Isolation and characterisation of the ovine gastrin-releasing peptide gene; abundant expression in the pregnant uterus and selective expression in fetal tissues

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Abstract

High concentrations of a peptide related to gastrin-releasing peptide (GRP) are produced in the utero-placental unit of the human and sheep and secreted into the general circulation. This suggests an endocrine role in addition to its role as a neurotransmitter/neuromodulator. The GRP is larger than the previously described form GRP(1–27) but it is not known whether the larger form is the product of a related GRP-like gene or differences in post-translational processing. We have therefore cloned the gene for the sheep homologue of the GRP gene and determined its distribution. Only a single GRP gene was found in the sheep. This had a similar organisation to the human GRP gene with three exons and two introns. The larger form of GRP in the pregnant endometrium therefore appears to be the result of an alteration in processing

of the GRP prohormone. The expression of GRP mRNA in the pregnant uterus was extraordinarily high comprising one-third of all mRNA synthesised by the pregnant endometrium. As the endometrial GRP mRNA arises solely from the glandular epithelium, the localised synthesis of GRP mRNA would be far higher. GRP mRNA was expressed in a wide variety of fetal tissues (fundus, colon, jejunum, ileum, duodenum, kidney, adrenal, lung, heart and pancreas) with a corresponding presence of GRP immunoreactivity. The expression of GRP in the fetal lung was biphasic with peaks at mid-term and near parturition but none in the adult supporting the concept of a specific developmental role of GRP in the lung.

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Introduction

A novel endocrine role for gastrin-releasing peptide (GRP) was proposed several years ago with the discovery of high levels of GRP immunoreactivity in the peripheral circulation and uteroplacental unit of pregnant ewes (Giraud *et al.* 1993, 1994, Fraser *et al.* 1994). Previous to this observation, GRP was thought to act mainly as a neurotransmitter and be localised almost exclusively to neurons and neuroendocrine cells (Bunnett 1994). Further investigation of the GRP immunoreactivity in the pregnant ovine uterus showed that the protein was significantly larger than the known biologically active forms GRP(1–27) and the decapeptide GRP(18–27) (Giraud *et al.* 1996). A similar GRP related molecule was found in the pregnant human (Whitley *et al.* 1996). However it is not known whether this larger form is the product of a related GRP-like gene, alternative GRP mRNA transcripts or differences in post-translational processing.

Quantification of the expression of endometrial GRP is of interest because we have demonstrated that GRP in the ovine endometrium is a tightly regulated process with increases in GRP mRNA during the ovine oestrous cycle (24-fold) and early pregnancy (13-fold between days 17 and 20) (Whitley *et al.* 1998) while both GRP mRNA and peptide expression are reduced following the administration of oestrogen and progesterone (Whitley *et al.* 2000). Our initial studies suggested that in the pregnant uterus, the proportion of mRNA that is GRP was extraordinarily high and may be a potential explanation for the excess of large molecular weight forms. We have therefore quantified the proportion of poly(A)⁺ RNA that is GRP mRNA in the late term endometrium by comparison to synthetic GRP mRNA standards and Northern analysis with a GRP antisense riboprobe.

While the endometrial glands appear to be the major source of the circulating GRP-like molecule as demonstrated by *in situ* hybridisation and radioimmunoassay (Whitley *et al.* 1998), GRP-like peptides may also be

synthesised in other tissues of the uteroplacental unit and act locally. Thus in this study we have also analysed expression of the GRP gene in a panel of fetal ovine tissues in late pregnancy. Finally we have examined the ontogeny of GRP mRNA expression in the fetal ovine lung to determine whether the expression of GRP mRNA in some adult lung cancers (Cuttitta *et al.* 1985) is a recapitulation of fetal expression.

Materials and Methods

Construction and screening of ovine endometrial cDNA library

Human GRP cDNA (kindly provided by J Battey, NIHCD, NIH) was labelled with α -[32 P]dATP using a random primer labelling system (rediprime II, Amersham Pharmacia Biotech, Castle Hill, NSW, Australia) and used to screen approximately 2×10^4 plaques of an ovine late term endometrium cDNA library prepared in phage λ gt11 as per manufacturer's instructions (Amersham Pharmacia Biotech). Putative clones were selected and rescreened until individual plaques were isolated. Phage DNA was purified for three separate clones and the cDNA insert amplified using λ gt11 forward and reverse primers and the proof reading Taq polymerase Pfu prior to cloning into plasmid pBS SKII (Stratagene, La Jolla, CA, USA) and determination of nucleotide sequence by automated DNA sequencing (ABI Prism 373 DNA sequencer, Perkin-Elmer, Foster City, CA, USA) with BigDye terminator chemistry and scanning laser detection.

Screening of ovine genomic DNA library

Approximately 5×10^5 recombinant λ EMBL3 bacteriophages representing a library of EcoRI fragments of sheep genomic DNA (supplied by Dr Louise Baker, Department of Veterinary Science, University of Melbourne) were screened by hybridisation with radiolabelled ovine GRP cDNA (isolated above) as described previously (Whitley *et al.* 1999). Following four rounds of screening, two individual positive plaques were isolated and phage DNA purified. Restriction maps were constructed by Southern hybridisation using fragments of ovine GRP cDNA. Exons 1 and 2 were located on one genomic fragment (phage denoted λ GRP7) and exon 3 on the other (phage denoted λ GRP8). Appropriate restriction fragments encompassing individual exons were subcloned and the nucleotide sequence determined as described above.

Southern hybridisation of ovine genomic fragments

Ovine genomic DNA was prepared from white blood cells (Ausubel *et al.* 2000) and 10 μ g subjected to restriction endonuclease digestion with BamHI, EcoRI or HindIII. Fragments were separated by 0.7% agarose gel

electrophoresis prior to vacuum transfer (VacuGene, LKB/AMRAD, Kew, Victoria, Australia) in 0.4 M NaOH to charged nylon membrane (Hybond N+; Amersham Pharmacia Biotech). Fragments with homology to the GRP cDNA were detected by hybridisation at 42°C in cDNA hybridisation buffer (50% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSC, 50 mM Na phosphate pH 6.8, 100 μ g/ml denatured salmon sperm DNA and 1% SDS) for 16 h with radiolabelled ovine GRP cDNA as probe. Unbound probe was removed by two rinses in a buffer containing 0.1 $\times$ SSC, 0.1% SDS and two successive washes at 60 °C in the same buffer and responsive fragments visualised by autoradiography to X-ray film (XAR-5, Kodak, Integrated Sciences, East Kew, Victoria, Australia).

Analysis of mRNA expression

Several methods were used for detection of mRNA expression depending on the relative abundance of GRP in the tissues analysed. For all samples, ovine tissue was removed soon after killing (all procedures were approved by the Animal Ethics Committee of the Austin and Repatriation Medical Centre) and stored frozen at -70 °C.

Preparation of total RNA

Total RNA was prepared from 200 mg frozen tissue using Trizol reagent (Gibco-BRL, Mulgrave, Victoria, Australia). Contaminating endogenous RNases were destroyed by treatment with 40 μ g proteinase K (Sigma Chemical Co., St Louis, MO, USA) in a reaction buffer consisting of 0.1 M Tris-Cl, pH 7.8, 0.05 M EDTA, 1% SDS for 30 min at 50 °C. Proteinase K was removed by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and the RNA precipitated with 0.3 M Na acetate, pH 5 and one volume of isopropanol as per standard procedures (Ausubel *et al.* 2000) and resuspended in sterile water. A 5 μ g sample of each RNA was subjected to denaturing electrophoresis through low formaldehyde containing agarose as previously described (Whitley *et al.* 2000) to check integrity and concentration estimation of the prepared RNA.

Northern analysis

Samples of total RNA were separated by agarose gel electrophoresis (as above) before vacuum transfer in 20 $\times$ SSC 300 mM NaCl, 30 mM sodium citrate) for 4 h to nylon membrane (Hybond N+). Following transfer, the membrane was rinsed briefly in 2 $\times$ SSC and air dried before fixing the nucleic acids to the membrane by treatment with u.v. light (Ultraviolet Cross-linker, Amersham Pharmacia Biotech) using the manufacturer's settings. Labelled riboprobes for GRP and GAPDH were

prepared and purified essentially as described previously (Whitley *et al.* 1998) using the MAXIscript kit (Ambion, Austin, TX, USA) including 0.85 μM [^{32}P]UTP and 2.15 μM unlabelled UTP. Recombinant plasmids containing ovine GRP cDNA (isolated above) or ovine GAPDH cDNA (exons A–H, a generous gift from Dr Louise Baker, Department of Veterinary Science, University of Melbourne) were digested with appropriate restriction enzymes to yield labelled antisense cRNA molecules of approximately 230 and 290 nucleotides (nt) respectively. Riboprobes were added (5×10^5 c.p.m./ml) to membranes sandwiched between two sheets of Whatman No. 1 filter paper that had been prehybridised for 16 h at 68 °C in RNA–RNA hybridisation buffer (55% formamide, 5 $\times$ Denhardt's solution, 2 $\times$ SSC, 50 mM Na phosphate pH 6.8, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, 100 $\mu\text{g}/\text{ml}$ *E. coli* tRNA and 1% SDS). Following hybridisation at 68 °C for 24 h, membranes were briefly rinsed twice in 0.1 $\times$ SSC, 0.1% SDS and washed for 15 min at 68 °C in the same solution. GRP or GAPDH mRNA was detected following autoradiography at –70 °C using XAR–5 film and two intensifying screens.

RT-PCR

The methods used have been described previously (Whitley *et al.* 2000) and are only summarised here. Prior to cDNA preparation for RT-PCR, poly(A)+ RNA was prepared from 75 μg total RNA using 1 mg oligo dT-linked magnetic beads according to the manufacturer's instructions (Dynal, Clifton Hill, Victoria, Australia). Approximately 100 ng poly(A)+ RNA and 4 ng pdN₆ were used for cDNA synthesis with Superscript reverse transcriptase according to the manufacturer's instructions (GibcoBRL, Life Technologies, Mulgrave, Victoria, Australia). A portion of prepared cDNA (1/20) was used in PCR for ovine GRP and ovine β -actin using oligonucleotide primers and conditions as described previously (Whitley *et al.* 2000). Products (1/5) were taken at the completion of PCR (40 cycles) and visualised by agarose gel electrophoresis and/or Southern hybridisation using oligonucleotide probes complementary to sequences located between primers.

Quantitative determination of GRP mRNA abundance

Synthesis of GRP mRNA Sense GRP mRNA was synthesised from linearised plasmid DNA (ovine GRP cDNA inserted into pGEM3Zf+) as template and T7 polymerase according to the manufacturer's instructions (MEGAscript kit, Ambion, Austin, TX, USA). Protein was removed by extraction of the reaction mix with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by extraction with chloroform:isoamyl alcohol (24:1) and precipitation of the nucleic acid with

one volume of isopropanol. A single product of 670 bases (corresponding to nts 8–678, GenBank Accession S75723) was visualised by denaturing polyacrylamide gel electrophoresis (5% acrylamide, 8 M urea).

Purification of poly(A) RNA Poly A+ RNA was purified from two different samples of approximately 900 μg total RNA (prepared from late term ovine pregnant endometrium as described above) using biotinylated oligo dT and streptavidin linked magnetic beads according to the manufacturer's instructions (PolyATtract, Promega, Annandale, NSW, Australia). Products of the first round of purification were subject to a second round of poly(A)+ purification to ensure removal of all ribosomal RNA molecules. Quality and quantity of double purified poly(A)+ RNA were assessed by denaturing formaldehyde agarose gel electrophoresis and spectrophotometry (GeneQuant Spectrophotometer; LKB/AMRAD) respectively.

Slot blot hybridisation The slot blot manifold was prepared and assembled according to the manufacturer's instructions (Schleicher and Schuell, Dassel, Germany). Twofold serial dilutions of synthetic GRP mRNA were performed in denaturing solution (60% formamide, 2.3 M formaldehyde, 1.2 $\times$ MOPS Buffer, 0.1 mg/ml tRNA) to give a range from 20 000 to 0.15 pg synthetic GRP mRNA. Dilutions of poly(A)+ RNA were performed in denaturing solution to give 10 000, 1000 and 100 pg RNA. RNA samples were heated at 65 °C for 15 min, placed on ice and four volumes of ice cold 20 $\times$ SSC added to each sample prior to vacuum transfer to positively charged nylon membrane (Hybond N+). Following two washes with 10 $\times$ SSC, the slot blot apparatus was disassembled and the membrane allowed to dry at room temperature. Nucleic acid was u.v. fixed onto the membrane and baked at 80 °C for 30 min. Sense GRP mRNA was detected by hybridisation with radiolabelled antisense GRP riboprobe (5×10^5 c.p.m./ml) as described above for Northern analysis and visualised after exposure to phosphor screens (Storm 840 Phosphorimager, Molecular Dynamics, Amersham Pharmacia Biotech) and quantified using ImageQuANT software (Molecular Dynamics). Results were subject to linear regression analysis and the amount of GRP mRNA in the two samples of poly(A)+ RNA determined by reference to a standard curve of log phosphor imager units versus log 10 pg sense GRP mRNA using SigmaPlot 5 software (SPSS, Chicago, IL, USA).

Results

Organisation of the ovine GRP gene

Figure 1 shows partial restriction maps of ovine genomic DNA encompassing exons 1, 2 and 3 of the GRP gene.

Figure 1 Genomic arrangement of the ovine GRP gene. Schematics for two ovine genomic DNA fragments cloned into λEMBL3 and encompassing GRP exons 1, 2 and 3; λGRP7 (entire 17 kbp insert) (A) and λGRP8 (2.0 kbp HindIII subfragment) (B). The positions of all restriction sites for BamHI (Bm), EcoRI (Ec), HindIII (Hd), SacI (Sc) and some restriction sites for endonucleases KpnI (Kp) and PstI (Pt) are shown above. The position of exons and repeat sequences are shown below. Maps were constructed using the Redasoft Plasmid 1.1 program (www.redasoft.com). (C) Southern hybridisation of ovine genomic DNA restricted with endonucleases BamHI, EcoRI and HindIII and probed with radiolabelled ovine GRP cDNA.

Exons 1 and 2 are separated by 6–7 kbp and were isolated on a single recombinant phage (Fig. 1A) whereas exon 3 was contained in a 2.0 kbp HindIII fragment isolated from a separate recombinant phage of approximate total size 16.5 kbp (Fig. 1B). Southern hybridisation of ovine genomic fragments digested by endonucleases BamHI, EcoRI and HindIII with radiolabelled ovine GRP cDNA as probe revealed multiple responsive fragments (Fig. 1C). Comparison of the HindIII responsive fragments to the isolated genomic fragments suggested the presence of a single GRP gene, i.e. the 2.3 and 2.0 kbp bands represent the HindIII fragments possessing exons 1 and 3 respectively while exon 2 is located within a fragment that must

be larger than 6.9 kbp which is seen as a faint band at approximately 7.0 kbp on Southern hybridisation (Fig. 1C).

Nucleotide sequence of the ovine GRP gene and cDNA molecules

The nucleotide sequence of the ovine GRP gene was determined (Fig. 2) and compared with the known ovine GRP cDNA sequence (Xiao *et al.* 1996, GenBank Accession S75723). Several discrepancies were observed at the extreme 5' UTR (shown in Fig. 2). Similarly, two of the three cDNA molecules isolated in this study also

Figure 2 Nucleotide sequence of the sheep GRP gene. Coding sequence for all 3 exons is shown (upper case) along with 2214 nt of the promoter, all of the 5' UTR, partial introns 1 and 2, and part of the 3' UTR. Numbering is with reference to the adenosine of translation start codon ATG as +1. Intron sequence is not included in the number shown above. The promoter TATAA sequence is boxed and three predicted transcription initiation start sites denoted P1, P2 and P3. A cAMP responsive element (CRE) is boxed. The extreme 5' UTR of four GRP cDNA molecules are aligned underneath the genomic DNA sequence and labelled S75723 (Xiao *et al.* 1996) E14, E2 and E5 (isolated in this study). The amino acid sequence is shown in single letter code below the nucleotide sequence. Amino acids corresponding to biologically active GRP1–27 and to its prohormone processing site are shown in bold and underlined respectively. The stop codon TGA is indicated (*). A putative polyadenylation site in the 3'UTR is shown in bold. A single nucleotide difference to the published cDNA sequence is shown at +642.

-2214 atcaaggcct gtgggtgcat cgattctttt gggaagcagc taaagggtta agattccatt taagaaagat
 -2144 tagatgaaat gttccccaag tggggaattc aatgaaggag taccctaatt tgtgccacag gataagaggg
 -2074 tgcaatgccc cactgagcca aagcaagtga cgtatctaag ccgaagacgg ggtgcagaaa gctgttagac
 -2004 caggggttta gagcagaagc tgaatgaac agaggagccc aggtgcctct tggcctgtaa cagacctaaag
 -1934 aaagagtggc ctgcctgaat tagctctttc tgtagaactg ttcccacatt ttattatatt tacagttgct
 -1864 ttcccttggt ctcatagtgg aaaaaacttt attaaaagat tctaaaataa tcttgattaa actaagggga
 -1794 aaggatattt ttctatcact taatctggag agagaaaggt ttaatgcccc tctgaaggca ccggttgacg
 -1724 aaagcattta gatgcgagca tttctcaggt tagctgttca ttcatacaaac agctgctgtg tgcactggg
 -1654 tccaggctct ttgcaggccc acagggggaat gagaccctgt ccctgagttc aggcgagctc ccaccaagt
 -1584 gggaggcagt gagcctacaa atggtcacct gatggtggaa catttgctgc ctaataagca gagcctggga
 -1514 acccagaggg atgaagtcaa agcccattgt ggtcttcccc ctcaattttc acttgagggc attccctcag
 -1444 gatcctatta ctctgttgcc tggctaataa tgggtgctct gaaattagtg ctgtgtttaa cttctagcc
 -1374 agacatagtc tcttccaaagc tcttccaaagc tcaagaacac tcagtcattg gacatgcaca tgtcaccatc
 -1304 tgaggctggc tcagggtcac tgggccacga gataaagaat atggtttcaa ctaatttggt atcaacggcc
 -1234 agtattgaca cggaacacca gcagggaccc accagcgagt gattcaccag cacttggtga ggaacaggga
 -1164 cgcaggagcg cagcaacaca ccccagctc tggcagccga atcgtgggca gcagcaccct ccacgctcc
 -1094 agtcgctgcg gcacggctat ggcgccacc cgctttgtat ccatggcatt gagaatctcg ggagttgagc
 -1024 cgggtggcct tcctcatctc cgagaacaga agaggcagag caaagatgcc cagccctaga ggagtggtga
 -954 aacagcagct gagggacagt ttgcgcctgc tggtaggcgc agcctaggaa accagcatag aagcttttc
 -884 cattaggcgg acgctgatga agggaaccag gagcgtagta aaactgttgc tagttccatc acattacacg
 -814 tctttccgcg tcagcttcc attgcacctc acgggcaggg acggaaaacc caggtcgggt gagggatttt
 -744 gctggaggcg gtgaaccggc gggggttagg ggcgctctgc aggaggcagg caaaggtctg gagtctcgcg
 -674 ggctaggacg cgaggaggga ggtggccccc agagcggaaa ggtctgagaa tcagaagtgc ttgcaattc
 -604 gggctccaat ccctccggc aaagctcggc ctaaaaacat ccatcctccg tgttcatccc tcttacaac
 -534 ccgcgctccg ccgcacctct cgccccggg gtcggggcgc cgcgctcttg ctccccctg ctggatccga
 -464 aggcagctag ccaggagcg ccctggccga gctgcgaaag gagggcttcg ctacattgtg ctggagcgac
 -394 ggcgtcgagg gtgggaagga gtccagtgtg ggggtggcgg cggtgtctac cgggaaagag agaggcgcg
 -324 ggggaacacc cgccttttc acccctgggc ggggaagatg atggttttcg gaggcggtg tcccagcgcc

 -254 ccggctctcg ccacaggg ag acgtca gacg gctcggggcc gcggcgagg agcgcctccc ccgcccgcg

 -184 tccacgcccc cctacccgcg ccgcccgggc ttgca TATA agccccggcg ggcggcggcc ggagcagagg

 -114 cgggcgggcg cgccggcgct tctgcgagct ccgggtcctg ggcttgctc cgcggggaga acgcgctttc
 S75723 .gca ..ag.....
 E14
 E2 ...c... ..
 E5cg.....

 -44 cgcccagcct atccgcggcg ctccgggcgc ctcccgcggg +1 taccATGCGC AGCCGCGAGG TCTCGCTCGT
 M R S R E V S L V
 +27 CCTGCTGGCC CTGGTCTCTT GCCCGGCGCC CCGGGGATCG GCCGCCCCGG TGACGGCTGG CAGAGCAGGC
 L L A L V L C P A P R G S A A P V T A G R A G
 +97 GCGCTGGCCA AGATGTACAC GCGCGGCAAC CACTGGGCGG TCGgtgagtg tccgcgcgcg tcgagtcgcc
 A L A K M Y T R G N H W A V
 gggggccagc.....intron 1 (6.7 kbp)..... cttctccat tctgcttttg aacagGACAC
 G H
 +146 TTAATGGGAA AAAAGAGTGT GGCAGAGTCC CCACAAC TTC GTGAGGAGGA GAGCCTGAAG GAGCAGCTGA
 L M G K K S V A E S P Q L R E E E S L K E Q L
 +216 GGGAGTACGC CCAGTGGGAA GAAGCGACCA GGAACCTTGCT AAGCCTCCTA CAAGCAAAGG TGGCCCAAGG
 R E Y A Q W E E A T R N L L S L L Q A K V A Q G
 +286 CCACCAGCCG CCTCGATGGG AGCCCCTGAG CATTACCCAG CCTGCCTGGG ATTCCAAGGA CGTCAGCAAC
 H Q P P R W E P L S I H Q P A W D S K D V S N
 +368
 +356 TTCAAAGACT CAGgttcgag actcaagggt acagagaaca taccaggggg gtggaggctg ggagagggtg
 F K D S
 gctggggcgg ggggtggaag.....intron 2.....ggactcttca ctgcagGTTT TCAGCGTGAA
 G S Q R E
 +383 GGAGGGAACC CCCAGCTGTA CTGAcgatga caatagccgc ctccctcaaa gaagcaaac aaaaccctta
 G G N P Q L Y *
 +453 agaaactgtg ttctacgggc gtcggttcta ctggatcatc aacagatttc ctttgtgcaa aataactaac
 +523 tattcttgta cttttcaact ttgactaaag tcatgatatt caagcagcat cttctggttt gaacttggtt
 +593 gctgtgaaaa atgtgtccata aagagtcttc taattaatgt tctttacatc taggcatctc attagatttc
 g
 +663 agtccccaac ctgttaccag tcacaataaa agctt

Figure 3 Alignment of mammalian proGRP amino acid sequences using the single letter code with identical sequences denoted by dots. Shown is the sheep sequence determined in this study, human form I (GenPept accession no. P07492) and rat (GenPept accession no. A40922). Numbering is for the sheep sequence. Consensus sequence for GRP peptides and the prohormone processing site are written in bold. The conserved motif KXXXR is shown below the aligned sequence.

diverged from the genomic sequence in this region (shown in Fig. 2). A single nucleotide difference to the published cDNA sequence was also observed in the 3'UTR at +642 (Fig. 2). For all molecules (genomic and four cDNA), there was no variation in the coding sequence (not shown). Intron–exon boundaries were inferred by alignment to the ovine GRP cDNAs and agreed with intron placement of the human GRP gene. All splice sites scored highly by computer analysis (CGG Nucleotide sequence analysis, program spl, <http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gfb.htm>).

The position of the ovine GRP gene promoter and transcription start site was inferred by comparison to cDNA molecules, human GRP gene promoter and by computational analysis of the genome sequence. Promoter prediction by neural network (<http://www-hgc.lbl.gov>) of 3.6 kbp sequence including exon 1 and 2.2 kbp upstream sequence returned four promoters with cut off scores above 0.85 which detects 0.1–0.4% false positives. Three of these were clustered around the cDNA start sites and are found 15, 26 and 45 nt downstream from the TATA box (denoted P1, P2 and P3 respectively; Fig. 2). A consensus sequence for a cAMP responsive element (CRE) was found at –229 to –236 upstream from the ATG translation start site. The identical sequence AGACGTCA is found in a similar position in the human GRP gene promoter (GenBank Accession S73265, Nagalla & Spindel 1994).

Repeat sequences

Several repeat sequences were identified using the RepeatMasker program (Smit AFA & Green P at <http://ftp.genome.washington.edu/RM/RepeatMasker.html>) and are denoted on the restriction maps (Fig. 1A and B). These include both human and ruminant specific elements. There is a long interspersed nuclear element (LINE), type L2b in the region upstream to exon 1, two ruminant specific short interspersed nuclear elements

(SINEs), types tA2 and A2 preceding exon 2 and another preceding exon 3 that interrupts a human MER5A type DNA element (Fig. 1A and B). Consistent with species relativity, the ovine sequences show 21–27% divergence to the human sequences and 4 or 13% divergence to the bovine derived sequences. The presence of these repeat sequences is consistent with the observation that eukaryotic genomes comprise large amounts of repetitive DNA, some representing interspersed repeats derived from inactive copies of a wide variety of transposable elements (Jurka 1998).

Alignment of mammalian proGRP molecules

Figure 3 shows alignment of the proGRP molecules for three known mammalian species; sheep, human and rat. The endoplasmic reticulum signal sequence is co-translationally cleaved and is not shown in this alignment. The amino acid sequence for toad proGRP is known (GenPept accession no. P29007) but is not included in this comparison as it is significantly different. Previous analysis of cDNA clones from a carcinoid lung tumour (Spindel *et al.* 1986) and small cell lung cancer cell line (Sausville *et al.* 1986) showed the existence of three distinct GRP mRNA transcripts arising from processing at alternative donor and acceptor splice sites at exons 2 and 3 respectively, of a single GRP gene. In Fig. 3 we have only included the product resulting from translation of human GRP mRNA form I as this form shows the highest homology to rat and sheep sequence and is the predominant form found in normal and neoplastic tissue (Spindel *et al.* 1987). The most extensive region of homology exists from +11 to +31 and encompasses the motif conserved for all gastrin-releasing peptides – WAVGHLM – representing the C-terminal region of biologically active GRP1–27 and GRP18–27. Four conserved basic amino acids are observed at +13, +17, +45 and +49 (numbering refers to sheep sequence). Cleavage at Arg–17 is known to

Figure 4 Expression of GRP mRNA (top panel) and β -actin mRNA (bottom panel) in a panel of ovine fetal tissues detected by RT-PCR. cDNA was prepared from jejunum (A), ileum (B), kidney (C), duodenum (D), fundus (E), lung (F), colon (G), heart (H), adrenal (I), liver (J), antrum (K) and pancreas (L). cDNA from adult pituitary (1/5 dilution) was used as positive controls (+). A water control was also included (-). Products for GRP were detected by Southern hybridisation using a gene specific oligonucleotide probe. Products for β -actin were detected by ethidium bromide staining. GRP-ir (pmol/g) for matching tissue samples are shown above the top panel.

yield GRP18–27. Interestingly these four conserved basic amino acids pair to form two copies of the motif KXXXR.

GRP mRNA expression in fetal sheep tissues

GRP mRNA expression was examined in a variety of fetal tissues isolated from a single animal (120 days gestation) using the sensitivity afforded by semi-quantitative RT-PCR (Fig. 4). Synthesis of GRP mRNA was detected in a wide range of tissues including kidney, adrenal, lung and parts of the gastrointestinal tract (duodenum, jejunum, ileum, fundus and colon). Some GRP mRNA was detected in fetal liver and pancreas but none in stomach antrum. RIA of matching tissue samples (method described in Whitley *et al.* 1998) revealed GRP-ir in all tissues tested (adrenal gland was not assayed) and in amounts roughly correlating with GRP mRNA detection (Fig. 4). For example GRP-ir was highest in ileum (B), kidney (C) and colon (G) (6.8, 6.7 and 6.7 pmol/g) where

GRP mRNA abundance was also high, and lowest in heart (H), liver (J) and pancreas (L) (1.3, 0.9 and 1.8 pmol/g) where GRP mRNA abundance was low. Two exceptions to this pattern were fetal lung (F) (5 pmol/g) and antrum (K) (3.9 pmol/g) where GRP mRNA synthesis was very low and undetectable respectively. The ontogeny of expression of GRP mRNA in the fetal lung was examined by Northern analysis modified for high sensitivity and maximum detection (Fig. 5). GRP mRNA abundance was detected in similar amounts in fetal lung at days 40 (not shown), 60 and 80 but not at days 100 and 120. Fetal lung obtained at days 140 and 145 gestation had the most GRP mRNA abundance whilst the adult lung had none under these experimental conditions.

Examination and quantification of GRP mRNA abundance in the pregnant endometrium

Northern analysis using poly(A)⁺ RNA isolated from adult tissues confirmed previous observations of high expression in ovine pregnant endometrium and myometrium (Whitley *et al.* 1998), lower expression in non-pregnant endometrium and myometrium and the absence or extremely low expression of GRP mRNA in hypothalamus, testes and amnion (Fig. 6). Inspection of the agarose gel following staining with ethidium bromide revealed a smear of poly(A)⁺ material in all lanes (as expected) and three distinct bands (Fig. 6, top, lane E) in the lanes representing poly(A)⁺ RNA prepared from late term pregnant endometrium and myometrium (Fig. 6). The band of lowest mobility represents residual 18S rRNA and the band of highest mobility corresponds in size to GRP mRNA at approximately 900 nt, as identified by Northern analysis (Fig. 6, middle). A third abundant mRNA of approximately 1500 nt was also observed but is so far unidentified. This observation (and many similar) suggested that GRP mRNA is a highly abundant transcript in ovine pregnant endometrium and myometrium. The proportion of poly(A)⁺ RNA that is GRP mRNA was determined by comparing the amount of GRP mRNA in double purified poly(A)⁺ RNA isolated from two separate samples of late term pregnant ovine endometrium to a standard curve of known amounts of synthetic sense GRP cRNA (Fig. 7A and B). GRP

Figure 5 Northern analysis showing the ontogeny of GRP mRNA expression (top panel) compared with the expression of GAPDH mRNA (bottom panel) in fetal lung taken at different stages of gestation (as indicated) and adult lung.

Figure 6 Northern analysis of GRP mRNA in sheep utero-placental tissues, hypothalamus and testes. Approximately 5 µg polyA⁺ RNA isolated from hypothalamus (A), testes (B), non-pregnant endometrium (C), non-pregnant myometrium (D), pregnant endometrium (E), pregnant myometrium (F) and amnion (G). Arrows indicate residual 18S rRNA (top) and GRP mRNA (bottom). The middle and bottom panels show the results of Northern analysis for GRP and GAPDH mRNA respectively.

mRNA was visualised by Northern hybridisation with radiolabelled antisense GRP riboprobe and phosphor imaging. Linearity of response was observed over two orders of magnitude, from 1.22 to 1250 pg GRP cRNA (Fig. 7C). Linear regression analysis of these points enabled determination of the amount of GRP mRNA in 100 and 1000 pg double purified poly(A)⁺ RNA from two separate samples of late term pregnant endometrium (the 10 000 pg point was outside the linear range). For sample 1 this was found to be 37 and 322 pg respectively and for sample 2 this was found to be 22 and 230 pg respectively. Thus for two individual samples the proportion of all mRNA species that is GRP mRNA is 35 and 23%.

Discussion

Previously we and others have reported the presence of a larger-than-expected GRP in the pregnant uterus of women and sheep (Giraud *et al.* 1996, Whitley *et al.* 1996, Xiao *et al.* 1996). Examination of the ovine molecule revealed several differences such as increased molecular weight and stability in plasma when compared with the well-known biologically active GRP1–27 and GRP18–27 (Giraud *et al.* 1996). In this study we sought to examine whether the larger-than-expected GRP was the product of a GRP-related gene or a product of alternative transcript arising from the GRP gene. Southern hybridisation of ovine genomic DNA fragments revealed several responsive bands that corresponded with the restriction pattern of the genomic fragments encompassing the ovine GRP gene. This result strongly suggested that the ovine genome possesses a single GRP or GRP-related gene. Nucleotide sequence analysis of three separate cDNA molecules isolated from an ovine endometrial cDNA library revealed some small differences to each other and to the previously published sequence (Fraser *et al.* 1994) in the 5' and 3' UTRs. However no differences were observed in the coding region and translation of any of these molecules would lead to a gene product predicted to be processed to GRP1–27 and GRP18–27. These results suggest that the larger-than-expected GRP observed in the pregnant uterus results from alternative processing of the GRP prohormone.

Normal processing of the GRP preprohormone is similar to other neuropeptides and involves co-translational cleavage of the signal peptide to yield pro-GRP which undergoes endoproteolytic cleavage at MGKK↓ by an as yet uncharacterised prohormone convertase. Carboxypeptidase E removes the basic residues and peptidyl α – amidating mono-oxygenase acts on the glycine to yield C terminally amidated GRP1–27. Further endoproteolytic cleavage at Arg-17 within the motif KXXXXR releases the decapeptide GRP18–27 (Bunnett 1994). For the larger-than-expected GRP to arise from alternative peptide processing, we predict an absence of the prohormone convertase activity at MGKK resulting in a C terminally extended form of GRP. This may result from lack of the enzyme in the pregnant uterine glandular epithelial cells or from a lack of access of the GRP processing convertase to the GRP prohormone perhaps coinciding with a switch from the regulated pathway to the constitutive pathway.

One possibility is that the larger-than-expected GRP observed in the pregnant ovine uterus is a usual intermediate in GRP prohormone processing. Comparison of the known mammalian proGRP amino acid sequences (Fig. 3) revealed two instances of the motif KXXXXR. Processing at the first yields GRP(18–27) while processing at the second KXXXXR could produce cleavage at Lys-45 to generate GRP1–44 from proGRP not cleaved at

Figure 7 Quantification of GRP mRNA in RNA isolated from late term ovine pregnant endometrium. Phosphor image analysis of Northern hybridisation with radiolabelled antisense GRP riboprobe to (A) standards of membrane bound synthetic sense GRP RNA and to (B) double purified poly(A)+ RNA isolated from two separate samples of late term pregnant endometrium. C, plot of linear range for log₁₀ PPI counts versus log₁₀ synthetic GRP RNA (●) and linear regression (straight line).

MGKK. This hypothesis is strengthened by the observation of a molecule of similar high molecular weight in the conditioned medium of the small cell lung carcinoma cell line NCI-H345 (Lebacqz-Verheyden *et al.* 1988). In that study, several post-translation processing products of proGRP produced by the SCLC cell line NCI-H345 were identified including small amounts of amidated GRP1–27. The major secreted product was identified as a C terminally extended form of GRP1–27 ending at about Glu-40. Several other groups have reported the presence of C terminal extended forms of proGRP in SCLC tumours and cell lines (Holst *et al.* 1989, Vangsted & Schwartz 1990). C terminal extended forms are biologically active (Mervic *et al.* 1991, Oiry *et al.* 2000).

In pregnant ovine endometrial cells, the very high abundance of GRP gene transcription could lead to the GRP processing and post-translational modifications being saturated so that partially processed product(s) become the major end product(s) and the amidated forms of GRP become minor end products. In the present study we determined the proportion of poly(A)+ RNA that is GRP mRNA to be 23 and 35% for two separate samples of late term pregnant ovine endometrium. Thus in this tissue, about one-third of all mRNA molecules are GRP

mRNA. We have previously shown, using *in situ* hybridisation, that only one cell type within ovine pregnant endometrium – the glandular epithelial cell – is responsible for GRP synthesis (Whitley *et al.* 1998). Accordingly within the glandular epithelial cells the proportion of all mRNA that is GRP mRNA would far exceed one-third. This is a truly remarkable abundance given that in most biological systems an mRNA species that constitutes 1% of the total pool is considered to be abundant and that the most abundant mRNA in the liver is albumin accounting for about 15% (Anderson *et al.* 1997).

The observation of variation in the 5' end of the cDNA molecules, and the strong correlation between the sequence and predicted promoter sites suggests that several different start sites may be used for transcription of the ovine GRP gene. This has been observed experimentally for the human GRP gene. In SCLC cell line NCI-H209 and normal fetal lung, transcription initiation was shown to start 25 nt downstream from the TATA box with a minor start site 13 nt downstream (Spindel *et al.* 1987). In a separate study, analysis by S1 nuclease protection and primer extension in two SCLC cell lines (NCI-H209 and NCI-H510) revealed a single transcription initiation start site at 32 nt downstream of the TATA box (Markowitz

et al. 1988). Computational analysis of the human GRP gene promoter using the neural network promoter prediction program predicts the start site at 11 nt downstream from the TATA box. Alignment of approximately 300 nt of ovine and human sequence surrounding the TATAA box of the GRP gene reveals extensive homology with similar positioning of the experimentally determined transcription initiation sites of human GRP gene and the predicted transcription initiation sites of the ovine GRP gene. Also found immediately upstream from the putative transcription initiation sites was a CRE site that was similarly positioned and identical in sequence to that observed in the human GRP gene (Nagalla & Spindel 1994). However that study found only slight effects on GRP mRNA expression in SCLC cell lines in response to forskolin and dibutyl cAMP suggesting that this CRE site is unresponsive to CRE binding proteins or that the effect is masked by the action of other regulatory factors. In the present study, we determined the nucleotide sequence for 2200 bp upstream of the putative transcription initiation sites as a starting point for functional analysis of the transcription factors responsible for the regulation of GRP mRNA expression.

Studies with GRP/bombesin and their antagonists have established a role for these peptides in normal fetal lung growth and maturation in a large variety of species including humans (Spindel *et al.* 1989, Sunday *et al.* 1990, Emanuel *et al.* 1999). GRP has been shown to be involved in differentiation of pulmonary neuroendocrine cells and is able to induce cell proliferation. In human, baboon, rhesus monkey and mice, peak GRP transcript levels occur during the canalicular phase of pulmonary development (Spindel *et al.* 1989, Sunday *et al.* 1990, Emanuel *et al.* 1999). This phase occurs around mid-gestation and is characterised by considerable extension and branching of the respiratory bronchioles and air-spaces with concomitant epithelialisation and capillary invasion (Reid 1984). In human fetal lung, this phase and GRP mRNA expression occur from approximately 16–30 week gestation (Spindel *et al.* 1989). In sheep fetal lung, the canalicular phase also occurs around mid-gestation the time when GRP mRNA expression was detected. The observation in the present study of two distinct periods of GRP mRNA expression (around days 60 and 140, term is 145 days) suggests that the fetal lung GRP also has distinct roles in lung branching morphogenesis (around mid-term) and in lung maturation (near parturition).

We used RT-PCR to examine the expression of GRP mRNA in tissue obtained from an ovine fetus at 120 days. Using this sensitive technique, expression of GRP mRNA was widespread occurring throughout the gastrointestinal tract (fundus, colon, jejunum, ileum, duodenum) and in various other tissues such as kidney, adrenal, lung, heart and pancreas. Very low expression was detected in the liver. No GRP mRNA expression was detected in 120 day fetal antrum in contrast to adult antrum where it

has been demonstrated that GRP mRNA is produced by nerve fibres within the antral mucosa and circular muscle layer (Bunnett 1994). Radioimmunoassay of each of these fetal tissues (except adrenal) revealed the corresponding presence of GRP-ir. These findings suggest that GRP may act in a local manner for many fetal tissues, possibly as a morphogen, similar to the activity described in fetal lung above and as recently shown for mouse and human colon (Jensen *et al.* 2001). In the latter study, an identical transient pattern of GRP and GRP-R expression was observed in the developing human and mouse colonic villi during similar stages of gastrointestinal tract formation. Aberrant expression of growth factors during cancer often represents neo-expression of a fetal developmental phenotype as has been demonstrated for GRP expression in lung cancer and colon cancer (Sausville *et al.* 1986, Carroll *et al.* 1999).

The major conclusion of the present study is that the larger-than-expected GRP observed in ovine (and human) pregnant uterus does not arise from a distinct GRP-like gene or from alternative transcripts derived from the GRP gene. This leads us to the hypothesis that this form of GRP results from an alteration in processing of the GRP prohormone. We propose that the larger-than-expected GRP molecule observed in the pregnant ovine endometrium represents a normal processing intermediate of proGRP and that this molecule becomes the major end product in pregnant ovine glandular epithelial cells. A similar observation has been made for prorenin which is the major end product of preprorenin processing in human utero-placental tissues in contrast to renal tissue where most prorenin is cleaved to renin (Poisner 1995). The mechanisms responsible for generation of the larger-than-expected GRP in utero-placental tissues remain to be determined. Possibilities include the over-abundance of GRP mRNA and subsequent large amounts of prohormone exhausting the processing machinery, an absence or inaccessibility of specific processing enzymes or a redirection of synthesis down the constitutive pathway (denying access to secretory vesicles containing the appropriate proGRP prohormone convertase). These studies are in progress as are those exploring potential biological functions of the uterine predominant form of GRP.

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