Identification and Characterization of the Gonadotropin-releasing Hormone Response Elements in the Mouse Gonadotropin-releasing Hormone Receptor Gene*

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The response of the pituitary gonadotrope to gonadotropin-releasing hormone (GnRH) correlates directly with the concentration of GnRH receptors (GnRHR) on the cell surface, which is mediated in part at the level of GnRHR gene expression. Several hormones have been implicated in this regulation, most notably GnRH itself. Despite these observations and the central role that GnRH is known to play in reproductive development and function, the molecular mechanism(s) by which GnRH regulates transcription of the GnRHR gene has not been well elucidated. Previous studies in this laboratory have identified and partially characterized the promoter region of the mouse GnRHR gene and demonstrated that the regulatory elements for tissue-specific expression as well as for GnRH regulation are present within the 1.2-kilobase 5'-flanking sequence. By using deletion and mutational analysis as well as functional transfection studies in the murine gonadotrope-derived αT3-1 cell line, we have localized GnRH responsiveness of the mouse GnRHR gene to two DNA sequences at −276/−269 (designated Sequence Underlying Responsiveness to GnRH-2 (SURG-2)), which contains the consensus sequence for the activating protein-1-binding site) and −292/−285 (a novel element designated SURG-1), and demonstrated that this response is mediated via protein kinase C. By using the electrophoretic mobility shift assay, we further demonstrate that a member(s) of the Fos/Jun heterodimer superfamily is responsible in part for the DNA-protein complexes formed on SURG-2, using αT3-1 nuclear extracts. These data define a bipartite GnRH response element in the mouse GnRHR 5'-flanking sequence and suggest that the activating protein-1 complex plays a central role in conferring GnRH responsiveness to the murine GnRHR gene.

The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH),1 plays a pivotal role in regulating mammalian reproductive development and function. Pituitary gonadotropes, which make up 8–15% of all cells in the anterior pituitary gland (1), express cell surface, G protein-coupled receptors specific for GnRH (2, 3). Activation of this receptor by GnRH stimulates intracellular signal transduction pathways to increase the synthesis and release of the pituitary gonadotropins, luteinizing hormone (lutropin; LH) and follicle-stimulating hormone (folliotropin; FSH) (4, 5). These hormones then enter the systemic circulation to regulate gonadal function, including steroid hormone synthesis and gametogenesis.

The biosynthesis and secretion of LH and FSH by pituitary gonadotropes are tightly regulated as evidenced by predictable and reproducible changes in circulating levels during the menstrual cycle. This regulation is dependent primarily on GnRH pulse amplitude and frequency, which varies with physiological state, with the rat estrous and human menstrual cycle, and with puberty, and the menopause. The response of pituitary gonadotropes to GnRH correlates directly with the concentration of GnRH receptors (GnRHR) on the cell surface, which are, in turn, regulated by a number of hormonal factors, most notably GnRH itself (6–9). The highest concentration of GnRHR in the pituitary gland is associated with a GnRH pulse frequency of 30 min and results in the optimum synthesis and release of LH. Lower concentrations of GnRHR are seen with GnRH pulse frequencies of 2 h and correlate with optimum synthesis and release of FSH (8–10). Continuous exposure to high concentrations of GnRH results in down-regulation of GnRHR mRNA (11). The difference in the concentration of GnRHR between high and low frequency GnRH pulses is 2–3 fold (8, 12). The difference in the concentration of GnRHR appears to be mediated at least in part at the level of GnRHR gene expression (6). GnRH regulation of GnRHR mRNA is well documented in rat pituitary cells (11). However, the cellular mechanism(s) by which GnRH regulates transcription of this and other genes has not been intensively investigated. This study was designed to identify and characterize the critical cis-DNA element(s) and cognate trans-factors that mediate the regulation of mouse GnRHR (mGnRHR) gene expression by GnRH.

Previous studies in this laboratory have identified and partially characterized the promoter region of the mGnRHR gene and demonstrated that the regulatory elements for tissue-specific expression as well as for GnRH regulation are present.

1 The abbreviations used are: GnRH, gonadotropin-releasing hormone; AP-1, activating protein-1; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GnRH, GnRH receptor; GnRHα, GnRH agonist; EMSA, electrophoretic mobility shift assay; mGnRHR, mouse GnRHR; PKA, protein kinase A; PKC, protein kinase C; RSV, Rous sarcoma virus; SF-1, steroidogenic factor-1; PMA, phorbol 12-myristate 13-acetate; ANOVA, analysis of variance; RE, response elements; DEMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; kb, kilobase; bp, base pair(s); mut, mutant; TSS, transcriptional start site.

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within a 1.2-kilobase (kb) 5′-flanking region of the mGnRHR gene (designated −1164/+62 relative to the major transcriptional start site (TSS)) (13). By using deletion and mutational analysis as well as functional transfection studies in the murine gonadotrope-derived αT3-1 cell line, we have localized GnRH responsiveness of the mGnRHR gene to two distinct DNA elements that appear to be both necessary and sufficient to mediate a full GnRH response. The first and critical element (5′-TATGAGTC-3′), designated the Sequence Underlying Responsiveness to GnRH-2 (SURG-2), lies at position −276/−269 and contains the consensus sequence for the canonical 12-O-tetradecanoylphorbol-13-acetate response element, also known as the activating protein-1 (AP-1)-binding site. The second element (5′-GCTAATTG-3′), designated SURG-1, lies at position −292/−285 and appears to be a novel enhancer element. The importance of these two elements in mediating GnRH responsiveness of the mGnRHR gene was confirmed by demonstrating that both SURG-1 and SURG-2 are capable independently of conferring activity on a heterologous minimal promoter but that both elements are required for a full response. Our data further suggest that the response of the mGnRHR gene promoter to GnRH is mediated via the protein kinase C (PKC), and not protein kinase A (PKA), signal transduction pathway. By using the electrophoretic mobility shift assay, we further demonstrate that a member(s) of the Fos/Jun heterodimer superfamily is responsible in part for the DNA-protein complexes formed on SURG-2, using αT3-1 nuclear extracts, and that such proteins are rapidly induced by GnRH stimulation. We propose therefore that GnRH-stimulated activity of the mGnRHR gene is regulated by two distinct elements within the GnRH gene promoter and that the key component of this mechanism involves the AP-1 protein complex that activates transcription in a cell-specific fashion.

EXPERIMENTAL PROCEDURES

Materials—Des-Gly3(9-D-Ala9)-GnRH-ethylamide (GnRH agonist (GnRHAg)), phorbol 12-myristate 13-acetate (PMA), forskolin, and 8-bromo-cAMP were obtained from Sigma. GF-109203X (bisindolylmaleimide II), 9-camphorsulfonic acid (CSA)-methyl-3-(1H-phenalenyl)maleimide, a selective inhibitor of PKC, and not protein kinase A (PKA), signal transduction pathway. By using the electrophoretic mobility shift assay, we further demonstrate that a member(s) of the Fos/Jun heterodimer superfamily is responsible in part for the DNA-protein complexes formed on SURG-2, using αT3-1 nuclear extracts, and that such proteins are rapidly induced by GnRH stimulation. We propose therefore that GnRH-stimulated activity of the mGnRHR gene is regulated by two distinct elements within the GnRH gene promoter and that the key component of this mechanism involves the AP-1 protein complex that activates transcription in a cell-specific fashion.

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m Na₂HPO₄ buffer (pH 7.3), 0.013 M 2-nitrophenyl-β-D-galactopyranoside, 0.1% (v/v) 10.0 M MgCl₂, 0.35% (v/v) β-mercaptoethanol, incubating overnight at 37 °C, and measuring colorimetrically at 410 nm in a Beckman DU640 spectrophotometer (Beckman, Fullerton, CA) after the addition of 100 µl of 1.0 mM sodium carbonate. Luciferase activity was normalized to expression of BSV-β-galactosidase.

Northern Blot Analysis—To investigate the effect of GnRHAg stimulation on GnRHR mRNA, α73-1 cells were treated with 100 nM GnRHAg or vehicle for varying time intervals (1, 2, 4, or 8 h), and total RNA was extracted from cells using the Qiagen “RNEasy” RNA extraction kit (Qiagen, Santa Clarita, CA). Total RNA (10 µg/lane) was separated by electrophoresis on a 1% agarose gel containing 6.7% formaldehyde prior to capillary transfer onto sheets of Nytran (Schleicher & Schuell) immobilization membrane. Northern blot analysis was performed under high stringency conditions using a [32P]UTP-labeled antisense riboprobe (5 × 10⁶ cpm/ml) prepared from the coding region (+173/+1153) of mGnRHR cDNA using T7 RNA polymerase (New England Biolabs, Inc., Beverly, MA). Washed blots were exposed to Kodak X-OMAT/AR film at −70 °C for 8–38 h. Rat cyclophilin antisense riboprobe was used as an internal standard. The intensity of the individual RNA bands was quantified in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) according to the protocol outlined by the manufacturer. Measurements were standardized for cyclophilin mRNA.

Preparation of Nuclear Extracts—α73-1 cells were grown to 20, 40, 60, and 80% confluence and treated with 100 nM GnRHAg or vehicle for varying time intervals (1 or 4 h). Thereafter, cells were harvested, and nuclear extracts were prepared by the method of Andrews and Faller (16).

Electrophoretic Mobility Shift Assay (EMSA)—Probe was prepared for EMSA by digestion of the pXP2 plasmid containing the −308/−220 fragment of the mGnRHR gene promoter with HindIII and XhoI, followed by 5′-end-labeling with [γ-32P]ATP by T4 polynucleotide kinase (New England Biolabs). Constructs were then purified using a Qiagen nucleotide removal kit. The binding reaction for EMSA was performed by incubating 50,000 cpm of DNA probe with 10 µg of nuclear extract and 1 µg of salmon sperm DNA in reaction buffer (20 mM HEPES (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 10 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 5% (v/v) glycerol) for 30 min at 4 °C. For competition studies, excess unlabeled DNA was added 5 min prior to the addition of probe. Protein-DNA complexes were resolved on 4% low ionic strength non-denaturing polyacrylamide gel electrophoresis in 0.5× Tris borate/EDTA buffer (45 mM Tris-HCl (pH 8.0), 45 mM boric acid, 1 mM EDTA). Gels were then dried for 1 h and subjected to autoradiography for 24–48 h. Antibody supershift experiments were performed using an anti-Fos antibody (Santa Cruz Biotechnology) which recognizes all members of the Fos oncprotein family. Similar experiments were carried out using an anti-Jun blocking antibody (Santa Cruz Biotechnology) raised against the common DNA binding domain of all members of the Jun family. Antibody, either anti-Fos (1 µl), anti-Jun (1, 2, 4, or 8 µl), or both, was added to the EMSA mixtures after 30 min of incubation at 4 °C for an additional 2 h prior to gel electrophoresis. When indicated, the intensity of the individual protein bands was quantified in a PhosphorImager (Molecular Dynamics). Measurements were standardized to background intensity.

Characterization of the Signal Transduction Pathway Involved in the GnRH Agonist-triggered Increase in mGnRHR Gene Expression—To identify the second messenger pathway(s) involved in the GnRHAg stimulation of the mGnRHR gene, α73-1 cells were transfected with GH₂-pXP2, GH₂/-308/-264 (wild type), GH₂/-308/-264 (wild type, GH₂/-308/-264/wild type, and GH₂/-308/-264/region E (SURG-1 mutant), or GH₂/-308/-264/region E (SURG-2 mutant) as described, and response to GnRHAg stimulation was measured in the presence or absence of selective agonists/antagonists. To investigate the role of the PKC signal transduction pathway, transfected cells were stimulated for 4 h with PMA (100 ng/ml), GnRHAg (100 nM), or both. Final concentrations of GnRHAg (Fig. 2A) and PMA (data not shown) were chosen to give maximal stimulation at 4 h. Similar experiments were carried out in the presence or absence of 1 µM GF-109203X (Sigma), a simplified derivative of staurosporine that acts as a competitive inhibitor for the ATP-binding site of PKC. This agent is selective for PKC isoforms α, β, ε, γ, δ, and ζ. It is used with PMA, phosphorylase kinase, various tyrosine kinases, and PKC isoform γ. In the latter experiments, cells were incubated with PMA or vehicle for 30 min immediately prior to as well as during the 4-h period of stimulation. Reagents were initially dissolved in dimethyl sulfoxide (Me₂SO) and subsequently diluted in culture medium to give a final concentration of 0.1% (v/v) Me₂SO in each experiment. To investigate the role of the PKA signal transduction pathway, transfected cells were stimulated for 4 h with forskolin (25 µM), GnRHAg (100 nM), or both. Identical studies were carried out using a second PKA agonist, 8-bromo-cAMP (1 mM). The effect of 1 µM SQ 22536 (Sigma), a selective inhibitor of PKA activity, was investigated in selected experiments.

Statistical Analysis—Transfections were performed in triplicate and repeated multiple times. Data in each experiment were normalized to the basal level of activity of either pXP2 or GH₂-pXP2 (designated 1-fold). Data were then combined across experiments. Results were expressed as mean ± S.E. for basal and GnRHAg-stimulated activities for each construct, and fold stimulation in response to GnRHAg was calculated. One-way analysis of variance (ANOVA) followed by post hoc comparisons with Fisher’s protected least significant difference test was used to assess whether changes in GnRH responsiveness among different GnRH promoter-luciferase reporter constructs were significant. Significant differences were designated as p < 0.05. When appropriate, data were analyzed by the Student’s t test for independent samples.

RESULTS

Northern Blot Analysis—A selected Northern blot of RNA extracted from α73-1 cells stimulated with GnRHAg (100 nM) or vehicle for varying time intervals (1, 2, 4, or 8 h) and hybridized with a mGnRHR antisense riboprobe is shown (Fig. 1A). The size of the major mGnRHR mRNA was 4.5 kb as previously reported (17) and that of cyclophilin mRNA was 0.8 kb. The intensities of individual bands were quantified in a PhosphorImager and corrected for total RNA content by using cyclophilin mRNA levels. Results (expressed as percent of time 0) demonstrate a significant increase in GnRHR mRNA in
response to GnRHAg stimulation that was maximal at 4 h (1.84 ± 0.2-fold stimulation; \( p < 0.01 \)) but decreased thereafter (Fig. 1B). These data are consistent with previous reports indicating that GnRH is capable of regulating GnRHR gene expression, both in the short term (up-regulation) and in the long term (down-regulation) (6, 11). The following study was then designed to identify and characterize the critical cis-DNA element(s) and cognate trans-factors responsible for this regulation in aT3-1 cells.

**Optimization of the GnRHAg Response**—To optimize conditions for GnRH responsiveness, αT3-1 cells were transiently transfected with the full-length 1.2-kb 5'-flanking region of the mGnRHR gene (−1164/+62), followed by 4 h treatment with increasing concentrations of GnRHAg (0, 10, 100, and 500 nM). Results are mean ± S.E. from multiple experiments. *, \( p < 0.0002 \) compared with no GnRHAg; ●, \( p = 0.012 \) compared with 10 nM GnRHAg. **, \( p < 0.001 \) compared with no GnRHAg. A, GnRHAg dose-response curve. αT3-1 cells were transfected for 4 h with the 1.2-kb 5'-flanking region of the mGnRHR gene (−1164/+62), followed by 4 h treatment with increasing concentrations of GnRHAg (0, 10, 100, and 500 nM). Results are mean ± S.E. from multiple experiments. Fold stimulation to GnRHAg is shown. **, \( p < 0.001 \) compared with multiple experiments. Fold stimulation to GnRHAg is shown. **, \( p < 0.001 \) compared with multiple experiments. Fold stimulation to GnRHAg is shown.

**Identification of Two GnRH Response Elements (GnRH-REs) in the Mouse GnRHR Gene Promoter**—Transfection of αT3-1 cells with −1164/+62 also demonstrated a modest increase in basal luciferase activity with time but showed no response to GnRHAg (Fig. 2C), suggesting that the GnRH response is cell-specific.
GnRH-REs in the Mouse GnRHR Gene Promoter

Luciferase activity between the constructs −765/+62, −387/+62, −341/+62, −300/+62, −232/+62, and −117/+62 was not different from each other nor from −1162/+62 but were all significantly different from pXP2 vector alone (p < 0.01; ANOVA) and the remaining constructs −232/+62, −117/+62, and −38/+62 (in which fold stimulation was not significantly different from each other nor from pXP2 vector alone) (Fig. 3). Despite a 2.4 ± 0.4-fold and 2.3 ± 0.4-fold stimulation in the −232/+62 and −117/+62 constructs, respectively, these measurements were not significantly different from pXP2 alone (p = 0.56 and p = 0.57, respectively; ANOVA; n = 13 separate experiments). Basal luciferase activity was not significantly different between the various constructs (data not shown). These data suggest the presence of an element(s) within the region −300/+232 of the mGnRHR gene promoter which is necessary for GnRH responsiveness.

To investigate further the importance of SURG-1 (SURG-1) contains an enhancer element which may be necessary for GnRH responsiveness. Transfection with the mutant construct of region C (GH50/region C, SURG-1mut) resulted in a 3.6 ± 0.4-fold increase in luciferase activity, which was significantly different from both GH50-pXP2 alone and from the wild type mutant, GH50/−308/+264 (p = 0.041 and p = 0.0003, respectively; ANOVA) (Fig. 5B). Once again, basal luciferase activity was not significantly different between the various constructs (data not shown). These data suggest that region E (SURG-2) in the putative AP-1-binding site was mutated, completely abrogated the GnRH Ag response (p < 0.0001 compared with wild type, but not significant compared with GH50-pXP2; ANOVA). Similarly, a single point mutation of the AP-1-binding site at position −269 (GH50/region E/mut) completely eliminated the GnRH Ag response, suggesting that region E at position −276/−269 of the mGnRHR gene promoter is critical for GnRH responsiveness. Transfection with the mutant construct of region C (GH50/region C, SURG-1mut) resulted in a 3.6 ± 0.4-fold increase in luciferase activity, which was significantly different from both GH50-pXP2 alone and from the wild type mutant, GH50/−308/+264 (p = 0.041 and p = 0.0003, respectively; ANOVA) (Fig. 5B). Once again, basal luciferase activity was not significantly different between the various constructs (data not shown). These data suggest that region E (SURG-2) contains the consensus sequence for the AP-1-binding site, is critical for GnRH responsiveness of the mGnRHR gene and that region C (SURG-1) contains an enhancer element which may be necessary for the full GnRH response.

To identify and characterize further the GnRH-RE(s) within the region −308/+264 of the mGnRHR gene promoter, five scanner-linker mutants of this region were synthesized as detailed above (Fig. 5A), placed upstream of the GH50 minimal promoter in GH50-pXP2, and transfected into αT3-1 cells. Wild type expression vector (GH50/−308/+264) resulted in a 7.8 ± 1.2-fold increase in luciferase activity in response to GnRH Ag stimulation, which was similar to that measured in the scanner-linker mutant constructs for regions A, B, and D (Fig. 5B). However, transfection with the mutant construct of region E (GH50/region E, SURG-2mut), in which the putative AP-1-binding site had been mutated, completely abrogated the GnRH Ag response (p < 0.0001 compared with wild type, but not significant compared with GH50-pXP2; ANOVA). Similarly, a single point mutation of the AP-1-binding site at position −269 (GH50/region E/mut) completely eliminated the GnRH Ag response, suggesting that region E at position −276/−269 of the mGnRHR gene promoter is critical for GnRH responsiveness. Transfection with the mutant construct of region C (GH50/region C, SURG-1mut) resulted in a 3.6 ± 0.4-fold increase in luciferase activity, which was significantly different from both GH50-pXP2 alone and from the wild type mutant, GH50/−308/+264 (p = 0.041 and p = 0.0003, respectively; ANOVA) (Fig. 5B). Once again, basal luciferase activity was not significantly different between the various constructs (data not shown). These data suggest that region E (SURG-2), which contains the consensus sequence for the AP-1-binding site, is critical for GnRH responsiveness of the mGnRHR gene and that region C (SURG-1) contains an enhancer element which may be necessary for the full GnRH response.
and this response appeared to be additive so that the activity of the reconstituted \( \text{GH}_{50}/\text{SURG}-2/\text{NotI}/\text{SURG}-1 \) construct was similar to that seen with the full-length mGnRHR promoter, \(-1164/-62 (13.7 \pm 3.0\text{-fold and } 13.6 \pm 3.0\text{-fold, respectively})\) (Fig. 6A). \( \text{GH}_{50}/\text{SURG}-2/\text{NotI}/\text{SURG}-2 \) gave a 72.7 \pm 26.5-fold response to GnRHaG stimulation, lending further evidence to the importance of SURG-2 in GnRH responsiveness in the mGnRHR gene. Similar studies using the full-length mGnRHR gene promoter containing a C269T point mutation of SURG-2 (\(-1164/-62/\text{SURG}-2\text{mut}\)) completely abrogated the response to GnRHaG stimulation (Fig. 6B), confirming that SURG-2 is critical for GnRH responsiveness. Mutation of SURG-1 in the...
full-length construct (−1164/+62/SURG-1mut), on the other hand, significantly diminished but did not abrogate the response (6.8 ± 0.9-fold as compared with 14.6 ± 1.7-fold response seen with −1164/+62; p < 0.001 compared with all other reactions). These data provide further evidence in support of the hypothesis that SURG-2 is a critical element for GnRH responsiveness of the mGnRHR gene but that the inclusion of SURG-1 is necessary for optimal response to GnRH.

Identification and Characterization of trans-Factors by EMSA—Using nuclear extracts from αT3-1 cells and 32P-end-labeled −308/−220 of the mGnRHR gene promoter as probe, two distinct protein-DNA bands could be identified on EMSA that were not present with probe alone (Fig. 7). Nuclear extracts from cells grown to approximately 40% confluence appeared to give optimum binding as compared with nuclear extracts derived from cells grown to approximately 20, 60, or 80% confluence (Fig. 7). These results are in keeping with data from transfection studies suggesting that cells grown to 40–50% confluence showed a marked reduction in GnRHAg responsiveness (data not shown). Further EMSA experiments were therefore standardized to nuclear extracts from αT3-1 cells grown to 40–50% confluence. GnRHAg stimulation (100 nM for 4 h) of αT3-1 cells prior to preparation of nuclear extract increased the intensity of the lower band by 1.9 ± 0.4-fold (p < 0.05; Student’s t test), suggesting the presence of a specific, GnRH-responsive protein within the complex (Fig. 7). A shorter GnRHAg stimulus (100 nM for 1 h) appeared to give similar results (1.8 ± 0.5-fold (data not shown)). The intensity of the upper band did not change significantly with GnRHAg stimulation.

Since SURG-2 containing the AP-1-binding site had been shown to be critical for GnRH responsiveness in the mGnRHR gene (above), anti-Fos and anti-Jun antibodies (Santa Cruz Biotechnology) were used in antibody-supershift EMSA experiments to identify and characterize further the trans-factors present within the protein-DNA complex. Supershift of the lower band with an anti-Fos antibody suggests the presence of a Fos protein within the complex (Fig. 8A). Similarly, incubation with an anti-Jun blocking antibody resulted in diminution in binding of the lower band, suggesting the presence of a Jun protein within the complex (Fig. 8B). However, this effect was moderate at best and required large amounts of anti-Jun antibody (8 µl/lane as compared with only 2 µl/lane for positive control). Positive control for the anti-Jun blocking antibody

**Fig. 6.** Confirming the importance of regions SURG-1 and SURG-2 on GnRHAg-stimulated luciferase activity in αT3-1 cells. A, GH50-linked constructs containing single or multiple copies of SURG-2 (region E, AP-1-binding site) and/or SURG-1 (region C) were synthesized as detailed above and transfected into αT3-1 cells. Measurements are expressed as fold stimulation of luciferase activity by GnRHAg (100 nM for 4 h). Results are mean ± S.E. from multiple experiments. *, p < 0.0001 compared with pXP2 (positive control). **p < 0.001 compared with all other reactions. ●, p < 0.001 compared with GH50, GH50/SURG-1, and GH50/SURG-2/Nat-1/SURG-2. B, similar experiments were carried out using the full-length (1.2 kb) mGnRHR gene promoter containing mutations of SURG-2 (−1164/+64/SURG-2mut) or SURG-1 (−1164/+64/SURG-1mut). *, p < 0.0001 compared with pXP2 (positive control). ●, p < 0.02 compared with −1164/+62 and −1164/+62/SURG-1mut but not significant compared with pXP2. ●, p < 0.01 compared with all other reactions.
FIG. 7. Identification of a GnRH-responsive trans-factor by EMSA. αT3-1 cells were grown to 20, 40, 60, and 80% confluence and treated with GnRHAg (100 nM) or vehicle for 4 h prior to preparation of nuclear extract. Using αT3-1 nuclear extracts and the −308/–220 PCR-generated fragment of the mGnRHR gene promoter as probe, EMSA identified two distinct protein-DNA complex bands that were not present in probe alone (designated by arrows). Nuclear extracts from cells grown to approximately 40% confluence appeared to give optimum binding as compared with nuclear extract derived from cells grown to 20, 60, or 80% confluence. Stimulation of αT3-1 cells by GnRHAg prior to preparation of nuclear extract increased the intensity of the lower band by 1.9 ± 0.4-fold as measured by a PhosphorImager.

The maintenance of normal reproductive function in all vertebrate species is dependent on the regulation of LH and FSH synthesis and release by pituitary gonadotropes. Although the synthesis and intermittent release of the pituitary gonadotropins are affected by a number of endocrine, paracrine, and autocrine factors, the most important influence appears to be that of GnRH (6–9). In this study, we have defined the dimeric GnRH-RE within the 1.2-kb 5′-flanking sequence of the mGnRHR gene, and we have demonstrated that the AP-1 complex plays a central role in conferring GnRH responsiveness to the mGnRHR gene. We have used αT3-1 cells, a well characterized mouse pituitary gonadotrope cell line, as a model for the analysis of
cis-regulatory elements in the mGnRHR gene. This cell line, obtained by targeted tumorigenesis in the mouse pituitary with the SV40 large T antigen driven by the human glycoprotein hormone α-subunit promoter (18), has been used to study many aspects of gonadotrope physiology. A number of studies have shown that αT3-1 cells constitutively express GnRHR and are capable of binding and responding to exogenous GnRH (13, 18). Characterization of this cell model has demonstrated many similarities in the GnRH response compared with that in mouse primary pituitary cells, including the specific intracellular signal transduction pathways activated, the degree of stimulation of the gonadotropin subunit promoter activities, and the presence of differential regulation of GnRHR and α-subunit gene promoter activities by GnRH (4, 14, 19). αT3-1 cells thus appear to be a useful model for the study of the regulation of expression of the GnRHR gene by GnRH.

The responsiveness of pituitary gonadotropes to GnRH correlates directly with changes in GnRHR concentrations. It has been suggested that the concentration of GnRHR on the cell surface is mediated in turn, at least in part, at the level of gene expression (6, 12). Data from Northern blot analyses presented above (Fig. 1), which demonstrate a significant increase in GnRHR mRNA in response to GnRH stimulation which was maximal at 4 h, would support this conclusion. These findings are consistent with previous reports in primary monolayer cultures of rat pituitary cells in which GnRHR mRNA levels were significantly increased by pulses of GnRH (10 nM, 5 min/pulse) at all pulse frequencies tested over a 24-h period (12). In contrast, Alarid and Mellon (20) found no change in GnRHR mRNA levels in αT3-1 cells in response to continuous exposure to GnRHAg for 1–24 h, and Mason et al. (21) demonstrated a time- and dose-dependent decrease in the level of GnRHR mRNA in αT3-1 cells in response to GnRH or GnRHAg. The disparity among these results may be related to cell culture conditions and the timing of GnRH stimulation. In our hands, optimal response of mGnRHR promoter-transfected αT3-1 cells

**FIG. 8.** Identification and characterization of trans-factors binding to critical cis-DNA elements in the mGnRHR gene promoter by EMSA. A, using nuclear extracts prepared from αT3-1 cells and the −308/−220 fragment of the mGnRHR gene promoter as probe, the two protein-DNA complex bands could again be identified by EMSA. Control was probe alone. An increase in intensity of the lower band was again seen with GnRHAg stimulation. Supershift of the lower band with anti-Fos antibody (Santa Cruz Biotechnology) suggests the presence of a Fos protein within this DNA-protein complex (see arrows). B, similar experiments were carried out using an anti-Jun blocking antibody (Santa Cruz Biotechnology). Results demonstrate a moderate but significant diminution in binding of the lower band suggesting the presence of Jun protein within the complex (see small arrow). To determine the specificity of the anti-Jun antibody, EMSA experiments were carried out using purified c-JUN protein (Promega) and the consensus AP-1-binding site (5'-TGAGTCA-3') as probe. Control was probe alone. Addition of excess anti-Jun antibody resulted in significant diminishment in the intensity of the lower band (see large arrow). Addition of excess anti-Fos antibody had no effect on binding (data not shown).
to GnRHAg stimulation was seen after 4 h transfection and 4 h GnRHAg stimulation. A longer transfection time resulted in increased basal expression of luciferase activity but a significantly diminished response to GnRHAg (Fig. 2B).

The mGnRHR gene has been isolated, and its major TSS has been identified (17, 22, 23). A 1.2-kb 5′-flanking region of the mGnRHR gene has been characterized and shown to be active in transfection studies (13). This region has also been used in transgenic mice to show that it is sufficient to mediate gonadotrope-specific expression in vivo (24). Preliminary studies on the 5′-flanking putative promoter region of the mouse, human, and sheep GnRHR genes reveal complex organization with multiple TSS that are occasionally associated with TATA boxes (13, 25). In the mGnRHR gene, the major TSS was shown to be located 62 nucleotides upstream of the translational start site by primer extension and ribonuclease protection analysis of αT3-1 gonadotrope mRNA (13). Functional analysis by transient transfection of αT3-1 cells with the 1.2-kb 5′-flanking region of the mGnRHR gene (−1164/+62) confirmed previous observations (13, 25) that this region contains an element(s) that is necessary for tissue-specific basal expression as well as for GnRH responsiveness. By using deletion and mutational analysis in αT3-1 cells, Duval et al. (26) recently identified a tripartite enhancer that appears to be responsible for regulating cell-specific basal expression of the GnRHR gene. Individual elements of this putative enhancer include binding sites for steroidogenic factor-1 (SF-1), AP-1, and a novel element designated GnRHR receptor activating sequence. Although a number of hormones, including GnRH (7–9), estradiol (9, 27), and activin A (28), either alone or in combination, are known to affect transcriptional activation of the mGnRHR gene, neither the cAMP-dependent protein kinase (PKA) signaling pathway in GnRH stimulation of the mGnRHR gene completely abrogated the GnRHAg-stimulated response, whereas mutation of SURG-1 diminished but did not completely abolish this stimulation (Fig. 6B). These data suggest that SURG-2 (AP-1-binding site) is critical to GnRH responsiveness in the mGnRHR gene, whereas SURG-1 acts as an enhancer element to facilitate full GnRH response. The putative repressor element in the region −264/−220 of the mGnRHR gene has not been further characterized.

Earlier studies identified a putative gonadotrope-specific element (5′-TGTCCTTG-3′) at position +48/+55 of the mGnRHR gene and suggested that this element may be important in conferring GnRH responsiveness (13). This sequence was first described in the human α-subunit gene as an element that binds the nuclear orphan receptor, SF-1 (29), and appears to be important for gonadotrope-specific expression of the α- (30) and LHβ-subunit genes (31). It has also been shown to be important in regulating cell-specific basal expression of the mGnRHR gene (26). Our studies, however, suggest that the putative gonadotrope-specific element is not involved in GnRHAg responsiveness of the mGnRHR gene. Functional transfection studies using serial 5′-deletion constructs of the 1.2-kb putative mGnRHR gene promoter (−1164/+62) showed that downstream constructs (−232/+62, −117/+62, and −38/+62) did not significantly stimulate luciferase activity in response to GnRHAg (Fig. 3). Results from mutational studies in the full-length −1164/+62 construct (Fig. 6B) further confirm these observations. These data are in keeping with observations made in SF-1 knock-out mice. Targeted disruption of the murine fzt-F1 gene encoding SF-1 results in adrenal and gonadal hypoplasia (32). Such SF-1 knock-out mice exhibit malformations of the ventromedial hypothalamus as well as selective deficiency of GnRH, LHβ, and FSHβ mRNA in the pituitary (32). However, treatment with GnRH results in partial restoration of gonadotropin subunit gene expression as well as detectable levels of mGnRHR mRNA (32). Taken together, these results along with our data suggest that SF-1 is not a critical element for mGnRHR gene expression.

Using nuclear extracts from αT3-1 cells, with or without GnRHAg stimulation, two distinct protein-DNA bands were identified on EMSA. Nuclear extracts from cells grown to approximately 40% confluence appeared to give optimum binding. These results are in keeping with data from transfection studies suggesting that cells grown to 40–50% confluence showed optimal response to GnRHAg stimulation. The lower protein-complex band on EMSA, but not the upper band, appeared to be GnRH-responsive. Using antibody blocking and supershift EMSA experiments, we have demonstrated that the lower band represents a complex containing a member(s) of the Jun/Fos heterodimer superfamily, also known as the AP-1 protein complex (Fig. 8). These data are consistent with the functional transfection studies demonstrating that the AP-1-binding site (SURG-2) is critical for GnRH responsiveness of the mGnRHR gene. Anti-Fos supershift EMSA experiments resulted in a supershift of the entire lower band. Anti-Jun blocking EMSA, on the other hand, resulted in only a modest inhibition in binding. It is possible that Jun may not itself bind to cis-regulatory elements and that AP-1 stimulation of mGnRHR gene

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Fig. 9. Role of selected signal transduction pathways in GnRHAg stimulation of the mGnRHR gene. A, to investigate the effect of PKA agonists and antagonists on GnRHAg-stimulated luciferase activity, αT3-1 cells were transfected with GH<sub>4</sub> wild type sequence (GH<sub>4</sub>-/−308/−264), or mutant constructs for regions C (SURG-1mut) or E (SURG-2mut) as described and stimulated for 4 h with PMA (100 ng/ml), GnRHAg (100 nM), or both. Measurements are expressed as fold stimulation of luciferase activity. Results are mean ± S.E. from multiple experiments. *, p < 0.04 compared with fold stimulation in the presence of GF-109203X. ■, p < 0.05 compared with fold stimulation in the presence of GF-109203X #, p < 0.01 compared with PMA- or GnRHAg-stimulated luciferase activity in wild type construct (GH<sub>4</sub>-/−308/−264). NS = not significant. B, similar experiments were performed to investigate the role of the PKA signal transduction pathway in GnRH stimulation of the mGnRHR gene. αT3-1 cells were transfected as described above and stimulated for 4 h with forskolin (25 μM), GnRHAg (100 nM), or both. *, p < 0.001 compared with all other reactions. ●, p < 0.04 compared with GH<sub>4</sub>-pXP2, SURG-1mut + forskolin ± SQ 22536. NS = not significant.
gene transcription may be mediated by Jun interaction with another protein(s), perhaps Fos, that directly binds to the proximal mGnRHR promoter. A similar observation was made by Brueder et al. (33) investigating the role of AP-1 in the repression of GnRH gene transcription in GT1-7 neuronal cells. Exactly which of the Jun and Fos family members make up this heterodimer complex has yet to be determined. The identity of the DNA-protein complex responsible for the upper band seen on EMSA has yet to be characterized but may represent a trans-factor binding to the SURG-1 cis-regulatory element.

Serum is a highly effective stimulus of primary response genes, including fos and jun. Endogenous steady-state levels of Fos and Jun in αT3-1 cells cultured in the presence of 10% fetal calf serum are relatively high (34). However, levels of c-fos, c-jun, as well as junB mRNA have been shown to decrease progressively over a 6-h period in αT3-1 cells cultured in serum-free medium. The final steady-state mRNA level of all three of these trans-factors after 6 h was around 3–5% of that observed in cells cultured in the presence of 10% fetal calf serum (34). In transcription studies detailed above, optimal response to GnRHAg stimulation was observed in αT3-1 cells cultured overnight in serum-free medium (data not shown). By having subsequently identified a member of the Jun/Fos heterodimer superfamily as the critical element in GnRH responsiveness of the mGnRHR gene, we hypothesize that an overnight incubation in serum-free medium might decrease basal (endogenous) levels of Jun and Fos, thereby allowing for an enhanced response to GnRHAg.

The expression of the GnRHR, α-, FSHβ-, and LHβ-subunit genes are all strictly regulated in pituitary gonadotropes by GnRH and other hormones. Separate and independent manipulation of each of these related genes in a single cell type requires a complex regulatory system. The individual components of this regulatory system are poorly defined but probably include activation of distinct signal transduction pathways, the presence of specific cis-regulatory elements in the promoter regions of each of these genes, and the incorporation of different trans-factors and/or coactivators/corepressors which differentially regulate gene transcription. The GnRH-RE(a) in each of the gonadotropin subunit genes have been partially characterized. In the LHβ gene, two putative Sp1-binding sites in the proximal promoter region appear to play an important role in conferring GnRH responsiveness (35), and two transcription factors, SF-1 and early growth response-1 (Egr-1), are known to be involved in tissue-specific expression of this gene (31, 36). There is no consensus Sp1-binding site in the mGnRHR gene. Analysis of the α-subunit promoter in αT3-1 cells and in transgenic mice (14, 37) have led to the identification of multiple cis-regulatory elements that appear to be important for tissue-specific basal expression of the α-subunit gene. Such elements include a binding site for a LIM homeodomain protein (38), several canonical E boxes (39), the αACT element that binds members of the GATA binding factor family (40), and the gonadotrope-specific element that binds the SF-1 transcription factor (29). A binding site for Ets factor (a family of transcription factors that have been implicated in mediating transcriptional responses to mitogen-activated protein kinase activation) has also been identified in the α-subunit gene promoter (41). The precise GnRH-RE(s) in the common α-subunit gene, however, has not been characterized. This may be due in part to the observation that the common α-subunit gene is less well regulated by GnRH as compared with the GnRHR, LHβ-, and FSHβ-subunit genes (10). Recent studies in the ovine FSHβ gene have identified two AP-1 enhancers in the proximal promoter that appear important for tissue-specific basal FSHβ expression in vivo (42). These same AP-1 elements also appear to mediate GnRH-stimulated transcription of the ovine FSHβ gene in primary cultures of ovine pituitary cells (43). There are no confirmed AP-1 consensus sequences in either common α- or LHβ-subunit gene promoters.

The mechanism(s) by which a common and ubiquitous cis-regulatory element, such as the AP-1-binding site, is able to regulate differentially both GnRHR and FSHβ-subunit genes within the pituitary gonadotrope cell remains unclear. A number of potential mechanisms exist. Specific homo- and heterodimer members of the AP-1 family, for example, may differentially regulate target genes through a common cis-regulatory element. Alternatively, the same AP-1 trans-factor may interact with different protein kinases and/or transcriptional coactivators/corepressors to affect distinct biological functions. Our data suggest another possibility, namely the incorporation of one or more secondary cis-elements, such as SURG-1 for the GnRHR gene (above). Exactly which of the AP-1 family members binds to the GnRHR and FSHβ-subunit promoter regions are not known. There is, however, evidence to suggest that different signal transduction pathways may be involved in the regulation of these two genes. Although GnRHAg stimulation of both FSHβ-subunit (43) and GnRHR genes (above) appears to be mediated through PKC, inhibition of PKC activity completely blocked GnRHAg-mediated stimulation of the GnRHR gene (above) but only partially blocked that of the FSHβ-subunit gene (43).

The single copy GnRHR gene is well conserved between the species, as its putative promoter sequence. Indeed, there appears to be 69–71% homology of the entire 1.2-kb 5′-flanking region among the mouse (13), rat (44), human (45, 46), and sheep genes (47). The sequence homology of SURG-1 and SURG-2 between various species (Fig. 10) shows a relatively high concordance between sequences in the mouse, rat, and human. The sheep GnRHR gene promoter is poorly characterized but does not appear to contain a consensus AP-1-binding site (47). It is likely that different mechanisms are involved in the GnRH-mediated activation of the GnRHR gene in different species. For example, the 5′-flanking region of the human GnRHR gene is far more complex than that of the other species (45, 46, 48). It is larger (~2.3 kb), contains multiple TSS, and numerous putative cis-regulatory sequences have been identified by sequence homology, including thyroid hormone-RE, glucocorticoid/progesterone-RE, cAMP-RE, PEA-3, AP-1, AP-2, and Pit-1 sites (46, 48).

The intracellular signal transduction pathways within pituitary gonadotropes, which are involved in regulating gonadotropin subunit and GnRHR gene transcription, are still not clearly described but likely include phosphoinositides, calcium, and cAMP as second messengers and/or mitogen-activated protein kinase cascades (see Ref. 2 for review). GnRH induction and basal regulation of the α-subunit gene seems to occur through the PKC/mitogen-activated protein kinase pathway, whereas induction of the LHβ gene is dependent on calcium influx (49). In this study, PMA stimulation of luciferase activity in αT3-1 cells transfected with −308/−264 of the mGnRHR gene was similar to that seen with GnRHAg (Fig. 9A). The addition of GF-109203X (Sigma), a inhibitor selective for the PKC isoforms α, β1, β2, γ, δ, and ε, resulted in complete abrogation of the response to either PMA or GnRHAg. Simultaneous stimulation with optimal doses of both PMA and GnRHAg resulted in an additive effect as compared with each agonist alone, which was similarly completely blocked by GF-109203X. These data suggest that both PMA and GnRHAg stimulation of the mGnRHR gene are mediated via PKC. The additive effect of PMA on GnRHAg stimulation implies either that these two agents act through different PKC isoforms or that they act
synergistically through the same PKC pathway. Whatever the mechanism, it is clear from the transfection data presented above that both agents act at least in part through the SURG-2 consensus sequence. Similar experiments were carried out using forskolin and 8-bromo-cAMP to investigate the role of the PKA signal transduction pathway in the GnRHAg response. Neither agonist was able to stimulate luciferase activity in αT3-1 cells transfected with −308/−264 nor were they able to influence GnRHAg-stimulated luciferase activity in such cells. Similarly, the addition of SQ 22351 (Sigma), a competitive inhibitor of adenylate cyclase, had no effect on GnRHAg stimulation (Fig. 9B). These findings were not unexpected given that no cAMP-response element-like sequence has been identified in the mGnRHR gene (13, 25), although there may be cAMP-response element-like elements in the rat and human GnRHR genes (44, 45). Taken together, these data suggest that the response of the mGnRHR gene to GnRHAg stimulation is mediated via PKC and not PKA. These observations are consistent with a number of previous reports suggesting that PKC and its activators increase GnRH binding activity in pituitary gonadotropes (50, 51) but in contrast with other studies in which phorbol esters did not affect levels of GnRH mRNA in αT3-1 cells, whereas forskolin decreased GnRH mRNA (20). Whether this discrepancy can be explained on the basis of post-transcriptional modification has yet to be determined. Although GnRH is known to induce levels of cAMP in gonadotropes both in vitro and in vivo (52, 53), GnRHAg stimulation of gonadotropin secretion appears to be independent of changes in cAMP (54). These data do not exclude the possibility that second messengers such as calcium and/or mitogen-activated protein kinase cascades may be involved in this response downstream of PKC. Indeed, the observations that both GnRH and PMA induce rapid increases in mRNA levels for primary response genes (including jun and fos) with a peak response at around 30 min (34), whereas maximal response of the mGnRHR gene to GnRHAg stimulation is achieved at around 4 h (above), suggest that a more complex intracellular signal transduction pathway may be involved.

While this paper was being completed, Lin and Conn (55) reported on transcriptional activation of the mGnRHR gene by GnRHAg and cAMP in GGH3 cells (GH3 cells stably expressing GnRHR). By using the same full-length mGnRHR promoter construct as that detailed above (13), the authors localized the major putative GnRH-RE(s) to the region −331/−255 (relative to the major TSS) of the mGnRHR gene promoter, which is in keeping with our data. In our studies, response to GnRHAg stimulation (100 nM for 4 h) ranged from 10- to 12-fold (Fig. 2–3) as compared with a 2-fold response to GnRHAg stimulation (Buserelin; 100 nM for 6 h) reported by Lin and Conn (55). This discrepancy may be accounted for by the use of different cell lines (αT3-1 cells and GGH3 cells, respectively) but is more likely due to differences in cell culture conditions. As demonstrated above (Fig. 2B), optimal response to GnRHAg stimulation was seen after 4 h transfection. Longer transfection times were associated with higher basal luciferase activity but a marked reduction in GnRHAg responsiveness. In the study by Lin and Conn (55), cells were transfected for 24 h. By using serial 5′-deletion constructs of the full-length (12 kb) mGnRHR gene promoter in transfection studies, the authors demonstrated a 1.5–2-fold response to both GnRHAg and dibutyryl-cAMP in the −255/+62 construct, which they suggested was statistically significant. Comparable studies detailed above (Fig. 3) demonstrated a similar 2.3–2.4-fold response to GnRHAg stimulation in the −232/+62 and −117/+62 constructs, but these measurements were not statistically different from pXP2 alone.

In summary, we have used deletion and mutational analysis as well as functional transfection studies in the murine gonadotrope-derived αT3-1 cell line to localized GnRH responsiveness of the mGnRHR gene to two DNA sequences at −276/−269 (SURG-2, the AP-1 consensus binding site) and −292/−285 (a novel element designated SURG-1), and we demonstrated that this response is mediated via PKC. By using EMSA, we further demonstrate that a member(s) of the Fos/Jun heterodimer superfamily is responsible for the DNA-protein complexes formed using αT3-1 nuclear extracts. These data define the dimeric GnRH-RE in the mGnRHR gene promoter and suggest that the AP-1 complex plays a central role in conferring GnRH responsiveness to the mGnRHR gene.

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