Myocyte Enhancer Factors-2B and -2C Are Required for Adhesion Related Kinase Repression of Neuronal Gonadotropin Releasing Hormone Gene Expression*

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Synthesis of the hypothalamic peptide, gonadotropin releasing hormone (GnRH), is paramount for reproductive function. GnRH neurons originate in the olfactory region and migrate into the forebrain during development. We recently implicated adhesion related kinase (Ark) in GnRH neuron development based on its differential expression in two GnRH producing cell lines, GT1-7 and Gn10. The Ark membrane receptor encodes an extracellular domain resembling cell adhesion molecules and an intracellular tyrosine kinase. Ark is expressed in Gn10 cells derived from migrating GnRH neurons but not GT1-7 cells of the post-migratory phenotype. Here, we show that Ark and GnRH transcripts are colocalized in the cribriform plate at midgestation, suggesting that Ark is expressed in migrating GnRH neurons in vivo. Furthermore, we have identified the GnRH gene as a downstream target of Ark signaling. Ark inhibits GnRH gene expression in GnRH neuronal cells via the coordinated binding of myocyte enhancer factor-2B and -2C (MEF-2B and -2C) and a putative homeoprotein within the proximal rat GnRH promoter. Given that MEF-2 proteins are widely expressed in the brain, these studies provide further evidence for MEF-2 action during neuronal development. Moreover, our studies elucidate a potential role for Ark in regulating GnRH gene expression during GnRH neuronal migration.

The precisely orchestrated synthesis and pulsatile release of gonadotropin releasing hormone (GnRH) from neurons of the hypothalamus is essential for reproductive competence. The GnRH neurons are unique among other neurons as they are born in the olfactory placode and migrate into the forebrain during development (1, 2). Although the GnRH neuronal pathway has been well characterized during development, the factors regulating GnRH gene expression along the migratory route remain to be identified.

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The scarcity (~800 neurons in the mouse) (2) and heterogeneity of the GnRH neuronal population has hindered in vivo as well as primary cell culture studies (3, 4). Thus, two immortalized GnRH neuronal cell lines have been generated by targeted tumorigenesis of the GnRH neurons (5, 6). The Gn10 GnRH neuronal line was developed by SV40 T-antigen immortalization of GnRH neurons at the time of migration and expresses low levels of GnRH (5). In contrast, the GT1-7 line was derived from an SV40 T-antigen targeted hypothalamic tumor of post-migratory GnRH neurons and synthesizes high levels of GnRH (6). The GT1-7 cells have been used extensively as a model system to identify potential regulators of GnRH gene expression and secretion in vivo (7–10).

Previously, we performed differential display on the two GnRH producing cell lines to identify novel factors regulating GnRH neuron migration and gene expression (11). Studies revealed that adhesion related kinase (Ark) was expressed in the Gn10 but not the GT1-7 cells. Axl, the human homolog of Ark, was originally isolated as a transforming gene in chronic myelogenous leukemia patients (12, 13). Subsequently, the Ark cDNA was cloned based on homology to the bek fibroblast growth factor receptor (14). Ark/Axl belongs to a novel subclass of receptor tyrosine kinases that includes Tyro3 and Mer (15). The extracellular domains of these receptors contain immuno-globulin and fibronectin III repeats reminiscent of cell adhesion molecules, while the intracellular portions encode a tyrosine kinase (12). Ark/Axl and Tyro3 are highly expressed in the brain, although their physiologic roles are largely unknown (16–22).

Growth arrest specific gene 6 (Gas6) functions as the ligand for Ark, Tyro3, and Mer (23–26). The murine Gas6 is a member of the vitamin K-dependent protein family and has 44% amino acid identity with human protein S, a negative regulator of blood coagulation. Like protein S, the carboxyl terminus of Gas6 shows similarity to steroid binding globulin; however, Gas6 does not contain the thrombin cleavage site present in protein S required for negative feedback in the blood coagulation cascade (27). Although related to protein S, Gas6 does not play a role in coagulation but rather activates anti-apoptotic and mitogenic pathways in various cell types (28–32). Our studies have shown that Gn10 GnRH neuronal cells are protected from serum withdrawal induced apoptosis by Gas6-Ark signaling pathways (33). However, Gas6-Ark signaling does not stimulate Gn10 cell proliferation. These data suggest that Ark may play a role in GnRH neuronal survival during development.

In vascular smooth muscle cells, Ark stimulates chemotaxis in response to Gas6 (34, 35) and also engages in homophilic and heterophilic interactions with adjacent cells (17, 36). Similarly, preliminary studies in our laboratory suggest that Gas6-Ark

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The abbreviations used are: GnRH, gonadotropin releasing hormone; Ark, adhesion related kinase; MEF, myocyte enhancer factor; DIG, digoxigenin; EMSA, electrophoretic mobility shift assay; HDAC, histone deacetylases.
signaling potentiates Gn10 neuronal migration (38). This diversity in Ark function argues that members of this receptor family may have multiple functions in vivo that may vary depending on the cell type. Indeed, mice harboring null mutations in Axl family receptors were recently generated (39). Animals lacking one or two of the receptors were generally healthy, while triple knockouts displayed multiple abnormalities. The most prominent defect was that of male sterility owing to defects in spermatogenesis. Whether other reproductive deficits exist in these animals has not been characterized.

Although several Ark signaling cascades have been defined (28–31, 33, 40), Ark-regulated genes have not been elucidated. In this report, we identified the GnRH promoter as a nuclear target of Ark signaling in GT1-7 GnRH neuronal cells. Our studies delineate a novel pathway whereby Ark negatively regulates GnRH gene expression via myocyte enhancer factors-2B and 2C (MEF-2B and -2C) in concert with a putative homeoprotein. The MEF-2 family of MADS (MCM1, agamous, deficiens, serum response factor) box transcription factors play critical roles in the development and differentiation of skeletal, cardiac, and smooth muscle (41, 42). In vertebrates, four mef2 genes have been identified, mef2a, -b, -c, and -d (41). In addition to muscle, MEF-2 expression has been widely documented in the developing brain, but MEF-2 functions in neuronal lineages are largely unknown (43–49). Recent studies have linked MEF-2-mediated transcription to calcium-dependent neuronal survival, but the natural MEF-2 promoter targets involved await identification (50, 51). Other than GnRH, the N-methyl-D-aspartic acid receptor subunit 1 (NR1) promoter is the only other neural specific gene known to be regulated by MEF-2 (52).

In general, activation of muscle-specific genes is governed by MEF-2 proteins in combination with the MyoD family of bHLH proteins (MyoD, myogenin, Myf5, and MEF4) (42). These factors activate transcription by binding either to individual E-box (bHLH consensus) or MEF-2 sites or via adjacent E-box/MEF-2 elements (41). Non-classical regulatory mechanisms also exist wherein MEF-2 interacts with non-MyoD family members such as Sp1 (53), Oct-1 (54), thyroid hormone receptor (55), GATA factors (56), or NFAT (57) to stimulate gene expression. MEF-2 regulation of neural genes has been proposed to occur via association with neural-specific transcription factors (58, 59).

Indeed, MEF-2 operates in cooperation with the neurogenic bHLH factor, MASH-1, to transactivate synthetic MEF-2 and E-box response elements (58, 59). That notwithstanding, the N-methyl-D-aspartic acid receptor gene NR1 promoter is synergistically activated in primary cortical neurons by MEF-2C and the ubiquitous homeoprotein, Octamer consensus oligomers were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Jun-MEF oligomer was purchased from Operon (Valencia, CA). The MEF-2B antibody was purchased from Transduction Laboratories (Lexington, KY). The MEF-2B antibody was provided by Ron Prywes (Columbia University), and the MEF-2C antibody was provided by John Schwarz (University of Texas Medical School, Houston, TX).

**Cell Culture—**GT1-7 (6) and Gn10 (5) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37 °C in humidified 5% CO2, 95% air.

**Plasmids—**The pα-LUC plasmid contains a trimered SV40 polyadenylation signal located upstream of inserted promoter sequences resulting in minimal background luciferase activity in the promoterless vector (61). A HindIII fragment containing nucleotides −3026 to +116 of the rat GnRH promoter was ligated into the HindIII site of pα-LUC, placing the GnRH promoter upstream of the luciferase (LUC) coding region (62). Promoter deletion constructs were generated using convenient restriction enzyme sites and ligated into the SmaI/HindIII sites of pα-LUC (62). The bGnRH-LUC construct was generated by ligating −3832/+5 of the human GnRH promoter into pα-LUC (63). The heterologous construct (−3333/−1660GnRH/RSV180-LUC), contains a 180-base pair promoter fragment (−130 to +50) within the 3’ long terminal repeat of the Rous sarcoma virus downstream of the GnRH promoter fragment, −3333/−16 (64). The MMTV-LUC contains the mouse MMTV long terminal repeat of the Rous sarcoma virus downstream of the pTK-LUC (65). T-REK-LUC contains a trimered 12-O-tetradecanoylphorbol-13-acetate response element upstream of pTK-LUC (66). pTK-LUC contains −109 to +18 of the HSV-TK gene and was provided by William Wood (University of Colorado Health Sciences Center). ERE-TK-LUC contains the estrogen response element from the Xenopus vitellogenin gene promoter upstream of pTK-LUC (66). PRK5-Ark encodes the mouse Ark cDNA (17) (provided by Paola Belgotta, New York University). MEF-2A, MEF-2B, MEF-2C, and MEF-2D cDNAs in pcDNA1 were provided by Eric Olson (University of Texas Southwestern Medical Center). The dominant negative pcDNA1-MEF2C-S387A was provided by Jiang Han (Scripps Research Institute) (67).

**Northern Analysis—**Total RNA from GT1-7, Gn10 cells, and E13 mouse brain regions was isolated using Trizol reagent (Life Technologies, Inc., Grand Island, NY). Five micrograms of total RNA was separated by electrophoresis on a 1.4% agarose gel containing formaldehyde and transferred to nitrocellulose. Antisense RNA probes for Ark and GnRH were synthesized using digoxigenin (DIG)-labeled nucleotides (Roche Molecular Biochemicals, Indianapolis, IN). The Ark probe contained the antisense sequence spanning nucleotides 401–819 of the Ark cDNA. The GnRH probe encoded the mouse antisense GnRH sequence. The probes were hybridized at 68 °C for 16 h in 50% formamide, 5 × SSC, 2% blocking reagent (Roche Molecular Biochemicals), 0.1% N-laurylsarcosine, and 0.2% SDS (probe concentrations, Ark, 0.4 μg/ml; GnRH, 0.1 μg/ml). The filters were washed twice in 2 × SSC, 0.1% SDS for 5 min at room temperature and twice in 0.1 × SSC, 0.1% SDS at 68 °C for 15 min. To visualize bound probe, a Roche Molecular Biochemicals Nucleic Acid Detection Kit was used. Filters were washed in 0.1 M maleic acid, 0.3% Tween 20, 0.15 M NaCl, pH 7.5, and blocked for 30 min in blocking buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, containing 1% blocking reagent (Roche Molecular Biochemicals)). The filters were incubated in anti-DIG-alkaline phosphatase conjugate (1:5000) diluted in blocking buffer for 30 min and washed twice in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5, for 15 min. Bound antibody was detected using an enzyme catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium salt.

**Transient Transfection—**GT1-7 cells were electroporated with 10 μg of the luciferase construct (rGnRH-LUC, hGnRH-LUC, ERE-TK-LUC, α-subunit-LUC, MMTV-LUC, TRE-K-LUC) (−3333/−1660GnRH/RSV180-LUC, or RSV180-LUC), 5 μg of pRK5-Ark, and 0.6 μg of RSVp5A to control for transfection efficiency. The total amount of plasmid was maintained constant at 20 μg with the inclusion of empty vector, pRK5. Cells were harvested 16–18 h post-transfection, and the lysate was assayed for luciferase and β-galactosidase activities as described previously (62). For the dominant negative MEF-2C studies, 5 μg of pcDNA1-MEF2C-S387A was used.

**Western Blot Analysis—**GT1-7 and Gn10 cells were washed twice in phosphate-buffered saline at 4 °C and lysed in 0.1 ml of cell lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton-100, 0.1% Nonidet P-40, 1% glyceral, 1 mM dithiothreitol, 10 mM Tris–Cl, pH 7.5, supplemented with complete protease inhibitor mixture from Sigma). Following addition of lysis buffer, the cells were sonicated for 10 pulses using a Branson sonifier 250 (Branson Sonic Power Co., setting, duty cycle 30 and output control 3). Cell debris was removed by centrifuga...
action at 14,000 × g for 10 min at 4 °C. The protein concentration of the supernatant was determined using the BCA protein assay kit (Pierce, Inc., Rockford, IL). Twenty micrograms of protein were resolved by SDS-polyacrylamide gel electrophoresis on 7.5–12% gels and transferred to Hybond polyvinylidene difluoride (Amersham Pharmacia Biotech). For immunoprecipitation, 1.5 mg of cell lysate was immunoprecipitated with 4G10 antibody. The membranes were blocked in 5% non-fat milk/TBS-T buffer (137 mM NaCl, 0.1% Tween 20, 20 mM Tris-Cl, pH 7.6) for 1 h at room temperature or overnight at 4 °C. Membranes were incubated for 1 h in primary antibody and washed three times in TBS-T for 15 min. The membranes were incubated in horse-radish peroxidase-linked secondary antibody for 20–60 min, washed three times in TBS-T for 15 min, and incubated in enhanced chemiluminescence (ECL) immunodetection reagents according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assay (EMSA)—The double-stranded GnRH oligomers used in the EMSA studies were described in Table I and were synthesized as complimentary pairs (Life Technologies, Inc.). Double stranded oligomers were end-labeled using the Klenow fragment of DNA polymerase I (Life Technologies, Inc.) and [α-32P]dCTP (PerkinElmer Life Sciences, 3000 Ci/mmol) to a specific activity of 10,000–30,000 cpm/ng. The labeled oligomers were purified using a Sephadex G-25 spin column (5-Prime, Boulder, CO). GT1-7 and Gn10 nuclear extracts were prepared as described (68). For EMSAs, nuclear extracts (5 μg) were incubated for 20 min at 4 °C in 1 mM dithiothreitol, 2.5 mM MgCl₂, 1% glycerol, 0.1 mg/ml bovine serum albumin, 10–20 ng/μl poly(dG-dC), 20 mM HEPES, pH 7.9, and 50–100,000 cpm of oligomer in a total volume of 20 μL. For competition experiments, the unlabeled DNA competitors were incubated with the nuclear extracts for 20 min at 4 °C followed by another 20-min incubation in the presence of labeled oligomers. Subsequently, the reaction mixtures were resolved by electrophoresis on 5% non-denaturing polyacrylamide gels containing 3% glycerol and 0.25 M NaCl. Gel mixtures were resolved by electrophoresis on 5% non-denaturing polyacrylamide gels containing 3% glycerol and 0.25 M NaCl. Following electrophoresis, the gels were run at 4 °C for 4 h at 250 V. Following electrophoresis, the gels were dried and exposed to film at −70 °C for 16–24 h. For the combined EMSA/Western analysis, protein-DNA complexes were eluted from dried EMSA gels in Laemmli sample buffer (69) and subsequently analyzed by Western blot as described above.

Promoter Mutagenesis—Rat GnRH promoter mutations were generated using the Quickchange Site-directed Mutagenesis System (Stratagene, La Jolla, CA) and (333/−16 GnRH/RSV180-LUC as the template. Mutant primers were synthesized by Life Technologies, Inc. The mutant primers were as follows: the Homeobox primer spanned the sequence −178/−122, the MEF-2 primer spanned the sequence −177/−97, and the E-box primer spanned the sequence −169/−94. Mutations that were generated in the GnRH promoter using these primers are indicated in Fig. 7A. The mutations were confirmed by DNA sequencing (UCHSC, Cancer Center Sequencing Core Facility).

Statistical Analysis—Data are shown as mean ± S.E. Data were compared using the Student’s t test, and p values of <0.05 were considered significant.

RESULTS

Ark Expression in Neuronal Cell Lines and in Vivo—Since Gn10 and GT1-7 cells were derived during two windows of GnRH neuronal development, we performed differential display on the cell lines to identify novel factors potentially involved in the migratory process and GnRH gene expression (11). Using this method, we demonstrated that Ark is expressed in the Gn10 cells derived during GnRH neuron migration but not the GT1-7 cells of the post-migratory phenotype (Fig. 1, A and B). To establish whether Ark was expressed in GnRH neurons in vivo, Northern analysis was performed on RNA isolated from mouse brain regions at embryonic day 13 (E13), a time when the GnRH neurons are migrating and concentrated in the cribriform plate area (70, 71). Both Ark and GnRH mRNA were detected in the cribriform plate RNA but not in the basal forebrain, brainstem, or telencephalon (Fig. 1B). Together, these data are consistent with the notion that Ark is expressed in migratory GnRH neurons during development.

Ark Inhibits GnRH Promoter Activity in GT1-7 Neuronal Cells—It has previously been noted that GnRH expression levels are markedly different between the two neuronal cell lines being high in the post-migratory GT1-7 and low in the migratory Gn10 cells (Fig. 1B) (5, 6). The divergent expression of GnRH and Ark between the neuronal cell lines suggested that one of Ark’s functions in GnRH neurons may be to limit GnRH gene expression during migration. To test this hypothesis, Ark was reintroduced into GT1-7 (Ark negative) cells with various promoter constructs, and transcriptional activity was assessed using a luciferase reporter. Ark repressed the full-length rat (rGnRH-LUC) and human (hGnRH-LUC) GnRH promoters to 39 and 27% of basal promoter activity, respectively (Fig. 2A). Conversely, Ark had no significant effect on other constructs tested including the vitellogenin gene estrogen response element (ERE-TK-LUC), the pituitary α subunit gene promoter (α-subunit-LUC), or the mouse mammary tumor virus promoter (MMTV-LUC). In contrast, Ark stimulated a 12-O-tetradecanoylphorbol-13-acetate response element (TRE-TK-LUC) by nearly 4-fold. To confirm Ark expression and activation of its tyrosine kinase domain in transfected GT1-7 cells, immunoprecipitation and Western blot analysis was performed. As shown in Fig. 2B, Ark was present in cells transfected with pRK5-Ark but not vector alone (pRK5). In addition, tyrosine-phosphorylated Ark was abundant in Ark-transfected GT1-7 cells. Thus, Ark expression in immortalized GnRH neurons results in activation of its tyrosine kinase domain in the absence of ligand, Gas6. Together these data demonstrate that Ark signaling results in promoter specific activation or repression.
Ark Repression Is Conferred via the Proximal GnRH Promoter—To map the GnRH promoter regions that conferred Ark responsiveness, five deletion constructs were tested in GT1-7 cells. Co-expression of Ark with the full-length rat GnRH promoter (rGnRH-LUC, −3026/+116) resulted in 60% inhibition of promoter activity (Fig. 3A). Deletion to −516 and −171 maintained Ark repression, while truncation to −126 resulted in loss of Ark regulation. Thus, the Ark responsive region lies between −171/−126 of the proximal rat GnRH promoter. The Transcription Element Search System (TESS) (72) was used to identify potential cis-regulatory elements between −171/−126 of the promoter (Fig. 3B). Putative binding motifs for members of several transcription factor families were identified including the basic helix loop helix, homeodomain, zinc finger, bZIP, and MADS families.

MEF-2B and 2C Bind the MEF-2 Site in the Proximal GnRH Promoter—The promoter deletion studies demonstrated that the proximal GnRH promoter between −171 and −126 conferred Ark inhibition. To identify potential nuclear targets of Ark signaling in the GnRH neuronal cell lines, EMSAs were performed with the −171/−126 promoter fragment. Initially, protein-DNA complexes from Gn10 (Ark positive) and GT1-7 (Ark negative) cells were compared (Fig. 4A). Factors from both Gn10 and GT1-7 cells bound the −171/−126 GnRH oligomer. However, the protein-DNA complexes observed from Gn10 cells (indicated by a bracket) were distinct from that of GT1-7 cells and vice versa, suggesting that factors unique to each of the neuronal cell lines bind this region of the GnRH promoter.

Although AP-1 and Sp1 consensus elements were detected within the Ark responsive promoter region (Fig. 3B), excess unlabeled AP-1 and Sp1 consensus oligomers had no effect on the Gn10 protein-DNA complexes (Fig. 4B). In contrast, formation of the Gn10 protein/DNA doublet was completely blocked upon exposure to an excess of the unlabeled MEF-2 site from the Jun promoter and substantially inhibited in the presence of the POU homeoprotein octamer consensus. These data suggested that homeoproteins and MEF-2 proteins were components of the Gn10 protein-DNA complexes.

To ascertain the promoter sequences necessary for the formation of the Gn10 complexes, mutant oligomers were tested in the gel shift (Fig. 4C, Table I). The protein/DNA doublet was detected with the wild type oligomer (−157/−121 GnRH) and Gn10 nuclear extracts. Mutation within the region containing homeoprotein consensus elements (Homeobox MT, −154 to −147) resulted in complete loss of both complexes. In contrast, only the lower complex was specifically abolished upon mutation of the MEF-2 site spanning −145 to −136 (MEF-2 MT). Mutation of the E-box motif (E-box MT, −129 to −124) within the oligomer had no effect on the protein/DNA doublet. An oligomer encoding both the Homeobox and MEF-2 mutations (Homeobox/MEF-2 MT) generated similar results to that of the Homeobox MT alone (not shown). Similarly, the combination MEF-2/E-box MT generated a protein-DNA complex identical to the MEF-2 MT alone (not shown). Taken together, these data suggested that homeodomain and MEF-2 proteins specific to Gn10 cells bind the homeobox and MEF-2 sites within the Ark-regulated promoter region.

Because the promoter deletion and gel shift studies suggested the participation of MEF-2 proteins in Ark repression, we examined MEF-2 expression in the GnRH neuronal cell lines (Fig. 5A). Four mef2 genes have been identified, mef2a, −2b, −2c, and −2d, and antibodies specific to each MEF-2 protein have been developed (60). All four MEF-2 proteins were expressed in the two cell lines. However, MEF-2B and −2C were expressed at higher levels in the Gn10 (Ark positive) cells than the GT1-7 (Ark negative) cells.

To identify the MEF-2 protein(s) binding to the MEF-2 element between −145 and −136, anti-MEF-2 antibodies were tested by EMSA. Addition of anti-MEF-2B and -2C antibodies to the reactions resulted in loss of the lower band, while the anti-MEF-2A and 2D antibodies had no effect on the complexes (not shown). To confirm the binding of MEF-2B and -2C, the lower band of the protein/DNA doublet was eluted and analyzed for the presence of MEF-2 proteins by Western blot (Fig. 5B) (69). MEF-2B and -2C were detected in the protein eluted from the gel, while MEF-2A and -2D were undetectable. Together, these data confirm that MEF-2B and -2C bind the MEF-2 site in the proximal GnRH promoter.

In an effort to identify the constituents of the upper Gn10 protein-DNA complex, additional EMSAs were performed. The promoter deletion studies and EMSAs suggested that a homeodomain protein may operate in conjunction with MEF-2B and -2C to regulate the GnRH promoter. Previous studies from our laboratory demonstrated that the POU homeoprotein, SCIP (Oct-6, Tst-1), repressed GnRH promoter activity in GT1-7 cells in part through the class III POU consensus spanning −154/−147 (9, 73). However, addition of POU homeodomain protein antibodies including anti-Oct-1, Oct-2, SCIP (Oct-6, Tst-1), or Brn-4 to the reaction mixtures did not supershift or obliterate the upper band (data not shown). Thus, further studies are necessary to identify the protein(s) binding adjacent to MEF-2B and -2C in Gn10 GnRH neuronal cells.

Ark Repression Is Partially Mediated by MEF-2 Proteins—To evaluate whether myocyte enhancer factors alone were sufficient to regulate the GnRH promoter, all four MEF-2s were
tested in the transfection assay. MEF-2A, 2B, -2C, and -2D each repressed GnRH promoter activity by approximately 20% (data not shown). In addition, various combinations of the MEF-2s were tested, and promoter inhibition remained at only 20% (data not shown), suggesting that MEF-2 proteins may require additional regulatory factors to modulate the GnRH promoter.

FIG. 3. Ark inhibition maps to the proximal GnRH promoter. A, GT1-7 cells were electroporated with the full-length rat GnRH promoter construct (rGnRH-LUC, −3026/+116) or a 5' deletion construct (−516, −171, −126, or −73) (10 μg), RSV-β-gal (0.5 μg), and pRK5 or pRK5-Ark (5 μg). Cells were harvested after 16 h and assayed for LUC and β-galactosidase activities. The LUC activity of cells that received empty vector, pRK5, RSV-β-gal, and the respective luciferase construct was set at 100% (Control). Data are presented as percent LUC activity of the respective control (n = 3–4 for each construct tested; *, p < 0.05, −171 construct compared with −126 construct by the Student's t test). B, putative cis-regulatory elements between −171 and −124 of the rat GnRH promoter. The figure was generated using the Transcription Element Search Software (TESS) allowing for 20% mismatches (72). Putative binding motifs for several transcription factor families were identified (transcription factor classes are indicated in italics): the basic helix loop helix family (E12, E47, MEF-1, TF43-S, sea urchin myogenic factor-1 (SUM-1), Myo-D), the zinc finger family (SP1, Krueppel (Kr)), the bZIP family (AP1, C/EBPα, and β), the MADS family (MEF-2), serum response factor (SRF), and the homeodomain protein family (MATα1–2, Bicoid (Bcd), fushi tarazu (Ftz), HoxD9, HoxD8, HoxD10, Oct-1, Oct-4, SCIP). The SCIP (Oct-6, Tst-1) site was identified as a class III POU element in previous studies by us and others (9, 73). DNA sequences shown in boxes were mutated for the EMSA and transfection assays shown in the following figures.

FIG. 4. MEF-2 and homeoproteins bind the Ark responsive region of the GnRH promoter. A, Gn10 or GT1-7 nuclear extracts (5 μg) were subjected to EMSA with a 32P-labeled oligomer, −171/−126, (the Ark responsive region of the GnRH promoter). The bracket denotes a protein/DNA doublet specific to Gn10 (Ark positive) cells. B, an EMSA was performed with Gn10 nuclear extracts and 32P-labeled oligomer, −171/-126. The protein/DNA doublet was competed with 200 times unlabeled AP-1, Sp1, Octamer, or MEF-2 consensus element. Oligomer sequences are presented in Table I. C, an EMSA was performed with Gn10 nuclear extracts and 32P-labeled oligomer, −171/−126. The protein/DNA doublet was competed with 200 times unlabeled AP-1, Sp1, Octamer, or MEF-2 consensus element. Oligomer sequences are presented in Table I. C, an EMSA was performed with Gn10 nuclear extracts and 32P-labeled wild type or mutant (MT) oligomers spanning −157/−121 of the rat GnRH promoter. Specific mutations are described in Table I.
To assess whether MEF-2 proteins were obligatory for Ark repression of GnRH gene expression, a dominant negative form of MEF-2C (MEF2C-S387A) (67) was tested (Fig. 6). MEF2C-S387A encodes a serine to alanine change at amino acid 387 within the transactivation domain. In the presence of the full-length rat GnRH promoter, Ark suppressed basal promoter activity by 55%. Dominant negative MEF-2C alone had no effect on basal promoter activity. However, in the presence of dominant negative MEF-2C, Ark inhibition of GnRH decreased to only 19%. Taken together, these data support that Ark-mediated repression of the GnRH promoter is governed in part, by MEF-2 proteins.

### Table I

| Oligomer        | Sequence                           |
|-----------------|------------------------------------|
| −171/−126       | ATTCACAATGTCGTTTTATTACCTTAGAATGGTGGCTTCAGC |
| −157/−121       | TTAAGTTGAACAGGCCAAGGTTATTAGCCCTAGAATGGTGGCTTCAGC |
| −157/−121 Homeobox MT | CCAAAAATTAGGAATCTTACCCACCCGAAGTCGACACCT |
| −157/−121 MEF-2 MT | GGCGGGCGGTCACTCCGGACATGGCTTCAGCTG |
| −157/−121 E-box MT | GGGCTGACATGGCAGTGAGAATGGTGGCTTCAGC |
| −157/−121 Homeobox/MEF-2 MT | CCAAAAATTAGGAATCTTACCACCCGAAGTCGACACCT |
| −157/−121 MEF-2/E-box MT | GGCGGGCGGTCACTCCGGACATGGCTTCAGCTG |
| AP-1            | GCCTTAGAGCTCCGCGGAAGT |
| Sp1             | GCCAGCTACGTATGCGCCCTT |
| Octamer         | ATTCGATCGGGGCTCAGGTCG |
| Jun-MEF2        | ACMCTCTCGTTATGTACAGTCT |

*Mutated nucleotides are presented in bold face type.

![Fig. 5. MEF-2B and MEF-2C bind the MEF-2 element in the proximal GnRH promoter.](image)

To assess whether MEF-2 proteins were obligatory for Ark repression of GnRH gene expression, a dominant negative form of MEF-2C (MEF2C-S387A) (67) was tested (Fig. 6). MEF2C-S387A encodes a serine to alanine change at amino acid 387 within the transactivation domain. In the presence of the full-length rat GnRH promoter, Ark suppressed basal promoter activity by 55%. Dominant negative MEF-2C alone had no effect on basal promoter activity. However, in the presence of dominant negative MEF-2C, Ark inhibition of GnRH decreased to only 19%. Taken together, these data support that Ark-mediated repression of the GnRH promoter is governed in part, by MEF-2 proteins.

![Fig. 6. Dominant negative MEF-2C partially blocks Ark inhibition of the GnRH promoter.](image)
wild type (−333/−16 GnRH)RSV180-LUC was inhibited by 43%. Mutations (MT) of the Homeobox, MEF-2, or E-box site individually had modest effects on Ark inhibition of GnRH (27, 26, and 32% inhibition, respectively), whereas Ark inhibition was completely abrogated in the presence of the GnRH promoter construct encoding both the Homeobox and MEF-2 motif mutations (Homeobox/MEF-2 MT). These studies confirm the functional requirement of both the MEF-2 and homeoprotein elements in Ark control of the GnRH promoter.

**DISCUSSION**

Prior to our studies, the nuclear effectors of Ark signaling were unknown. Herein, we have shown that Ark inhibits transcription of GnRH, the gene whose coordinated activation and inhibition is absolutely essential for normal reproductive function. The fact that Ark is expressed in migratory (Gn10 cells) rather than postmigratory (GT1-7 cells) immortalized GnRH neurons, and Ark transcripts were detected in E13 mouse cribriform plate tissue containing GnRH neurons argues that Ark is expressed in migrating GnRH neurons during development. Regarding this possibility, it should be noted that the GnRH neuronal population has not been carefully examined in the Axl family member null mice (39). Given that our studies implicate Ark function in GnRH neurons, studies are underway to determine the temporal colocalization of Ark and GnRH in the GnRH neuronal population during development.

The observation that reintroduction of Ark into post-migratory GT1-7 cells inhibited GnRH promoter activity prompted investigation of the promoter regions involved. Ark repression mapped to the sequences lying between −171 and −126 of the proximal GnRH promoter. Proteins specific to the Gn10 (Ark expressing) cells bound this region of the promoter and were competed by unlabeled octamer and MEF-2 consensus elements, but not AP-1 or Sp1 elements, thus implicating MEF-2 and homeodomain proteins in Ark regulation of the GnRH promoter. Mutagenesis of individual E-box, MEF-2, and homeoprotein consensus elements within this region had modest effects on Ark inhibition of the GnRH promoter. However, Ark regulation was completely abrogated in the presence of a GnRH promoter construct encoding both the homeoprotein and MEF-2 site mutations. Thus, the coordinated binding of both a myocyte enhancer factor(s) and a homeoprotein(s) were required for Ark inhibition. The gel shift analysis combined with the dominant negative MEF-2C studies implicated MEF-2B and -2C in Ark regulation rather than MEF-2A and -2D. Moreover, the partial effectiveness of dominant negative MEF-2C in blocking Ark repression confirmed that MEF-2 action was necessary but not sufficient in this pathway.

Although the promoter mutagenesis confirmed that a homeoprotein operated in collaboration with MEF-2B/2C to downregulate GnRH gene expression, the identity of this factor is still under investigation. Several types of homeoprotein consensus elements were detected between −158 and −143, including binding sites for Hox, bicoid, and POU homeodomain proteins (Fig. 3B) (72). Previous studies from our laboratory showed that SCIP (Oct-6) repressed GnRH gene expression via proximal promoter elements (9, 73). Based on the EMSA data, however, the protein does not appear to be the POU homeoprotein.
tein(s), Oct-1 (8), Oct-2 (8), SCIP (Oct-6) (9), or Brn-42 expressed in GnRH neuronal cells. Because the mutagenesis also disrupted the Iox and bicoid motifs, our studies cannot eliminate these types of homeoproteins as potential candidates involved in Ark repression. Indeed, studies by us and others have demonstrated that the cis-acting element spanning −154 to −147 of the rat GnRH promoter is required for basal promoter activity in GT1-7 cells (63, 74). Furthermore, this site appears to integrate signals for both activation and inhibition of GnRH promoter activity, as Otx-2, a bicoid-like homeoprotein, stimulates the GnRH promoter via this region in GT1-7 cells (74). Thus, the identity of the homeoprotein(s) interacting with MEF-2B and 2C in Gn10 and GT1-7 cells may provide important insights related to both positive and negative regulation of the GnRH promoter.

Although MEF-2 isoform expression has been demonstrated both spatially and temporally in the embryonic brain (43–49), unraveling MEF-2 functions in neuronal lineages has just begun. The finding that MEF-2 regulated transcription is associated with neuronal survival implicates anti-apoptotic genes as potential MEF-2 targets (50, 51). Prior to GnRH, however, the only neural promoter having been identified as a MEF-2 target was the N-methyl-d-aspartic acid NR1 receptor promoter (52). In fetal cerebrocortical cultures, Sp1 and MEF-2C interacted to synergistically activate the NR1 gene. Given that MEF-2C and Sp1 were also expressed endogenously in these fetal cerebrocortical cultures suggests that these two factors regulate NR1 expression in vivo. Coupled with our studies of the GnRH promoter, these data provide further evidence that MEF-2 proteins may play pivotal roles in neuronal development.

The complexities of MEF-2-mediated gene regulation have been extended with recent work from Lu et al. (75). In cardio-myocytes, MEF-2 was shown to be maintained in an inactive state when associated with the class II histone deacetylases 4 and 5 (HDAC4 and -5) that deacetylase histones resulting in transcriptional repression. Under hypertrophic conditions, HDAC dissociation from MEF-2 was mediated via CaMK signaling, while subsequent MEF-2 activation occurred by mito-gen-activated protein kinase phosphorylation within the MEF-2B and 2C in Gn10 and GT1-7 cells may provide important insights related to both positive and negative regulation of the GnRH promoter.

Therefore, if Ark stimulates HDAC association with MEF-2 proteins bound to the GnRH promoter, signal-dependent recruitment of HDACs is likely to occur through a different mechanism than that of HDAC release. Ultimately, studying the Ark signaling pathways that converge on MEF-2B/C and the putative homeoprotein will further elucidate the general mechanisms governing negative regulation of gene expression as well as expand our understanding of how the GnRH gene is modulated across development.

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