The Genomic Structure of the Rat Corticotropin Releasing Factor Receptor

A MEMBER OF THE CLASS II G PROTEIN-COUPLED RECEPTORS*

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Isolation and structural characterization of the rat corticotropin releasing factor receptor (CRFR) gene was performed to determine the exon/intron organization of the coding region and the potential for splice variants. The CRFR gene contains 13 exons and 12 introns, and the positions of the exon/intron junctions are similar to those of other Class II G protein-coupled receptor genes including the parathyroid hormone and glucagon receptors. The promoter resides within 593 base pairs of the initiation codon and the major transcriptional start site at nucleotide -238. This domain does not possess a TATA box but contains multiple Sp1 and AP-2 sites upstream and downstream of the major transcriptional start site. Intron junctions were identified in the extracellular, transmembrane (TM), and cytoplasmic (C) domains of the CRFR, giving the potential for differential signal transduction by splice variants. CRFR cDNAs derived from rat Leydig cell mRNA included the pituitary Form A, which spans exons 1–13, and two splice variants with deletion of exon 3 or exons 7, 11, and 12. An evolutionary link between the intron-less TM/C module of the glycoprotein hormone receptors and the intron-containing TM/C module of the CRFR is suggested by the common position of the luteinizing hormone receptor Form D alternate acceptor splice site and the CRFR intron 12.

Corticotropin releasing factor (CRF)[1] is a principal neuroregulator within the hypothalamo-pituitary-adrenal axis that coordinates endocrine, behavioral, and autonomic responses to stress. CRF is also an important anti-reproductive hormone that inhibits luteinizing hormone/human chorionic gonadotropin (cAMP)-stimulated androgen production in the rat testis through inhibition of the catalytic subunit of adenylate cyclase (1). High affinity CRF receptors have been characterized in several target tissues including the pituitary (2) and testicular Leydig cells (3). Recent molecular cloning of the pituitary CRF receptor cDNA from human (4), rat (5), and mouse (6) indicate that the expressed protein is a member of the G protein-coupled seven-transmembrane receptor (GPCR) family and has been shown to couple to both adenylate cyclase and phospholipase C (7), stimulating cAMP production and phosphoinositide hydrolysis. The CRF receptor exhibits significant homology to the parathyroid (8) and glucagon (9) receptor family that have been designated as members of Class II (10, 11) in the GPCR superfamily. This class exhibits little primary sequence similarity to the Class I GPCR, and members contain specific conserved amino acid motifs in the extracellular and transmembrane domains (11). In addition, genomic structures of the rat parathyroid (12) and glucagon (13) receptors suggest that some members of the Class II family differ from the catecholamine and the glycoprotein hormone receptor families by the presence of introns in both the NH_2-terminal extracellular and the transmembrane/cytoplasmic domains.

Several variant CRF receptor clones have been identified that carry insertions and deletions of both the extracellular and seven-transmembrane module (7, 14). This suggests the presence of multiple introns in the CRF receptor gene and the expression of splice variants that potentially may differ in ligand specificity and modes of signal transduction. This was particularly relevant to our studies in the rat Leydig cell where the CRF receptor did not stimulate cAMP production or appear to be coupled to G_s (1, 3) and suggested either a tissue-specific post-translational modification of the CRFR, different G proteins or inhibitors, or the presence of a splice variant of the CRF receptor in the Leydig cell. The structural organization of the CRF receptor gene was determined for subsequent elucidation of isoforms in order to clarify the nature of the Leydig cell receptor.

MATERIALS AND METHODS

Isolation of the Rat CRF Receptor Gene—A rat liver lambda dash genomic library (Stratagene, La Jolla, CA) was screened with ^{32}P-labeled DNA containing the following segments of the rat CRFR (5) DNA sequence: $(-14/+361$ bp), $(215/1318$ bp), $(-78/+60$ bp), $(-78/-49$ bp), and $(-55/-26$ bp). Positive clones were subjected to restriction enzyme mapping, Southern analysis, and DNA sequencing as described previously (15).

Mapping of 5'-flanking Region of Rat CRF Receptor Gene—A 741-bp *Alu*I digestion fragment of clone I (see Fig. 1) was used to analyze the 5'-flanking sequence of the CRF receptor. This was subcloned into a *Sma*I site of pGEM4z. 135- and 606-bp *Pst*I restriction fragments were then subcloned into pBluescript (Stratagene, La Jolla, CA). Isolated clones were sequenced by the dideoxynucleotide chain termination method (U. S. Biochemical Corp.).

Primer Extension and S1 Nuclease Mapping—Rat pituitary poly(A)$^+$ mRNA (10 μg) was used for primer extension and S1 nuclease mapping studies to determine transcriptional start sites (16). Two synthetic oligonucleotide DNA primers that include the initiation codon, coding region, and 5'-flanking domain $(-23/+6)$, $(+33/+59)$, and a genomic DNA fragment containing the 5'-flanking region $(-554/+6)$, isolated by

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[1] The abbreviations used are: CRF, corticotropin releasing factor; GPCR, G protein-coupled receptor; CRFR, corticotropin releasing factor receptor; bp, base pair(s); PCR, polymerase chain reaction; PTH, parathyroid hormone; PTHR, parathyroid hormone receptor; TM, transmembrane domain; TM/C, transmembrane/cytoplasmic; LHR, luteinizing hormone receptor.

FIG. 1. **Organization of the rat CRFR gene.** *a*, genomic clones (I–IV) containing the 5′- and 3′-flanking regions and the receptor coding region. *b*, structure of the CRFR gene. *Top*, position of CRFR introns in relation to extracellular and transmembrane domains. *S*, signal (○); *EC*, extracellular; *il*, intracellular loop; *el*, extracellular loop cytoplasmic domain after TMVII (1–1246): initiation to terminating codons. *Middle*, position of CRFR introns relative to the rat PTHR (12) and glucagon receptor (13) genes. *E1–E3*, *G*, *M1–M7*, *EL2*, and *T* are PTHR exon labels (12) that correspond to CRFR exons 1 to 13. The *asterisks* indicate insertion. *Bottom*, position of CRFR introns relative to CRFR human and rat cDNA clones. *Dot*, missing exon or sequence. |, primer start or stop site. *r(A)*, pituitary cDNA (accession number L24096) (5); *r(B)* and *r(C)*, CRFR PCR products identified in the Leydig cell; *h(1)*, *h(2)*, and *h(3)*, type 1 (accession number L23332), type 2 (accession number L23333), and variant (accession number U16273) human CRFR.
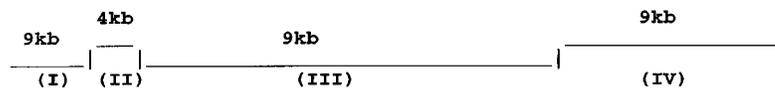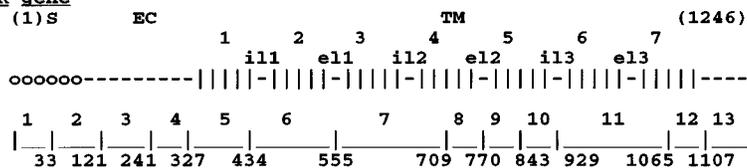
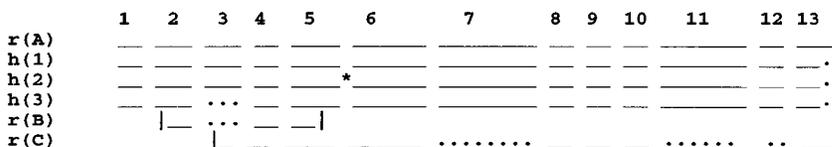PCR, were end labeled with [γ-^{32}P]ATP and used for primer extension and S1 nuclease mapping, respectively (16).

Promoter Activity of the CRF Receptor Gene—Two luciferase reporter gene constructs were synthesized for analysis of the CRFR gene promoter activity. A 610-bp *Pst*I restriction fragment of the 5′-flanking region or a 668-bp *Kpn*I restriction fragment of the 5′-flanking region was subcloned into LucLink v2.2 (a gift from Dr. R. Maurer, Oregon Health Sciences University) containing the luciferase reporter gene (17). Transient co-transfection of each of these constructs and the β-galactosidase gene into mouse Leydig tumor cells and Chinese hamster ovary cells was performed as described previously (17). Luciferase activity was measured and normalized with β-galactosidase activity.

Isolation of Rat Leydig Cell CRFR cDNA Clones by Reverse Transcription-PCR—Poly(A)$^+$ RNA was isolated from purified rat Leydig cell by centrifugal elutriation (InVitrogen, San Diego, CA). Synthetic oligonucleotide primers tailed with *Xba*I and *Hin*dIII restriction enzyme cleavage sites were designed to conform to the DNA sequence of the rat pituitary CRF receptor cDNA. Primer 1, (−27/−7 bp); Primer 2, (60/82 bp); Primer 3, (195/215 bp); Primer 4, (340/360 bp); and Primer 5, (1298/1312 bp). The poly(A)$^+$ RNA was reverse transcribed using Primer 1 or 2 (Life Technologies Inc.). Primer pairs (1–5, 2–4, and 3–5) were used for the first round of PCR amplification. The products of this reaction were size selected and used as templates for two additional rounds of amplification. These clones were digested with *Xba*I and *Hin*dIII and ligated into pGEM4z (Promega). Clones were sequenced by the dideoxynucleotide chain termination method. Primer pair (1–5) yielded the Form A pituitary receptor. Primer pair (2–4) yielded the Form B receptor with deletion of exon 3, and pair (3–5) yielded the Form C receptor with deletions of exons 7, 11, and 12. A recombinant Form A/C CRFR was constructed by ligating the 5′ 302-bp *Pvu*II fragment of Form A with the 3′ 711-bp *Pvu*II fragment of the Form C receptor and was subcloned into pGEM4z. Sequence analysis was performed with the program Pile-Up in the Genetics Computer Group (Madison, WI).

RESULTS AND DISCUSSION

Isolation of the Rat CRF Receptor Gene—The rat CRF receptor gene was isolated from the rat liver lambda dash genomic library using rat pituitary CRFR (Form A) cDNA (accession number L24096 (5)) and synthetic oligonucleotide probes. Genomic DNA fragments were excised from four phage clones (Fig. 1) with *Eco*RI, and Southern analysis indicated that the CRF receptor coding region is contained within clones I–IV. The coding region was estimated to span at least 36 kilobases and contains 13 exons separated by 12 introns (Fig. 1). All of
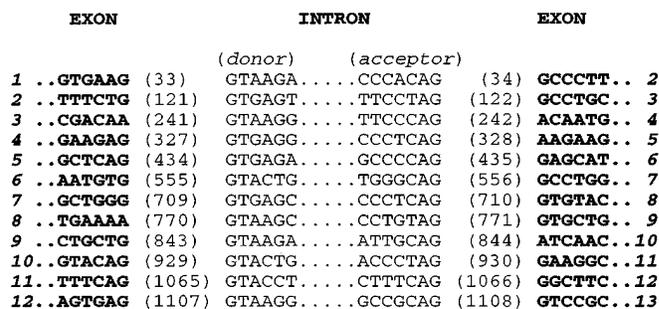
FIG. 2. **The Rat CRFR gene structure.** Sequence position of exons and introns with the A from the initiation codon ATG as +1.

the intron-exon junction sites correspond to the consensus splice sequence (GT-AG) (Fig. 2), and the coding sequence of the gene is identical to the deduced amino acid sequence of the rat pituitary CRF receptor cDNA (accession number L24096) (5) (Fig. 3, *r(A)*).

The reported CRF receptor cDNA variants were found to correspond to the deletion of complete exons (Figs. 1 and 3). Exon 3 of the extracellular domain is deleted from a human variant 3 (accession number U16273) (14) (Fig. 3, *h(3)*). A second variant, the human CRF Type 2 receptor (accession number L23333) (7) contains an 87-nucleotide insertion after exon 5 of the rat CRF receptor gene (Figs. 1 and 3, *h(2)*). To determine whether this additional exon or a highly similar sequence was contained within the rat intron between exons 5 and 6, PCR and hybridization studies using the oligonucleotide sequence of the 87-bp insert and genomic clone 3 was performed. We were unable to locate this fragment in the noncoding intron 5 of the rat gene, indicating that this specific splice variant may not be expressed in the rat.

5′ End of CRF Receptor Gene—The 722 bp of 5′ noncoding sequence of the CRFR gene contains multiple Sp1 and AP-2 sites but no typical TATA box (Fig. 4). Primer extension analysis reveals heterogenous start sites with extension from two different primers (−23 to +6bp) and (+33 to +59 bp), where +1

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       (-78)                                                                                              (1)                    (22)
r(A)   GACCGCAGCC GCCCGCCCTC CGCTCTGGGA TGTCGGAGCG ATCCAGGCAT CCAGGACGCT GACGGAGCGA GCCCGAGGAT GGGACGGCGC CCGCAGCTCC
h(1)   .......... .......... .......... .......... .CCCGGGCAT TCAGGACGGT AGCCGAGCGA GCCCGAGGAT GGGAGGGCAC CCGCAGCTCC
h(2)   .......... .......... .......... .......... .......... .......... .......... .......AT GGGAGGGCAC CCGCAGCTCC
h(3)   .......... .......... .......... .......... .......... .......... AGCCGAGCGA GCCCGAGGAT GGGAGGGCAC CCGCAGCTCC

       (23)          ▪1                                                                                            ▪2(122)
r(A)   GGCTCGTGAA GGCCCTTCTC CTTCTGGGGC TGAACCCTGT GTCCACCTCC CTTCAGGATC AGCGCTGTGA GAACCTGTCC CTGACCAGCA ATGTTTCTGG
h(1)   GTCTCGTCAA GGCCCTTCTC CTTCTGGGGC TGAACCCCGT CTCTGCCTCC CTCCAGGACC AGCACTGCGA GAGCCTGTCC CTGGCCAGCA ACATCTCAGG
h(2)   GTCTCGTCAA GGCCCTTCTC CTTCTGGGGC TGAACCCCGT CTCTGCCTCC CTCCAGGACC AGCACTGCGA GAGCCTGTCC CTGGCCAGCA ACATCTCAGG
h(3)   GTCTCGTCAA GGCCCTTCTC CTTCTGGGGC TGAACCCCGT CTCTGCCTCC CTCCAGGACC AGCACTGCGA GAGCCTGTCC CTGGCCAGCA ACATCTCAG.

       (123)                                                                                                         (222)
r(A)   CCTGCAGTGC AATGCATCCG TGGACCTCAT TGGCACCTGC TGGCCCCGGA GCCCTGCGGG CCAGTTGGTG GTCCGACCCT GCCCTGCCTT TTTCTACGGT
h(1)   ACTGCAGTGC AACGCATCCG TGGACCTCAT TGGCACCTGC TGGCCCCGCA GCCCTGCGGG GCAGCTAGTG GTTCGGCCCT GCCCTGCCTT TTTCTATGGT
h(2)   ACTGCAGTGC AACGCATCCG TGGACCTCAT TGGCACCTGC TGGCCCCGCA GCCCTGCGGG GCAGCTAGTG GTTCGGCCCT GCCCTGCCTT TTTCTATGGT
h(3)   .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........

       (223)            ▪3                                                                                          (322)
r(A)   GTCCGCTACA ACACGACAAA CAATGGCTAC CGGGAGTGCC TGGCCAACGG CAGCTGGGCA GCCCGTGTGA ATTATTCTGA GTGCCAGGAG ATTCTCAACG
h(1)   GTCCGCTACA ATACCACAAA CAATGGCTAC CGGGAGTGCC TGGCCAATGG CAGCTGGGCC GCCCGCGTGA ATTACTCCGA GTGCCAGGAG ATCCTCAATG
h(2)   GTCCGCTACA ATACCACAAA CAATGGCTAC CGGGAGTGCC TGGCCAATGG CAGCTGGGCC GCCCGCGTGA ATTACTCCGA GTGCCAGGAG ATCCTCAATG
h(3)   .......... .........A CAATGGCTAC CGGGAGTGCC TGGCCAATGG CAGCTGGGCC GCCCGCGTGA ATTACTCCGA GTGCCAGGAG ATCCTCAATG

       (323)       ▪4                                                                                              (422)
r(A)   AAGAGAAGAA GAGCAAAGTA CACTACCATG TTGCAGTCAT CATCAACTAC CTGGGTCACT GCATCTCCCT GGTAGCCCTC CTGGTGGCCT TTGTCCTCTT
h(1)   AGGAGAAAAA AAGCAAGGTG CACTACCATG TCGCAGTCAT CATCAACTAC CTGGGCCACT GTATCTCCCT GGTGGCCCTC CTGGTGGCCT TTGTCCTCTT
h(2)   AGGAGAAAAA AAGCAAGGTG CACTACCATG TCGCAGTCAT CATCAACTAC CTGGGCCACT GTATCTCCCT GGTGGCCCTC CTGGTGGCCT TTGTCCTCTT
h(3)   AGGAGAAAAA AAGCAAGGTG CACTACCATG TCGCAGTCAT CATCAACTAC CTGGGCCACT GTATCTCCCT GGTGGCCCTC CTGGTGGCCT TTGTCCTCTT

       (423)          ▪5                                                                                            (435)
r(A)   CTTGCGGCTC AG........ .......... .......... .......... .......... .......... .......... ..........G
h(1)   TCTGCGGCTC AG........ .......... .......... .......... .......... .......... .......... ..........G
h(2)   TCTGCGGCTC AGGCCAGGCT GCACCCATTG GGGTGACCAG GCAGATGGAG CCCTGGAGGT GGGGGCTCCA TGGAGTGGTG CCCCATTTCA GGTTCGAAGG
h(3)   TCTGCGGCTC AG........ .......... .......... .......... .......... .......... .......... ..........G

       (436)                                                                                                        (535)
r(A)   AGCATCCGGT GCCTGAGAAA CATCATCCAC TGGAACCTCA TCTCGGCTTT CATCCTACGC AACGCCACGT GGTTTGTGGT CCAGCTCACC GTGAGCCCCG
h(1)   AGCATCCGGT GCCTGCGAAA CATCATCCAC TGGAACCTCA TCTCCGCCTT CATCCTGCGC AACGCCACCT GGTTCGTGGT CCAGCTAACC ATGAGCCCCG
h(2)   AGCATCCGGT GCCTGCGAAA CATCATCCAC TGGAACCTCA TCTCCGCCTT CATCCTGCGC AACGCCACCT GGTTCGTGGT CCAGCTAACC ATGAGCCCCG
h(3)   AGCATCCGGT GCCTGCGAAA CATCATCCAC TGGAACCTCA TCTCCGCCTT CATCCTGCGC AACGCCACCT GGTTCGTGGT CCAGCTAACC ATGAGCCCCG

       (536)               ▪6                                                                                       (635)
r(A)   AGGTGCACCA GAGCAATGTG GCCTGGTGTA GGTTGGTGAC AGCCGCCTAC AATTACTTCC ATGTAACCAA CTTCTTCTGG ATGTTCGGTG AGGGCTGCTA
h(1)   AGGTCCACCA GAGCAACGTG GGCTGGTGCA GGTTGGTGAC AGCCGCCTAC AACTACTTCC ATGTGACCAA CTTCTTCTGG ATGTTCGGCG AGGGCTGCTA
h(2)   AGGTCCACCA GAGCAACGTG GGCTGGTGCA GGTTGGTGAC AGCCGCCTAC AACTACTTCC ATGTGACCAA CTTCTTCTGG ATGTTCGGCG AGGGCTGCTA
h(3)   AGGTCCACCA GAGCAACGTG GGCTGGTGCA GGTTGGTGAC AGCCGCCTAC AACTACTTCC ATGTGACCAA CTTCTTCTGG ATGTTCGGCG AGGGCTGCTA

       (636)                                                                              ▪7                        (735)
r(A)   CCTGCACACA GCCATTGTGC TCACGTACTC CACCGACCGT CTGCGCAAGT GGATGTTCGT CTGCATTGGC TGGGGTGTAC CTTTCCCCAT CATTGTGGCT
h(1)   CCTGCACACA GCCATCGTGC TCACCTACTC CACTGACCGG CTGCGCAAAT GGATGTTCAT CTGCATTGGC TGGGGTGTGC CCTTCCCCAT CATTGTGGCC
h(2)   CCTGCACACA GCCATCGTGC TCACCTACTC CACTGACCGG CTGCGCAAAT GGATGTTCAT CTGCATTGGC TGGGGTGTGC CCTTCCCCAT CATTGTGGCC
h(3)   CCTGCACACA GCCATCGTGC TCACCTACTC CACTGACCGG CTGCGCAAAT GGATGTTCAT CTGCATTGGC TGGGGTGTGC CCTTCCCCAT CATTGTGGCC

       (736)                  ▪8                                                                                    (835)
r(A)   TGGGCCATTG GGAAGCTGCA CTACGACAAT GAAAAGTGCT GGTTTGGCAA ACGTCCTGGG GTATACACTG ACTACATCTA CCAGGGCCCC ATGATCCTGG
h(1)   TGGGCCATTG GGAAGCTGTA CTACGACAAT GAGAAGTGCT GGTTTGGCAA AAGGCCTGGG GTGTACACCG ACTACATCTA CCAGGGCCCC ATGATCCTGG
h(2)   TGGGCCATTG GGAAGCTGTA CTACGACAAT GAGAAGTGCT GGTTTGGCAA AAGGCCTGGG GTGTACACCG ACTACATCTA CCAGGGCCCC ATGATCCTGG
h(3)   TGGGCCATTG GGAAGCTGTA CTACGACAAT GAGAAGTGCT GGTTTGGCAA AAGGCCTGGG GTGTACACCG ACTACATCTA CCAGGGCCCC ATGATCCTGG

       (836)       ▪9                                                                              ▪10 (935)
r(A)   TCCTGCTGAT CAACTTTATC TTTCTCTTCA ACATTGTCCG CATCCTCATG ACCAAACTCC GGGCATCCAC TACATCTGAG ACCATTCAGT ACAGGAAGGC
h(1)   TCCTGCTGAT CAATTTCATC TTCCTTTTCA ACATCGTCCG GATCCTCATG ACCAAGCTCC GGGCATCCAC CACGTCTGAG ACCATTCAGT ACAGGAAGGC
h(2)   TCCTGCTGAT CAATTTCATC TTCCTTTTCA ACATCGTCCG CATCCTCATG ACCAAGCTCC GGGCATCCAC CACGTCTGAG ACCATTCAGT ACAGGAAGGC
h(3)   TCCTGCTGAT CAATTTCATC TTCCTTTTCA ACATCGTCCG CATCCTCATG ACCAAGCTCC GGGCATCCAC CACGTCTGAG ACCATTCAGT ACAGGAAGGC

       (936)                                                                                                        (1035)
r(A)   TGTGAAGGCC ACTCTGGTGC TCCTGCCCCT TCTGGGCATC ACCTACATGT TGTTCTTCGT CAACCCTGGA GAGGACGAGG TCTCCAGGGT CGTCTTCATC
h(1)   TGTGAAAGCC ACTCTGGTGC TGCTGCCCCT CCTGGGCATC ACCTACATGC TGTTCTTCGT CAATCCCGGG GAGGATGAGG TCTCCCGGGT CGTCTTCATC
h(2)   TGTGAAAGCC ACTCTGGTGC TGCTGCCCCT CCTGGGCATC ACCTACATGC TGTTCTTCGT CAATCCCGGG GAGGATGAGG TCTCCCGGGT CGTCTTCATC
h(3)   TGTGAAAGCC ACTCTGGTGC TGCTGCCCCT CCTGGGCATC ACCTACATGC TGTTCTTCGT CAATCCCGGG GAGGATGAGG TCTCCCGGGT CGTCTTCATC

       (1036)                       ▪11                        ▪12                                                  (1135)
r(A)   TACTTCAACT CTTTTCTGGA GTCCTTTCAG GGCTTCTTTG TGTCTGTGTT CTACTGTTTT CTGAACAGTG AGGTCCGCTC CGCTATCCGG AAGAGGTGGC
h(1)   TACTTCAACT CCTTCCTGGA ATCCTTCCAG GGCTTCTTTG TGTCTGTGTT CTACTGTTTC CTCAATAGTG AGGTCCGTTC TGCCATCCGG AAGAGGTGGC
h(2)   TACTTCAACT CCTTCCTGGA ATCCTTCCAG GGCTTCTTTG TGTCTGTGTT CTACTGTTTC CTCAATAGTG AGGTCCGTTC TGCCATCCGG AAGAGGTGGC
h(3)   TACTTCAACT CCTTCCTGGA ATCCTTCCAG GGCTTCTTTG TGTCTGTGTT CTACTGTTTC CTCAATAGTG AGGTCCGTTC TGCCATCCGG AAGAGGTGGC

       (1136)                                                                                                       (1235)
r(A)   GTCGGTGGCA GGACAAGCAC TCCATCAGAG CCCGAGTGGC CCGAGCTATG TCCATCCCCA CCTCCCCGAC CAGAGTCAGC TTTCACAGCA TCAAGCAGTC
h(1)   ACCGGTGGCA GGACAAGCAC TCGATCCGTG CCCGAGTGGC CCGTGCCATG TCCATCCCCA CCTCCCCAAC CCGTGTCAGC TTTCACAGCA TCAAGCAGTC
h(2)   ACCGGTGGCA GGACAAGCAC TCGATCCGTG CCCGAGTGGC CCGTGCCATG TCCATCCCCA CCTCCCCAAC CCGTGTCAGC TTTCACAGCA TCAAGCAGTC
h(3)   ACCGGTGGCA GGACAAGCAC TCGATCCGTG CCCGAGTGGC CCGTGCCATG TCCATCCCCA CCTCCCCAAC CCGTGTCAGC TTTCACAGCA TCAAGCAGTC

       (1236)        ‾‾‾‾                                                                                 (1322)
r(A)   CACAGCAGTG TGAGCTCCAGG CCACAGAGCA GCCCCCAAGA CCTGAGGCCG GGGAGATGAT GCAAGCTCAC TGACGAGCCA GTCTGC
h(1)   CACAGCAGTC TGA....... .......... .......... .......... .......... .......... ......
h(2)   CACAGCAGTC TGA....... .......... .......... .......... .......... .......... ......
h(3)   CACAGCAGTC TGA....... .......... .......... .......... .......... .......... ......
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FIG. 3. **Comparison of the sequence of rat and human CRF receptors.** ▪ indicates intron positions (numbers according to rat CRFR sequence). The Initiation and termination codons are *underlined* and *overlined*, respectively. *r(A)*, rat pituitary Form A CRF holoreceptor (accession number L24096); *h(1)*, human type I CRF receptor (accession number L23332); *h(2)*, human type 2 CRF receptor (accession number L23333); and *h(3)*, human variant CRF receptor (accession number U16273).

is the A of the initiation codon ATG (Fig. 5, *A* and *B*). The nucleotide position -238 ± 2 was identified as the major transcript start site with both primers. The $-23/+6$ primer produced an extension product at 244 bp that corresponds to position -238 (Fig. 5*A*, *lane 3*), and the primer $+33/+59$ gave an extension product at 297 that also corresponds to position -238 (Fig. 5*A*, *lane 1*). An additional start site was identified at -174 only with the $(+33/+59)$ primer (Fig. 5*A*, *lane 1*, 233 bp). S1 nuclease mapping using the PCR fragment ($+6$ to -554) as probe showed a 244-bp protected fragment, which demonstrates a start site at -238 bp, consistent with primer extension (Fig. 5*B*). The major initiation site at -238 bp is approximately 20 bp downstream of an AP-2 site (18) and 60 bp downstream of an Sp1 consensus element (Fig. 4). Transient

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expression of two CRFR/luciferase reporter gene constructs with −651 or −593 bp (ATG initiation codon is +1) of the CRFR 5′-flanking region into the mouse Leydig tumor cells and Chi-

nese hamster ovary cells confirmed that a promoter domain is located within 593 bp of the 5′-flanking domain. The addition of the domain between −593 and −651 increased promoter activity in both cell types by 1.6-fold, indicating that either an enhancer or a second promoter is located between −593 and −651 bp (Fig. 5C).

Similarity of the CRF Receptor Gene to the Glucagon Receptor Gene Family—The CRF receptor gene exhibits substantial similarity to the glucagon/PTH (12, 13) receptor gene family that is characterized by the presence of introns within its transmembrane/cytoplasmic module, highly conserved cysteines in its extracellular domain, and a highly conserved first intracellular loop (12). The exon/intron junctions of the CRF, PTH (12), and glucagon (13) receptor genes are remarkably similar in alignment (Genetics Computer Group; gap weight = 3; gap length weight = 0.1) following the signal peptide in exons 1 and 2 (Fig. 6). The CRF receptor exon/intron junctions are aligned to that of the PTH and glucagon receptors after exons 3, 5, 7, 8, 9, 10, and 12. CRFR is similar to PTHR in that amino acids 457–509 are divided into exons 11 and 12, whereas this domain in the glucagon receptor is a single exon (Fig. 6). All members of this family induce cAMP generation and phosphoinositide hydrolysis (7, 19) and thus the similarity of the TM domains and intracellular loops. With the exception of the cysteines in extracellular loops 1 and 2, there is no visible similarity within the extracellular loops among the three receptors, and these domains may contribute to ligand specificity. Extracellular loop 3 has been shown to be of importance to PTH

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                                                         AP-2
-722   CTAGGTGCTTCTCAACCAGGCCTAGTGACAGGCNCCACTTCGCCAGCGCA

-672   TCTCAGAGCNCTNNNTCACTGGGTACNAGCACCTCGTCTCGGTGCTGGAG

-622   AAGGTGAGTGGGCCTGGGTGTCGCCGCCCTGCAGCCTCAATTTCTATGCC

-572   TGGGTCTCCGGACCGCTCAGGGCTGATATAGACCTTCTGGGCCACAAGCC
                           AP-2
-522   GGGTTAGCTG CGGCGGNCGCCACCGCTGCNNNNNNCTCGCGCACTGCCG

-472   CAAGCCGGGGTGCGGGGAGACGAGGTTCAG CACGGCCAGGTGCGGGGAT

-422   CCGGCCCGGGTTTGGGCAGTGGACCGAGGAGGCGGGAGCCNNCAGCCAGC

-372   GGAGGGGCGAGGAGCGGAGGAGGGACCGCAGGGGAGAGGAGGGAGAAAGA
                           SP-1
-322   GGAGGGGAGCGAGGCGAGGGGGCGGGGAGCCGAGGCGAGAGGCAGGCGGA
         AP-2                               tss
-272   CCGTGGGGGAGGCGACTGGGCAAGGCGGGATCCAGGCAGTAGAGAGGTGN
                                                        tss
-222   NGGCCCGGCTGAGTCGGGAGAAGGCTACCAGACTNNNNNNGAAGGGCGAG
                Sp-1    AP-2
-172   CCAGAGCTGCCGGTGGGGCGGGGCGGGGCGGAAGGGTGCGAAGCCCGGCA
                   AP-2                  AP-2
-122   CCTGGGCGCAGATCACGGCCCGGGGAGACCGGCCGCCCACCGGAGACCGC
            AP-2
-72    AGCCGCNCGCCCTCCGCTCTGGGATGTCGGAGCGATCCAGGCATCCAGGA
                 IC
-22    CGCTGACGGAGCGAGNCCGAGGATGGGACGGCGCCCGCAG
                          +1
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FIG. 4. **The 5′-flanking region of the rat CRFR gene.** The *overlined* regions indicate consensus elements. *tss*, transcript start sites. *N*, undetermined nucleotide.

FIG. 5. **Primer extension (A) and S1 nuclease mapping (B) using designated oligonucleotide primers and genomic probes.** Bands at 297 (*A, lane 1*) and 244 (*A, lane 3; B, lane 1*) represent −238 bp, and the band at 233 (*A, lane 1*) represents −174 bp. Yeast tRNA controls (*A, lanes 2* and *4; B, lane 2; C*, summary of primer extension data and promoter activity relative light unit/unit β-galactosidase activity).

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                    _____SIGNAL PEPTIDE_____|
crfr(A)  MGRRPQLRLVK*1 ALLLLGLNPVSTSLQDQRCENLSLTSNVS*2      GLQCNASVDLIGTCWPRSPAGQLVVRPCPAFFYGVRYNTT*3
    (B)                    //-------------------*2          ..........................................
    (C)                                                                               //--------*3
pthr     oooooooooo   ooooooooooooooooooooooooooooooo*      -RP-LPEW-N-V.---LGAP-EV-AV---DYI-DFNHK*
glur     oooooooooo   oooooooooooooYS--CHH----LPPPT*        E-V--RTF-KYS.---DT-PNTTANIS--.WYLPWYHK*VQ
                                    ++   ++++    +            +   +   +++     +    ++ +

                                                                         _____TM 1_____
crfr(A)  NN.GYRECLANGSWA.......ARVNYSECQEILNEE*4            KKSKV...HYHVAVIINYLGHCISLVALLVAFVLFLRL*5
    (B)  d----------------------------------*4              --------------//
    (C)  ----------------------------------*4              -------------------------------------*5
pthr     GH.A--R-DR----EVVPGHNRTWA-----LKFMTN-TRER*         EVFDRLG.......M-YTV-YSM--AS-T---VLILAYF*
glur     HRLVFKR-GPD-Q-.VRGPRGQSWRDA-Q--MDDD-IEVQ*          -GVAKMYSSYQ...VMYTV-YSL--G---L-L-IL-G-*
             +++ +   +   +          + +                       +++     +++ + ++ ++ +

               _____TM2_____                        _____TM3_____             _____TM4__
crfr(A)  RSIRCLRNIIHWNLISAFILRNATWFV.....VQLTVSPEVHQSNV*6   AWCRLVTAAYNYFHVTNFFWMFGEGCYLHTAIVLTYSTDRLRKWMFVCIGW*7
    (C)  --------------------------------------------*6     vylspslwlgplgsctttmksaglanvlgytlttstrap//
pthr     -RLH-T--Y--MHMFLS-M--A-SI--(ins1)-VPPP-AAAAVGY*    -G--VAVTFFL-FLA--YY-ILV--L----SLIFMAFFSEKKYL-G-TIF--*
glur     -KLH-T--Y--G--FAS-V-KAGSVL-(ins2).VS-CLSDG*AV      -G--VA-VIMQ-GIIA-YC-LLV--V--YSLLSI-TFSEKSFFSLYL----*
          + +++ ++ ++        + ++ ++  +      +               + ++++    +   + ++ +   ++ ++ +++     +++        ++

            ___TM4_____
crfr(A)  GVPFPIIVAWAIGKLHYDNEK*8 CWFGKRPGVYTDYIYQGPMILVLL*9  INFIFLFNIVRILMTKLRASTTSET...IQY*10
pthr     -L-AVFVAV-VGVRATLA-TG*  --.DLSS-HK.KW-I-V-ILASVV*   L---LFI--I-V-A----ETNAGRCDTRQ--*
glur     -S-LLFVIP-VVVKCLFE-VQ*  --.TSNDNMGFWW-LRI-VL-AI-*   ---FIFAR-IHL-VA----HQMHYADYKF..*
          + +     +  +++++  +     ++         ++ + + ++ ++    +++    ++++++  +++      +++ ++

             _____TM6_____              _TM7___    _____
crfr(A)  RKAVKATLVLLPLLGITYMLFFVNPGEDEVS..RVVFIYFNSFLESFQ*11  GFFVSVFYCFLNSE*12  VRSAIRKRWRRWQDKHSIR.
pthr     --LLRS----V--F-VH-TV-MAL-YTEVSGTLWQIQMHYEMLFN---*    ----AII---C-G-*    -QAE---S-S--TLALDF..
glur     -L-RS.--T-I----VHEVV-AFVTD-HAQGTL-STKLF-DL-FS---    -LL-A-L-----K-*    -QAELLR------EGKALQE
          + +    ++ ++++ ++    ++            +      +++       +  + + +++ + +     ++  + + + ++

crfr(A)  ARVARAMSIPTSPTRVSFHSIKQSTAV///
pthr     K-K--SG-SSY-YGPMVS-TSVTNVGPRAGLSLPLSPRLPPATTNGHSQLPGHAKPGAPATETETLPVTMAVPKDDGFLNGS
glur     E-M--S......-HGSHMAPAGTCHGDPCEKLQL.MSAGSSSGTGCEPSAKTSLASSLPRLADSPT///
             + +      +

pthr     CSGLDEEASGSARPPPLLQEEWETVM///

INSERTS (ins)
pthr 1. KDAVLYSGFTLDEAERLTEEELHIIA
glur 2. IDWLLKTRYSQKIGDDLS
```
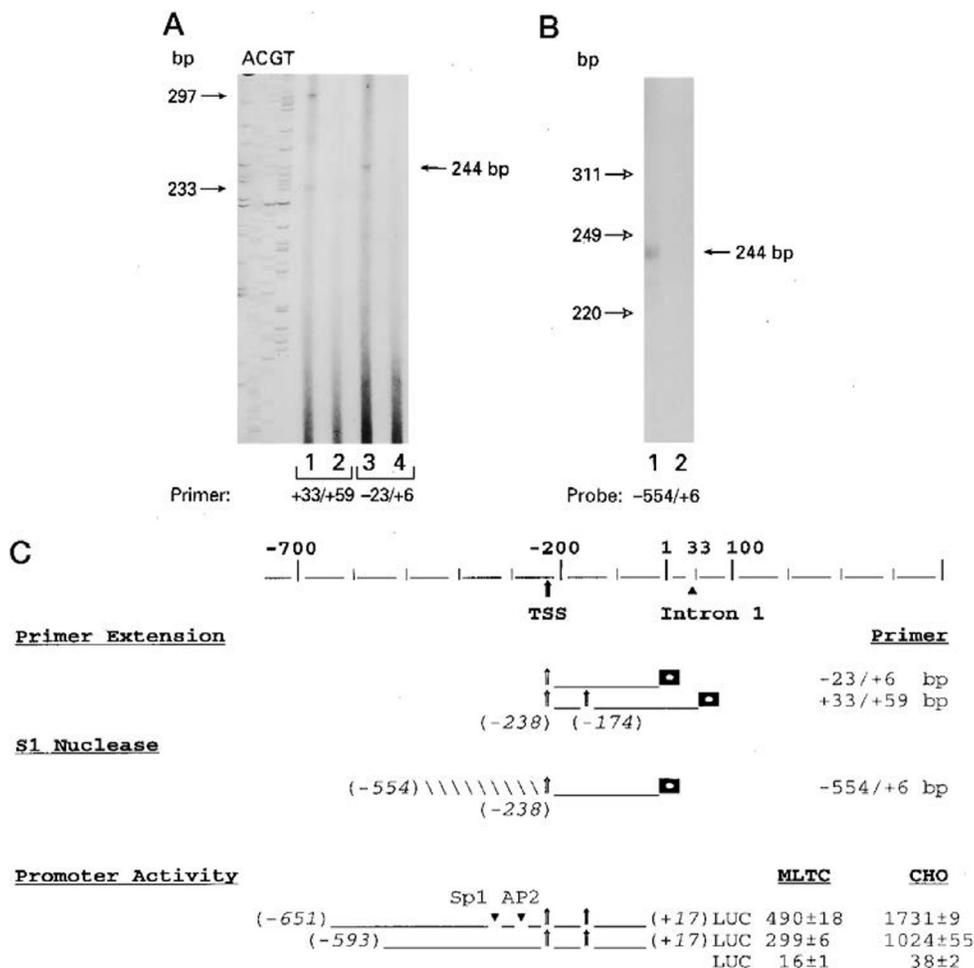
FIG. 6. **Exon/intron pattern of the rat CRF, PTH, and glucagon receptor family.** Amino acid sequences of rat CRFR (5) (accession number P35353), rat PTHR (8) (accession number P25961), and rat glucagon receptor (9) (accession number P30082). CRFR transmembrane domains are designated as in Ref. 4). *Asterisk*, intron; *dot*, gap; *dash*, identity to CRFR; //, 5′,3′ end of sequenced PCR product that corresponds to primer; ///, end of cDNA; *lowercase letters*, novel sequences from exon deletion; +, position of amino acid similarity; ○, no visible homology.

binding to the PTHR (20) and extracellular loop 1 as well as TM3, TM4, and TM6 for glucagon binding to the glucagon receptor (21). The potential for receptor splice variants that exhibit differential modes of signal transduction is indicated by the presence of introns in the TM/cytoplasmic module and has been shown to be physiologically relevant in a member of the glucagon family, the pituitary adenylate cyclase-activating polypeptide receptor (22). Variants containing combinations of two additional exon cassettes in the third intracellular loop have been identified and sequenced, each exhibiting different degrees of G_s and phosphoinositide coupling (23). These pituitary adenylate cyclase-activating polypeptide receptor exons are inserted at a position that aligns with the CRFR, PTHR, and glucagon receptor exon 10-11 junction (Fig. 6) within the third intracellular loop.

CRFR cDNA Isolated from the Rat Leydig Cell—We proceeded to isolate and sequence CRFR PCR products from rat Leydig cell mRNA to determine which CRFR forms are present in this cell type. The assumption is a derivation from the rat CRFR Form A gene described in this study, and several PCR primers were constructed using the sequence of the rat pituitary Form A receptor cDNA (Fig. 3, *r(A)*) (5). cDNA clones were amplified with select CRF receptor primers by reverse transcription-PCR and sequenced (Fig. 7). Although all Leydig cell CRFR mRNA were expressed at very low levels in comparison with the pituitary, the predominant form of the Leydig cell receptor mRNA was identified as the rat pituitary CRF receptor sequence (Figs. 3, *r(A)*, and 7, *FORM A*).

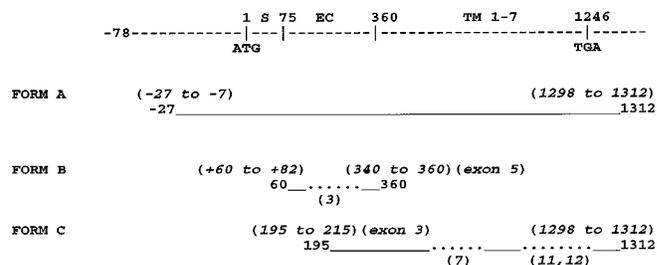Two splice variants (B and C) were also recovered from rat

FIG. 7. **CRFR forms identified in the rat Leydig cell by reverse transcription-PCR.** *Form A*, rat pituitary holoreceptor. *Forms B* and *C*, PCR products. *Dot*, missing exons 3, 7, 11, or 12. No additional 3′ Form B or 5′ Form C was recovered using primers derived from the Form A sequence, although unique additional 3′ and 5′ sequences cannot be ruled out. *S*, signal; *EC*, extracellular domain.

Leydig cell mRNA but at very low expression levels. The first variant Form B that was recovered from the Leydig cell with PCR primers from (+60 to +82) in exon 2 to (340–360 bp) within exon 5 amplified a cDNA fragment that contains a deletion of exon 3 (Fig. 7). This deletion is in frame, resulting in the substitution of amino acids 41–71 for an aspartic acid (Fig. 6, *crfr (B)*). This variant may be of significance, because the exon 3 deletion was also observed in the human Form 3 (Figs. 1 and 3 *h(3)*) and does not change the reading frame or result in premature termination of the coding sequence. The positions of the initiation and termination codons in CRFR Form B have not been identified. PCR primers from (−27 to −7) did not amplify this clone, indicating that the signal sequence of this
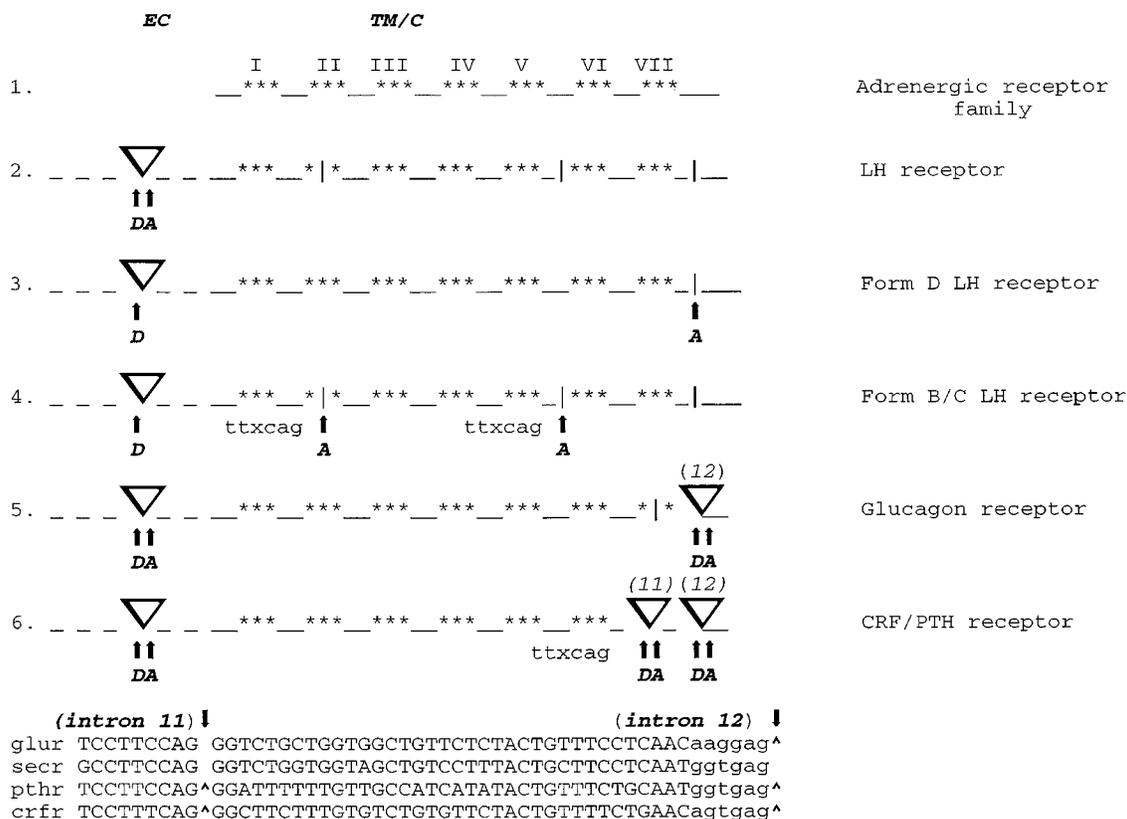
```
           EC                    TM/C
                        I    II  III   IV   V     VI  VII
   1.                  __***__***__***__***__***__***__***___        Adrenergic receptor
                                                                           family

   2.  _ _ _  ▽  _ _ __***__*|*__***__***__***_|***__***_|__        LH receptor
             ↑↑
             DA

   3.  _ _ _  ▽  _ _ __***__***__***__***__***__***__***_|___       Form D LH receptor
             ↑                                         ↑
             D                                         A

   4.  _ _ _  ▽  _ _ __***__*|*__***__***__***_|***__***_|___       Form B/C LH receptor
             ↑      ttxcag ↑              ttxcag ↑
             D             A                     A
                                                         (12)
   5.  _ _ _  ▽  _ _ __***__***__***__***__***__***__*|* ▽          Glucagon receptor
             ↑↑                                        ↑↑
             DA                                        DA
                                                     (11)(12)
   6.  _ _ _  ▽  _ _ __***__***__***__***__***__***_ ▽ _ ▽          CRF/PTH receptor
             ↑↑                             ttxcag ↑↑  ↑↑
             DA                                    DA  DA
```

```
        (intron 11)↓                                (intron 12) ↓
   glur TCCTTCCAG GGTCTGCTGGTGGCTGTTCTCTACTGTTTCCTCAACaaggag^
   secr GCCTTCCAG GGTCTGGTGGTAGCTGTCCTTTACTGCTTCCTCAATggtgag
   pthr TCCTTCCAG^GGATTTTTTGTTGCCATCATATACTGTTTCTGCAATggtgag^
   crfr TCCTTTCAG^GGCTTCTTTGTGTCTGTGTTCTACTGTTTTCTGAACagtgag^
```

FIG. 8. **Proposed mechanism of CRFR Class 2 intron 11–12 insertion in evolution from the intronless exon 11 of the LH receptor (*row 2*) at sites that resemble LHR alternative splice sites either in sequence (*Forms B* and *C*) or position (*Form D*).** *EC*, intron containing extracellular domain (*dashes*). *Asterisks*, TMI–TMVII. ▽, intron. *D* ↑ and *A* ↑, donor and acceptor splice sites at intron to produce wild type receptor (*row 2*) or alternative splice sites positions within exon (|) to produce LHR splice variants B, C, or D (*rows 3* and *4*). The alternative splice cleavage sites in the Class 1 GPCR may have been retained as sites of intron insertion in the glucagon receptor (*glur*) and CRFR/PTHR (*rows 5* and *6*). *Bottom*, TMVII domain of the CRFR, PTHR, glucagon receptor, and secretin receptors. Similarity of 3′ exon/intron 11 insertion sequence between the CRFR, PTHR (12), and the glucagon receptor (that does not contain intron 11) to the LHR Form B and C alternative splice site sequence TTXCAG within intron j (15). The rat genomic sequence of the secretin receptor (28) is not available. Similarity in position of the LHR Form D splice site to CRFR intron 12 immediately after TMVII (*capital letters*) is shown. See "Results and Discussion." Introns are represented as *carets*. *glur*, accession number L31574; *pthr*, accession numbers L31392 and L31393.

clone (exon 1) differs from that of the Form A CRFR (Fig. 6). However, Form B does contain the NH_2-terminal of the mature receptor at leucine 25, because the PCR product begins at valine 21. PCR primers constructed from the CRFR Form A cDNA sequence at the putative 2nd transmembrane domain in exon 6 at 552 or 451 bp or TM3 (731 bp) in exon 7 did not amplify the variant Form B, implying that this cDNA may contain a novel carboxyl-terminal sequence.

Because the exon 5-intron 5 donor splice site is apparently susceptible to alternative splicing (Fig. 3, *h(2)*) (7), it is possible that this is the specific site of divergence of the Form B variant from Form A. With a putative continuation into the intron, a termination codon (TGA) is present at position 2 of the 5′-flanking intron 5 sequence (TGA) (Fig. 2, *exon 5*), leaving a single transmembrane domain with a single arginine (AGG) in the putative cytoplasmic domain.

A second rat Leydig cell variant CRFR Form C cDNA was amplified with a 5′ primer beginning in exon 3 at position 215 and continues to exon 13 with deletions of exon 7, 11, and 12 (Figs. 1, 3, 6, and 7). PCR with 5′ primers from exons 1 or 2 did not recognize or amplify this variant, indicating a novel amino-terminal sequence. The CRFR Form C PCR product begins with amino acid 73 in exon 3. Deletion of exon 7 results in a frameshift with premature termination after a unique 39-amino acid tail that leaves only two hydrophobic domains (Fig. 6). It remains to be proven whether this form of the CRFR is responsible for the unique properties of the Leydig cell CRFR

(1, 3). The absence of initiating methionines in the PCR products Form B and C CRFR did not permit expression studies with these clones.

A comparison of splice variants CRFR Form B and Form C and the human variant 3 (14) suggests that exon 3 may be of particular importance to receptor function, because two of the PCR products exclude exon 3, and the third appears to begin with exon 3. Exon 3 contains three of the five extracellular cysteines that are also conserved in the PTH and glucagon receptors (Fig. 6). Functional studies that did not address changes in receptor number or expression levels of the CRFR variant 3 (Fig. 3, *h(3)*) indicate a significant decrease in CRF binding activity (14). However, specific mutations of the conserved cysteines in exon 3 of the PTH receptor indicate that these cysteines are essential for receptor processing and expression on the cell surface (20), and mutant PTHR binding activity was commensurate with lower expression levels. It is interesting to note that the sequence differences between the pituitary Form A and several of the reported human and rat variants (NH_2-terminal signal and exon 3) appear to impact on surface expression.

The genomic structure of the mammalian GPCR are diverse and vary from intronless seven-transmembrane/cytoplasmic (TM/C) modules without an NH_2-terminal extracellular domain (24) (Fig. 8, *row 1*) to intron containing NH_2-terminal protein binding modules conjugated to intronless TM/C domains (16, 25) (Fig. 8, *row 2*) to the intron containing EC/TM/C

domains of the CRFR receptor (Fig. 8, *row 6*). The presence of an alternative acceptor splice site at position 1895 within the intronless LHR exon 11 module to produce the soluble splice variant, initially identified in the pig as Form D (26) may provide some insight into the origin of the intron containing CRFR TM/C module, because it is positioned at the same location as intron 12 of the CRFR, immediately following TMVII (Figs. 8, *row 2*, and 6). The alternative splice sites in the LHR coding region substitute for the 3′ end of the intron within the spliceosome (15), serve as a cleavage site and possibly as a site of intron insertion in evolution (Fig. 8, *row 3*). Studies with the Tetrahymena Group I intron show intron insertion at RNA splice sites via RNA intermediates, and reverse transcription resulting in incorporation of the intron into the gene is proposed (27). It is interesting to note that CRFR exons 1, 4, 5, 10, 11, and 12 terminate in the 3′ intron acceptor consensus element AG, (Fig. 2, *column 1 versus column 3*) indicating that these positions may have served as alternate acceptor (or intron insertion points) within an intronless coding region. The 3′ nucleotides of CRFR exons 11 and 12 are not unique to the CRFR. These are conserved in the PTHR (12) and glucagon (13) receptor genes and are also present in the secretin receptor at this aligned position (28) (Fig. 8, *bottom*). The 3′ element of CRFR exon 11 conforms to the LHR TTXCAG 3′ intron J (between 959 and 960 of the LHR) splice element and the alternative splice sites that produce LHR forms B and C (15). This position is not an intronic site in the glucagon receptor (13) but is in the PTHR (12) and CRFR (Figs. 6 and 8, *rows 5* and *6*). Thus, the glucagon receptor may be an intermediate form between the LHR and CRFR.

REFERENCES

1. Ulisse, S., Fabbri, A., Tinajero, J. C., and Dufau, M. L. (1990) *J. Biol. Chem.* **265,** 1964–1971
2. Vale, W., Spiess, J., Rivier, C., and Rivier, J. (1981) *Science* **213,** 1394–1397
3. Ulisse, S., Fabbri, A., and Dufau, M. L. (1989) *J. Biol. Chem.* **264,** 2156–2163
4. Chen, R., Lewis, K. A., Perrin, M. H., and Vale, W. W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8967–8971
5. Perrin, M. H., Donaldson, C. J., Chen, R., Lewis, K. A., and Vale, W. W. (1993) *Endocrinology* **133,** 3058–3061
6. Vita, N., Laurent, P., Lefort, S., Chalon, P., Kaghad, M., Le Fur, G., Caput, D., and Ferrara, P. (1993) *FEBS Lett.* **335,** 1–5
7. Xiong, Y., Xie, L. Y., and Abou-Samra, A.-B., (1995) *Endocrinology* **136,** 1828–1834
8. Abou-Samra, A.-B., Juppner, H., Force, T., Freeman, M. W., Kong, X.-F., Schipani, E., Urena, P., Richards, J., Bonventre, J. V., Potts, J. T., Jr., Kronenberg, H. M., and Segre, G. V. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2732–2736
9. Jelinek, L. J., Lok, S., Grant, F. J. Rosenberg, G. B., Smith, R. A., Bensch, P. A., Sheppard, P. O., O'Hara, P. J., Foster, D. C., Kuijper, J. L., Biggs, S. H., Walker, K. M., Chen, L. H., McKernan, P. A., and Kindsvogel, W. (1993) *Science* **259,** 1614–1616
10. Svoboda, M., Ciccarelli, E., Tastenoy, M., Cauvin, A., Stievenart, M., and Christophe, J. (1993) *Biochem. Biophys. Res. Commun.* **191,** 479–486
11. Burbach, J. P., and Meijer, O. C. (1992) *Eur. J. Pharmacol.* **227,** 1–18
12. Kong, X. F., Schipani, E., Lanske, B., Joun, H., Karperien, M., Defize, L. H., Juppner, H., Potts, J. T., Jr., Segre, G. V., Kronenberg, H. M., and Abou-Samra, A. B. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1290–1299
13. Maget, B., Tastenoy, M., and Svoboda, M. (1994) *FEBS Lett.* **351,** 271–275
14. Ross, P. C., Kostas, C. M., and Ramabhadran, V. (1994) *Biochem. Biophys. Res. Commun.* **205,** 1836–1842
15. Tsai-Morris, C. H., Buczko, E., Wang, W., and Dufau, M. L. (1990) *J. Biol. Chem.* **265,** 19385–19388
16. Tsai-Morris, C. H., Buczko, E., Wang, W., Xie, X.-Z., and Dufau, M. L. (1991) *J. Biol. Chem.* **266,** 11355–11359
17. Tsai-Morris, C. H., Xie, X., Wang, W., Buczko, E., and Dufau, M. L. (1993) *J. Biol. Chem.* **268,** 4447–4452
18. Imagawa, M., Chiu, R., and Karin, M. (1987) *Cell* **51,** 251–260
19. Wheeler, M. B., Lu, M., Dillon, J. S., Leng, X.-H., Chen, C., and Boyd, A. E. (1993) *Endocrinology* **133,** 57–62
20. Lee, C., Gardella, T. J., Abou-Samra, A.-B., Nussbaum, S. R., Segre, G. V., Potts, J. T., Kronenberg, H. M., and Juppner, H. (1994) *Endocrinology* **135,** 1488–1495
21. Buggy, J. J., Livingston, J. N., Rabin, D. U., and Yoo-Warren, H. (1995) *J. Biol. Chem.* **270,** 7474–7478
22. Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., and Journot, L. (1993) *Nature* **365,** 170–175
23. Journot, L., Waeber, C., Pantaloni, C., Holsboer, F., Seeburg, P. H., Bockaert, J., and Spengler, D. (1995) *Biochem. Soc. Trans.* **23,** 133–137
24. Dohlman, H. G., Bouvier, M., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1987) *J. Biol. Chem.* **262,** 14282–14288
25. Koo, Y. B., Ji, I., Slaughter, R. G., and Ji, T. H. (1991) *Endocrinology* **128,** 2297–2308
26. Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Thi, M. T. V. H.-L., Jolivet, A., Guichon-Mantel, A. Sar, S., Jallal, B., Garnier, J., and Milgrom, E., (1989) *Science* **245,** 525–527
27. Woodson, S. A., and Cech, T. R. (1989) *Cell* **57,** 335–345
28. Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K., and Nagata, S., (1991) *EMBO J.* **10,** 1635–1641