Direct Binding of AP-1 (Fos/Jun) Proteins to a SMAD Binding Element Facilitates Both Gonadotropin-releasing Hormone (GnRH)- and Activin-mediated Transcriptional Activation of the Mouse GnRH Receptor Gene*

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The response of pituitary gonadotropes 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 gene expression. Several factors are known to affect expression of the mouse GnRHR (mGnRHR) gene, including GnRH and activin. We have previously shown that activin augments GnRH-mediated transcriptional activation of mGnRHR gene, and that region −387/−308 appears to be necessary to mediate this effect. This region contains two overlapping cis-regulatory elements of interest: GnRH activating sequence (GRAS) and a putative SMAD-binding element (SBE). This study investigates the role of these elements and their cognate transcription factors in transactivation of the mGnRHR gene. Transfection studies confirm the presence of GnRH- and activin-response elements within −387/−308 of mGnRHR gene promoter. Competition electrophoretic mobility shift assay experiments using −335/−312 as probe and αT3–1 nuclear extract or SMAD, Jun, and Fos proteins demonstrate direct binding of AP-1 (Fos/Jun) protein complexes to −327/−322 and SMAD proteins to −329/−328. Further transfection studies using mutant constructs of these cis-regulatory elements confirm that both are functionally important. These data define a novel cis-regulatory element comprised of an overlapping SBE and newly characterized non-consensus AP-1 binding sequence that integrates the stimulatory transcriptional effects of both GnRH and activin on the mGnRHR gene.

A functional hypothalamic-pituitary-gonadal axis is critical to mammalian reproductive development and function. At the level of the anterior pituitary, GnRH1 binds to a specific, G protein-coupled, heptahelical receptor on the surface of pituitary gonadotropes, known as the GnRH receptor (GnRHR) (1, 2). Activation of these receptors stimulates intracellular signal transduction pathways to increase synthesis and release of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (3, 4). 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 is highly regulated, and is dependent primarily on the amplitude and frequency of pulsatile GnRH from the hypothalamus (5). The response of pituitary gonadotropes to GnRH correlates directly with the concentration of GnRHR on the cell surface, which is mediated in part at the level of GnRHR gene expression (6). Previous studies in this laboratory have identified and partially characterized the promoter region of the mouse GnRHR (mGnRHR) gene (7) and demonstrated that the regulatory elements for tissue-specific expression as well as for GnRH regulation are present within a 1.2-kb 5’-flanking region of the mGnRHR gene (designated −1164/+62 relative to the major transcriptional start site) (7, 8). Several factors are known to affect expression of the GnRHR gene, including GnRH (9–13) and activin (14–16). Recent studies out of this (13, 16) and other laboratories (15, 17) have begun to define how such factors interact at a cellular level to affect transcription of the mGnRHR gene. For example, GnRH-responsiveness of the mGnRHR gene has been localized to two distinct DNA elements: the consensus AP-1 binding site (5’-TGAGTCA-3’ at position −274/−268 and a novel enhancer element (5’-GCTAATTG-3’ at position −292/−285, designated Sequence Underlying Responsiveness to GnRH-1 (SURG-1) (13). More recently, we have shown that activin augments GnRH-mediated transcriptional activation of the mGnRHR gene and that region −387/−308 appears to be necessary to mediate this effect (16). This region contains two overlapping cis-regulatory elements of interest: GnRH Receptor Activating...

* The abbreviations used are: GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; AP-1, activating protein-1; ARF, activin-responsive factor; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GRAS, GnRH receptor-activating sequence; EMSA, electrophoretic mobility shift assay; mGnRHR, mouse GnRHR; RSV, Rous sarcoma virus; SBE, SMAD binding element; SURG-1, sequence underlying responsiveness to GnRH-1; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PI, propidium iodide; ANOVA, analysis of variance; Act-R, activin receptor.

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Sequence (5'-CTAATTACATAACA-3' (GRAS)) at position −329/−318 (15, 18) and a putative SMAD-binding element (5'-GTTCTAG(T/C-3') (SBE)) at position −331/−324 (19). This study was designed to investigate the role of these cis-regulatory elements and their cognate transcription factors in transcrip- tional activation of the mGnRHR gene.

In functional transfection studies using murine gonadotrope-derived oT3–1 cells, GnRH agonist stimulation of region −387/−308 of the mGnRHR gene promoter resulted in a significant 3.8-fold increase in activity, which was further increased 2.7-fold (to 10.4-fold) following activin treatment. Activin treat- ment alone increased promoter activity by 2.2-fold. Competition EMSA experiments using region −335/−312 of the mGnRHR gene promoter as probe and nuclear extract from oT3–1 cells or SMAD, Jun, and Fos proteins demonstrated direct binding of AP-1 (Fos/Jun) protein complexes to −327/−322 (5'-AGTCTAC-3') and SMAD proteins to −329/−328 (5'-CT-3'). Further transfection studies using mutant constructs of these cis-regulatory elements in −387/−308 demonstrate that disruption of either complex eliminated both GnRH and activin responsiveness of this region. These data demonstrate that both GnRH- and activin-mediated transcriptional activation of the mGnRHR gene are mediated, at least in part, by direct binding of AP-1 (Fos/Jun) and SMAD protein complexes to the overlapping GRAS/SBE element of the mGnRHR gene promoter.

**EXPERIMENTAL PROCEDURES**

**Materials—Des-Gly^7-[D-Ala]^4-GnRH-ethylamide (GnRH agonist) was obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human activin A and follistatin were a gift of Dr. A. F. Parlow and the National Hormone and Pituitary Program (Minneapolis, MN). Anti-SMAD3 and anti-SMAD4 antibodies were obtained from Zymed Laboratories, Inc. (San Francisco, CA). Biotinylated anti-rabbit IgG was obtained from Vector Laboratories, Inc. (Burlingame, CA). All oligonucleotides were prepared by Invitrogen (Gaithersburg, MD). oT3–1 and LTF2 cells were generously provided by Dr. Pamela Mellon (University of California-San Diego, San Diego, CA).

**Reporter Plasmids and Expression Vectors—A fusion construct was prepared by ligation of the 1.2 kb 5'-flanking region of the mGnRHR gene (designated −1164/+62) into the luciferase reporter plasmid, pXP2, as previously described (7, 13). The nucleotide sequence of the mGnRHR gene promoter used in these studies is based on previous work in this laboratory (7), with −1 assigned to the nucleotide immediately 5' of the major transcriptional start site. Polymerase chain reaction (PCR)-generated fragments of the mGnRHR gene promoter were synthesized using selected sense/antisense primers with the −1164/+62 construct as a template, placed in control of the rat growth hormone gene minimal promoter (GH [−358/−328]) designated GHclus, and inserted upstream of the luciferase reporter in pXP2, as previously described (7, 13). The nucleotide sequence of the mGnRHR gene promoter used in these studies is based on previous work in this laboratory (7), with −1 assigned to the nucleotide immediately 5' of the major transcriptional start site. Polymerase chain reaction (PCR)-generated fragments of the mGnRHR gene promoter were synthesized using selected sense/antisense primers with the −1164/+62 construct as a template, placed in control of the rat growth hormone gene minimal promoter (GH [−358/−328]) designated GHclus, and inserted upstream of the luciferase reporter in pXP2, as previously described (13). These constructs were designated GHclus/−358/-328 and GHclus/−387/-308. Two 2-bp mutants of the putative SBE/GRAS element were prepared in the GHclus/−387/-308 construct of the mGnRHR gene promoter using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with selected sense and antisense mutant oligonucleotides. These mutant constructs were designated GHclus/−358/-308/GRAS-3 (AG replacement of CT at −329/−330), GHclus/−358/-308/GRAS-4 (CT replacement of AG at −327/−328) (refer to Fig. 5A below). An expression vector expressing β-galactosidase driven by the Rous sarcoma virus promoter (RSV-β-galactosidase) was used as an internal standard and control. SMAD2, SMAD3, and SMAD4 expression vectors in pCS2 and A3-Luc-pGL2 (3' activin response element (GTCT) in pGL2) were gifts of Dr. Malcolm Whitman (Harvard Medical School, Boston, MA) (20). The identity of all reporter constructs was confirmed by sequencing using the dyeoxynucleotide chain termination method.

**Cell Culture and Transient Transfection—** oT3–1 (mouse gonado- trope) cells were maintained in monolayer culture in high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (Invitrogen) at 37 °C in humidified 5% CO_2/95% air. For transient transfection studies, cells were divided into six-well culture plates and cultured overnight in DMEM in the absence of serum or antibiotics. Under these condi- tions, cells were 60–80% confluent. Cells were then transfected with reporter plasmids containing mGnRHR gene promoter constructs of interest by calcium phosphate co-precipitation, as previously described (13). Following transfection, cells were incubated for 48 h and treated with or without activin A (20 ng/ml) for the last 20 h of the 48 h transfection time period, and the response to GnRH agonist stimulation (100 nM for 4 h) was measured. Prior studies in this laboratory have shown that the presence or absence of activin during the 4-h GnRH agonist stimulation did not affect the response to GnRH agonist stimu- lation (16). As such, activin was not added during this incubation. The final concentration of activin A used in these experiments was based on prior studies demonstrating that 20 ng/ml is sufficient to mediate an effect on the mGnRHR gene promoter in transient transfection experi- ments (13, 14, 16).

**Identification of Endogenous SMAD Proteins by Fluorescence Immuno- cytochemistry—** We have used fluorescence immunocytochemistry to confirm expression of SMAD2 and SMAD3 in both oT3–1 and LTF2 mouse gonadotrope-derived cell lines under basal conditions and to investigate the effect of activin and GnRH treatment on SMAD expres- sion. Cells were cultured on coverslips containing 2% fetal calf serum to 40–60% confluence on glass cover slips placed in six-well tissue culture plates. Cells were treated with activin A (20 ng/ml), GnRH agonist (100 nm), or vehicle for 4, 24, or 48 h. Thereafter, cells were fixed with 4% paraformaldehyde in PBS prior to immunofluorescence staining as previously described (21). Briefly, after incubation with PBS containing 10% goat serum for 1 h at 4 °C in a humidified chamber, specific rabbit polyclonal antisera against SMAD2 or SMAD3 (final concentration 5 mg/ml (Zymed Laboratories)) were applied to the cells and incubated overnight at 4 °C. Thereafter, cells were washed in PBS, and biotinylated anti-rabbit IgG (1:200 dilution, Vector Laboratories) was applied for 1 h at 4 °C followed by fluorescein avidin D cell sorter (1:200 dilution, Vector Laboratories) for a further 1 h at 4 °C. After washing with PBS, cells were incubated with propidium iodide (PI, 0.5 mg/ml in PBS) for 5 min at room temperature to stain the nuclei. Finally, cells were washed with distilled water, and the cover slips were mounted onto glass slides using Vectashield mounting medium (Vector Laboratories). Negative controls used antisera pre-absorbed with SMAD2 or SMAD3 peptide (R&D Systems, 50 mg/ml) for 2 h. Rat granulosa cells were used as a positive control (21). Slides were visualized using a Leica DMBRE microscope outfitted with a light/darkfield and 510- and 590-nm fluorescence filters, and images were overlaid using KS300 computer software (DFI Technologies, Inc., Sacramento, CA).

**Effect of SMAD Overexpression on Activin- and GnRH-mediated Transcriptional Activation of the mGnRHR Gene Promoter—** To investigate the effect of SMAD overexpression on transcriptional activation of the mGnRHR gene promoter, expression vectors encoding wild-type or SMAD mutants were transiently transfected into oT3–1 cells. Cells were transfected with GHclus/−358/−328 (4 μg/well) plus SMAD3 and SMAD4 expression vectors or pCS2 alone (4 μg/well) for 48 h and treated with or without activin A (20 ng/ml for the last 20 h of the 48 h transfection) and/or GnRH agonist (100 nm for 48 h after the 48 h transfection).
transfection). To confirm that the SMAD proteins overexpressed in oT3–1 cells were able to exert a functional effect, the SMAD expression vectors were each co-transfected with a reporter construct (A3-Luc-pGL2) known to be responsive to SMAD3 and SMAD4 (20).

**Preparation of Nuclear Extracts**—oT3–1 cells were grown to 60–80% confluence and treated with GnRH agonist (100 nM), activin A (20 ng/ml), or vehicle for 1, 4, or 24 h. Thereafter, cells were harvested, and nuclear extracts were prepared by the method of Andrews and Fuller (22).

**Preparation of AP-1 and SMAD Proteins**—Purified c-Jun protein was obtained from Promega (Madison, WI). Because purified c-Fos proteins were not commercially available, the cDNA sequences encoding c-Fos, FosB, Fra-1, and Fra-2 were isolated and amplified by reverse transcription-PCR from RNA prepared from oT3–1 cells and subcloned into pCS2 expression plasmids, and proteins were prepared by in vitro translation using the T7T Coupled Reticulocyte Lysate Systems kit (Promega) according to the manufacturer’s protocol. In vitro translated SMAD2, SMAD3, and SMAD4 proteins were similarly prepared using the same SMAD-pCS2 expression plasmids as those used in the transfection studies. The identity of the inserts in each of the pCS2 expression vectors was confirmed by DNA sequencing using the dideoxynucleotide chain termination method (data not shown). The presence and identity of the in vitro translated proteins were confirmed by preparing 35S-labeled controls with each in vitro translation experiment, visualizing the resultant protein on a 12% gel, and verifying the size of the protein using standard size markers (data not shown).

**Electrophoretic Mobility Shift Assay**—Probes were prepared for EMSA by annealing of complementary oligonucleotides representing selected regions of the mGnRHR gene promoter, followed by 5′-end labeling with [γ-32P]ATP (PerkinElmer Life Sciences, Boston, MA) by T4 polynucleotide kinase (New England BioLabs, Inc., Beverly, MA). The binding reaction for EMSA was performed by incubating 50,000 cpm of DNA probe with 5 μg of nuclear extract and 2 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. On occasion, EMSA experiments were performed using purified c-Jun protein (Promega) and/or in vitro translated c-Fos, FosB, Fra-1, Fra-2, SMAD2, SMAD3, or SMAD4 proteins in place of nuclear extract. For competition studies, excess unlabeled (cold) DNA was added 5 min prior to the addition of probe. Oligonucleotides used for competition experiments included regions −261 to −35 (T3) (see Fig. 5A), −335 to −312 (T3−1) (the SBE/GRAS element, 5′-ATGTGCTCTAGGCATAACAGAATCAACAACG-3′ (see Fig. 5A)), −335 to −312/GRAS-1 through −335 to −312/GRAS-9 (see Fig. 5A), −281 to −261 (AP-1) of mGnRHR gene promoter, and CE3 (5′-GGCTCGGTTTTTATTGCT-3′) as an unrelated sequence. Protein-DNA complexes were resolved on 5% 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). The gels were then dried for 1 h and subjected to autoradiography for 24–48 h.

**Statistical Analysis**—Transfections were performed in triplicate and repeated multiple times. Data in each experiment were expressed as luciferase/β-galactosidase activity. Data were combined across experiments, and the results were expressed as means ± S.E. for basal and activin agonist- and/or activin-stimulated activities for each construct and -fold stimulation in response to agonist 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 agonist and/or activin 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**

**Effect of Activin on Transcriptional Activation of the mGnRHR Gene Promoter**—We have previously shown that activin A augments GnRH-mediated transcriptional activation of the mGnRHR gene promoter in oT3–1 cells (16). In these experiments, however, activin A alone had no effect (16). We have attributed this to the transfection paradigm used in these prior experiments (viz. activin A treatment at a final concentration of 20 ng/ml for 20 h followed by a 4-h calcium-phosphate transfection and a 4-h treatment with 100 nM GnRH agonist). To investigate further the effect of activin on transcriptional activation of the mGnRHR gene promoter, we chose to modify our transient transfection conditions. A transfection time course using region −387 to −308 of the mGnRHR gene promoter upstream of the GH50 heterologous promoter in pXP2-Luc demonstrated a significant response to 20 ng/ml activin A treatment at 48 h (1.8 ± 0.2-fold; p < 0.05, ANOVA), whereas the responses at 4 h (1.3 ± 0.2-fold) and 20 h (1.2 ± 0.3-fold) were not significantly different from GH50−/−/−387 to −308 control (Fig. 1A). This time course is consistent with prior studies from our laboratory, demonstrating maximal stimulation of GnRH mRNA levels by activin at 36 h in oT3–1 cells (14). Similar results were observed with region −387 to −264 of the mGnRHR gene promoter, although the magnitude of the response to GnRH agonist was greater, because this region contains both the −387 to −264 region of interest as well as the previously described bipartite GnRH-response element, AP-1 and SURG-1 (13) (data not shown). No difference was noted between treatment with activin A (National Hormone and Pituitary Program) or activin B (R&D Systems) (data not shown). In light of these data and prior studies showing no difference between 20 and 50 ng/ml activin A treatment (16), all subsequent experiments were performed using a transfection time of 48 h and activin A at a final concentration of 20 ng/ml for the last 20 h of the transfection.

In functional transfection studies using oT3–1 cells and a transfection time of 48 h, GnRH agonist stimulation of GH50−/−387 to −308 resulted in a 3.8 ± 0.2-fold increase in luciferase activity as compared with vector alone (p < 0.0001, ANOVA), which was further increased 2.7-fold (to 10.4 ± 1.6-fold) following activin treatment. Activin treatment alone significantly increased promoter activity by 2.2 ± 0.2-fold (p < 0.05, ANOVA) (Fig. 1B). The addition of follistatin (100 ng/ml) to the activin-containing treatment mixture for the final 20 h of the 48-h transfection followed by GnRH agonist stimulation (100 nM for 4 h) resulted in complete abrogation of the activin response and of the synergistic response to GnRH agonist and activin. Follistatin also significantly decreased basal luciferase activity (Fig. 1B). However, the fold response to GnRH agonist alone was unaffected (Fig. 1B). These data suggest that the activin response of the mGnRHR gene promoter as well as the synergistic response to GnRH-agonist and activin is mediated by activin-activin receptor binding, a process that can be inhibited by follistatin.

**Effect of SMAD Overexpression on Activin- and GnRH-mediated Transcriptional Activation of the mGnRHR Gene Promoter**—Because activin acts primarily by activating activating-responsive SMAD transcription factors (viz. SMAD2, SMAD3, and SMAD4) in the cytoplasm of target cells, we chose to investigate the effect of SMAD overexpression on activin- and GnRH agonist-mediated transcriptional activation of the mGnRHR gene. All SMAD proteins were shown to be functionally active by demonstrating a significant increase in luciferase activity in oT3–1 cells transfected with SMAD expression vectors and the A3-Luc-pGL2 reporter plasmid (data not shown), which is known to be responsive to SMAD2, SMAD3, and SMAD4 (20). We have previously shown that overexpression of SMAD2 or SMAD3 along with SMAD4, but not overexpression of any one of the SMAD proteins alone, significantly increased transcriptional activation of the GH50−/−387 to −264 construct of the mGnRHR gene promoter (16). Whether this is true also of region −387 to −308 of the mGnRHR gene promoter has not previously been investigated.

Consistent with the data presented in Fig. 1 (A and B), activin and GnRH agonist treatment of oT3–1 cells transiently transfected with the GH50−/−387 to −308 construct of the mGnRHR gene promoter and pCS2 plasmid resulted in a significant 2.2 ± 0.3-fold and 3.2 ± 0.4-fold increase in luciferase activity, respectively.
**AP-1 and SMAD Proteins Bind to SBE in mGnRHR Gene Promoter**

Effect of Activin and GnRH Treatment on SMAD Expression in Mouse Pituitary Gonadotrope Cells—Prior studies have shown that mouse gonadotrope cell lines are able to respond to stimulation with exogenous activin (14–16). Although the ability of such cells to respond to activin suggests that they likely express activin-responsive SMAD transcription factors (viz. SMAD2, SMAD3, and SMAD4), to our knowledge this has not previously been demonstrated. Using fluorescence immunocytochemistry, we have confirmed the presence of endogenous SMAD3 (Fig. 2) and SMAD2 (data not shown) in aT3–1 cells. Negative controls using anti-SMAD antisera pre-absorbed with SMAD peptide showed no binding (data not shown). Rat granulosa cells were used as a positive control (data not shown) (see Ref. 21). Moreover, computer-generated overlay images demonstrated that SMAD3 and SMAD2 are present predominantly in the cytoplasm under basal conditions and that stimulation with activin resulted in nuclear translocation of SMAD proteins. This was evident after 4 h of activin stimulation (Fig. 2) but persisted up to 48 h of treatment (data not shown). Surprisingly, some nuclear translocation of SMAD proteins could also be seen after GnRH agonist stimulation for 4 h (but not at 20 or 48 h), although to a lesser extent. There was no obvious increase in the intensity of staining with the anti-SMAD antisera after treatment with exogenous activin (14–16) and GnRH agonist was maintained in the setting of SMAD3 and SMAD4 overexpression (at 2.3 ± 0.6-fold and 3.4 ± 0.9-fold, respectively). Interestingly, the magnitude of the synergistic response to both activin and GnRH agonist in the setting of SMAD3 and SMAD4 overexpression was significantly increased by 7.8 ± 1.1-fold as compared with GnRH agonist alone (to 26.4-fold compared with control (Fig. 1C)). These data demonstrate that the response of the mGnRHR gene promoter to activin and GnRH agonist stimulation can be further augmented by overexpression of SMAD transcription factors.

**Identification and Characterization of Transcription Factors Binding to the SBE/GRAS Element by EMSA—Using nuclear extracts.**

respectively. Moreover, concurrent activin treatment resulted in a 3.1-fold augmented response to GnRH agonist stimulation (Fig. 1C). However, combined overexpression of SMAD3 and SMAD4 resulted in a significant 10.1-fold increase in basal activity of the GH50/–387/–308 construct as compared with pCS2 overexpression alone (Fig. 1C). Despite the increase in basal activity, the response of this construct to treatment with activin or GnRH agonist was maintained in the setting of SMAD3 and SMAD4 overexpression (at 2.3 ± 0.6-fold and 3.4 ± 0.9-fold, respectively). Interestingly, the magnitude of the synergistic response to both activin and GnRH agonist in the setting of SMAD3 and SMAD4 overexpression was significantly increased by 7.8 ± 1.1-fold as compared with GnRH agonist alone (to 26.4-fold compared with control (Fig. 1C)).

**Fig. 1. Effect of activin and GnRH agonist on transcriptional activation of –387/–308 of the mGnRHR gene.** A, to optimize the transfection experimental paradigm, aT3–1 cells were transfected with pXP2-GH50 (designated GH50) or pXP2-GH50/–387/–308 (designated GH50/–387/–308) for 4, 20, or 48 h. Cells were treated with or without activin A (20 ng/ml for 20 h), GnRH agonist (100 nM for 4 h), or both as described. Measurements are expressed as luciferase/β-galactosidase. Results are mean ± S.E. from four separate experiments. Response to agonist stimulation is shown. *p < 0.05 compared with GH50 and controls within each group. **p < 0.01 compared with all controls. †p < 0.05 compared with control. ‡p < 0.0001 compared with all other groups. B, aT3–1 cells were transfected with pXP2-GH50 or pXP2-GH50/–387/–308 for 48 h. Cells were treated with or without activin A (20 ng/ml for 20 h), GnRH agonist (100 nM for 4 h), or both as described. Experiments were carried out in the presence or absence of follistatin (100 ng/ml for 20 h). Measurements are expressed as luciferase/β-galactosidase. Results are mean ± S.E. from four separate experiments. Response to agonist stimulation is shown. *p < 0.05 compared with pXP2-GH50 control (with and without follistatin) and pXP2-GH50/–387/–308 control with follistatin. †p < 0.05 compared with pXP2-GH50 (with and without follistatin) as well as pXP2-GH50/–387/–308 control and activin alone (with and without follistatin). #p < 0.05 compared with all other reactions. *p < 0.0001 compared with all other reactions. C, to investigate the effect of SMAD overexpression on activin- and GnRH-mediated transcriptional activation of the mGnRHR gene, expression vectors encoding SMAD3 and SMAD4 or pCS2 control were transfected into aT3–1 cells along with GH50/–387/–308 for 48 h. Cells were treated with activin A (20 ng/ml for 20 h), GnRH agonist (100 nM for 4 h), or both as described. Measurements are expressed as luciferase/β-galactosidase. Results are mean ± S.E. from multiple experiments. *p < 0.05 compared with control and activin A alone within each group. †p < 0.05 compared with control within each group. ‡p < 0.03 compared with all other reactions within each group. **p < 0.0001 compared with control in GH50/–387/–308 + pCS2.
extracts from αT3-1 cells and 32P-end-labeled −335/−312 of the mGnRHR gene promoter as probe, two protein-DNA complexes could be identified in EMSA that were not present with probe alone (Fig. 3A). GnRH agonist treatment of αT3-1 cells prior to preparation of nuclear extract resulted in an increase in intensity in the existing bands and in the appearance of two additional bands that were evident after 1 h and were absent after 24 h of GnRH agonist stimulation. The intensity of all four bands seen on EMSA using nuclear extracts from αT3-1 cells treated with GnRH agonist for 1 h were diminished in competition EMSA experiments using 500-fold excess unlabeled (cold) −335/−312 probe (SBE/GRAS) but not with unrelated sequences (CE3), confirming the specificity of the binding (Fig. 3A). No competition EMSA studies using unlabeled −281/−261 (containing the AP-1 binding site) suggest that the lower two bands may represent AP-1 (Fos/Jun) protein complexes (Fig. 3A). This observation is consistent with EMSA supershift experiments that clearly demonstrate, using an anti-Fos antibody (Santa Cruz Biotechnology), a supershifted complex arising from one or both of the lower two bands (16).

Similar EMSA experiments using nuclear extracts from αT3-1 cells treated with activin and region −335/−312 of the mGnRHR gene promoter as probe again demonstrated two protein-DNA complexes that were not present with probe alone (Fig. 3B). These complexes appeared to increase in intensity with activin treatment for 1 h. Interestingly, activin treatment for 24 h resulted in the appearance of an additional band (designated by the large arrow in Fig. 3B).

Competition and supershift EMSA experiments suggesting that the lower two protein-DNA complexes binding to region −335/−312 of the mGnRHR gene promoter may represent AP-1 (Fos/Jun) protein complexes (above) do not confirm direct binding to DNA. Similar results would be expected if Fos/Jun proteins were serving as co-activators to promote transcription of the mGnRHR gene without binding DNA directly. We therefore performed EMSA experiments using purified c-Jun protein (Promega) and/or in vitro translated c-Fos, FosB, Fra-1, and Fra-2 proteins with 32P-end-labeled −335/−312 of the mGnRHR gene promoter as probe (Fig. 4). Direct binding of c-Jun is evident and likely represents a c-Jun/c-Jun homodimer. Because Fos proteins are not able to bind to DNA as monomers or homodimers, no protein-DNA complexes were evident when in vitro translated Fos proteins were added alone. However, the addition of c-Jun along with Fos proteins demonstrated direct binding of Fos/Jun proteins to region −335/−312 of the mGnRHR gene promoter. Closer examination reveals the presence of two separate protein-DNA complexes; the upper (weaker) complex likely represents a c-Jun/c-Jun homodimer and the lower (stronger) complex likely represents a c-Jun/Fos heterodimer (Fig. 4).

Localization of Transcription Factor Binding to the SBE/GRAS Element by EMSA—To further characterize and localize transcription factor binding, additional EMSA experiments were performed using purified c-Jun (Promega) and in vitro translated FosB, c-Fos, Fra-1, and Fra-2 proteins and 32P-end-labeled −335/−312 of the mGnRHR gene promoter as probe (Fig. 5B). Serial 2-bp mutant oligonucleotides of region −335/−312 (designated μGRAS-1 through -6 in Fig. 5A) were used for cold competition. Reticulocyte lysate control showed several minor nonspecific bands that were not present in probe alone. Consistent with data presented in Fig. 4, in vitro translated FosB alone was unable to bind region −335/−312 of the mGnRHR gene promoter but did bind in the presence of c-Jun, likely as a c-Jun/FosB heterodimer (Fig. 5B). Competition with excess unlabeled mutant oligonucleotides of −335/−312 demonstrated direct binding of this protein complex to the region defined by μGRAS-4, -5, and -6, which corresponds to −327/−322 (5′-AGTCAC-3′) of the mGnRHR gene promoter. Direct binding of other AP-1 protein complexes (viz. c-Jun/c-Fos, c-Jun/Fra-1, and c-Jun/Fra-2) were localized to the same region (data not shown).

Similar EMSA experiments were performed using in vitro translated SMAD3 in place of the purified Fos/Jun proteins (Fig. 5C). Again, reticulocyte lysate control showed several nonspecific bands that were not present in probe alone. Fig. 5C demonstrates direct binding of in vitro translated SMAD4 to region −335/−312 of the mGnRHR gene promoter. Moreover, competition with excess unlabeled mutant oligonucleotides of −335/−312 demonstrated direct binding of this protein complex to the region defined by μGRAS-3, although some binding to the regions defined by μGRAS-2 and μGRAS-4 was also evident (Fig. 5C). These mutants correspond to region −331/−326 (5′-GTCTAG-3′) of the mGnRHR gene promoter. Similar EMSA experiments demonstrated direct binding of SMAD3, but not SMAD2, to region −335/−312 of the mGnRHR gene promoter, and localized SMAD3 binding to the same sequence (data not shown). These experiments provide definitive evidence for direct binding of purified SMAD4 and SMAD3 to a...
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Experiments were performed using 32P-end-labeled arrows and that were not present in probe alone (lanes 3–5) for 1 h (lane 3), 4 h (lane 4), or 24 h (lane 5) prior to preparation of nuclear extract revealed further DNA-protein complexes (arrows 3 and 4) that were apparent after 1 h of GnRH agonist treatment but which disappeared with prolonged treatment. Specific binding was confirmed by competition with 500-fold excess unlabeled (cold) 335/-312 probe (lane 6) but not unrelated sequence (CE3, lane 6). Competition with unlabeled AP-1 consensus binding sequence diminished the intensity of the lower two (but not upper two) bands (lane 7). B, similar EMSA experiments were performed using 32P-end-labeled 335/-312 as probe and αT3-1 nuclear extracts without (lanes 2) and with activin A treatment (20 ng/ml) for 1 h (lanes 3), 4 h (lane 4), or 24 h (lane 5). Two protein-DNA complex bands were again identified using αT3-1 nuclear extracts (lane 2) that were not present in probe alone (lane 1) and are designated by arrows 1 and 2. Treatment of αT3-1 cells with GnRH agonist (100 nM for 1 h) with 32P-end-labeled μGRAS oligonucleotides (Fig. 5A) as probe. Mutation of the 2-bp regions 329/-328 (designated 335/-312/μGRAS-3) and 327/-326 (335/-312/μGRAS-4) showed complete elimination of the upper and middle two protein-DNA complexes, respectively (data not shown). Taken together, these data suggest that the uppermost protein-DNA complex formed after GnRH agonist treatment appears to contain SMAD3 and SMAD4 proteins, and that the middle protein-DNA complexes likely contain Fos/Jun proteins.

Confirmation of the Functional Importance of the SMAD and AP-1 Binding Sites within Region −387/−308 of the mGnRHR Gene Promoter—To investigate the functional importance of the SMAD and AP-1 binding sequences identified above, further EMSA experiments were performed using αT3-1 nuclear extract without and with GnRH agonist treatment (100 nM for 1 h) with 32P-end-labeled μGRAS oligonucleotides (Fig. 5A) as probe. Mutation of the 2-bp regions 329/-328 (designated 335/-312/μGRAS-3) and 327/-326 (335/-312/μGRAS-4) showed complete elimination of the upper and middle two protein-DNA complexes, respectively (data not shown). Taken together, these data suggest that the uppermost protein-DNA complex formed after GnRH agonist treatment appears to contain SMAD3 and SMAD4 proteins, and that the middle protein-DNA complexes likely contain Fos/Jun proteins.

Further competition EMSA experiments were performed again using region 335/-312 of the mGnRHR gene promoter as probe but with αT3-1 nuclear extract in place of SMAD or Jun/Fos proteins (Fig. 5D). Competition with excess unlabeled mutant oligonucleotides of region 335/-312 demonstrated binding of the upper protein-DNA complex to the region defined by μGRAS-3 and of the middle protein-DNA complexes to the region defined by μGRAS-4, -5, and -6 (Fig. 5D). These binding sites correspond to regions −329/-328 (5′-CT-3′) and −327/-322 (5′-AGTCAC-3′) of the mGnRHR gene promoter, respectively. The lower complex is competed by all mutant constructs, although some residual binding is evident in the lanes representing competition with μGRAS-7 and μGRAS-8 (Fig. 5D). To confirm the importance of the SMAD and AP-1 binding sequences identified above, further EMSA experiments were performed using αT3-1 nuclear extract with and without GnRH agonist treatment (100 nM for 1 h) with 32P-end-labeled μGRAS oligonucleotides (Fig. 5A) as probe. Mutation of the 2-bp regions 329/-328 (designated 335/-312/μGRAS-3) and 327/-326 (335/-312/μGRAS-4) showed complete elimination of the upper and middle two protein-DNA complexes, respectively (data not shown). Taken together, these data suggest that the uppermost protein-DNA complex formed after GnRH agonist treatment appears to contain SMAD3 and SMAD4 proteins, and that the middle protein-DNA complexes likely contain Fos/Jun proteins.
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**Fig. 5.** Localization of AP-1 (Fos/Jun) and SMAD binding within region -335/-312 of the mGnRHR gene promoter. A. To further characterize and localize transcription factor binding, serial 2-bp mutant oligonucleotides of region -335/-312 of the mGnRHR gene promoter were prepared as shown. B, competition EMSA experiments were performed using purified c-Jun protein (Promega) and/or in vitro translated FosB with 32P-end-labeled -335/-312 of the mGnRHR gene promoter as probe. Reticulocyte lysate control (lane 2) showed several minor nonspecific bands that were not present in probe alone (lane 1). As demonstrated in Fig. 4, FosB will bind in the presence of c-Jun (designated by an arrow in lane 4) but not in the absence of Jun protein (lane 3). Competition with 500-fold excess unlabeled (cold) mutant oligonucleotides of region -335/-312 (designated μGRAS-1 through -9) demonstrated that only μGRAS-4, -5, and -6 failed to bind μGRAS-1 to the DNA-protein complex. C, similar competition EMSA experiments were performed using in vitro translated SMAD4. Reticulocyte lysate control (lane 2) again showed several nonspecific bands that were not present in probe alone (lane 1). Specific SMAD4 binding is designated by an arrow (lane 3). Competition with 500-fold excess unlabeled mutant oligonucleotides of -335/-312 (designated μGRAS-1 through -9) demonstrated that μGRAS-3 failed to compete for the DNA-protein complex, although a small amount of SMAD4 binding to GRAS-2 and -4 was also evident. D, to localize binding of αT3-1 nuclear proteins to region -335/-312 of the mGnRHR gene promoter, EMSA experiments were performed using αT3-1 nuclear extract with and without GnRH agonist treatment (100 nM for 1 h) and 32P-end-labeled -335/-312 of the mGnRHR gene promoter as probe. Competition with 500-fold excess unlabeled (cold) mutant oligonucleotides of -335/-312 of the mGnRHR gene promoter (designated μGRAS-1 through -9) demonstrated that μGRAS-3 failed to compete for the upper protein-DNA complexes (designated arrows 3 and 4), whereas μGRAS-4, -5, and -6 failed to compete for the middle protein-DNA complex (designated arrow 2).

**Fig. 6.** Confirmation of the functional importance of the SBE/GRAS sequence on transcriptional activation of the mGnRHR gene promoter. Mutation constructs of region -335/-308 of the mGnRHR gene promoter in pXP2-GH50 were transfected into αT3-1 cells for 48 h. Cells were stimulated with or without activin A (20 ng/ml for 20 h), GnRH agonist (100 nM for 4 h), or both as described. Measurements are expressed as luciferase/β-galactosidase. Results are mean ± S.E. from multiple separate experiments. Response to agonist stimulation is shown. *, p < 0.05 compared with all reactions in GH50, GH50/GRAS-3, and GH50/GRAS-4. **, p < 0.03 compared with GH50/GRAS-3. †, p < 0.05 compared with GH50/GRAS-3. ‡, p < 0.001 compared with all other reactions.

data demonstrate that both the SMAD and AP-1 binding sequences within -335/-312 are functionally important for basal, GnRH-stimulated, and activin-stimulated transcriptional activity.

**DISCUSSION**

The mGnRHR gene (24–26) as well as its promoter (7, 8, 27) have been isolated and characterized. We have previously shown that activin augments GnRH-mediated transcriptional activation of the mGnRHR gene and that region -335/-308 of the 1.2 kb 5'-flanking sequence appears to be necessary to mediate this effect (16). This region contains two overlapping cis-regulatory elements of interest: GRAS at -329/-318 (15, 26) as well as its promoter (7, 8, 27).
and a putative SBE by sequence homology at −331/−324 (19). In this study, we demonstrate that activin and GnRH act both individually and synergistically through −387/−308 of the promoter to activate mGnRHR gene expression. We further demonstrate direct binding of the activin-responsive SMAD transcription factors (SMAD3 and SMAD4) and of AP-1 (Fos/Jun) protein complexes to −335/−312 of the mGnRHR gene promoter and localize this binding to −329/−328 (5′-CT-3′) and −327/−322 (5′-AGTCAC-3′), respectively. Further functional studies in αT3-1 cells demonstrate that both of these cis-regulatory elements are functionally important for basal, GnRH-stimulated, and activin-stimulated transcriptional activity. These data confirm the presence of an SBE and define a novel AP-1 cis-regulatory element in the mGnRHR gene promoter. Activin, a member of the transforming growth factor-β superfamily (28, 29), acts by binding directly to activin receptor II (Act-RII), a serine-threonine kinase, on the cell surface, thereby increasing association with Act-RI. Formation of this complex leads to phosphorylation of Act-RI, followed by activation and nuclear translocation of cytoplasmic SMAD transcription factors, where they bind to DNA through a defined SBE (5′-GTCTAG(NC)C-3′) (19) and act, either alone or in combination with other factors, to regulate gene transcription (30). Follistatin refers to a family of highly glycosylated polypeptides, structurally unrelated to activin, that act primarily as activin-binding proteins and inhibit activin action by preventing interaction between activin and Act-RII (31, 32). Pituitary gonadotropes and gonadotrope cell lines secrete activin and follistatin (33, 34), and both αT3-1 and LβT2 mouse gonadotrope cell lines are known to express activin receptors and to respond to stimulation with exogenous activin (14, 16, 34–36). GnRH stimulation of ovine FSHβ and rat LHβ gene promoters in LβT2 cells is inhibited by follistatin (36), and treatment with an activin-blocking antibody decreases FSH secretion (37, 38), suggesting that the GnRH response in these cells may depend on endogenously produced activin. Taken together, these data suggest that activin and follistatin play an important role in regulating gonadotropin and GnRHR gene expression.

Functional transfection studies were carried out in αT3-1 cells, a well characterized mouse pituitary gonadotrope cell line (39) that has been shown to be a useful model for the study of GnRHR gene expression (13, 15–17). In contrast to prior studies that used a 4-h transfection time (13, 16), current studies used a 48-h transfection time so as to optimize the response of region −387/−308 of the mGnRHR gene promoter to activin stimulation (Fig. 1A). These data are consistent with the known response of the GnRHR gene to activin stimulation in αT3-1 cells (14). Follistatin treatment completely abrogated the effect of activin on mGnRHR gene expression as well as the augmented response to GnRH agonist stimulation in the presence of activin (Fig. 1B). Moreover, in keeping with prior studies in LβT2 cells (36), treatment of αT3-1 cells with follistatin diminished basal mGnRHR gene expression (Fig. 1B), suggesting that endogenous activin may be important for mGnRHR gene expression. These results were not unexpected, because this region had previously been shown to be necessary for the ability of activin to augment GnRH-mediated transactivation in this gene (16) and because it contains the GRAS sequence, which is known to be an activin response element (15). What was unexpected, however, was that this region also appeared to contain one or more GnRH-response elements. GnRH agonist stimulation of −387/−308 resulted in a 3.8-fold increase in activity, which was further increased 2.7-fold (to 10.4-fold) following activin treatment (Fig. 1B). Moreover, the synergy between activin and GnRH agonist on mGnRHR gene expression was further augmented by overexpression of SMAD3 and SMAD4 proteins (Fig. 1C), suggesting that this synergy likely involves the SMAD signal transduction pathway. This statement is supported also by immunocytochemical studies demonstrating nuclear translocation of cytoplasmic SMAD3 (Fig. 2) and SMAD2 (data not shown) with activin and, unexpectedly, also with GnRH agonist treatment.

To characterize further the GnRH-response element(s) within −387/−308, EMSA experiments were performed using αT3-1 nuclear extracts. GnRH agonist treatment of αT3-1 cells prior to preparation of nuclear extract resulted in an increase in intensity of the existing protein-DNA complexes and in the appearance of two additional complexes that were most intense after 1 h of GnRH agonist stimulation. The identification of GnRH agonist-responsive and -induced protein-DNA bands suggests that GnRH acts through one or more cis-regulatory elements in this region. Competition EMSA experiments with unlabeled AP-1 consensus sequence (Fig. 3A) as well as previously reported EMSA supershift experiments using anti-Fos antibody (16) suggest that AP-1 (Fos/Jun) proteins are present in the DNA-protein complexes binding to −387/−308. Interestingly, EMSA experiments using nuclear extract from αT3-1 cells treated with activin demonstrated an increase in intensity of the two protein-DNA complexes corresponding to the putative AP-1-containing complexes that was most apparent after 1 h of activin treatment and in the appearance of an additional band after 24 h (Fig. 3B), which has yet to be characterized. These data suggest that the crosstalk between activin and GnRH signaling likely converges at a common cis-regulatory element interacting with both SMAD and AP-1 (Fos/Jun) proteins. Functional cooperation between SMAD and AP-1 proteins has been previously described. Zhang et al. (40) showed that SMAD3 and SMAD4 act in concert with c-Jun/c-Fos to induce transcriptional activation of a synthetic reporter (four tandem AP-1 binding sites from the collagenase I promoter) in response to transforming
growth factor-β. This interaction has also been demonstrated in the c-Jun promoter (41). However, the one or more molecular mechanisms responsible for such an interaction have not previously been delineated.

To determine whether AP-1 binding to −335/−312 is direct or indirect, EMSA experiments were performed using purified c-Jun protein (Promega) and/or indirect, EMSA experiments were performed using purified Fra-1, and Fra-2 proteins in place of the experiments using these mutant oligonucleotides localized Fos/Jun binding site (19). Results of competition EMSA experiments using these mutant oligonucleotides (designated μGRAS-1 through -9 (Fig. 5A)) were designed in the wild type −335/−312 sequence for use in competition EMSA experiments. These were similar to the mutant oligonucleotides used in the original description of the GRAS element (18), with the exception of μGRAS-5 (which was found to give an additional protein-DNA complex on EMSA that was not present in the wild type sequence (data not shown), and was therefore redesigned). Competition EMSA experiments using these mutant oligonucleotides localized Fos/Jun protein binding to the region defined by μGRAS-4, -5, and -6 (Fig. 5B), which corresponds to −327/−322 (3'-AGTCA-5') of the mGnRHR gene promoter. These data therefore define a novel AP-1 binding element in the 5'-flanking sequence of the mGnRHR gene (Table 1) (42–50).

To determine whether SMAD transcription factors bind directly to this DNA sequence, EMSA experiments were performed using in vitro translated SMAD proteins. Results showed direct binding of SMAD3 and SMAD4 (but not SMAD2) to −335/−312. Competition EMSA experiments using unlabeled μGRAS-1 through -9 localized SMAD4 (Fig. 5C) and SMAD3 (data not shown) binding primarily to the region defined by μGRAS-3, although a small amount of binding to μGRAS-2 and -4 was also evident. This sequence corresponds to −331/−326 (3'-GTCTAG-5') of the mGnRHR gene promoter, which is the same location as the putative SBE defined by sequence homology (19). Results of competition EMSA experiments using atT3–1 nuclear extract in place of in vitro translated SMAD and AP-1 proteins suggest that the upper DNA-protein complex represents SMAD3 and SMAD4 binding primarily to −329/−328 (3'-CT-5') and the middle two bands represent AP-1 (Fos/Jun) proteins binding to −327/−322 (3'-AGTCA-5') (Fig. 5D). Functional transfection studies using mutant constructs of the newly identified SMAD and AP-1 binding sequences demonstrated that mutation of either one of these elements significantly decreased the basal luciferase activity and completely abrogated both the GnRH agonist and activin response (Fig. 6). These data confirm that both the SMAD and AP-1 binding sequences within −335/−312 are functionally important for basal as well as GnRH agonist- and activin-stimulated activation of the mGnRHR gene.

There is considerable evidence to suggest that SMAD proteins exert their transcriptional effects only after binding to one or more “transcriptional partners” to form a multifactor complex known as the Activin-Responsive Factor (ARF) (20, 51–53). In this study, we have demonstrated that AP-1 (Fos/Jun) proteins are required as part of the ARF binding to −327/−322. A proposed model for the ARF protein complex binding to the SBE/GRAS element of the mGnRHR gene promoter is shown in Fig. 7. Detailed analysis of the GRAS element in the mGnRHR gene promoter has shown that there is a 4-bp sequence (5'-AACA-3') at position −321/−318 immediately downstream from the AP-1 binding site that is necessary for activin responsiveness (Fig. 7) (15, 18). It is possible that one or more additional transcription factors are required that bind to both this cis-regulatory element and to the SMAD and/or AP-1 proteins to effect maximal response of the mGnRHR gene to activin stimulation. Indeed, a recent report suggests that a forkhead transcription factor, FoxL2/PFrk, may bind to this sequence (54).

In summary, we have used functional transfection studies and competition EMSA experiments to define a novel cis-regulatory element within −387/−308 of the mGnRHR gene promoter. This element is comprised of an overlapping SBE and newly characterized non-consensus AP-1 binding sequence that mediates transactivation of the mGnRHR gene by both GnRH and activin and suggests that this effect may be mediated through binding of a multifactor ARF protein complex, which includes AP-1 (Fos/Jun) and SMAD proteins. In addition to their effect on the GnRHR gene, both activin and GnRH are known to regulate the expression of gonadotropin subunit genes (especially FSH-β and LH-β) in mouse pituitary cell lines (36) and cultured human pituitary cells (55). Whether a molecular mechanism similar to that described above for the GnRHR gene is applicable also to gonadotrope subunit gene expression in pituitary gonadotropes has yet to be investigated.

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### Table 1

| Promoter        | Functional non-consensus AP-1 sites | Reference |
|-----------------|-------------------------------------|-----------|
| CONSENSUS       | TGAG/C/TCA                          | (42)      |
| Interleukin-2   | AGAG/TCA                            | (43)      |
| SV40            | TGAG/TCA                            | (44)      |
| Adenovirus E3   | TGAG/TCA                            | (45)      |
| Interleukin-2   | TCAG/TCA                            | (46)      |
| c-Fos          | TGAG/TCA                            | (47)      |
| Polyoma virus  | TGAG/TCA                            | (48)      |
| HPV 16         | TGAG/CAC                            | (49)      |
| pUC            | TGAC/CAC                            | (50)      |
| Transferrin receptor | TGAC/CAC                         | (51)      |
| TGF-β          | TGAG/CAC                            | (52)      |
| GnRH receptor  | CTAG/CAC                            | This report |

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The above text contains a table and references, formatted in a structured manner. The content is a continuation of a previous discussion on transcription factors and their interactions with DNA sequences, particularly focusing on the GnRH receptor (GnRHR) gene. It describes the methods used to identify regulatory elements and transcription factor binding sites, and discusses the roles of SMAD and AP-1 proteins in the activation of the GnRHR gene. The text also highlights the importance of activin-stimulated activation of the GnRHR gene and the role of the ARF (Activin-Responsive Factor) complex in this process. The references at the end provide additional support for the findings presented.
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