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TRANSCRIPTION INITIATION IS LOCALIZED TO A TATALESS REGION IN THE OVINE β1 ADRENERGIC RECEPTOR GENE

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Summary : We cloned and sequenced the ovine beta$_1$ adrenergic receptor (β_1AR) gene and promoter region. The transcription start site was localized by RNase protection and primer extension to a GC-rich region. The predominant initiation sequence did not resemble an initiator element and there was no upstream TATA box. Sequence analysis revealed several potential thyroid hormone and glucocorticoid regulatory elements. Identification of the promoter structure of this important gene will help define its novel regulation during development.

The β_1 adrenergic receptor is a member of the seven transmembrane, G protein-coupled receptor family (1). While these receptors share structural and functional similarities, their gene structure and regulatory characteristics are varied. Alpha$_2$, β_1 and β_2 adrenergic receptors are intronless while α_1, β_3 and D_{1a} receptors have introns in their 5' untranslated regions (2-4) The β_2 adrenergic receptor, which was the first G-coupled neurotransmitter receptor to be cloned and sequenced, has been extensively studied including, identification of its transcription start site, core promoter elements and definition of glucocorticoid and cyclic AMP regulatory elements within the promoter region (5,6). In contrast, attempts to characterize the β_1AR gene had been hampered by the inability to define the transcription start site (7).

We have shown that neither thyroid hormones nor glucocorticoids alter the number of β_1AR in fetal sheep, however, there is an increase in receptor number if the animals are treated in the newborn period (8,9). This is interesting because both glucocorticoid and thyroid hormone receptors are expressed in fetal sheep (10,11) and there are other genes which are steroid hormone responsive at these gestational ages (12). In order to explore more precisely its mechanisms of transcriptional control, we

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sought to clone and characterize the ovine β_1AR and 5' flanking region in order to begin studies of the transcriptional control of this important gene.

METHODS

We screened a sheep genomic EMBL3 library using a human β_1 receptor cDNA probe (kindly supplied by Dr. Robert Lefkowitz) and 2 different oligonucleotides chosen to correspond to discrete regions of the beta$_1$ receptor. Oligonucleotides corresponding to nucleotides 354-390 and 1044-1071 of the human gene and a 0.8kb PvuII fragment of the beta$_1$ cDNA, from human sequence -88 to 779, were used to identify clones containing sequences involved in ligand specificity. Among 800,000 clones only a single clone hybridized to all three of the probes from which a 5kb insert was excised with BglII and subcloned. Nested deletions were created by ExoIII digestion (Promega, *Erase-a-Base*™) and then sequencing was carried out on the deleted inserts. All sequence assembly and analysis was carried out on a Digital VAX computer using programs in the Genetics Computer Group (GCG) package. Standard methods for RNA extraction and separation were used. RNase protection was carried out with a series of an antisense ribonucleotide probes derived from sequences upstream from the translational start site (13). The synthesized riboprobes had an average specific activity of ~2.5 x 10^8 cpm/µg and were hybridized with total RNA extracted from sheep heart or brain tissue overnight at 45 °C. Following hybridization, samples were digested with *RNase*$_1$ (Promega). The RNase-resistant hybrids were resolved by electrophoresis in an 8% polyacrylamide/8M urea gel. RNase protection assays were repeated several times to confirm the results. Reverse transcription for primer extension on poly A+ RNA extracted from fetal brain frontal cortex and heart was done with MMLV reverse transcriptase (USB).

RESULTS

The 5kb BglII fragment contained the entire coding sequence in a single exon and over 2 kb of 5' flanking region. Comparison of the predicted amino acid sequence of the β_1AR from human, rat and sheep revealed overall amino acid identity of 85-90% which approached 100% within the transmembrane spanning regions, Figure 1 (14,15). Sequence homology was only 65-70% compared to the β_2 receptor of rat and human and less when compared to alpha, dopamine and serotonin receptors.

RNase protection was first carried out with a series of probes spanning the 5' sequence from -1157 to +175 relative to the initiator methionine, Figure 2A-D. The largest probe did not overlap the transcription start site and was fully protected except for deletion of vector sequences added during transcription, 2A lanes 8-10. The smaller probes (lanes 2 & 7) yielded protected fragments (lanes 1,5,6) which predicted the same transcription start site more than 550bp upstream from the initiator methionine. Another probe was used in RNase protection assay, 2B lane 1. The gel was purposefully overexposed to make sure no transcripts were overlooked. A single cluster of protected fragments corresponding to the previous results was identified, 2B lane 2. To map the precise location of transcription initiation, reverse transcription and then primer extension was carried out using a primer downstream from the identified sites. The predominant start site was at -660 bp relative to the translation start site with less prevalent start sites at -661 and -655 bp, as shown in 2D. A schematic illustration of the riboprobes and the location of the primer employed is shown in 2C. The same sites were seen in samples from both brain

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          1                                                  50
Humβ1AR   MGAGVLVLGA SEPGNLSSAA PLPDGAATAA RLLVPASPPA SLLPPASESP
Ovineβ1   MGAGALALGA SEPCNLSFAA PVPDGAATAA RLLVPXSPLR LAADLGQRGT
Ratβ1AR   MGAGALALGA SEPCNLSSAA PLPDGAATAA RLLVLASPPA SLLPPASEGS

          51                                                 100
Humβ1AR   EPLSQQWTAG MGLLMALIVL LIVAGNVLVI VAIAKTPRLQ TLTNLFIMSL
Ovineβ1   PLLSQQWTVG MGLLMAFIVL LIVAGNVLVI VAIAKTPRLQ TLTNLFIMSL
Ratβ1AR   APLSQQWTAG MGLLLALIVL LIVVGNVLVI VAIAKTPRLQ TLTNLFIMSL
                                  I
          101                                                150
Humβ1AR   ASADLVMGLL VVPFGATIVV WGRWEYGSFF CELWTSVDVL CVTASIETLC
Ovineβ1   ASADLVMGLL VVPFGATIVV WGRWEYGSFF CELWTSVDVL CVTASIETLC
Ratβ1AR   ASADLVMGLL VVPFGATIVV WGRWEYGSFF CELWTSVDVL CVTASIETLC
                 II                                    III
          151                                                200
Humβ1AR   VIALDRYLAI TSPFRYQSLL TRARARGLVC TVWAISALVS FLPILMHWWR
Ovineβ1   VIALDRYLAI TSPFRYQSLL TRARARALVC TVWAISALVS FLPIFMQWWG
Ratβ1AR   VIALDRYLAI TSPFRYQSLL TRARARALVC TVWAISALVS FLPILMHWWR
                                              IV
          201                                                250
Humβ1AR   AESDEARRCY NDPKCCDFVT NRAYAIASSV VSFYVPLCIM AFVYLRVFRE
Ovineβ1   DKDAKASRCY NDPECCDFII NEGYAITSSV VSFYVPLCIM AFVYLRVFRE
Ratβ1AR   AESDEARRCY NDPKCCDFVT NRAYAIASSV VSFYVPLCIM AFVYLRVFRE
                                        V
          251                                                300
Humβ1AR   AQKQVKKIDS CERRFLGGPA RPPSPSPSPV PAPAPPPGPP RPAAAAATAP
Ovineβ1   AQKQVKKIDS CERRFLSGPA RLPSPALS.. .......... .PGAPLPAAA
Ratβ1AR   AQKQVKKIDS CERRFLSGPP RPPSPAPS.. .........P SPGPPRPADS

          301                                                350
Humβ1AR   LANGRAGKRR PSRLVALREQ KALKTLGIIM GVFTLCWLPF FLANVVKAFH
Ovineβ1   VANGRANKRR PSRLVALREQ KALKTLGIIM GVFTLCWLPF FLANVVKAFH
Ratβ1AR   LANGRSSKRR PSRLVALREQ KALKTLGIIM GVFTLCWLPF FLANVVKAFH
                                        VI
          351                                                400
Humβ1AR   RELVPDRLFV FFNWLGYANS AFNPIIYCRS PDFRKAFQGL LCCARRAARR
Ovineβ1   RDLVPDRLFV FFNWLGYANS AFNPIIYCRS PDFRKAFQRL LCCARRAACG
Ratβ1AR   RDLVPDRLFV FFNWLGYANS AFNPIIYCRS PDFRKAFQRL LCCARRAACR
                           VII
          401                                                450
Humβ1AR   RHATHGDRPR ASGCLARPGP PPSPGAASDD DDD...DVVG ATPPARLLEP
Ovineβ1   SHGAAGDPPR AAGCLAVARP SPSPGAASDD DDDDDEDDVG AAPPVRLLQP
Ratβ1AR   RRAAHGDRPR ASGCLARAGP PPSPGAPSDD DDDD....AG ATPPARLLEP

          451                                                500
Humβ1AR   WAGCNGGAAA .DSDSSLDEP CRPGFASESK V......... .........
Ovineβ1   WAGYNGGAAA .NSDSSPDEP SRPGCGSESK V......... .........
Ratβ1AR   WAGCNGGTTT VDSDSSLDEP GRQGFSSESK V......... .........
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Figure 1. Alignment of the amino acid sequences of human, ovine and rat β_1AR. Transmembrane domains are shown by the Roman numerals.

and heart although an additional fragment >1500 bp in length was seen doing RNase protection with samples of RNA from heart. Becsuse this fragment was larger than the probe, we attributed the result to an artifact of incomplete digestion.

The entire sequence of the 2300 bp 5' flanking region is shown in Figure 3 with the primary transcription start site indicated by the ➨. There was no upstream TATA box. The 5' flanking sequence was searched for nucleotide sequences of identified regulatory elements using GCG programs and the Transcription Factor Database. The location of putative regulatory elements and sequence of interest are identified and described in the Legend for Figure 3 (16).

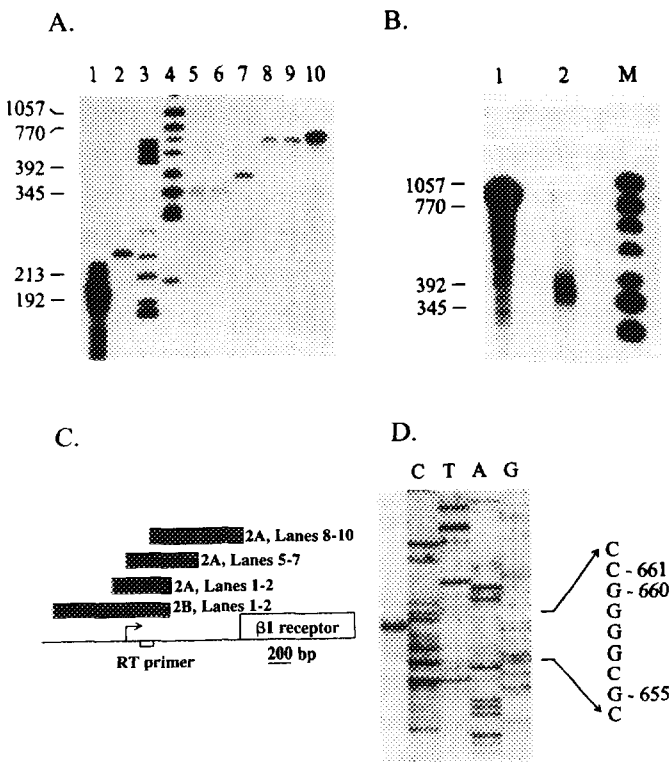

Figure 2. A) RNase protection was carried out with 3 different riboprobes (lanes 2,7,10). Protected fragments (lanes 1,5,6) predicted the same transcription start site more than 550bp upstream from the initiator methionine. B) RNase protection with extended version of the probe used in 2A Lane 2 (Lane 1). Cluster of protected fragments 375bp upstream (lane 2). HincII digest of ϕx174 (lane M). C) Schematic of RNase protection results. D) Primer extension fragments are seen at -655, -660 and -661bp adjacent to sequence ladder. The major start site is at -660bp.

DISCUSSION

We describe here the cloning and sequence of the ovine beta$_1$ adrenergic receptor (β_1AR) gene and promoter region. The transcription start site was defined using RNase protection and confirmed by primer extension. There were several closely spaced transcription initiation sites arising within a GC-rich region. The predominant initiation sequence did not resemble an initiator element and there was no upstream TATA box. Smale et al have demonstrated that transcription initiation in some TATAless genes starts from an initiator element which conforms to the consensus sequence PyPyA$_{+1}$NT/APyPy (17). There is frequently an Sp1 site 40-60 bp upstream from the initiator which has been shown to augment transcription efficiency. Nothing resembling an initiator element was identified in the region of any of the transcription start sites of the ovine β_1AR. Sp1 consensus sequences were located 10-15 and 60-70 bp upstream from the predominant start site. There were also several downstream Sp1 sites.

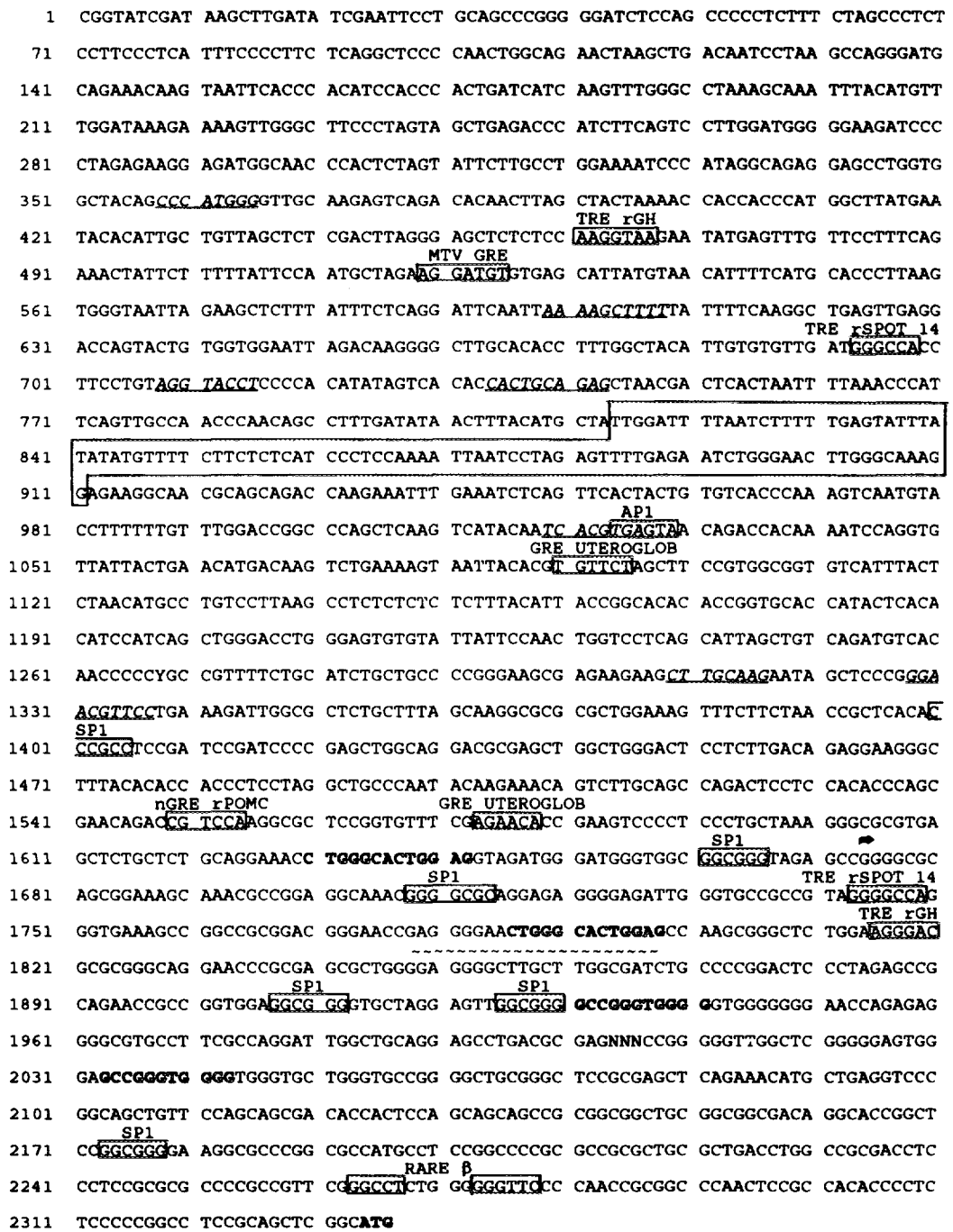

Figure 3.Ovine β_1 adrenergic receptor gene 5' flanking sequence. Putative regulatory elements in either orientation are indicated. Abbreviations are in the text. Palindromic sequences of 8 or more bp are in italics and underlined. Two sets of repeats are in bold. The location of the oligonucleotide used for primer extension is overlaid by ~~~~~~~. The predominant transcription initiation site is indicated by an overlying bold arrow. The large box indicates the location of the 98 bp element common to both the ovine and human β_1AR genes.

Transcription start sites have recently been identified for the β_1AR in the mouse and rat (18,19). In the mouse, reverse transription of brain RNA was used to identify an apparent start site in a GC rich region 451 bp upstream from the initiator methionine codon (18). Rnase protection was not used to confirm transcript sizes in any other tissue. The transcription start site of the rat β_1AR promoter in C6 glioma cells has been identified by RNase protection to reside in a TATAless region 250 to 280 bp upstream from the translation start site (19). Primer extension using several different primers over this region gave contradictory results, however, and deletion of the start site and surrounding sequences did not affect basal transcriptional activity. The region -306 to -232 in the ovine β_1AR promoter has 76% nucleic acid homology with the sequence surrounding the cluster of start sites identified in the report on the rat gene. We examined the areas identified in the mouse and rat genes in detail with two of our riboprobes (Figure 2A lanes 5-10) and, even with overexposed gels, we saw no evidence for transcription initiation in these regions. These contradictions may be explained by differences in the initiation site of the rat β_1AR gene in C6 cells or it may be due to species differences.

TATAless promoters and multiple transcription initiation sites are common to "housekeeping" genes but they are also seen in other important, polymerase II promoters including: the human androgen receptor (20), rat IGF binding protein 2 (21), the human integrin β_3 gene (22), mouse neurofilament heavy gene (23) and the Wilm's tumor gene (24). General transcription factors, including Sp1 and AP2, are important in regulation of core promoter activity of several of these genes. The human D_{1A} receptor promoter lacks both TATA and initiator elements (4). Core promoter activity has been localized to a 240 bp stretch of sequence which contains AP2 and Sp1 binding sites, and both of these general transcription factors appear to be involved in D_{1A} regulation (25).

The sequence $^{5'}$TGTTCT$^{3'}$, which is the most conserved portion of well-characterized glucocorticoid response elements (GRE), was found at -584 bp and an MTV-GRE important for glucocorticoid mediated repression of MTV transcription was found at -1155 bp. Half GRE's have been demonstrated to mediate the negative regulation of rat proopiomelanocortin gene expression (26), enhanced mouse α-amylase 2 gene expression (27) and delayed glucocorticoid responsiveness of the rat α_{2u} globulin gene (28). We identified the rat POMC "negative" GRE at -126. We also found several elements that could bind thyroid hormone receptor including: an elements partially homologous to the TRE of the rat Spot 14 gene located at -982 bp and to rat growth hormone at -1213 bp (29). We also noted a number of 8-10 bp palindromic sequences and several 11-13 bp repeats, some of which may be binding sites for yet uncharacterized transcription factors.

Overall sequence homology of the ovine promoter with the human β_1AR promoter sequence was 62% and with the rat β_1AR promoter sequence it was 58% when compared by the GAP program from GCG. The $^{5'}$TGTTCT$^{3'}$ portion of the GRE consensus sequence was present in all three β_1 promoters. We also identified a 98 bp region with 87% identity between the ovine and human sequences, Figure 3.

The functional significance of this region is not known and this region of the rat gene has not been sequenced.

In summary, we report the cloning and sequence analysis of the ovine β_1 adrenergic receptor and its 5' flanking region. We demonstrated that transcription initiation begins from a region well upstream (~660 bp) from the translation start site arising within an area with surrounding Sp1 sites. There is no consensus TATA box or initiator element within the transcription start site. Studies from our laboratory and others' demonstrate that this gene is hormonally regulated however the timing of hormone responsiveness is delayed compared to other genes. We identified several putative regulatory elements in the proximal promoter. The role of any of these elements will await definitive identification by functional assays. These results provide the basis for study of this potentially unique form of developmentally regulated transcription.

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