Characterization and nucleotide sequence of the gene for the common α subunit of the bovine pituitary glycoprotein hormones

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ABSTRACT

The gene coding for the common α subunit of the bovine pituitary glycoprotein hormones was isolated from a bovine genomic library. The gene spans roughly 16.5 kbp, contains three intervening sequences, and codes for a message of approximately 730 nucleotides. The complete coding region of the gene was sequenced as well as 315 nucleotides of 5' flanking sequence and the entire intron C. Only a single base difference was found when the sequence of the gene was compared with that of the cDNA. Genomic blotting experiments suggest the presence of a single α subunit gene. Comparison of the bovine and human α subunit genes indicated that the high level of homology observed in the coding regions has been maintained throughout the 5' and 3' untranslated regions, and at least 90 nucleotides of the 5' flanking regions. Additionally, there is an 18 base pair sequence present in both the 5' flanking and 5' untranslated regions of the gene that is homologous to a region of the chick ovalbumin gene. This ovalbumin sequence has been suggested as a binding site for the progesterone receptor-complex.

INTRODUCTION

The pituitary hormones LH², FSH, TSH, and the placental chorionic gonadotropins hCG and eCG, are all members of the glycoprotein hormone family (for review see ref. 1). Each hormone is dimeric, containing a common α subunit and unique β subunit which confers biological specificity. Homologies in the amino acid sequences of the different β subunits, as well as limited homology of the α subunit with the β subunits, have led to the proposal that these hormones evolved from a common ancestral gene (2). Recent evidence indicates that the human α subunit is encoded by a single gene (3), whereas at least one of the β subunit genes (β -hCG) is a member of a multigene family (4). In humans, the unique α subunit gene is expressed in both pituitary and placenta. It is not known whether these structural features have been maintained in other mammalian species.

Characteristically, intracellular levels of pituitary free α subunit exceed the intracellular levels of mature glycoproteins (5-9). This imbalance has led to the hypothesis that assembly of the hormones is limited by the appearance of each β subunit and further, that synthesis of the α and β

subunits are independently regulated (10-12). Whether synthesis of the glycoprotein hormone subunits are regulated during transcription, during post-transcriptional processing or transport, or during translation remains a major unanswered question. Further definition of these mechanisms requires the isolation and characterization of cDNA and genomic clones for the common α subunit and for the β subunits.

Recently we reported the isolation and sequence characterization of a bovine α subunit cDNA clone (13). Comparison of the bovine and human α subunit DNA sequences revealed that the 3'-untranslated regions have been highly conserved between these two species, but not in rodents (13). In the present study, we used the bovine α subunit cDNA to isolate the corresponding gene sequence from a bovine genomic library. Our results indicate the presence of a single α subunit gene in the bovine genome. Though the human α subunit gene is also present as a single copy (3), the bovine gene is approximately twice the size of the human, 16.5 kilobase pairs (kbp) compared to 9.4 kbp. This difference is primarily due to an increase in the size of the first intron which in the bovine gene is roughly 13.5 kbp. A comparison of the level of homology in the 5'-flanking, 5'-untranslated, and the 3'-untranslated regions between the bovine and human α subunit genes revealed the same or slightly greater homology than found in the coding regions of these two genes. The functional significance of these homologies is not apparent.

METHODS

Screening the Bovine Genomic Library

The construction of the genomic library has been described in detail elsewhere (14). Briefly, the library was formed by partial Mbo I digestion of bovine placental DNA, followed by ligation into the Bam HI "arms" of the λ vector Charon 28. A portion of the amplified library was screened (15,16) for clones which contain sequences that hybridize with a bovine α subunit cDNA (clone pB α , ref. 13). The cDNA insert for pB α consists of three Pst I fragments of 340, 171, and 101bp (13). These fragments were purified and nick-translated (17) using 40 μ Ci of each (α -³²P)dNTP(800Ci/mmol, Amersham) to obtain an approximate specific activity of 5×10^7 cpm/ μ g.

Oligodeoxynucleotides and Primer-Extended cDNA Synthesis

Two oligodeoxynucleotides were synthesized by the phosphite chemical method (18). Oligonucleotide I was a mixture of four tetradecamers which contained possible codons for amino acids 30 to 34 of the α subunit. Oligonucleotide II was 24 nucleotides in length and contained codons for amino acids -17 to -10 of the bovine α subunit signal sequence. To prepare uniformly labeled cDNA to be used to probe for the 5'-most exon, which is lacking in the cDNA, 30 pmols of either oligonucleotide were used with 40 μ Ci

of (α - ^{32}P)dCTP(Amersham, 800Ci/mmol) to prime synthesis from 10 μg of bovine pituitary polyadenylated RNA with reverse transcriptase (19). Preparation of end-labeled cDNAs for use in the chemical sequencing reactions utilized oligonucleotide II which was labeled using T4 polynucleotide kinase and (γ - ^{32}P)ATP (Amersham, 3000Ci/mmol) and extended with reverse transcriptase. The labeled cDNAs were further purified by electrophoresis through urea-acrylamide gels (19).

DNA Sequence Analysis

The majority of the α subunit gene was sequenced by the M13-dideoxy chain terminator technique (20). Sequencing templates were generated by subcloning restriction fragments into the M13 cloning vectors mp8 and mp9 (21). The universal sequencing primer (P-L Biochemicals) or α -specific synthetic oligonucleotides were used as primers in the dideoxy reactions. Partial sequence determination of the 5'-most exon was performed according to the procedure of Maxam and Gilbert (22) using a cDNA which was extended from the oligonucleotide II primer.

Genomic Blot

Bovine pituitary genomic DNA (14) was digested with a variety of restriction endonucleases for 4 hours at 37°C (approximately 10 units of enzyme per μg of DNA). To monitor complete digestion, and to provide size markers, 50ng of bacteriophage λ DNA was included in the reactions. The DNAs were sized by electrophoresis through 0.8% agarose gels, transferred to Gene Screen (NEN) using the method described by the manufacturer, and fixed to the filters by baking for 3 hours at 80°C in a vacuum oven. The filters were pre-hybridized for 3 hours at 42°C in 50% formamide, 20mM Pipes(pH 6.4), 0.8M NaCl, 2mM EDTA, 0.5% SDS, and 100 $\mu\text{g}/\text{ml}$ salmon sperm DNA. Hybridization was performed in the same buffer for 40 hours at 42°C.

To prepare single-stranded radioactive probes, M13 clones of the three Pst I restriction fragments of pB α (0.5 μg) were annealed to the universal sequencing primer and a complementary strand synthesized in the presence of 80 μCi of (α - ^{32}P)dCTP(3000Ci/mmol) with the large fragment of E. coli DNA polymerase (Boehringer Mannheim) (23,24). After one hour at room temperature, the DNA strands were denatured by addition of NaOH to 0.1M and separated by electrophoresis in a 0.7% agarose gel. The labeled DNA strand was excised, added to prehybridization solution, and placed in boiling water to melt the agarose. This solution was then added directly to the prehybridized filter. After 48 hours at 42°C, the filter was removed and washed twice at room temperature in 2X SSC (1X SSC is 0.15M sodium chloride, 0.015M sodium citrate), 0.1% SDS, followed by two washes in 0.2X SSC, 0.1% SDS at 65°C. Filters were exposed to Kodak XAR-5 X-ray film between two Dupont Lightning-Plus intensifying screens for two days at -80°C.

RESULTS AND DISCUSSION

Screening of approximately 5×10^5 recombinant bacteriophage with nick-translated bovine α subunit cDNA (pB α) resulted in the identification of 5 presumptive clones. Restriction endonuclease maps for three of these clones were determined by digesting whole DNA or purified fragments with either one, or with two different restriction enzymes (fig. 1). The extensive overlap of restriction sites between the three λ clones suggests that sequence rearrangements did not occur during construction of the genomic library. To locate the regions which contain α subunit coding sequences, clone $\alpha 2$ was digested with several restriction enzymes and analyzed by blot hybridization (25). The hybridization probe, nick-translated pB α , lacks 120 bases of the 5' end. All the hybridization occurred within the 9 kbp Bam HI fragment (fig. 1; hybridization data not shown). This fragment was subcloned into pBR322 and designated pB λ A2-1 (fig. 1). An identical pattern of hybridization was observed using clone $\alpha 4$.

To determine whether the 5' end of the α subunit gene was contained within the 9 kbp Bam HI fragment of pB λ A2-1, a 5'-specific cDNA fragment was synthesized by extension from the α -specific oligonucleotide I using reverse transcriptase and bovine pituitary polyadenylated RNA. This 5' probe failed to show any additional hybridization to clones $\alpha 2$ or $\alpha 4$. However, the primer-extended probe did hybridize to an additional restriction fragment of clone

Figure 1. Restriction endonuclease maps of the α subunit genomic clones. A Sma I-Bam HI fragment from the lambda clone $\alpha 1$ and a Bam HI-Bam HI fragment from lambda clone $\alpha 2$ were subcloned into pBR322 (pB λ A1-2, pB λ A2-1, respectively). The regions of the DNA which were sequenced are shown in the lower expanded section. The arrows indicate the direction and length of the individual sequencing reactions. Exons are represented by the solid shaded boxes. Only the Alu I and Sau 3A sites used in the nucleotide sequencing reactions are shown. H=Hind III, B=Bam HI, E=Eco RI, S=Sau 3A, A=Alu I, P=Pst I, Pvu=Pvu II.

$\alpha 1$. This hybridization occurred between the 5'-most Bam HI site and the λ arm of $\alpha 1$ (data not shown). To further map the 5' end of the gene, the Sma I-Bam HI fragment of $\alpha 1$ was subcloned into pBR322 (fig. 1). The Sma I site is in the λ arm approximately 3 kbp upstream from the λ DNA-insert DNA junction. This clone was designated p λ A1-2 (fig. 1). Blot analysis of restriction fragments of this subcloned DNA localized the 5' end of the α subunit gene within 900 base pairs 3' to the Bgl II site (fig. 1).

The majority of the α subunit gene was sequenced using the strategy shown in figure 1. The gene spans roughly 16.5 kbp and consists of four exons and three intervening sequences (A-C) which are approximately 13.5 kbp, 1.1 kbp, and 0.6 kbp, respectively, in length. The positions of introns B and C were defined by comparing the genomic sequences with the previously obtained α subunit cDNA sequences. All of the introns are positioned so that they begin with the dinucleotide GT and end with AG. To position intron A it was necessary to sequence the mRNA which spans this intron-exon junction since this region is lacking in the cDNA. This was accomplished by utilizing the method of Maxam and Gilbert (22) to sequence a cDNA which was synthesized using the α -specific oligonucleotide II to prime bovine pituitary polyadenylated RNA (data not shown). The partial sequence of the primer-extended cDNA was then compared to the sequence determined 3' to the Bgl II site in λ clone $\alpha 1$. These results thus specified the positions of both ends of intron A. The location of the 5' end of the mRNA, which was determined from the size of the primer-extended cDNA, exactly matches the cap site determined for the human α subunit gene (data not shown). Together, these results indicate that the first exon is 93 nucleotides long, with the cap site occurring 314 base pairs downstream from the Bgl II site.

Comparison of the nucleotide sequence of the gene with that of the cDNA reveals only one silent nucleotide difference (fig. 2). This occurs at the third nucleotide of amino acid nine which in the cDNA is an adenine. The sequence of the gene provides the complete signal peptide sequence and the entire 3' untranslated region. Comparison of the bovine α subunit signal sequence with those of the human, rat and mouse demonstrated nucleotide homologies of 86%, 82% and 86% respectively. This nucleotide sequence homology in the signal sequence is comparable to that obtained for the remainder of the coding regions (13). The 3'-untranslated region contains the conserved nucleotide sequence AATAAA, thought to be necessary for polyadenylation of the message (26). Furthermore the sequence TTTTAAATCATC is present in both the human and bovine α subunit mRNAs 3' to the hexanucleotide sequence. Fiddes and Goodman (3) have proposed that this A+T-rich sequence, which is homologous to a consensus sequence identified as a putative polyadenylation site (27), specifies the end of the α subunit gene. If so, the 3'-untranslated region of

5'---agatcctgttttaggaagtgcaactttcaggatgtgtgtataaacttgaaaaattatgtagaaaataacagagttctctgtctcataggctgaacctgcagtcaca
ccatctgaaatggctcaaaacaaaaataatctaaggctgaacaagataagataaaattgatgtcatggttaattgtgaagtaaccattcaatctgggtggaatttactgtga
tcoctggacttagatgtaggtgggaacatgcagcttttatgactctgtgtatataagacaggtgaggactttattaaaa GCAGTTGCTGAGAAATCACAAGACAAATATAAAT
CTTCTCAGATCCAGTCAACTGCCCTGACTACATCTGCAAAAATCCAGAGGACG gtaaaatgaatgaataattgggggatttttaattgaggggtaaaaatatttgaggatacaa
gggagctggtgtgcataaaccttagtaagaagagagacaggcatttatgcatctctcttcagcactcatgtgaattgggaggaagctgcacaaactgtgtctgtgtatc-----
-----Intron A > 13 kbp-----agcaatgttaaaactactggtgtgtttatgocgttgaagcaattttatagtaa
-24 -20 -10
MET Asp Tyr Tyr Arg Lys Tyr Ala Ala Val Ile Leu Ala Ile Leu Ser Leu Phe Leu Gln Ile
cttttttccatgtctgttttgcag AAGAGCC ATG GAT TAC TAC AGA AAA TAT GCA GCT GTC ATT CTG GGC ATT TTG TCT CTG TTT CTG CAA ATT
+1
Leu His Ser Phe Pro Asp Gly Glu Phe Thr Met Gln
CTC CAT TCC TTT OCT GAT GGA GAG TTT ACA ATG CAG G gtatgtgoccaactgggtctgcag-----Intron B = 1.1 kb-----
-----ctgtagtcaggacaacagatattgtgtgtgaagatgaacatacaacaaaaagtatttttttttaactgccaatagaaaaaagaatatttgtgt
ccaataaagaagatcattcagacttttttttaaaaaattcttttttgattttttactcttttgctttttctttctttctttcttctcttttttaattgtgttttttt
10 20 30
Gly Cys Pro Glu Cys Lys Leu Lys Glu Asn Lys Tyr Phe Ser Lys Pro Asp Ala Pro Ile Tyr Gln Cys Met Gly Cys Cys
tttggatag GC TGT OCT GAA TGC AAG CTA AAA GAA AAC AAA TAC TTC TCC AAG CCA GAT GCT CCA ATC TAT CAG TGC ATG GGG TGC TGC
40 50 60
Phe Ser Arg Ala Tyr Pro Thr Pro Ala Arg Ser Lys Lys Thr Met Leu Val Pro Lys Asn Ile Thr Ser Glu Ala Thr Cys Cys Val Ala
TTC TCC AGG GCA TAC CCC ACT CCA GCG AGG TCT AAG AAC ACA ATG TTG GTC CCC AAG AAC ATC ACC TGG GAA GCT ACA TGC TGT GTG GGC
70
Lys Ala Phe Thr Lys
AAA GCA TTT ACC AAG gtgaggacctaaagggcccaagaaacttttaacagttcaaccagagaaatgttccacagcacatccatggcttcagocagaggtagtaagtacc
agtccttcaatgggcatgttagacaaagggctgagctgtgtgtctttaaccaagacagtcagocacttctgggcagaggtggtgactatccagaatgaagacagtgacagaggtg
ataaggggcaggttgggagaaaacatagaatacttatcagtgcaacttaagcttttaggaatgaaatatacagctgaaaggagactatgtgaatcaaacagcatctgttttttaa
gtacttatgtattgtttggctgcacaggtcttagttgtagcactcagtatgtggatctttagttgtggcaggogggatctagtctctgaccagggatggaacctgggtccoctgc
actgagagctagagacctagocactggaccagacagaagtccatatagaagcatttatcagttcagctggctacagactattttactgaacagttttcaattttgttagoctgoc
tatctatccatctgocctgtctatcatctatctttcttccctccggttag Ala Thr Val Met Gly Asn Val Arg Val Glu Asn His Thr Glu Cys His
GCC ACA GTG ATG GGA AAT GTC AGA GTG GAG AAC CAC ACC GAG TGC CAC
80
90 96
Cys Ser Thr Cys Tyr Tyr His Lys Ser CC AM
TGC AGC ACT TGT TAT TAT CAC AAA TCC TAATAGTTTGCAGTGGGCTTGCTGATGATGCTGACTTGTCTCAAAAGGAAAATAATTTGTCCAGTGTCTATGGCTTTTGTA
GATAAAACCTCTCTTCTGCTGACATACCAATTTTAACCTGCTTTGAGATATACAGCTTTATGCTTTTCTCTTATCTCAATAATAACAGTAGTCTGTATCTTTTCATTGTA
AATGAATATGCAATTAGCATGACCAATAAAAGCTGATTCAGCTGGAATAAAGCTCTTTTAAATCATC actctatcactgaattc---3'
↑

Figure 2. Partial nucleotide sequence of the gene for the α subunit of the bovine glycoprotein hormones. The regions of the DNA that code for the mature mRNA are shown in capital letters. The CAAT, TATA and AATAAA sequences are boxed. The ends of the cDNA clone (pB α) are indicated by arrows. The sequences complementary to the synthetic oligonucleotide primers are underlined. The single nucleotide in the gene which differs from that found in the cDNA is indicated by an asterisk.

Figure 3. Autoradiograph of a genomic blot of bovine pituitary DNA (10 μ g) which had been digested by the restriction endonucleases indicated.

the bovine α subunit mRNA contains 271 nucleotides, generating a mature transcript of approximately 730 nucleotides, excluding the poly A tail.

To determine if there are multiple copies of the bovine α subunit gene, we analyzed restricted genomic DNA by blot hybridization. The genomic DNA was digested with either Bam HI, Hind III or Eco RI, transferred to Gene Screen and probed using labeled single-stranded DNA derived from pB α M13 subclones (see methods). Autoradiography demonstrated a single band in the Bam HI and Eco RI digests of approximately 9 kbp and 11 kbp respectively, and three bands in the Hind III digest of 2.8, 1.1, and 0.9 kbp (fig. 3). The 5'-most exon is not detected in these experiments since this region of the gene is lacking in the cDNA which was used to probe these blots. These results are identical to those predicted from the sequenced gene, and suggest that the bovine α subunit gene is present as a single copy as was similarly shown for the human gene (3). If additional genes were present it is highly unlikely that the flanking regions would be so conserved as to retain all of these restriction sites. Additionally, genomic titration experiments further support the conclusion that the bovine α subunit gene is present as a single copy (data not shown).

The completion of the sequence of the bovine α subunit gene allows for a comparison with the sequence of the human gene (3). Both genes are composed of four exons and three introns. The positions of the introns have been highly conserved, with the first intron of each occurring within the 5'-untranslated region. However, the human gene spans 9.4 kbp compared to 16.5

Figure 4. Comparison of the 5'-flanking regions of the α subunit genes of the bovine and human glycoprotein hormones. Homologous bases are indicated with *. Gaps which have been introduced for maximum alignment are indicated by dashes. The sequences homologous to the putative chick progesterone receptor-complex binding site are underlined. The numbers refer to the position of nucleotides in the bovine sequence with the cap site designated +1.

kbp for the bovine gene. This large difference in size is primarily due to an increase in the length of intron A which in the bovine gene spans 13.5 kbp compared to 6.4 kbp in the human. Comparison of the bovine and human nucleotide sequences (with the introduction of small gaps for maximum alignment) shows that the high level of homology found for the coding regions (82%) has also been maintained in both the 5' and 3' untranslated regions. This is in contrast to the α subunit mRNAs of rodents, where the 5' and 3' untranslated regions have diverged considerably from their human or bovine counterparts, while the homology in the coding regions has been maintained. While evolutionary conservation of nucleotides within different regions of mRNA has been suggested to be an indication of functional significance (28), the role of 5' and 3' untranslated sequences in bovine or human α subunit mRNA remains to be established.

In characterizing the bovine α subunit gene, we also determined the nucleotide sequence 315 base pairs immediately upstream from the start site for RNA transcription. Inspection of this region revealed the sequence TATATAA positioned 30 nucleotides upstream from the cap site. This sequence conforms to the "TATA" box consensus sequence which has been demonstrated to be a part of the promoter sequence for eukaryotic RNA polymerase II (27). Additionally, the sequence ATTCAATCA located at position -110 is homologous to 6 of the 9 bases of the consensus "CAAT" box often found 70 to 80 nucleotides upstream from the start of RNA transcription (27). The high degree of homology (80%) shared between the bovine and human α subunit genes is also present throughout the 90 bases of 5'-flanking sequence which has been

reported for the human gene (fig. 4). Furthermore, there is an 18 base pair sequence in the 5' flanking region of the bovine gene between -182 and -165 which is homologous to another sequence found in the 5' untranslated region of both the bovine and human α subunit genes between nucleotides +20 and +37. In the bovine, both of these 18 base pair sequences are A+T-rich (80%), and share at least 70% homology with a similar 18 base pair sequence localized between positions -135 and -247 of the chick ovalbumin gene. In the chick, this A+T-rich sequence has been suggested as a binding site for the progesterone receptor-complex (29). Since there is increasing evidence that expression of the α subunit gene is regulated by gonadal steroids (24,30,31), it will be of interest to determine whether these A+T-rich regions of the bovine α subunit gene are required for hormonal regulation.

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²Abbreviations. LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; hCG, human chorionic gonadotropin; eCG, equine chorionic gonadotropin.

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