is required for sulphation of carbohydrate moieties. Comparison of the possum gonadotrophin α -subunit 3' untranslated region (UTR) sequence with the 3' UTRs of

eutherian α -subunit transcripts revealed sequence homology. In particular, an 18 nucleotide imperfect palindromic sequence present in the possum 3' UTR, with the potential to form a hairpin loop, was found to be evolutionarily conserved and present in five out of seven eutherian α -subunit 3' UTR sequences. *In situ* hybridization localized the transcripts to a sub-population of anterior pituitary cells presumed to be gonadotrophs and thyrotrophs. In summary, these results indicate considerable conservation of the structure and function of the gonadotrophin α -subunit protein since the divergence of the marsupial and eutherian mammalian lineages.

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INTRODUCTION

The mammalian subclass Marsupialia comprises a diverse fauna whose evolutionary lineage parallels that of the eutherian (placental) mammals from which it branched some 130 million years ago (Janke *et al.* 1997). Marsupial and eutherian mammals are distinguished principally by their differing reproductive strategies. In contrast to the extended period of intrauterine development characteristic of eutherian mammals, marsupials give birth to altricial young at a comparatively early stage of development. This 'early' birth is then followed by an lengthy lactation period during which the marsupial young are protected in a maternal abdominal pouch (Low 1978, Tyndale-Biscoe & Janssens 1988).

In recent years, increased interest in marsupial reproductive biology has arisen from both ecological and economic concerns; in Australia from a wish to

conserve endangered native species, and in New Zealand from a need to reduce the population of the introduced brushtail possum (*Trichosurus vulpecula*), which damages native forests and acts as a feral reservoir for bovine tuberculosis (Burbidge & McKenzie 1989, Cowan 1996, Cowan & Tyndale-Biscoe 1997).

In mammals, the two gonadotrophic hormones secreted from the anterior pituitary, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are required for ovarian follicle maturation and oocyte release in females, and spermatogenesis in males (Hseuh *et al.* 1989, Richards 1994, Kendall *et al.* 1995, Kumar *et al.* 1997). In addition, gonadotrophins stimulate gonadal steroidogenesis and participate in regulating the mammalian ovarian cycle (Richards 1994). The gonadotrophins act by binding to specific membrane-bound receptors, activating second messenger pathways, and thereby influencing the

physiology of the target cell (Richards 1994). Structurally, both FSH and LH are heterodimers of two dissimilar, non-covalently associated glycoproteins. The α -subunit is common to both FSH and LH and to an additional pituitary hormone, thyroid-stimulating hormone (TSH), while the β -subunit is hormone specific and determines receptor binding specificity (Ryan *et al.* 1987, Combarrous 1992, Nagaya & Jameson 1994).

Sequence information, both nucleotide and amino acid, is available for the gonadotrophins of a wide range of vertebrates (Combarrous 1992). However, comparatively little is known about the structure and function of marsupial gonadotrophins (Moore *et al.* 1997). Such information is needed to guide the development of biological control strategies based on perturbing possum reproductive endocrine function and, in addition, may provide insights into the evolution of the marsupial and eutherian reproductive systems. The cDNA sequence and pattern of gonadotrophin α -subunit gene expression in the brushtail possum pituitary is reported here.

MATERIALS AND METHODS

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Pituitaries were collected from euthanased possums and stored at -70°C . Total RNA was extracted from the pituitary tissue using guanidine isothiocyanate-phenol (Chomczynski & Saachi 1987; TRIzol reagent, Gibco BRL, Grand Island, NY, USA) and oligo(dT)-primed first strand cDNA synthesized using reverse transcriptase (Superscript II, Gibco-BRL). PCR primers were designed from conserved nucleotide sequences identified by comparison of published eutherian gonadotrophin α -subunit cDNA sequences (human (Fiddes & Goodman 1979), mouse (Chin *et al.* 1981), rat (Godine *et al.* 1982), bovine (Erwin *et al.* 1983, Nilson *et al.* 1983), porcine (Hirai *et al.* 1989), ovine (Bello *et al.* 1989)): forward primer 5'-ATGGATTACTACAGAAAATATGCAGCTGTC-3', reverse primer 5'-TCCCATCACTGTGCGCTTGGTAAATGCTTTGGCCACACAG-3'. An amplification product of the predicted size (299 bp) was produced using the following PCR conditions: 94°C , 1 min, one cycle; 94°C , 30 s, 45°C , 30 s, 72°C , 30 s, 35 cycles; 72°C , 10 min, one cycle. The identity of the PCR product was determined by DNA sequencing, and gene specific primers (GSPs) were designed for both 3' and 5' RACE (rapid amplification of cDNA ends) (Frohman *et al.* 1988). A single 3' RACE GSP (5'-CTGCATATTTTTCACCTCCTTCCCAGAT

GGGGAGT-3') produced a specific amplification product of 0.6 kb. The 5' RACE required three nested GSPs (GSP1 5'-TACATTGGCAAATGGGAGCT-3'; GSP2 5'-TGTTCTCCTTCAGCTTGC GTTCTG-3'; GSP3 5' CATAATAAACA CCCCATGTGG-3') to produce a specific amplification product of 0.2 kb. The PCR conditions used for the RACE reactions 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^{GTG}, FMC BioProducts, Rockland, ME, USA). Amplification products of the anticipated size were cut from the gel, ligated into the T-tailed cloning vector pMOS-Blue (Amersham, Amersham, Bucks, UK) and transformed into competent *E. coli* DH5 α cells (Hanahan 1985). Plasmids having inserts were first identified by restriction enzyme digestion, purified for DNA sequencing by alkaline lysis (Boehringer Mannheim, Mannheim, Germany) and sequenced using an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). All enzymes were used according to manufacturer specifications (Boehringer Mannheim). Sequence changes due to *Taq* DNA polymerase-catalysed replication errors were identified by comparison of sequences from a minimum of three independent PCRs. Nucleotide sequences common to at least two clones from independent PCRs were considered correct because of the low probability of *Taq* DNA polymerase introducing identical alterations at the same nucleotide position in two independent PCR amplifications.

In situ hybridization

The *in situ* hybridization methodology has been described previously (Tisdall *et al.* 1994). The cDNA sequence to be used as a probe was amplified by RT-PCR from possum pituitary cDNA and cloned into pGem3Z (Promega, Madison, WI, USA). Radioactively labelled ($[\alpha\text{-}^{32}\text{P}]$ uridine triphosphate) sense and antisense RNA transcripts were synthesized from linearized plasmid templates using SP6 or T7 RNA polymerase (Promega). Paraformaldehyde-fixed, paraffin wax-embedded possum pituitary tissue sections (5 μm) were cut and transferred to Superfrost-Plus glass slides (Erie Scientific, Portsmouth, NH, USA). The sections were deparaffinized 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 10 min, acetylated in 0.1 M triethanolamine, pH 8.0, 0.25% (v/v) acetic anhydride (twice for 5 min, room temperature) and then washed in $2 \times \text{SSC}$ before dehydration through an alcohol series to absolute ethanol. Hybridization was performed in 50% (w/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, $1 \times \text{Denhardt's}$ 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. Following hybridization the slides were washed sequentially in $5 \times \text{SSC}$ (50°C , 15 min, twice); $2 \times \text{SSC}/50\%$ (v/v) formamide (65°C , 15 min, twice) and $2 \times \text{SSC}$ (37°C , 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 , 15 min, twice), $2 \times \text{SSC}$ (37°C , 15 min) and $0.2 \times \text{SSC}$ (37°C , 15 min). The slides were dehydrated through an alcohol series, air-dried, coated with LM-1 emulsion (Amersham) and exposed at 4°C for 8 days. The emulsion was developed and the sections stained with haematoxylin and viewed using light and dark field illumination on a Olympus BH-2 microscope. Hybridization of duplicate sections with a ^{32}P -labelled sense riboprobe was used as a negative control.

RESULTS

The possum gonadotrophin α -subunit cDNA nucleotide sequence, and corresponding predicted amino acid sequence, are shown in Fig. 1. Determination of the cDNA sequence identity was by comparison with published eutherian gonadotrophin α -subunit gene sequences and comparison of the predicted amino acid sequence with *N*-terminal sequence obtained from purified possum gonadotrophin α -subunit (Moore *et al.* 1997). All sequence data shown were derived from a minimum of three clones derived from separate amplification reactions to allow for sequence alterations that may have been introduced during *Taq* DNA polymerase catalysed amplification reactions.

The cDNA sequence, including the polyA tail, was found to be 756 nucleotides in length and contained a 363 nucleotide open reading frame (positions 118–480) which encoded the 120 amino

acid α -subunit precursor protein. The length of the gonadotrophin α -subunit gene transcript in adult possum pituitary, as determined by Northern hybridization, was approximately 0.7 kb (data not shown), confirming that the majority of the α -subunit mRNA sequence has been obtained. The boundary between the 24 amino acid signal and 96 amino acid mature protein sequences was determined by comparison of the predicted precursor protein sequence with 11 residues of *N*-terminal sequence from purified possum gonadotrophin α -subunit, with which there was complete sequence identity (Moore *et al.* 1997). As partial *N*-terminal degradation may have occurred during the α -subunit purification, the signal peptide/mature protein boundary assignment was confirmed by comparison with eutherian mammal gonadotrophin α -subunit homologues (Fig. 2). The mature protein had two potential *N*-linked glycosylation sites (sequence motif Asn-Xxx-Ser/Thr; Pless & Lennarz 1977) at residues asparagine +56 and +82 and ten evolutionarily conserved cysteine residues (+11, +14, +32, +35, +36, +63, +64, +86, +88, +91) (Fig. 1) (Pierce & Parsons 1981). Figure 2 shows a comparison of the possum α -subunit protein with its eutherian homologues using the conserved cysteine residues for alignment. Quantitative comparisons of the possum α -subunit cDNA nucleotide and predicted amino acid sequences with seven eutherian α -subunit sequences (human, mouse, rat, bovine, porcine, ovine and donkey) revealed mean homologies of 82.6% (coding region nucleotide sequence), 88.8% (precursor protein) and 89.7% (mature protein).

Nucleotide sequence homology between the possum and eutherian transcripts extended outside the coding region. In particular the possum 3' untranslated region (3' UTR) sequence 610–724 showed a high level of homology with a segment of the 3' UTRs of ovine (71.3%), bovine (70.4%), porcine (69.6%) and horse (69.0%) α -subunit cDNA sequences, with a maximum of two nucleotide insertions being required to gain alignment. Within this conserved region were two overlapping imperfect palindromic sequences 5'-TCTTTTCA TTTGAAATGA-3' (nucleotides 658–675) and 5'-TTTTCATTTGAAATGAAAA-3' (nucleotides 660–678). Alignment of the possum, ovine, human, bovine, porcine and horse α -subunit 3' UTR sequences indicated partial evolutionary conservation of these imperfect palindromic sequences (Fig. 3). Another feature present in the possum 3' UTR, located approximately eight nucleotides 3' to the imperfect palindromes, was an 'AU-rich' sequence, 5'-AUUUA-3' (nucleotides 684–688), which also appeared to be evolutionarily conserved (Fig. 3).

FIGURE 1. The cDNA and predicted protein amino acid sequences of the possum gonadotrophin α -subunit gene. Coding and signal sequences were identified by homology with eutherian mammal α -subunit sequences and confirmed by *N*-terminal sequencing of purified possum FSH α -subunit protein. Potential *N*-linked glycosylation sites (Asn-X-Thr/Ser; Pless & Lennarz 1977) are double-underlined (Asn +56, Asn +82) and ten cysteine residues in the mature protein (residues +11, +14, +32, +35, +36, +63, +64, +86, +88 and +91) are marked (●). A consensus polyadenylation sequence (AATAAA, nucleotides 719–724) is single-underlined. An eighteen nucleotide sequence that may form a hairpin loop (nucleotides 658–675) is shaded. The *N*-terminal amino acid of the mature protein is indicated, Phe (+1), as is the termination codon (***). The locations of the PCR primers used are indicated by heavy lines above the sequence with an arrow-head denoting the 3'-terminus. Abbreviations: FP, forward primer; RP, reverse primer; 3' RACE GSP, 3' RACE gene specific primer; 5' RACE GSP1, first 5' RACE gene specific primer; 5' RACE GSP2, second (nested) 5' RACE gene specific primer; 5' RACE GSP3, third (nested) 5' RACE gene specific primer. (GenBank Accession No. AF004520.)

In situ hybridization of adult possum pituitary sections with α -subunit antisense riboprobes produced a punctate pattern of expression with a sub-population of cells within the anterior pituitary showing high levels of gene expression (Fig. 4). The α -subunit-expressing cells appeared unevenly distributed through the anterior pituitary with a higher density consistently found towards the peripheral layers (Fig. 4C and D). No α -subunit mRNA was detected in the posterior pituitary. Hybridization

with a sense (negative control) riboprobe consistently produced a very low density of randomly distributed silver grains, indicating no specific hybridization (not shown).

DISCUSSION

A surprising finding arising from the quantitative comparisons of the possum and eutherian α -subunit

	-24	-20	-10	+1	10	20	30	40	50	60	70	80	90	96
possum	MDYYRKYAAVILATLSVFLHILHSFPDGEFIMQGCPECKLKENKYFSKVGAPIYQCMGCCFSRAYPTPARSKKTMVLVFKNITSEATCCVAKATKATVMGNKAVENHTECHCSTCYHKS													
human	Y KY IF VT VF HV A V... D T QE EF QEGAP L								L	Q Y ES	SYNRV V	GFKV	A H	Y S
mouse	Y KY VI VM MF HI L GDFIL G K KE KY KLGAP Y								A	P I EA	AFTKA V	NARV	E H	Y S
rat	Q RY VI VM MY HI L GDLIL G K KE KY KLGAP Y								A	P I QA	SFTKA V	NARV	D H	Y S
bovine	Y KY VI TI LF QI F GEFTM G K KE KY KPDAA Y								A	P I EA	AFTKA V	NVRV	E H	Y S
porcine	Y KY VI AI VF QI F GEFTM G K KE KY KLGAP Y								A	P I EA	AFTKA V	NARV	E H	Y S
ovine	Y KY AI AI LF QI F GEFTM G K KE KY KPDAP Y								A	P I EA	AFTKA V	NVRV	E H	Y S
donkey	Y KH VI AT VF HI F GEFTT D K KK KY KLGVP Y								A	P I EA	AFIRV L	NIRL	Q Y	H I

FIGURE 2. Alignment of the possum gonadotrophin α -subunit amino acid sequence with homologues from seven eutherian mammals (human (Fiddes & Goodman 1979), mouse (Chin *et al.* 1981), rat (Godine *et al.* 1982), bovine (Erwin *et al.* 1983, Nilson *et al.* 1983), porcine (Hirai *et al.* 1989), ovine (Bello *et al.* 1989), donkey (Chopineau & Stewart 1996)). Positions at which all the sequences are identical are indicated by spaces. Where one or more of the eutherian sequences differs from the possum sequence the differing amino acid is underlined. A four space insertion, indicated by ..., is included to align the 116 amino acid human sequence with the other mammalian sequences. The positions of conserved cysteine residues (●) and potential N-linked glycosylation sites (double-underlined) are indicated. Signal sequences are shaded. All the amino acid sequences shown are derived from the cDNA sequences referenced.

sequences was that the mean percentage identity of seven eutherian α -subunit mature protein sequences (human, mouse, rat, bovine, porcine, ovine, donkey) compared amongst themselves, 84.9%, was lower than that from the eutherian versus possum comparison, 89.7%. This differential was still present if the human sequence was omitted from the comparison, with the mean sequence identity of the eutherian mature proteins being 90.2% and the mean sequence identity of the eutherian and possum sequences 92.4%. Thus there appear to have been strongly conservative selection pressures acting on the gonadotrophin α -subunit gene during the 130 million years separating the marsupial and eutherian lineages. Presumably the requirement that the gonadotrophin α -subunit must interact appropriately with three different β -subunits (FSH β , LH β , TSH β), and with the corresponding

receptors, placed considerable restrictions on viable sequence changes (Combarnous 1992).

The evolutionary sequence conservation was not evenly distributed through the gonadotrophin α -subunit. In particular residues +31 to +67 showed considerable conservation, wherein the only differences from the possum sequence are in the human (four amino acid differences) and rat (single difference) protein sequences. The remaining five eutherian gonadotrophin α -subunit sequences (mouse, bovine, porcine, ovine and donkey) were identical within this region. Included within this highly conserved region was one of two evolutionarily conserved potential N-linked glycosylation sites, Asn (+56), the other being Asn (+82), and the peptide sequence Pro-(Leu or Ala)-Arg-Ser-Lys-Lys (+44 to +49) which is thought to be required for sulphation of N-linked carbohydrate moieties (Mengeling

possum	UAG ₄₈₀ - (N) ₁₇₃ - 654	UGGCUCUUUUCAUUUGAAUUGAAAAACAGAAUUUA ₆₈₈	- (N) ₃₀ - AAUAAA - (N) ₁₄ - A _x
ovine	UAA ₄₃₃ - (N) ₁₈₂ - 616	UUGAUCUUUUCAUUUGGAAUGAAUUAUGGCAUUUA ₆₅₀	- (N) ₃₀ - AAUAAA - (N) ₁₅ - A _x
human	UAA ₄₀₁ - (N) ₁₈₂ - 584	UAGUUCUUUUCAUUUGGAAUGAAUACAGCAUUUA ₆₁₇	- (N) ₁₅ - AAUAAA - (N) _x - A _x
bovine	UAA ₄₄₀ - (N) ₁₈₂ - 623	UUGAUCUUUUCAUUUGGAAUGAAUUAUGGCAUUUA ₆₅₇	- (N) ₃₀ - AAUAAA - (N) ₂₀ - A _x
porcine	UAA ₄₄₅ - (N) ₁₈₄ - 630	UQGCUCUUUUCAUUUGGAACGAAUUGCAGCAUUUA ₆₆₄	- (N) ₂₈ - AAUAAA - (N) _{>24} - A _x
horse	UAA ₃₁₁ - (N) ₁₈₂ - 494	UAGCUCUUUUGAUUUGGAAUGAAUACAGCAUUUA ₅₂₉	- (N) ₃₀ - AAUAAA - (N) ₁₆ - A _x

FIGURE 3. Comparison of the 3' UTR sequences of six mammalian gonadotrophin α -subunit mRNAs. The nucleotide numbering shown is from the following references: possum (this work), ovine (Bello *et al.* 1989), bovine (Erwin *et al.* 1983), porcine (Hirai *et al.* 1989), horse (Stewart *et al.* 1987). The human sequence is a composite, with the sequences in normal type from Fiddes & Goodman (1979) (cDNA sequence) and nucleotides in bold type from exon 4 of the genomic sequence (Fiddes & Goodman 1981). The donkey gonadotrophin α -subunit sequence has not been included as insufficient 3' UTR sequence has been reported (Chopineau & Stewart 1996). Nucleotides proposed to form the stem portion of a potential hairpin loop are shaded and nucleotides differing from the possum sequence are underlined. An 'AU-rich' (i.e. AUUUA) sequence is present 7–8 bases 3' to the potential hairpin sequence. Translation termination codons (UAG and UAA) are shown at the 5' end of the 3' UTR sequences and polyadenylation sequences (AAUAAA) at the 3' end.

FIGURE 4. *In situ* hybridization of adult possum pituitary sections with a radioactively labelled possum gonadotrophin α -subunit antisense riboprobe corresponding to nucleotides 181–719 of Fig. 1. A,C, bright-field photomicrographs; B,D, dark-field photomicrographs. Gonadotrophin α -subunit gene expressing cells were identified throughout the anterior pituitary, though at higher density towards the periphery (C,D). No α -subunit mRNA was detected in cells of the posterior pituitary. A,B, 35 $\times$ magnification; C,D, 125 $\times$ magnification; bar=100 μ m. ap, anterior pituitary; pp, posterior pituitary.

et al. 1995). In those species examined, glycosylation of the gonadotrophin α -subunit is not essential for receptor binding, but is required for maximal activation of the secondary messenger pathway and biological activity (Sairam 1989, Bishop *et al.* 1994, Nagaya & Jameson 1994). Furthermore, variation of the terminal residues of the carbohydrate moieties, including sulphation, may modulate hormonal bioactivity by influencing such parameters as receptor binding affinity, secondary messenger activation and metabolic clearance rate (Wilson *et al.* 1990, Ulloa-Aguirre *et al.* 1995, Arey *et al.* 1997).

The presence of ten evolutionarily conserved cysteines in the possum gonadotrophin α -subunit sequence, potentially forming five intramolecular disulphide bonds, indicated that the secondary structure of possum α -subunit resembles that of eutherian α -subunits (Pierce & Parsons 1981, Combarrous 1992). Amino acids at the C-terminus of the α -subunit, positions +75 to +96, are thought to be of particular significance in the interaction of gonadotrophin hormones with their receptors (Combarrous 1992, Yoo *et al.* 1993) and within this region possum gonadotrophin α -subunit conforms

		ΔG (kcal/mole)
possum	<pre> UU A U C-G U-A U-A U-A U U C-G U-A 5' UGGC AAAA3' </pre>	-5.5
ovine	<pre> UU A U C-G U-G U-A U-A U U C-G U-A 5' UUGA AAUA3' </pre>	-5.0
human	<pre> UU A U C-G U-G U-A U-A U U C-G U-A U-A 5' CUAG-UACA3' </pre>	-7.2
bovine	<pre> UU A U C-G U-G U-A U-A U U C-G U-A 5' UUGA AAUA3' </pre>	-5.0
porcine	<pre> UU A U C-G U-G U-A U-A U C C-G U-A 5' UCGC AAUG3' </pre>	-4.8
horse	<pre> UU A U *G G U-G U-A U-A U U C-G U-A 5' UAGC AAUA3' </pre>	-1.0

to the mammalian consensus, with the human and donkey sequences showing the greatest deviation from the consensus. The only amino acid residue unique to the possum gonadotrophin α -subunit protein sequence was the valine at position +25. However this is a conservative difference, with the mouse, rat, rabbit, porcine, equine and whale sequences all having a leucine at the equivalent position (Combarrous 1992). Consistent with the high level of homology between the possum and eutherian α -subunit amino acid sequences was the finding that possum FSH can bind to both bovine and recombinant human FSH receptors (Moore *et al.* 1997).

Outside the coding region the possum gonadotrophin α -subunit cDNA sequence had standard features characteristic of eukaryotic mRNAs. The sequence surrounding the translation start codon 5'-AGAGCCATGG-3' (nucleotides 112-121), resembled the vertebrate translation initiation consensus sequence, 5'-GCC(A/G)CCATGG-3' (Kozak 1987), and a polyadenylation sequence, 5'-AATAAA-3', was present (nucleotides 719-724) fourteen bases 5' to the polyA tail (Proudfoot 1990).

Sequence homology between possum and eutherian α -subunit transcripts extended into the 3' UTR. Such sequence conservation between mammalian gonadotrophin α -subunit 3' UTRs has been noted previously (Nilson *et al.* 1983, Hirai *et al.* 1989). Two overlapping imperfect palindromic sequences, nucleotides 658-675 and nucleotides 660-678, present in the possum α -subunit 3' UTR could potentially form hairpin loops in the mRNA transcripts with predicted free energies of formation (ΔG) of -5.5 and -5.4 kcal/mol respectively, as calculated using the RNA folding computer program mfold (Fig. 5) (Walter *et al.* 1994). Comparison of the 3' UTR sequences of the possum, ovine, human, bovine and porcine α -subunit mRNAs indicated that the imperfect palindrome formed by

FIGURE 5. Potential hair-pin loop secondary structures formed by a conserved 18-20 nucleotide sequence present in the 3' UTR of mammalian gonadotrophin α -subunit mRNA sequences: possum (nucleotides 654-678, Fig. 1), ovine (nucleotides 616-641, Bello *et al.* 1989), human (nucleotides 583-610, Fiddes & Goodman 1979), bovine (nucleotides 623-648, Erwin *et al.* 1983), porcine (nucleotides 630-655, Hirai *et al.* 1989) and horse (nucleotides 494-518, Stewart *et al.* 1987). The base-pairing alignments and theoretical Gibbs free energy (ΔG) values were calculated using the computer program mfold (Walter *et al.* 1994, <http://www.ibc.wustl.edu/~zucker/mfold/>). The ΔG value of the horse 3' UTR potential hairpin is less favourable than the others due to a G (asterisk), rather than a C, in the stem portion of the hairpin.

nucleotides 658–675 is evolutionarily conserved and that similar hairpin secondary structures could potentially form in all five transcripts (Figs 3 and 5). However, the potential hairpin loop sequence was not present in all reported mammalian α -subunit mRNA sequences; a G in the horse 3' UTR removes a potential base pair in the hairpin stem reducing the predicted ΔG value to -1.0 kcal/mol, while the rat and mouse transcripts entirely lack the sequence (Chin *et al.* 1981, Godine *et al.* 1982). Conservation of mRNA secondary structure may indicate functional significance, and a role for hairpin loop structures in regulating mRNA stability in some transcripts is well established (Rajagopalan & Malter 1997). It is noteworthy that the position of the possum α -subunit putative hairpin loop with respect to the translation termination codon was also conserved, varying between 177 and 188 nucleotides in the six α -subunit 3' UTR sequences shown in Fig. 3. Furthermore, in five of the six 3' UTR sequences in Fig. 3, the putative hairpin sequence was located six or eight nucleotides 5' to the sequence AUUUA (AUUUUA in horse) which itself was positioned approximately 30 nucleotides 5' to the polyadenylation signal AAUAAA. In contrast the human 3' UTR sequence had 15 nucleotides separating the sequence AUUAA and the polyA tail. A role for such AU-rich elements (AREs) in influencing mRNA stability has been proposed (Shaw & Kamen 1986, Sachs 1993), with recent work indicating that the AUUUA sequence may form a necessary but not sufficient core sequence that destabilizes some mRNAs (Zubiaga *et al.* 1995, Lagnado *et al.* 1994). It has been noted previously that the prevalence of (U)_nA sequences, a possible indicator of mRNA stability, suggested that the porcine, human, bovine and equine gonadotrophin α -subunit transcripts may be less stable than those of rat and mouse (Wreschner & Rechavi 1988, Hirai *et al.* 1989).

The pattern of gonadotrophin α -subunit gene expression in the adult possum pituitary was similar to that described previously for eutherian mammals, with *in situ* hybridization identifying a punctate distribution of cells in the anterior pituitary synthesizing α -subunit mRNA. As the gonadotrophin α -subunit is common to the hormones FSH, LH and TSH, the hybridizing cells are probably gonadotrophs, producing FSH and LH, and thyrotrophs, producing TSH.

In summary, a cDNA sequence of the possum gonadotrophin α -subunit gene has been determined. The coding sequence showed a high level of homology, at both the nucleotide and predicted amino acid levels, with the gonadotrophin α -subunit genes of eutherian mammals. From the

perspective of designing possum biocontrol strategies, the high level of evolutionary conservation of the gonadotrophin α -subunit amino acid sequence indicates that gonadotrophins may be unsatisfactory 'target molecules' as they offer little species specificity. A possible hairpin loop region within the possum gonadotrophin α -subunit mRNA 3' UTR has been described and the evolutionary conservation of this sequence indicates possible functional significance, perhaps influencing mRNA stability. Expression studies using the α -subunit cDNA indicated close similarity with other mammals.

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