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The Rat Growth Hormone-Releasing Hormone Receptor Gene: Structure, Regulation, and Generation of Receptor Isoforms with Different Signaling Properties*

TERESA L. MILLER†, PAUL A. GODFREY†‡, VENITA I. DEALMEIDA, AND KELLY E. MAYO

Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208

ABSTRACT

The interaction of GHRH with membrane-bound receptors on somatotroph cells of the anterior pituitary is an important step in the regulation of GH synthesis and secretion. The identification of a G protein-coupled receptor for GHRH has made it possible to investigate the pathway by which GHRH regulates pituitary somatotroph cell function. To initiate an analysis of the mechanisms regulating expression and function of the GHRH receptor, the structure of the gene and its promoter region were analyzed. The coding sequence of the rat GHRH receptor gene is contained within 14 exons spanning approximately 15 kb of genomic DNA. Four transcription start sites are located within 286 bp upstream of the initiation codon. The 5′ flanking region of the GHRH receptor gene acts as a functional promoter in rat pituitary tumor GH3 cells, and basal promoter activity is enhanced in GH3 and COS7 cells by cotransfection of an expression construct encoding the pituitary-specific transcription factor Pit-1. The rat GHRH receptor gene is subject to at least 1 alternative RNA processing event that generates 2 receptor isoforms differing by 41 amino acids within the third intracellular loop (IL) of the protein. The short isoform of the GHRH receptor is predominant in pituitary cells. The MtT/S pituitary tumor cell line was found to express the GHRH receptor, and different populations of these cells produce predominantly the long or short isoforms of the receptor messenger RNA, suggesting that the alternative splicing can be regulated. Functional analysis of the two GHRH receptor isoforms demonstrates that both bind GHRH, but only the short isoform signals through a cAMP-mediated pathway. Neither receptor isoform is able to stimulate calcium mobilization from internal stores after GHRH treatment. Our findings indicate that the pituitary-specific transcription factor Pit-1 is involved in the somatotroph-specific expression of the GHRH receptor gene and that functionally distinct receptor proteins are generated by an alternative RNA processing mechanism. (*Endocrinology* **140:** 4152–4165, 1999)

GH IS NECESSARY for the regulation of linear growth in mammals (1). Hypothalamic control of GH synthesis and secretion is modulated primarily by the opposing actions of the neuropeptides GHRH and somatostatin on the somatotroph cells of the anterior pituitary (2). The somatotroph-specific expression of GH in the developing pituitary is initiated by expression of the pituitary-specific transcription factor Pit-1 (3). Mutations in the Pit-1 gene (4) or in the gene encoding its transcriptional regulator, Prophet of Pit-1 (5), lead to the absence of GH-, PRL-, and TSH-secreting cells and produce a dwarf phenotype in both mouse and human (4, 6, 7). Mutations in the Gsh-1 homeobox gene disrupt expression of GHRH in the arcuate nucleus of the hypothalamus, leading to a decrease in GH- and PRL-secreting cells in the anterior pituitary and a dwarf phenotype (8). Similar pituitary dysfunction and dwarfism are observed in the *little* mouse, as a result of a point mutation in the GHRH receptor gene (9, 10), suggesting that integrity of the GHRH signaling pathway is necessary for expansion of the somatotroph cell lineage.

The interaction of GHRH with its receptor stimulates increased intracellular levels of cAMP (11). Somatotroph hypoplasia and dwarfism are observed when the GH promoter is used to target a dominant negative form of the cAMP response element binding protein (CREB) (12) to the somatotroph cells of the pituitary, whereas chronic stimulation of adenylyl cyclase in transgenic mice overexpressing GHRH (13) or a GH-cholera toxin transgene (14) leads to pituitary hyperplasia and gigantism. Somatotroph proliferation in response to GHRH has been observed in primary pituitary cultures (15) and in patients with GHRH-secreting tumors (16, 17) or pituitary tumors expressing a constitutively active form of the α-subunit of the stimulatory G protein (18), suggesting that overstimulation of the GHRH signaling pathway may lead to inappropriate cell proliferation.

GHRH is structurally related to a family of neurosecretory peptides that includes vasoactive intestinal peptide (VIP), pituitary adenylyl cyclase activating peptide (PACAP), glucagon, glucagon-like peptide 1 (GLP-1), gastric inhibitory polypeptide (GIP) and secretin (19). The receptors for these neuropeptides are also highly related and are grouped into family B (20, 21) of the G protein-coupled receptor (GPCR) superfamily. The GHRH receptor complementary DNA (cDNA) has previously been cloned (11, 22) and the encoded protein found to share several conserved features with other

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Address all correspondence and requests for reprints to: Dr. Kelly E. Mayo, Northwestern University, Department of Biochemistry, Hogan Science Building 3–100, 2153 North Campus Drive, Evanston, Illinois 60208. E-mail: k-mayo@nwu.edu.
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† Contributed equally to this work.
‡ Present address: Howard Hughes Medical Institute, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115.

GPCR family B members, such as the presence of one or more N-linked glycosylation sites, six highly conserved cysteine residues, and an absolutely conserved aspartic acid residue within the relatively large aminoterminal extracellular domain (21, 23). The integrity of the conserved aspartic acid residue has been shown to be critical for the interaction of GHRH, VIP, and glucagon with their respective receptors and is the site of receptor mutation in the *little* mouse (9, 24–26).

The gene structure of family B GPCRs is highly conserved and seems to be complex, relative to all other GPCRs (27–30). Partial characterization of the rat GHRH receptor gene suggested there was alternative splicing of an exon coding for an additional 41 amino acids within the third IL of the protein (11). The IL domains of family B GPCRs are involved in G protein coupling, and disruption of these loop regions by alternative splicing alters G protein activation (31, 32). For example, various isoforms of the PACAP receptor are generated by alternative splicing of two exons within the third IL domain, leading to differential coupling to the adenylyl cyclase and phospholipase C signaling pathways (31). This suggests the possibility of alternative effector coupling between the two isoforms of the related GHRH receptor. To understand more fully the regulation of the GHRH receptor gene and the functional differences between the two GHRH receptor isoforms, we isolated and characterized the rat GHRH receptor gene, analyzed the promoter region, and examined the biological activities of the two receptor isoforms. We demonstrate that the coding region of the GHRH receptor gene contains 14 exons, spanning 15 kb of DNA, and that the transcription of the gene is regulated by the POU-homeodomain protein, Pit-1. We also show that the two receptor isoforms are differentially expressed in the pituitary and in a pituitary tumor cell line, and they differ in their ability to stimulate cAMP production in response to GHRH.

Materials and Methods

Plasmids and reagents

The pGEM plasmids were obtained from Promega Corp. (Madison, WI), pcDNA-3 was from Invitrogen (Carlsbad, CA), and pA3-Luc was provided by Dr. William Wood, University of Colorado (33). All DNA restriction and modifying enzymes were purchased from Promega Corp., except for AmpliTaq, which was purchased from Perkin-Elmer-Cetus Corp. (Branchburg, NJ). Oligonucleotides were synthesized by the Northwestern University Biotechnology Facility, and peptide hormones were from Peptides International (Louisville, KY) or Peninsula Laboratories, Inc. (Belmont, CA). Radiolabeled nucleotides were from ICN Biomedicals, Inc. (Irvine, CA), and 3-[^{125}I]iodotyrosyl^{10}-hGHRH (1–44)-amide and ^{35}S cysteine-methionine were from Amersham Pharmacia Biotech (Arlington Heights, IL). All chemicals and tissue culture reagents were from Sigma Chemical Co. (St. Louis, MO) or Life Technologies, Inc. (Grand Island, NY) unless otherwise noted, and autoradiographic film was from Eastman Kodak Co. (Rochester, NY). The rat genomic DNA library (λCRG) was provided by Dr. Carolyn Bruzdzinski, University of Illinois at Chicago (34). The Pit-1 expression constructs were provided by Dr. Holly Ingraham, University of California at San Francisco (35). The monoclonal antibody 12CA5 directed against the hemagglutinin (HA) epitope was provided by Dr. Robert Lamb, Northwestern University; and the polyclonal antibody against the human GHRH receptor was provided by Dr. Bruce Gaylinn, University of Virginia, Charlottesville, VA. The muscarinic M3 receptor clone was provided by Dr. Marlene Hosey, Northwestern University Medical School.

Animals and cell lines

Tissues were isolated from adult male Sprague Dawley rats (Harlan Bioproducts for Science, Indianapolis, IN), housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care, under a 14-h light, 10-h dark cycle, with water and food provided *ad libitum*. The experimental protocols used in these studies were approved by the Northwestern University Institutional Animal Care and Use Committee. Primary rat pituitary cell cultures were isolated and maintained as previously described (36). COS7 cells were obtained from Dr. Daniel Linzer, Northwestern University; GH3 cells were obtained from Dr. Ursala Kaiser, Brigham Women's Hospital at Harvard Medical School; GHFT1 cells were obtained from Dr. Pamela Mellon, University of California at San Diego; and P0 cells were a gift from Dr. Piotr Chomczynski, Molecular Research Center, Inc. (Cincinnati, OH). The TG-Pit-5–7 cell line was generated in our laboratory from dispersed MT-GRF transgenic mouse pituitary tumor cells. MtT/S cells were purchased from the Riken Cell Bank (Tsukuba, Japan) and maintained as previously described (37). All other cell lines were maintained in DMEM (Sigma Chemical Co.) containing 5% (Hela T4 cells) or 10% (COS7, GH3, P0, GHFT1, and TG-Pit-5–7 cells) FCS (Life Technologies/BRL) plus 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc.).

Isolation and mapping of GHRH receptor genomic clones

Rat GHRH receptor genomic clones were isolated using a genomic library derived from rat hepatoma cells (λCRG) screened with radiolabeled probes derived from GHRH receptor cDNA clones RPR64, RPR11, and RPR20 (11), using standard hybridization methodology (38). DNA was prepared from isolated plaques and mapped using a variety of restriction endonucleases. Contiguous overlapping clones of a 25-kb region, contained within the genomic bacteriophage clones G1, G2 and G7, were subcloned into the vectors pGEM3Z, pGEM5Z, pGEM7Z; and selected clones were sequenced using the dideoxy nucleotide chain-termination method (Amersham Pharmacia Biotech). The sequences were analyzed with the Geneworks sequence analysis software package (Intelligenetics, Inc., Mountain View, CA) and compared with the cDNA to determine the location of the exon/intron boundaries and the sizes of the introns.

RNA analysis

Total RNA was isolated from tissue or cells using acidic phenol extraction of guanidine isothiocyanate lysates, as described (39). For RT-PCR assays, approximately 2.5 μg total RNA from primary pituitary cells or pituitary cell lines was reverse transcribed using AMV reverse transcriptase and amplified using PCR, for 26 cycles at an annealing temperature of 65 C, with AmpliTaq and primers for the GHRH receptor (RPRJA; 5′-CATCTCCTAGGTCCAAACCAGC-3′ and RPRJC; 5′-GAAGTTCAGGGTCATGGCCATA-3′), ribosomal protein L19 (RPL19A; 5′-CTGAAGGTCAAA GGGAATGTG-3′ and RPL19B; 5′-GGACAGAGTCTTGATGATCTC-3′) or GH (RMGHA; 5′-CCAT GCCCTTGTCCAGTCTG-3′ and RMGHB; 5′-TGCCCTCTTCCAGGTCCTTC-3′), as previously described (36). For Northern RNA analysis, approximately 20 μg total RNA from rat pituitary or liver tissue was separated by electrophoresis on denaturing 1.5% agarose/formaldehyde gels (38). RNAs were transferred to nylon membranes, covalently cross-linked by UV irradiation, and detected by hybridization to probes *Hin*cII/*Sac*I, *Sac*I/*Nco*I, and RPR20 derived from the 5′ regions immediately upstream of the initiation codon of the GHRH receptor gene. Hybridization to a cDNA probe for ribosomal protein S2 (40) was used to normalize RNA loading. Autoradiographic exposures were for 24 h (GHRH receptor) or 2 h (RPS2). For primer extension studies, oligonucleotide primer 5′-CACAGGTTCAGCAAGCAGAGGACCC-3′ (KM2), which is located 50 bp downstream of the initiation codon in the first coding exon of the GHRH receptor gene and 143 bp downstream of the longest known cDNA (22), was synthesized and end-labeled with T4 polynucleotide kinase and (γ-^{32}P)deoxy-ATP, as described (38). Approximately 20 μg total RNA from rat pituitary or liver tissue were hybridized for 20 h with 50,000 cpm/ml of the appropriate end-labeled oligonucleotide in 150 mM KCl, 10 mM Tris-HCl, 1 mM EDTA. Hybridization reactions were precipitated, and extension products were generated using 5 U of AMV reverse-transcriptase in 330 mM Tris-HCl (pH

8.3), 150 mM $MgCl_2$, 8 mM dithiothreitol (DTT), 20 μg/μl actinomycin D, 20 μM nucleotide triphosphate mix (38). Samples were size separated on 5% polyacrylamide/8 M urea denaturing gels (38), dried and exposed to Kodak X-OMAT AR film for 18 h. To confirm the colinearity of the primer extension products with genomic DNA, 1 μg total RNA was analyzed by RT-PCR for 20 cycles with AmpliTaq, an annealing temperature of 65 C, and the KM2 primer in combination with an oligonucleotide corresponding to the 5′ end of the longest primer extension product, 5′-TTCTTGCTGTCAGG TTAGGC-3′ (PG2), using conditions previously described (36). The RT-PCR products were separated on 4% (3% NuSieve/1% SeaKemGTG) (FMC Bioproducts, Rockland, ME) agarose gels, transferred to nylon membranes, and hybridized to a radiolabeled *Sac*I/*Nco*I fragment located immediately upstream of the initiation codon. RT-PCR products were cloned into pGEM7Z and sequenced to verify their identity.

GHRH receptor promoter constructs and cell transfections

An *Nco*I fragment of the GHRH receptor promoter (−1672 to +286, relative to the farthest upstream transcriptional start site) was cloned into the *Sma*I site of pGEM7Z after cutting with *Bst*XI and filling in the ends with T4 DNA polymerase to remove the ATG initiation codon. *Nco*I, *Eco*RI, *Hin*dIII, *Bgl*II, and *Sac*I deletion fragments of the GHRH receptor promoter having identical 3′ ends were inserted into the *Kpn*I site of pA3-Luc (33). The restriction enzyme sites used to generate the deletion constructs Nco −1672, Eco −1187, Hind −885, Bgl −714, and Sac +62 are numbered relative to the most 5′ transcriptional start site identified by primer extension. Transfections were performed in GH3 and COS7 cells using liposomes (41). Briefly, GH3 cells (2×10^5 cells/well) or COS7 cells (1×10^5 cells/well) were grown overnight in 12-well plates, then transfected for 4–6 h with 2.5 μg of each plasmid DNA in serum-free Opti-MEM containing lipid reagent, and allowed to recover for 16 h in complete media. The transfected cells were washed with 1× PBS, and proteins were extracted in cold lysis buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 1 mM DTT, 0.1% Triton-X 100]. Lysate was mixed with assay buffer [25 mM HEPES (pH 7.8), 15 mM $MgSO_4$, 1 mM DTT, 1 μg/ml BSA, 5 mM ATP, 0.2 mM luciferin] and analyzed for luciferase activity using a luminometer (Analytical Luminescense Laboratory, San Diego, CA).

Expression of epitope-tagged GHRH receptor clones

The partial rat GHRH receptor cDNA clones originally described (11) were recombined into full-length cDNA clones in pGEM5Z, generating the clones RPRC18 and RPRC2, which are identical in sequence except for the insertion of the 123 bp derived from exon 11 in the region encoding the third IL in clone RPRC2. Using PCR, sequences encoding an influenza virus HA epitope were fused to the C-terminal end of the receptor (42). A recognition site for the enzyme *Xba*I was engineered on the 3′ side of the HA epitope and contained the stop codon. The products were digested with *Eco*RI and *Xba*I and cloned, in context with the receptor, into the expression vector pcDNA-3 to generate full-length clones for the epitope-tagged receptors (RPRC18HA and RPRC2HA).

All binding and signaling experiments were performed using Hela T4 cells transfected with the epitope-tagged GHRH receptors, RPRC18HA and RPRC2HA, using the Vaccinia Virus-T7 polymerase hybrid expression system, as described (43, 44). The cellular localization of the receptors was determined by indirect immunofluorescence using a human GHRH receptor polyclonal antibody. Hela T4 cells, grown on coverslips and transfected with the various receptors, were fixed with 2% paraformaldehyde and incubated for 4 h at 4 C with a 1:1000 dilution of the polyclonal antiserum in PBS/0.1% BSA, in the presence or absence of 0.1% saponin. After washing, the coverslips were incubated at 4 C for 60 min with 2 μg/ml fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS containing 0.2% whole goat serum, in the presence or absence of saponin. The coverslips were then washed with PBS and mounted using fluorescein isothiocyanate-Guard (Testog Inc., Chicago, IL). The images of cells are optical sections obtained using a confocal laser scanning microscopy with a Carl Zeiss LSM 410 confocal microscope and a 40× objective. All samples were scanned through the central plane of the cell using the same settings of contrast and magnification, and the signal was averaged over 4 frames to reduce background. Expression levels of the two receptor isoforms were assessed by immunoprecipitation of the epitope-tagged receptor proteins from metabolically labeled cells with 12CA5 monoclonal antibody against the HA epitope, as described (44). For glycosylation analysis, immunoprecipitated proteins were digested for 4 h at 37 C with 0.2 U peptide-N-glycosidase F (Roche Molecular Biochemicals, Indianapolis, IN) in a buffer containing 20 mM Na_2HPO_4 (pH 8.0), 20 mM EDTA, 1% NP40, 1 μg/ml leupeptin, 0.1 μg/ml pepstatin A, and 1 μg/ml aprotinin.

Functional analysis of the rat GHRH receptor proteins

Binding-competition assays were performed using approximately 50 μg of membrane protein per reaction, prepared from transfected cells, as described (44). The membrane proteins were incubated with (3-[I^{125}]iodotyrosyl^{10})hGHRH (1–44)-amide present at a concentration of 70 pM, in the absence or presence of increasing concentrations of the unlabeled hormone. The nonspecific binding, determined as the percent of input counts bound in the presence of 1 μM unlabeled hormone, was subtracted from all raw data to give the specific bound counts. The binding-competition data were fit to a one-site competition equation, and ED_{50} values were determined using the program Prism (GraphPad Software, Inc., San Diego, CA). For the measurement of cAMP responses, cells were transfected and treated with hormones, and the deproteinized lysates were used to assay cAMP as described (44). The assays were performed with triplicate samples, and the mean ± SEM of the intracellular cAMP was plotted as a function of the concentration of the ligand. The dose-response curves were fit to a sigmoidal dose-response equation and ED_{50} values determined using the program Prism (GraphPad Software, Inc.).

Intracellular calcium mobilization in response to GHRH treatment was measured using fluorimetry. Transfected cells were lightly trypsinized and washed with DMEM containing 5% serum. They were then washed with loading buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 1 mM $CaCl_2$, 1% BSA containing 147 μg/ml probenecid) and resuspended in 5 ml loading buffer containing 11.5 μg of the calcium indicator Fluo-3AM (Molecular Probes, Inc., Eugene, OR). After incubation for 30 min, with gentle shaking at room temperature, to load the cells with the dye, the cells were washed twice with loading buffer to remove excess dye; and the concentration of cells was adjusted to $0.5–1 \times 10^6$ cells/ml. Fluorometric emission, at 530 nM, was monitored in response to 0.25 μM GHRH and 40 μM digitonin, using an excitation wavelength of 505 nM. Before the addition of GHRH, the basal emission was measured and subtracted from all emission values before plotting.

Results

The rat GHRH receptor gene contains 14 exons

Five bacteriophage clones (G1, G2, G3, G4, and G7), isolated from a rat genomic DNA library and spanning a total of 25 kb, were found to contain sequence information corresponding to the coding sequence of the rat GHRH receptor cDNAs identified previously (11). Clones G1–4 were obtained by screening with cDNAs RPR20 and RPR11, and clone G7 was obtained by screening with cDNA RPR64 (11). Based on partial restriction endonuclease mapping, G2 extends at least 8.2 kb upstream of the translation initiation site, and G7 extends at least 1.0 kb downstream of the rat GHRH receptor coding sequence. The bacteriophage clones G1, G2, and G7, as well as DNA subclones corresponding to *Eco*RI restriction enzyme fragments derived from them, are summarized in Fig. 1.

The coding region of the rat GHRH receptor gene is contained within 14 exons that span at least 15 kb of genomic DNA. The exons vary in length from 42–456 bp and are separated by introns that vary in size from 111 bp to >2 kb. A diagram of the GHRH receptor gene structure aligned with the major restriction enzyme recognition sites and the *Eco*RI

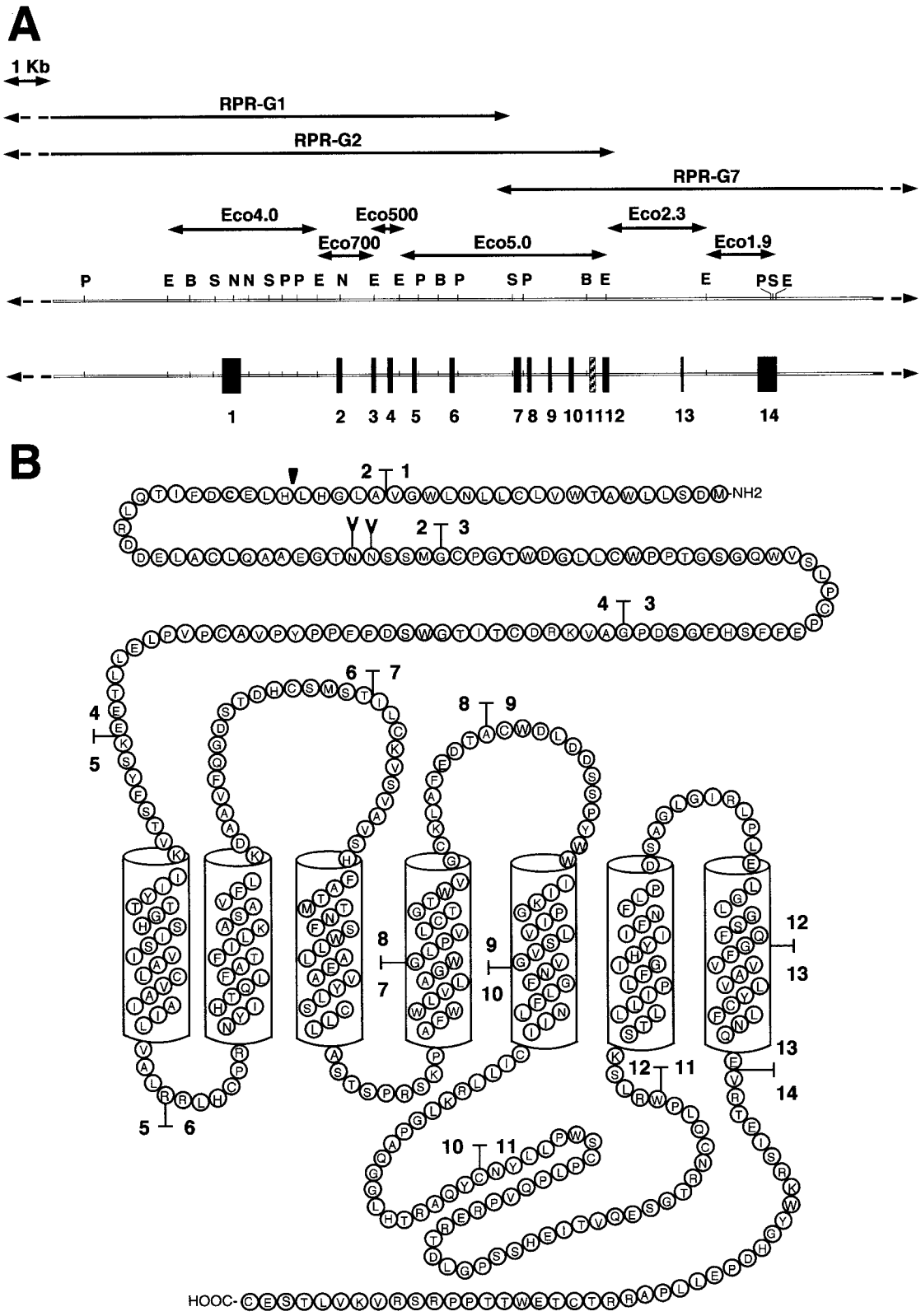

FIG. 1. Schematic representation of the rat GHRH receptor genomic locus. A, Restriction enzyme map of the GHRH receptor locus, with *arrows* representing the bacteriophage λ clones RPR-G1, RPR-G2, RPR-G7, and the major *Eco*RI fragments obtained from direct digestion of the λ clones (P, *Pst*I; E, *Eco*RI; B, *Bgl*II; S, *Sac*I; N, *Nco*I). The structure of the GHRH receptor gene containing 14 exons (1–14), including the alternatively spliced exon 11 (*hatched box*), is aligned below the restriction enzyme map. B, Schematic of the rat GHRH receptor protein, showing the location of the exon-intron boundaries (⊤). The *arrow* marks the approximate site of signal sequence cleavage, and the branched structures represent two consensus sites for N-linked glycosylation.

genomic subclones is shown in Fig. 1. The length of the exons and introns of the rat GHRH receptor gene, along with the splice donor and splice acceptor junction sequences, are summarized in Table 1. The coding sequence for the aminoterminal extracellular domain of the rat GHRH receptor is contained within exons 1–5, whereas exons 5–14 encode the transmembrane (TM), IL, extracellular loop (EL), and carboxylterminal domains (Fig. 1B). There are two consensus polyadenylation signals located 362 bp and 490 bp 3′ of the termination codon, both of which are used based on the analysis of cDNA clones (11, 22). Introns interrupt the coding sequences of EL1, IL1, EL2, IL3, TM4, TM5, and TM7. Exon 11, previously identified during the characterization of rat GHRH receptor cDNA clones (11), codes for amino acids that are alternatively spliced into IL3. This exon is followed by a nonconsensus splice donor sequence (Table 1). All of the exons, most of the introns, and 2 kb of 5′ flanking DNA were sequenced on both strands.

Identification of multiple transcriptional start sites

To determine where GHRH receptor transcripts were initiated, RNA blots were hybridized to clone RPR20, which contains the most 5′ portion of the GHRH receptor cDNA, or to two genomic subclones, *Nco*I/*Sac*I and *Hin*cII/*Sac*I immediately 5′ to the initiation codon (Fig. 2). Both the RPR20 cDNA clone (III) and the *Nco*I/*Sac*I genomic clone (II), which extends 207 bp 5′ of RPR20, detected GHRH receptor transcripts in pituitary RNA; but the *Hin*cII/*Sac*I genomic clone (I), which extends 425 bp upstream of the *Nco*I/*Sac*I region, did not detect GHRH receptor transcripts (Fig. 2). Primer extension analysis was used to define more precisely the sites of GHRH receptor transcript initiation. Four primer-extended products of 336, 253, 143, and 134 nucleotides were generated using the primer KM2 (Fig. 3A). To test whether these primer extension products were colinear with the GHRH receptor genomic DNA, RT-PCR analysis with the primer KM2 in combination with PG2, an upstream primer corresponding to the 5′ end of the longest primer extension product (see Fig. 4), was performed. The expected 336-bp RT-PCR product was detected using the KM2 and PG2 primers when pituitary RNA, but not liver RNA, was used as the substrate (Fig. 3B). Sequencing of this product showed it to be identical to and colinear with the genomic DNA sequence, containing 286 bp upstream of the initiation codon plus the sequence encoding the first 17 amino acids. The primer extension analysis, therefore, identified four transcriptional start sites located 286, 203, 93, and 84 bp upstream of the translation initiation codon.

Expression of GHRH receptor promoter constructs

A number of putative transcription factor binding sites are present within the upstream sequence of the rat GHRH receptor gene (Fig. 4), including potential binding sites for GATA factors, CREB, C/EBPα, Ets-1, Pit-1, and the nuclear receptors for estrogens and glucocorticoids (ER, GR) (45). The 5′ flanking region of the GHRH receptor gene was analyzed for basal promoter activity by fusing varying amounts of 5′ flanking DNA to the luciferase reporter gene (Fig. 5A). DNA constructs were transfected into rat pituitary GH3 cells or monkey kidney COS7 cells, and cellular proteins were isolated for luciferase assays. Transfection of 1.7 kb of GHRH receptor promoter DNA (Nco −1672) resulted in low, but detectable, luciferase activity in GH3 cells, which was 4.5-fold greater than the activity observed after transfection of the parent vector pA3-Luc (Fig. 5B). Transfection of Nco −1672 into the nonpituitary cell line COS7 resulted in equivalent activity to the parent vector pA3-Luc (Fig. 5C), suggesting no basal promoter activity in this nonpituitary cell line.

Because Pit-1 has been reported to regulate the GHRH receptor gene (22) and several potential Pit-1 binding sites were identified in the promoter region of the rat GHRH receptor gene, we tested the ability of Pit-1 to stimulate promoter activity in transfected cells. Cotransfection of a Pit-1 expression construct (Pit-1+) increased the activity of the Nco −1672 construct 1.4-fold in GH3 cells (which express endogenous Pit-1) and 7.2-fold in COS7 cells (which do not express endogenous Pit-1). No induction of GHRH receptor promoter activity was observed when a Pit-1 expression construct deleted for the sequences encoding the POU-homeodomain DNA binding region (Pit-1ΔHD) was cotransfected with the Nco −1672 construct.

Cotransfection of the Pit-1+ and Pit-1ΔHD expression constructs with a series of GHRH receptor promoter deletion

TABLE 1. Summary of the rat GHRH receptor splice junctions

Exon	Length (bp)	Splice donor	Intron length (bp)	Splice acceptor
1	343	GGAGTTgtaagtagcc	2183	aaacccatagGCACTG
2	103	CCATGGgtaggaaggg	111	ctccacttagGATGCC
3	108	ACCCAGgtgagtggcc	577	ttgcctctagGGGCTG
4	98	GAGGAGgtaactagct	999	ttgtccccagAAGTCT
5	98	TCTCAGgttcgtcatt	793	tgggttccagGAGGCT
6	132	TCCACTgtaagggcta	>2000	ctgctcttagATTCTG
7	154	GCTGGGgtacctggga	235	tcactcacagGACTCC
8	61	CACTGCgtgagtagag	421	ttcttcccagGTGCTG
9	70	GTTGGGgtcagtttct	591	tcgtttccagGTGAAC
10	92	GTACTGgtaccattca	540	tcttgtccagCAACTA
11	123	ACCATGgcgagtggct	270	cttccccaagGCGGCT
12	129	TTCCAGgtgagagtcc	1223	tgatccacagGGTTTT
13	42	CAAGAGgtaagtgtgc	>1600	agttctagagGTGAGG
14	456			

The DNA sequences at the exon and intron boundaries for exons 1–14 are shown with the splice donor and acceptor sites *highlighted*. Note the nonconsensus splice donor sequence (gc, rather than gt) following exon 11.

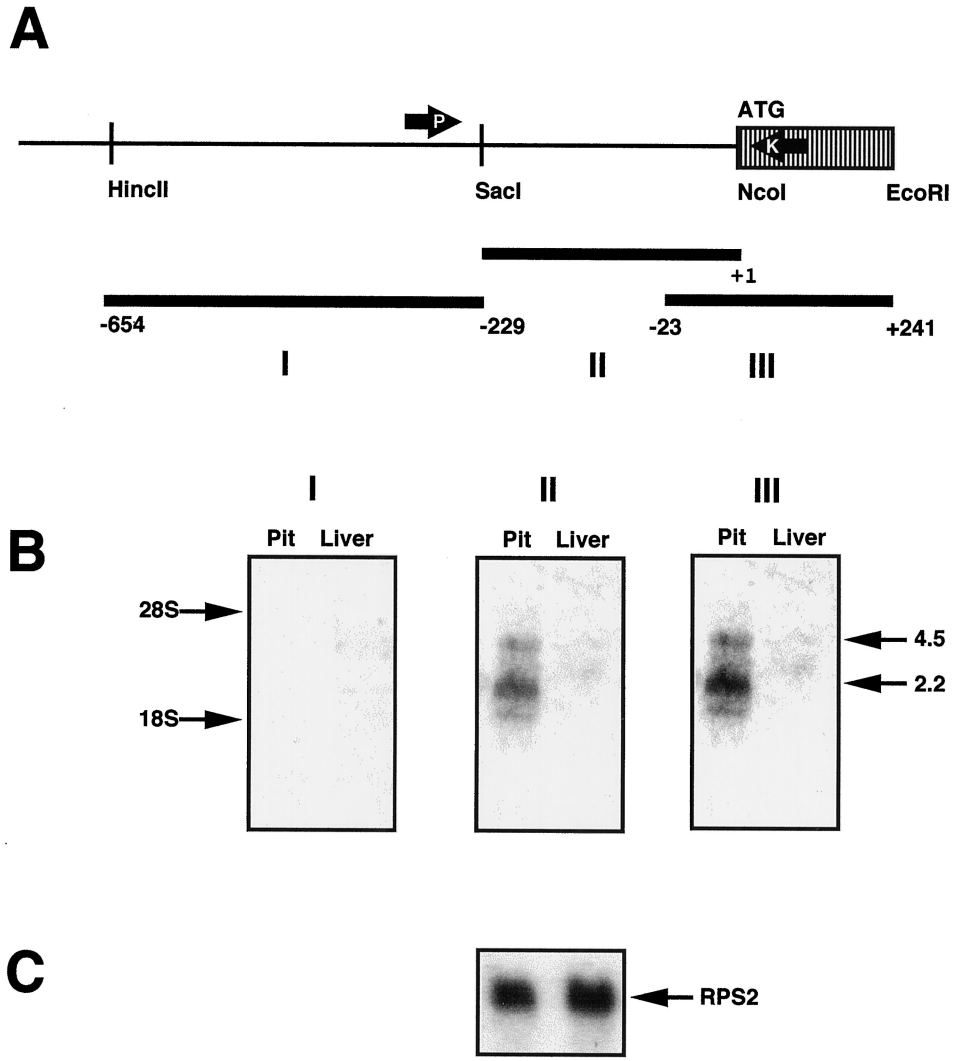

FIG. 2. RNA blot analysis of the 5′ portion of the rat GHRH receptor gene. Twenty micrograms of total RNA from rat pituitary or liver were separated on a 1.5% agarose/formaldehyde gel and hybridized to three probes specific for the 5′ regions immediately upstream of the initiation codon. A, Schematic showing the location of the three probes relative to the translation start site and the position of the KM2 (K) and PG2 (P) primers used in the subsequent transcription start site analysis; B, hybridization of the 4.5- and 2.2-kb GHRH receptor mRNAs to the *Hin*cII/*Sac*I (I), *Sac*I/*Nco*I (II), and RPR20 (III) probes, with the positions of the 28S and 18S ribosomal RNAs marked; C, hybridization to the RPS2 control. This analysis represents a single RNA blot that was stripped and reprobed for each round of hybridization.

constructs (Eco −1187, Hind −885, Bgl −714, and Sac +62) into GH3 and COS7 cells was used to more precisely define the regions of the GHRH receptor gene involved in mediating basal and Pit-1-inducible promoter activity. The GHRH receptor promoter region deleted to 1.2 kb (Eco −1187) retained substantial basal activity in GH3 cells (70% of Nco −1672) and gave a similar 1.6-fold induction upon cotransfection with the Pit-1+ expression construct (Fig. 5B). The GHRH receptor promoter region deleted to −885 bp (Hind −885) gave reduced basal activity in GH3 cells (50% of Nco −1672) but maintained induction in the presence of the Pit-1+ expression construct (2.0-fold). Substantially reduced basal promoter activity (35% of Nco −1672) was detected with the GHRH receptor promoter deleted to −714 bp (Bgl −714), however Pit-1 induction of promoter activity (1.4-fold) was still maintained. Significantly, both basal activity and Pit-1 modulation of the promoter were abolished with the GHRH receptor promoter deleted to a region 62 bp downstream of the most 5′ transcriptional start site (Sac +62) (Fig. 5B). No basal activity of any GHRH receptor promoter deletion construct over that of the pA3-Luc vector was observed in COS7 cells; however, cotransfection of the Pit-1+ expression construct induced activity of the Eco −1187 (5.1-fold), Hind −885 (5.2-fold) and Bgl −714 (5.0-fold) GHRH receptor promoter constructs, whereas the Sac +62 promoter construct was not Pit-1 responsive (Fig. 5C).

Functional characterization of GHRH receptor isoforms

The short GHRH receptor isoform is the predominant messenger RNA (mRNA) detected in primary pituitary cells by RT-PCR with primers that span exons 7–14 (Fig. 6). This is also the predominant transcript detected in pituitary tissue (not shown). In screening pituitary-derived cell lines for expression of the GHRH receptor, we identified a cell line, MtT/S (37), that expresses both GH and GHRH receptor mRNA (Fig. 6). These cells express the receptor mRNA at levels equivalent to (Fig. 6A) or greater than (Fig. 6B) primary pituitary cells, which are a mixture of somatotroph and other cell types. The MtT/S cell line is a mixed population of attached cells and floating cell clusters. Populations enriched for attached cells (MtT/S-M) express relatively more of the long mRNA isoform, whereas populations enriched for the floating cells (MtT/S-F) express relatively more of the short

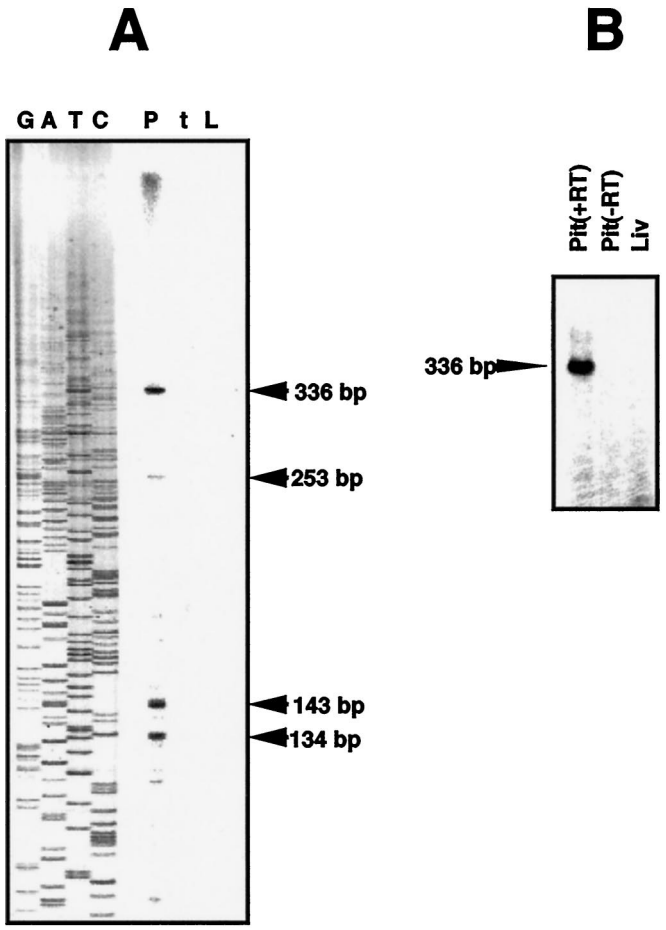

FIG. 3. Identification of multiple transcriptional start sites 5′ of the rat GHRH receptor coding sequence. A, Primer extension reactions, using the KM2 primer, were carried out using RNA isolated from rat liver (L), or rat pituitary (P), with transfer RNA (t) as the negative control. A sequencing ladder (G, A, T, and C), of a different fragment than the primer extended products, was used to determine sizes of the extended products. Primer extension reactions using the KM2 primer generated a 336-nucleotide extension product from pituitary RNA, which corresponds to 286 bp upstream of the initiation codon. Shorter extension products (of 253, 143, and 134 nucleotides) were also detected. B, RT-PCR reactions of rat pituitary or liver RNA, using the KM2 primer in combination with the PG2 primer, were separated by agarose gel electrophoresis and probed with the internal *Sac*I/*Nco*I fragment used in Northern blot analysis. A 336-bp RT-PCR product was detected with pituitary RNA as the template, and the identity was verified by subcloning and sequencing.

mRNA isoform (Fig. 6B). Cloning and sequencing of the MtT/S long-isoform PCR product verified that it was identical to the alternatively spliced long isoform of the receptor previously identified in pituitary by cDNA cloning (11).

To test the functional properties of these two receptor isoforms, full-length rat GHRH receptor expression clones were constructed by recombining the *Eco*RI fragments of the GHRH receptor cDNA clones RPR20 (5′ end) and RPR64 (3′ end) with RPR11 or RPR13, encoding the short and long receptor isoforms, respectively (11). The two GHRH receptor isoforms were expressed in HeLa T4 cells by transient transfection using a Vaccinia Virus-T7 polymerase hybrid expression system (43, 44). To ensure that equivalent levels of receptor protein were expressed in this system, the two rat GHRH receptor isoforms were tagged at their C-terminal ends with the amino acids encoding an influenza virus HA epitope (YPYDVPDYA) (42), allowing detection of receptor protein with a monoclonal antibody against the epitope. We have previously shown that introduction of this C-terminal epitope-tag does not affect the activity of the human GHRH receptor (44).

The expression levels of both rat GHRH receptor isoforms were determined by immunoprecipitation of metabolically labeled proteins with antibodies to the HA-epitope tag (Fig. 7A), and both isoforms were found to be expressed at similar levels. Treatment of the immunoprecipitated proteins with peptide-N-glycosidase F decreased the size of both isoforms, suggesting the presence of N-linked glycosylation. Immunofluorescence detection of the receptor protein in intact and permeabilized cells, using a polyclonal antibody against the N terminus of the GHRH receptor, demonstrated that both receptor isoforms were localized to the cell surface (Fig. 7B). The ligand-binding properties of the rat GHRH receptor isoforms were analyzed by measuring binding of (3-[^{125}I]iodotyrosyl^{10})GHRH (1–44)-amide to cellular membranes derived from cells transiently transfected with either the long or the short GHRH receptor isoform (Fig. 7C). Both receptor isoforms showed comparable levels of binding that could be competed in the presence of increasing amounts of cold GHRH in a dose-dependent manner, with ED_{50} values of approximately 1.05 and 1.5 nM for the short and long isoforms of the GHRH receptor, respectively.

The cloned human GHRH receptor stimulates cAMP production after GHRH binding (11). Dose-dependent accumulation of cAMP was therefore monitored in cells transfected with the two isoforms of the rat GHRH receptor in response to treatment with rat GHRH (Fig. 8A). Cells expressing the short isoform gave a robust accumulation of cAMP, with an ED_{50} of 6.3 nM. This ligand-mediated cAMP accumulation was not observed in cells expressing the long isoform of the rat GHRH receptor. Many of the receptors in family B couple to G proteins that activate phopholipase C, as well as adenylyl cyclase (31, 32). Studies in primary pituitary cells have detected Ca^{2+} mobilization in response to GHRH treatment (46), suggesting that the GHRH receptor may activate phospholipase C or Ca^{2+} channels, in addition to coupling to adenylyl cyclase. Because the long isoform of the GHRH receptor did not stimulate cAMP production in response to GHRH, the ability of the receptor to signal through the phospholipase C pathway in response to ligand was assessed by measuring Ca^{2+} mobilization with the calcium dye Fluo-3AM. Neither rat GHRH receptor isoform stimulated Ca^{2+} mobilization in transfected cells in response to GHRH treatment (Fig. 8B), although a control muscarinic M3 receptor gave a Ca^{2+} response upon carbachol treatment (47). The functional integrity of the GHRH receptor in the cells used for the Ca^{2+} assay was monitored by assessing cAMP accumulation in response to GHRH. Both the human GHRH receptor and the short isoform of the rat GHRH receptor were capable of activating adenylyl cyclase in response to ligand.

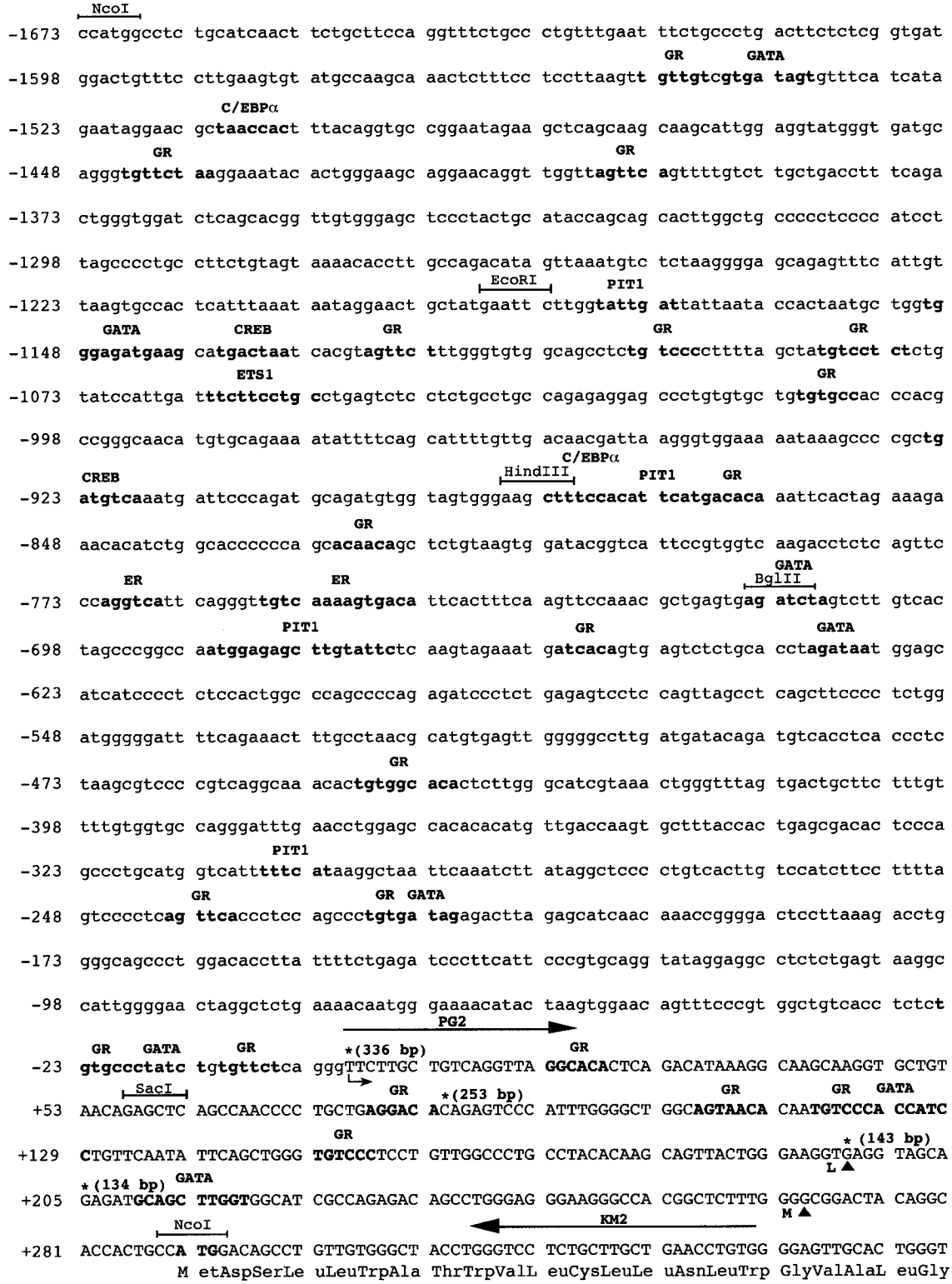

FIG. 4. Sequence of the 5′ flanking region of the rat GHRH receptor gene. The nucleotide sequence of the 5′ flanking region, in context with the protein coding region, is shown. *Lowercase* letters represent the 5′ flanking sequences, *capital* letters designate the transcribed regions, and numbers to the *left* designate upstream and downstream sequences, relative to the major transcriptional start site (+1). A three-letter amino acid code is used to show the coding sequence, and the initiation codon is *highlighted*. The *arrows* designate the orientation and location of primers used in these studies. The ends of the primer extension products and their corresponding sizes are marked (*). *Arrowheads* mark the location of the known cDNAs previously reported by Mayo (11) (M) and Lin *et al.* (22) (L). Potential regulatory elements present in the 5′ flanking sequences of the GHRH receptor gene are *highlighted*, and the restriction sites used to generate promoter constructs are labeled. This sequence has been deposited to the GenBank (accession no. AF121969).

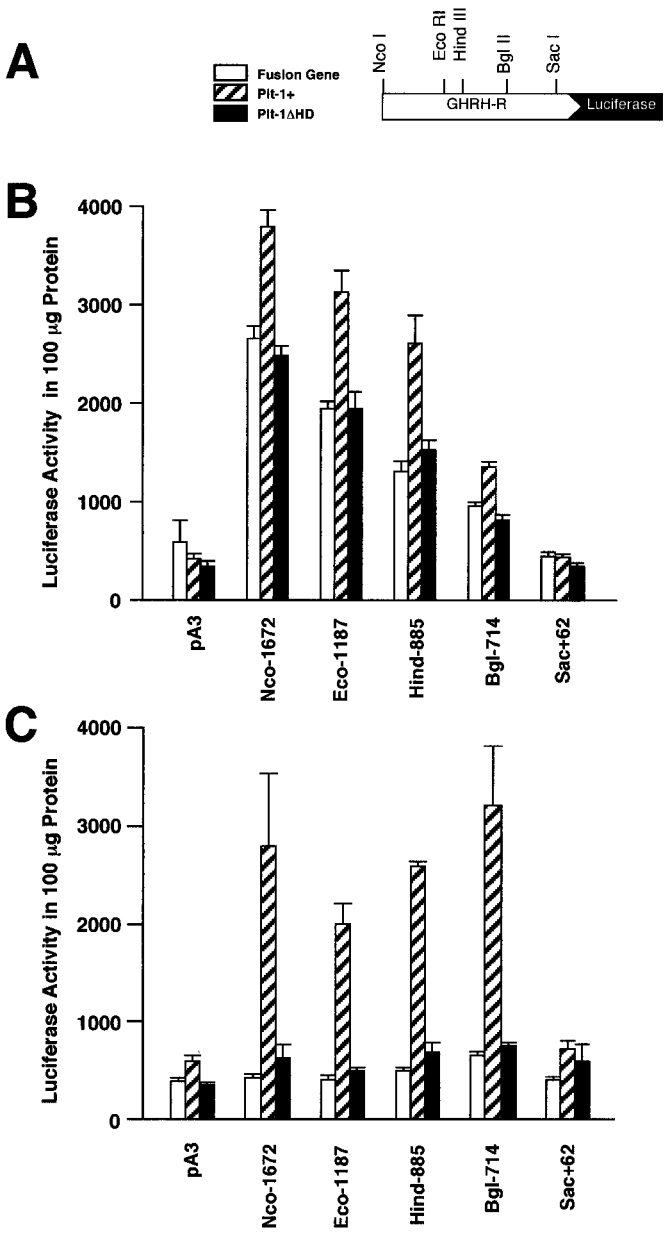

FIG. 5. Basal and Pit-1-regulated activity of the rat GHRH receptor promoter in GH3 and COS7 cells. A, Map of the GHRH receptor promoter-luciferase fusion gene, shown with the location of the restriction enzyme sites used to generate the deletion constructs. Nco −1672, Eco −1187, Hind −885, Bgl −714, and Sac +62 deletions of the rat GHRH receptor 5′ region linked to the pA3-luciferase vector were transfected alone or together with one of two Pit-1 expression constructs (Pit-1+, Pit-1ΔHD) into GH3 (B) and COS7 (C) cells. The *bars* represent the mean of triplicate cell lysates measured for luciferase activity, as relative light units (RLU), in 100 μg protein, with the SEM indicated by the *error bars*. RSV-luciferase transfected into GH3 or COS7 cells yielded 3.9×10^5 and 3.4×10^5 RLU in 100 μg protein, respectively. The data are representative of at least three independent experiments.

Discussion

GHRH is a peptide hormone synthesized in the hypothalamus that acts through a GPCR on pituitary somatotroph cells to stimulate GH synthesis and secretion (1, 23). The importance of the GHRH receptor signaling pathway is indicated by the observation that a single amino acid substitution in the *little* mouse receptor that disrupts ligand binding (24) results in a dwarf phenotype (9, 10). Similar inactivating mutations resulting in GH deficiency have also been identified in the human GHRH receptor (48–50). Conversely, enhanced growth phenotypes are evident when GHRH is overexpressed, either through the introduction of a GHRH transgene (13) or through excess production from GHRH-secreting tumors (16, 17, 51). To understand more fully the regulation of the GHRH receptor and the mechanisms by which GHRH stimulates GH production within the pituitary, we have characterized the rat GHRH receptor gene, analyzed the promoter region, and evaluated the function of two GHRH receptor isoforms expressed from this gene.

The rat GHRH receptor gene is complex, encompassing 14 exons. Although this organization is atypical for GPCRs in general, it is not unusual for the GPCRs of family B. The genes for the calcitonin, VIP, PTH, GIP, glucagon, PACAP, and CRF receptors have all been characterized and display a similar organization (27–30). Alignment of the genes for these receptors with the rat GHRH receptor gene suggests that many of the intron/exon junctions and the length of the exons have been conserved, whereas the length of the introns is highly variable. Numerous introns interrupt the coding sequence of these receptor genes, and there seems to be no organization, with regard to particular exons encoding specific functional domains of the protein. The rat GHRH receptor gene contains introns that interrupt the aminoterminal extracellular domain, IL1, IL3, EL1, EL2, TM4, TM5, and TM7. The location of introns within the coding region of the rat GHRH receptor gene is identical to the mouse GHRH receptor gene (10). Two receptor isoforms, differing by 41 amino acids, are generated from the rat GHRH receptor gene by differential splicing of exon 11 within IL3 (11). The large GHRH receptor isoform is much less abundant than the short receptor isoform in normal pituitary, suggesting that the nonconsensus splice donor sequence (gc, rather than gt) following exon 11 may result in less efficient splicing of this exon. The observation that the immortalized somatotrophic cell line MtT/S expresses predominantly the long or short GHRH receptor isoform in different cell population isolates may indicate that splicing of exon 11 is regulated in these cells. Alternatively, because the MtT/S cell line was derived from an estrogen-induced mammotrophic tumor (37), it is possible that this cell line has a mutation or allelic variation in the splicing regulatory regions. A partial structure of the human GHRH receptor gene has recently been reported (52), and it displays a similar organization to that of the rat gene. However, the analysis only extended as far downstream as the exon coding for TM5, precluding any analysis of potential alternatively spliced exons within IL3.

We sequenced 1.9 kb of 5′ flanking DNA of the rat GHRH receptor gene and have found it to contain multiple potential transcription factor binding sites. Three major transcription initiation sites were identified 286, 93, and 84 bp upstream of the initiator methionine, whereas one minor start site was observed at 203 bp 5′ of the ATG. Only one transcription initiation site was identified for the human GHRH receptor

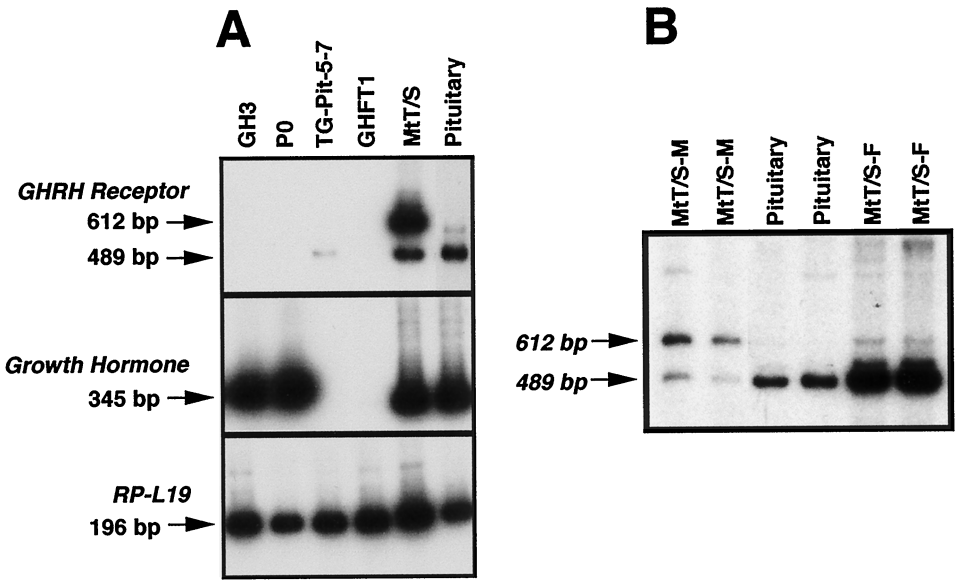

FIG. 6. Expression of rat GHRH receptor isoform mRNAs in pituitary and pituitary cell lines. A, RNA samples from primary cultured pituitary cells and various somatotrophic cell lines were reverse transcribed and amplified by PCR for 26 cycles with primers specific for GHRH receptor (*top panel*), GH (*middle panel*) or ribosomal protein L19 (*bottom panel*); B, RNA samples from primary pituitary cells, MtT/S cells enriched for attached cells (MtT/S-M), and MtT/S cells enriched for floating cells (MtT/S-F) were analyzed, by RT-PCR, for expression of GHRH receptor isoform mRNAs. The *arrows* mark the expected sizes of the 489-bp and 612-bp PCR products generated from the short and long isoforms of the GHRH receptor mRNA, respectively. The GH PCR product is 345 bp, and the RPL19 PCR product is 196 bp.

gene, and it is located 40 bp upstream of the translation start site (52). There are no consensus TATA or CAAT box motifs near any of the transcription initiation sites. The absence of TATA and CAAT box motifs is a feature shared with other related family B GPCR genes (27–30). A 714-bp GHRH receptor gene promoter directed strong basal expression in the rat pituitary tumor cell line GH3.

The GHRH receptor gene is expressed predominately in the anterior pituitary, as assessed by RNA blot hybridization and *in situ* hybridization (11, 22). The mechanisms that regulate this tissue-specific expression pattern are largely unknown, although the pituitary-specific transcription factor Pit-1 has been implicated (10, 22). Pit-1 is a major modulator of GH gene expression (3, 53) and is necessary for appropriate development of the somatotroph cells of the pituitary (4). In particular, mutations in the Pit-1 gene of both the Snell and Jackson dwarf mice lead to pituitary hypoplasia, which is distinguished by the absence of somatotroph, lactotroph, and thyrotroph cells. Expression of the GH and GHRH receptor genes is not detected in the pituitaries of these dwarf mice (22), suggesting that the expression of Pit-1 is necessary for the expression of both the GH and GHRH receptor genes. The 714-bp GHRH receptor promoter contains two potential Pit-1 binding sites, and it is able to direct Pit-1-dependent expression within pituitary (GH3) and nonpituitary (COS7) cell lines. The higher inducibility, but lower basal activity, observed in COS7 cells is likely caused by the absence of any endogenous Pit-1 in these cells; whereas the higher basal activity, but lower Pit-1 induction, seen in GH3 cells is probably attributable to the presence of endogenous Pit-1 expression in this pituitary cell line. Inclusion of additional sequences upstream of this region, which contain two more potential Pit-1 binding sites, increases basal and Pit-1 inducible expression of the rat GHRH receptor promoter constructs. It was previously reported, using transient transfection assays in COS7 cells, that coexpression of Pit-1 activates a 1.4-kb promoter fragment of the mouse GHRH receptor gene (10). Studies of the human GHRH receptor promoter region (52) have identified four potential binding sites for Pit-1. These authors determined that the minimal region required for basal expression of the human GHRH receptor promoter in GH4 cells was 202 bp upstream of the transcriptional start site. This region of the human GHRH receptor promoter was also sufficient to direct Pit-1-stimulated expression in COS7 cells.

The Pit-1 transcription factor is necessary, but not sufficient, for the tissue-specific expression of the GH, PRL, and TSH genes, suggesting that the synergistic interaction of Pit-1 with other transcriptional regulators may be required. A variety of *trans*-acting factors synergize or interfere with Pit-1 to appropriately regulate gene expression, including Ets-1 (54), C/EBPα (55), GATA (56), and the nuclear receptors ER and GR (57, 58). Pit-1 has been implicated in the regulation of the GH, PRL, and TSH genes in combination with C/EBPα (55), Ets-1 (54), and GATA (56) transcription factors, respectively. Binding sites for GATA (-1538, -1145, -712, -632, -221, -16, $+126$, $+215$), Ets-1 (-1058), and C/EBPα (-1508, -880) are located within the 5′ flanking sequences of the rat GHRH receptor gene and, therefore, may be involved in the pituitary-specific expression of the GHRH receptor gene. Conversely, there may be unique transcription factors interacting with Pit-1 that are required for tissue specific expression of the GHRH receptor gene.

Expression of the rat GHRH receptor gene is dynamically regulated. Corticosteroids up-regulate GHRH receptor mRNA expression, both *in vivo* and in cultured anterior pituitary cells (36, 59, 60). Similarly, thyroid hormone treatment of cultured anterior pituitary cells (60) or hypothyroid rats (61) stimulates GHRH receptor mRNA levels. Conversely, estrogens inhibit GHRH receptor mRNA expression (59) and may be involved in the sexually dimorphic regulation of GHRH receptor gene expression that has been observed in the rat (62). An increase in GHRH receptor mRNA levels is observed at the onset of sexual maturation in male rats (63), suggesting a role for testosterone in the higher level of GHRH receptor expression in the male rat (62). Characterization of the human GHRH receptor gene has demonstrated that corticosteroids increase, and estrogens decrease,

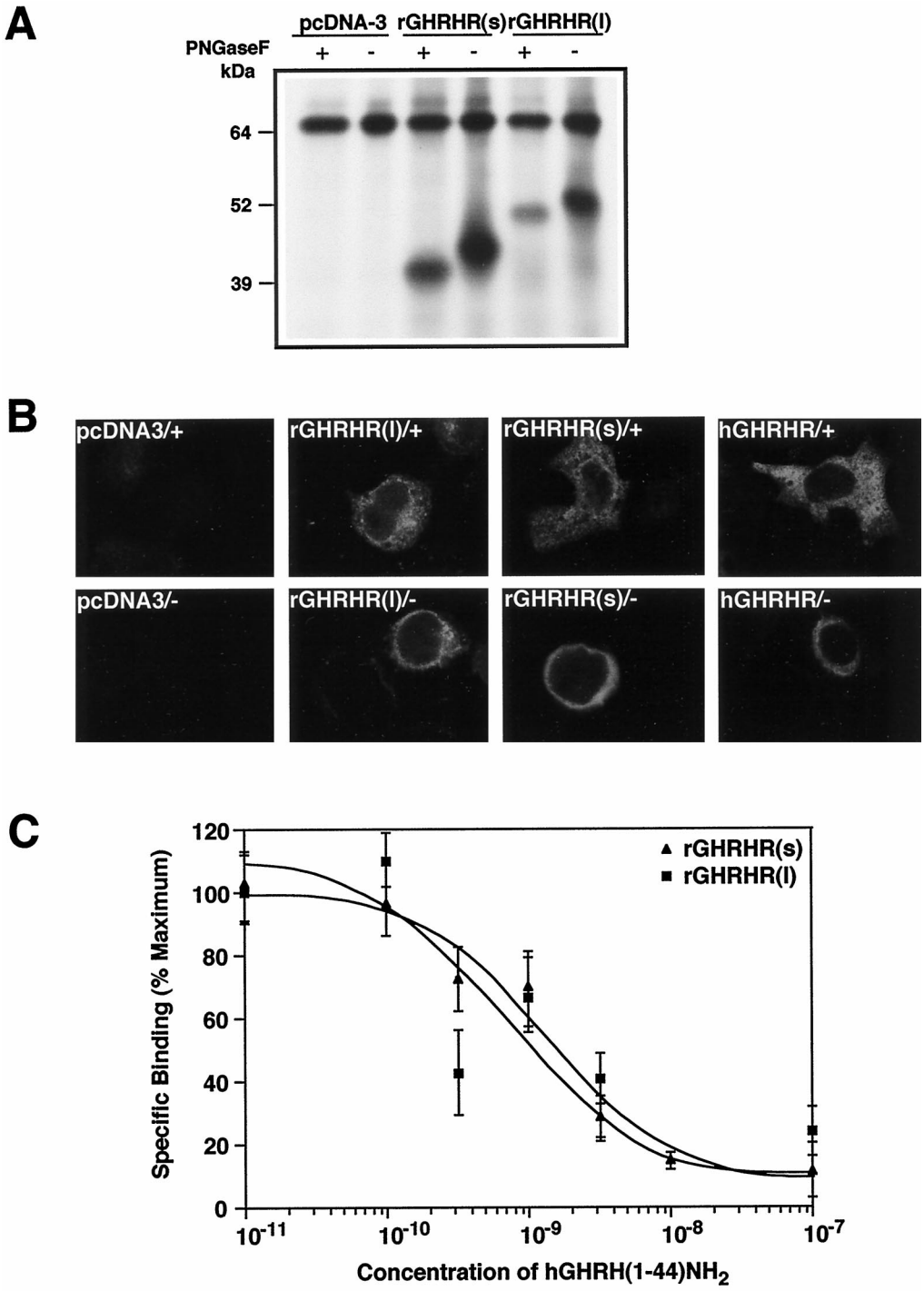

FIG. 7. Expression levels and GHRH binding of epitope-tagged isoforms of the rat GHRH receptor (rGHRHR). A, Equivalent amounts of protein from metabolically labeled HeLa T4 cells transfected with pcDNA-3 or the HA-tagged GHRH receptor constructs were immunoprecipitated, using the monoclonal antibody 12CA5 against the HA epitope-tag, and separated by SDS-PAGE on a 10% gel. The lanes loaded with samples treated with peptide-*N*-glycanase-F are denoted with a (+) sign on the *top*, and the sizes of the molecular mass standards included on the gel are shown on the *left*. B, Indirect immunofluorescence of HeLa T4 cells transfected with the indicated constructs was performed using the polyclonal antibody against the N terminus of the GHRH receptor. The panels on the *top* that are denoted with a (+) sign are images of cells permeabilized with 0.1% saponin, whereas those on the *bottom* denoted with a (−) sign are images of intact cells. All images were scanned using a confocal microscope under the same contrast settings for equivalent times, and the panels shown are representative of at least 20 fields scanned in 2 independent experiments. C, Dose-dependent competition of 3-[I^{125}]iodotyrosyl^{10}hGHRH (1–44)-amide binding to membranes of cells expressing the 2 isoforms of the rat GHRH receptor was performed. The relative amount of input radioligand bound in the presence of increasing concentrations of the respective unlabeled competitor is shown for the 2 constructs, with ED_{50} values of 1.5 nM and 1.05 nM for the long and short receptor isoforms, respectively. Data points represent the mean of triplicate samples, with the SEM indicated by the *error bars*, and the figure is representative of 2 independent experiments.

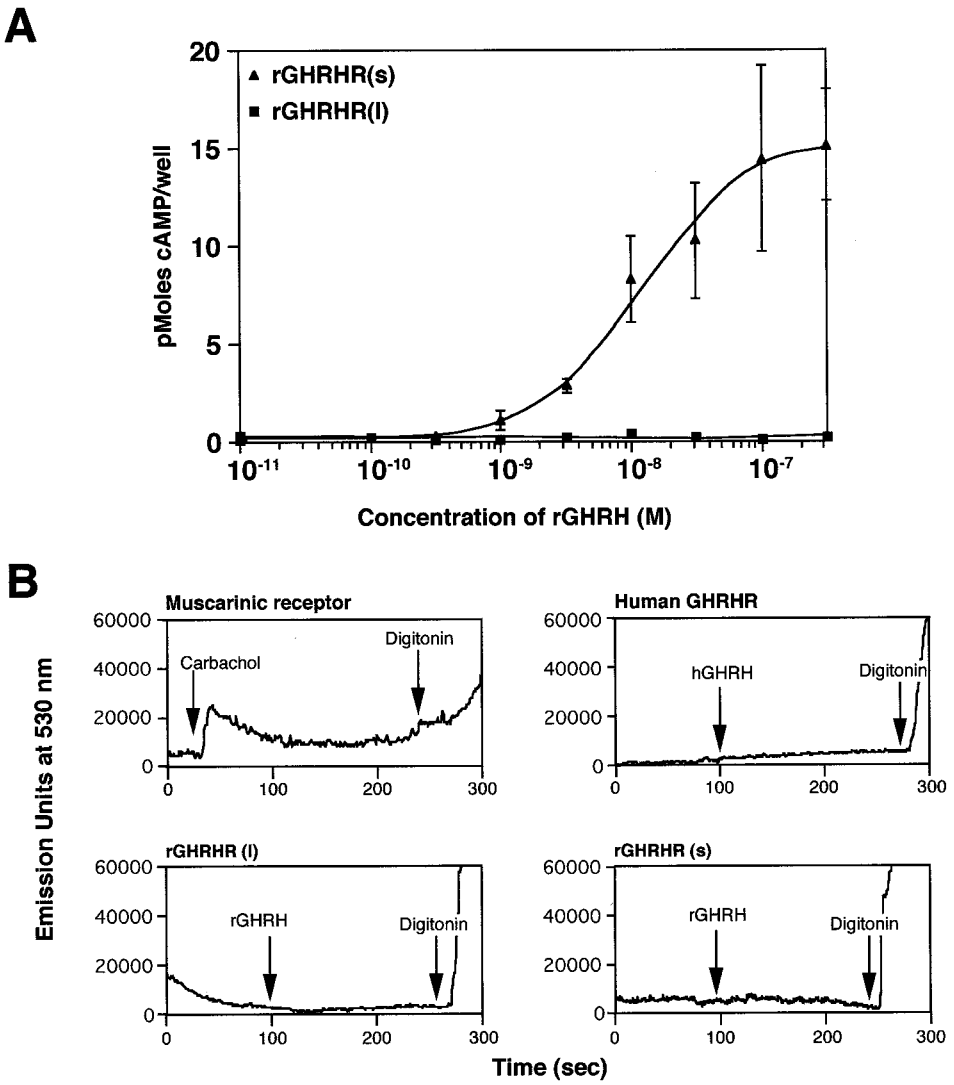

FIG. 8. Signal transduction of the two rat GHRH receptor isoforms in response to GHRH. A, Dose-dependent cAMP accumulation in HeLa T4 cells expressing isoforms of the rat GHRH receptor after treatment with GHRH. The amount of intracellular cAMP accumulated in cells treated with GHRH corresponds to an ED_{50} of 6.3 nM for the short GHRH receptor isoform. Data points represent the mean of triplicate samples, with the SEM indicated by the *error bars*, and the figure is representative of at least three independent experiments. B, GHRH-induced Ca^{2+} flux in HeLa T4 cells expressing the two isoforms of the rat GHRH receptor or the human GHRH receptor was measured by fluorimetry using Fluo-3AM as the indicator. The concentration of GHRH used was 250 nM. The muscarinic M3 receptor, transfected into HeLa T4 cells and treated with 25 μM carbachol, was used as a positive control. Data points represent the relative increase in the emission at 530 nM, as a function of time after ligand stimulation, and the figure is representative of two independent experiments.

expression of GHRH receptor promoter constructs (52). The rat GHRH receptor gene contains potential response elements for the nuclear hormone receptors GR (multiple half-sites) and ER, which may be involved in mediating the observed regulatory effects of these hormones. Decreased expression of receptor mRNA is detected after GHRH treatment of primary pituitary cells grown in culture (64) or chronic passive immunization of neonatal rats with GHRH (65). These effects of GHRH are likely to be mediated through cAMP-dependent pathways; and, in that respect, it is interesting that elements that recognize the CREB family of transcription factors are present in the rat GHRH receptor promoter region.

The IL domains and the C-terminal tail of the GPCRs have been implicated in coupling to the associated G protein, and alterations within these regions are known to disrupt or augment the signaling ability of the receptor (66). An activating mutation in IL1 or TM2 of the PTH receptor leads to ligand-independent stimulation of cAMP but not inositol phosphate production (67). Conversely, alternative splicing of 16 amino acids within IL1 of the calcitonin receptor inhibits receptor coupling to both the adenylyl cyclase and phospholipase C pathways and inhibits receptor internalization (68, 69). Deletion of residues within IL2 of the glucagon receptor has been shown to affect agonist activation of adenylyl cyclase (70). Alternative RNA splicing within IL3 of the PACAP receptor leads to differential G protein coupling to the adenylyl cyclase and phospholipase C pathways (31, 71, 72). Significantly, alternative RNA processing, leading to premature termination of the human GHRH receptor at IL3, resulting in an inability to transduce any signal, has been reported in normal pituitary (73) and in pituitary adenomas (74). The predominant isoform of the rat GHRH receptor contains 25 amino acids in IL3, and GHRH binding activates the adenylyl cyclase pathway. The insertion of 41 amino acids into this region, in the long isoform of the receptor, seems to disrupt receptor signaling through the adenylyl cyclase pathway, although the receptor is expressed on the cell surface and binds GHRH. Because alternative RNA processing in this region has been reported to lead to differential coupling to the adenylyl cyclase and phospholipase C pathways in the PACAP receptor (31), we tested the ability of the

2 GHRH receptor isoforms to elicit calcium mobilization upon ligand stimulation. We were unable to detect any calcium mobilization from internal stores in response to GHRH treatment of HeLa T4 cells transiently transfected with either isoform of the rat GHRH receptor. The N-terminal portion of IL3 adjacent to TM5 is predicted to form an amphipathic helix in GPCRs that interacts with the Gα-subunit (75). Experimentally introduced mutations in this region of the GLP-1 receptor have been shown to affect signaling (76). The insertion of 41 amino acids into the C-terminal portion of IL3 in the long isoform of the rat GHRH receptor may prevent signaling by disrupting the protein conformation required for productive interaction of IL3 with the stimulatory G protein.

The identification and characterization of the rat GHRH receptor gene is expected to advance our understanding of GHRH receptor gene regulation and provide insight into the mechanisms of GHRH action on the pituitary somatotroph cell. Given the emergent involvement of the GHRH receptor in diseases of GH secretion, these studies of the receptor gene are also expected to provide an important foundation for the investigation of diseases impacting the GH axis.

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