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A Specific Helical Orientation Underlies the Functional Contribution of the Activin Responsive Unit to Transcriptional Activity of the Murine Gonadotropin-Releasing Hormone Receptor Gene Promoter

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Activin responsiveness of the murine GnRH receptor (*GnRHR*) gene promoter requires two spatially distinct regulatory elements termed the GnRH receptor activating sequence or GRAS and the downstream activin regulatory element or DARE. While GRAS interacts with multiple transcription factors, DARE activity requires tandem homeodomain binding motifs (TAAT) and displays specific binding to the LIM homeodomain protein LHX3. Herein, we find that both the murine *GnRHR* gene promoter and DARE fused to a minimal heterologous promoter are responsive to LHX3 overexpression. A dominant-repressor of LHX3 attenuates transcriptional activity of the murine *GnRHR* gene promoter but had no impact on activin responsiveness. Thus, LHX3 would not appear to be the protein mediating activin responsiveness of this promoter. Within DARE itself, the tandem TAAT motifs are separated by 4 bp. Although this arrangement differs from the prototypical P2 or P3 binding sites characterized for paired-like homeodomain proteins and from the directly abutting TAAT motifs found for LHX3, a LIM-class homeodomain protein, we find that separation of the TAAT sites by 5 and 10 bp decreases *GnRHR* promoter activity to a level similar to promoters containing loss of function mutations in either the proximal or distal TAAT motif. Thus, the juxtaposition of the TAAT sites is critical for the functional activity of DARE. That activin responsiveness of the *GnRHR* promoter requires both GRAS and DARE suggests that these elements may be both functionally and structurally coupled. As to the latter, GRAS and DARE are separated by 20 bp, thus placing the elements on the same side of the helical backbone. To determine if this spatial organization is functionally relevant, multiples of 5 bp were inserted or deleted between GRAS and DARE. Any insertion or deletion that resulted in a half-turn alteration in the helical positioning between the two elements reduced promoter activity. Thus, an important spatial relationship underlies functional cooperation between GRAS and DARE and the emergence of a complex activin responsive unit (ARU) within the mouse *GnRHR* promoter.

Key Words: GnRH receptor; activin; follistatin; transcription.

Introduction

The binding of the hypothalamic peptide GnRH to specific, high-affinity receptors located on gonadotrope cells in the anterior pituitary gland is, perhaps, the central event in the regulation of reproductive function in mammals *(1,2)*. Accordingly, much effort has been expended toward understanding the physiological consequences of regulation of GnRH and the GnRH receptor (GnRHR). In regard to the latter, much progress has been made in elucidating the molecular mechanisms underlying cell-specific and hormonal regulation of *GnRHR* gene expression. Central to progress in this arena is the relatively robust transcriptional activity of the murine *GnRHR* gene promoter in the gonadotrope-derived αT3-1 cell line *(3,4)*. This paradigm has allowed for the development of an increasingly refined map of the key regulatory elements that mediate transcriptional activity of the murine *GnRHR* gene. In this regard, over 5 yr ago we defined a complex "tripartite" enhancer that mediates "basal" activity and endocrine responsiveness of the *GnRHR* gene promoter *(5)*. The key components of this enhancer include a binding site for the nuclear orphan receptor steroidogenic factor-1 (SF-1), a canonical AP-1 element, and an element we termed the GnRH receptor activating sequence or GRAS *(5–7)*. At least two of these elements also contribute to endocrine regulation of the *GnRHR* gene. Responsiveness to GnRH is dependent on recruitment of Jun/Fos family members to the AP-1 site *(7)*, whereas

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activin responsiveness is dependent on GRAS *(8)*. It has, however, become clear that the functional organization of the murine *GnRHR* gene promoter is far more complex, and an increasingly broader array of regulatory elements appears to be necessary for full transcriptional activity of this promoter. In particular, recent work has established the identity of binding sites for homeodomain proteins including LHX3 and Oct-1 *(9,10)*. Interestingly, at least two of these sites localized to tandem homeodomain sites termed the downstream activin regulatory element or DARE. As the name implies, this element, like GRAS, is necessary for activin responsiveness of the *GnRHR* gene promoter *(11)*. Thus, activin regulation of the murine *GnRHR* gene appears to require two spatially distinct regulatory motifs. An interesting insight that has emerged from these studies is that activin input is key to transcriptional activity of the murine *GnRHR* gene promoter. While clearly implicated in regulation of FSH synthesis and secretion *(12–14)*, several studies have established that activin, a member of the TGFβ family of growth and differentiation factors, regulates the number of GnRH receptors on gonadotropes *(15–17)*. The activin binding protein, follistatin, modulates the biological effects of activin by sequestering and preventing activin binding to its cognate receptor *(18)*. Both activin and follistatin are produced by gonadotropes in the anterior pituitary gland *(19)*. Follistatin is also produced by folliculostellate cells *(20)*. Thus, autocrine and paracrine mechanisms are an important component of activin signaling in the anterior pituitary.

It is well established that activin stimulates transcription of the *GnRHR* gene *(5,8,15,21,22)* and that both activin and activin receptors are expressed in the αT3-1 cell line *(15,18,19)*. This in itself presents an interesting problem in that αT3-1 cells exist in a constitutively activin-stimulated state. Thus, the challenge is to discriminate between the contribution of any regulatory element to activin-dependent and activin-independent transcriptional activity of the *GnRHR* promoter. Our approach has been based on testing for reduced promoter activity after administration of follistatin *(8)* or adenoviral-mediated overexpression of follistatin *(11)*. This approach was used to define the contribution of GRAS and, more recently, DARE to the activin responsive phenotype of the murine *GnRHR* gene promoter.

Consistent with its contribution to activin responsiveness, GRAS interacts with Smad3 and Smad4; however, the functional activity of GRAS depends not only on Smad binding but also AP-1 and FoxL2, a member of the forkhead family of transcription factors *(23)*. The definition of DARE as a necessary component of the complex activin responsive enhanceosome in the *GnRHR* gene promoter suggests that a functional interaction must exist between GRAS and DARE. In this regard, it is interesting to note that GRAS and DARE are separated by 20 bp or two complete turns of the helix. Herein we ask if this spatial arrangement is important for the functional contribution of the GRAS–DARE enhanceosome to transcriptional activity of the murine *GnRHR* gene promoter. Based on scanning mutagenesis, two core homeodomain motifs (TAAT) separated by four bp appear to be key to the functional activity of DARE. Altering the identity of these four intervening nucleotides does not affect the functional activity of DARE *(11)*; however, the presence of the tandem homeodomain motifs is striking. Thus, we sought to determine if the juxtaposition of the two TAAT sites is important for the functional activity of DARE. Collectively, the goal of these studies is to refine our understanding of the functional organization of the *GnRHR* gene promoter that begins to define not just the identity of key regulatory elements but the spatial organization of these elements that leads to enhanceosomes whose properties are dependent on specific spatial combinations of regulatory elements. Finally, the LIM homeodomain transcription factor LHX3 has recently been implicated as a binding component at DARE *(9,11)*; however, the potential contribution of LHX3 to the activin responsive properties of DARE was not specifically addressed. In the present studies, we sought to determine if a dominant-repressor of LHX3 termed LHX3a-KRAB *(24)* attenuates the activin response of the murine *GnRHR* gene promoter.

Results

DARE Confers LHX3 Responsiveness on a Heterologous Promoter

LHX3 has been implicated as a binding component at DARE *(9)*. To determine if DARE can serve as a functional response element for LHX3, we first tested the –600 wild-type promoter for LHX3 responsiveness. Briefly, αT3-1 cells were co-transfected with expression vectors for LHX3 and the –600 murine *GnRHR* promoter. Three hours after transfection, cells were infected with adenovirus constructs expressing either follistatin (Ad-follistatin) or GFP (Ad-GFP) as previously described *(11)*. This analysis revealed a small but significant ($p < 0.05$) increase in promoter activity associated with LHX3 overexpression in both the presence and absence of follistatin (Fig. 1). We next asked if DARE alone displays an LHX3-responsive phenotype when placed in the context of a minimal, heterologous promoter. We find that DARE alone or multimerized as three direct repeats confers LHX3 responsiveness on the minimal promoter from the rat prolactin (Prl) gene *(5)* (Fig. 2). In contrast, the transcriptional activity of a construct containing three copies of GRAS fused to the Prl promoter was not affected by LHX3 overexpression. Interestingly, in stark contrast to the 3XGRAS vector, neither the 1X DARE nor 3X DARE construct displayed responsiveness to follistatin. Thus, while DARE is sufficient for LHX3 responsiveness, an activin responsive phenotype appears to emerge only in combination with GRAS.

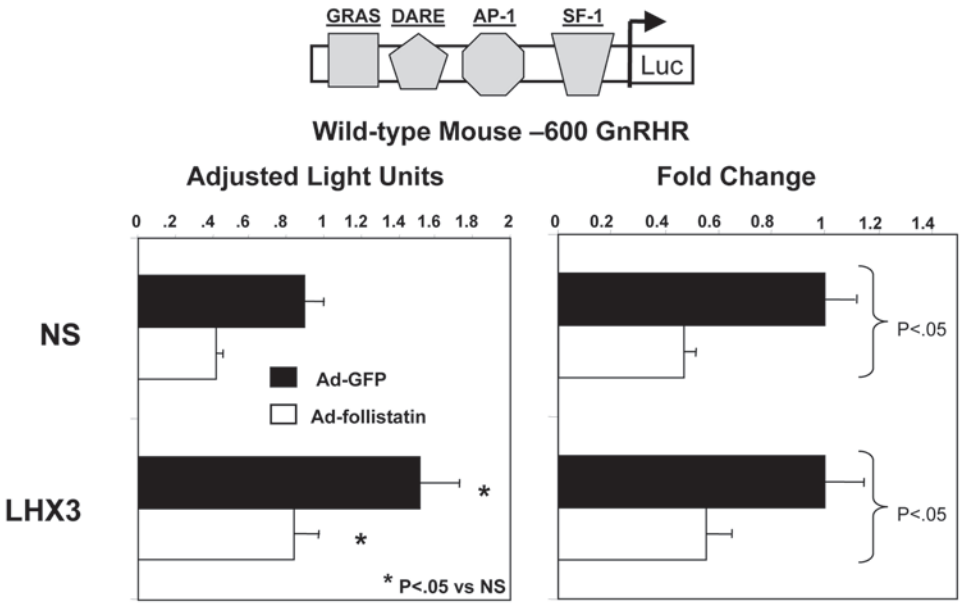

Fig. 1. Overexpression of LHX3 stimulates the wild-type mouse *GnRHR* promoter. The mouse wild-type *GnRHR* promoter was co-transfected into αT3-1 cells with either a non-specific expression vector or expression vectors for LHX3. After 3 h, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 h, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. *Represents values different ($p < 0.05$) between non-specific expression vector and LHX3.

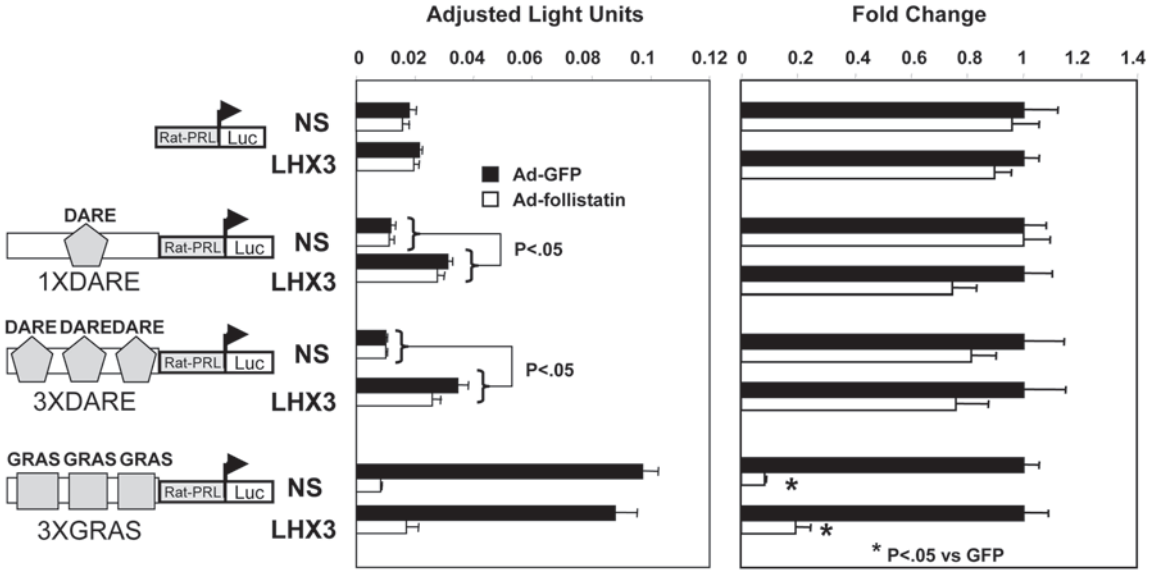

Fig. 2. Overexpression of LHX3 can activate minimal promoter constructs through DARE. Minimal promoter constructs (rat prl empty, 1XDARE, 3XDARE, and 3XGRAS) were transiently co-transfected into αT3-1 cells with either a nonspecific or LHX3 expression vector. After 3 h, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-Follistatin. After an additional 48 h, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. *Represents values different ($p < 0.05$) between Ad-GFP vs Ad-Follistatin.

A Dominant Repressor of LHX3 Attenuates Transcriptional Activity of the Mouse GnRHR *Gene Promoter*

If LHX3 represents a key player in mediating the functional contribution of DARE, then overexpression of dominant-negative forms of this protein should be revealed as a loss of transcriptional activity of the *GnRHR* gene promoter. To test this, we evaluated the impact of an LHX3 fusion protein in which the coding sequence of LHX3 is fused to the transcriptional repressor domain, or Kruppel domain, of the human KOX1 gene (KRAB) *(24)*. We find that the LHX3a–KRAB fusion protein attenuates the activity of the wild-type mouse *GnRHR* gene promoter by approx 65%

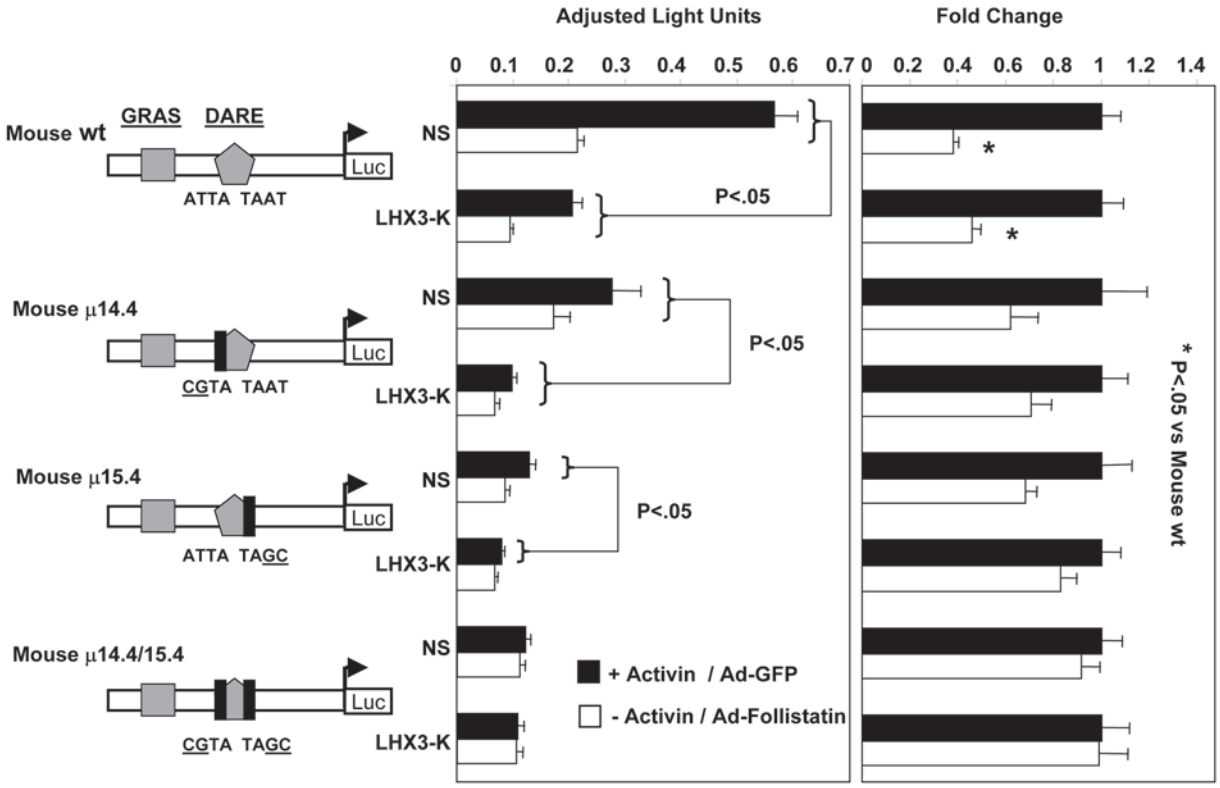

Fig. 3. Overexpression of a dominant negative LHX3 fusion protein can decrease expression of constructs through DARE. The wild-type mouse, μ14.4, μ15.4, and μ14.4/μ15.4 were transiently co-transfected into αT3-1 cells with either a nonspecific or LHX3a–KRAB expression vector. After 3 h, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 h, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. *Represents values different ($p < 0.05$) between Ad-GFP vs Ad-Follistatin.

(Fig. 3). There was, however, no impact of LHX3–KRAB on the follistatin response of the –600 promoter. The ability of LHX3a–KRAB to repress promoter activity was attenuated with mutation of either the distal (μ14.4) or proximal (μ15.4) TAAT sites and lost when both sites are mutated (μ14.4/μ15.4). Thus, the repressor activity of LHX3a–KRAB on the murine *GnRHR* gene promoter is, at least partially, mediated at DARE. It is important to note, however, that the activin responsive phenotype of the GRAS-DARE enhanceosome does not appear to be significantly compromised in the presence of the LHX3a–KRAB dominant-repressor.

Altering the Spatial Orientation Between the Tandem TAAT Sites Attenuates the Functional Contribution of DARE to GnRHR *Promoter Activity*

The 4 bp separation of the TAAT motifs in DARE would not place this element as one of the prototypical P2 or P3 binding sites characterized for paired-like homeodomain proteins *(25)*. Likewise, it differs from the dual TAAT motifs characterized for LIM-homeodomain family member LHX3, which directly abut *(26,27)*. Nevertheless, the juxtaposition of the two TAAT motifs is striking. Thus, we sought to determine if this spatial relationship organization is relevant to the functional contribution of DARE by placing either 5 or 10 bp between the two TAAT repeats in DARE. Transcriptional activity of the spacer mutants was compared to the –600 wild-type murine GnRHR promoter and the same promoter region containing loss of function mutations in either one or both of the TAAT motifs *(11)* using the identical transfection/infection paradigm described above. Insertion of either 5 or 10 bp between the two TAAT repeats in DARE led to a 50% reduction in transcriptional activity of the –600 promoter in the Ad-GFP infected cells (presence of activin) (Fig. 4). Thus, the spatial orientation between the two TAAT repeats within DARE appears to be critical for the functional activity of this element. It is interesting that the decrease in promoter activity associated with the spacer mutations is similar to the impact of mutating either one of the two core TAAT motifs (μ14.4 and μ15.4) suggesting that the spatial separation yields a phenotype equivalent to the contribution of one homeodomain site within DARE. Consistent with this notion, similar to the effects of the μ14.4 and μ15.4 mutations, the follistatin response of the spacer mutants was attenuated but not lost. Specifically, transcriptional activity of the spacer mutants was reduced by 30% in the presence of follistatin as compared to the approximately 60% reduction characteristic of the wild-type pro-

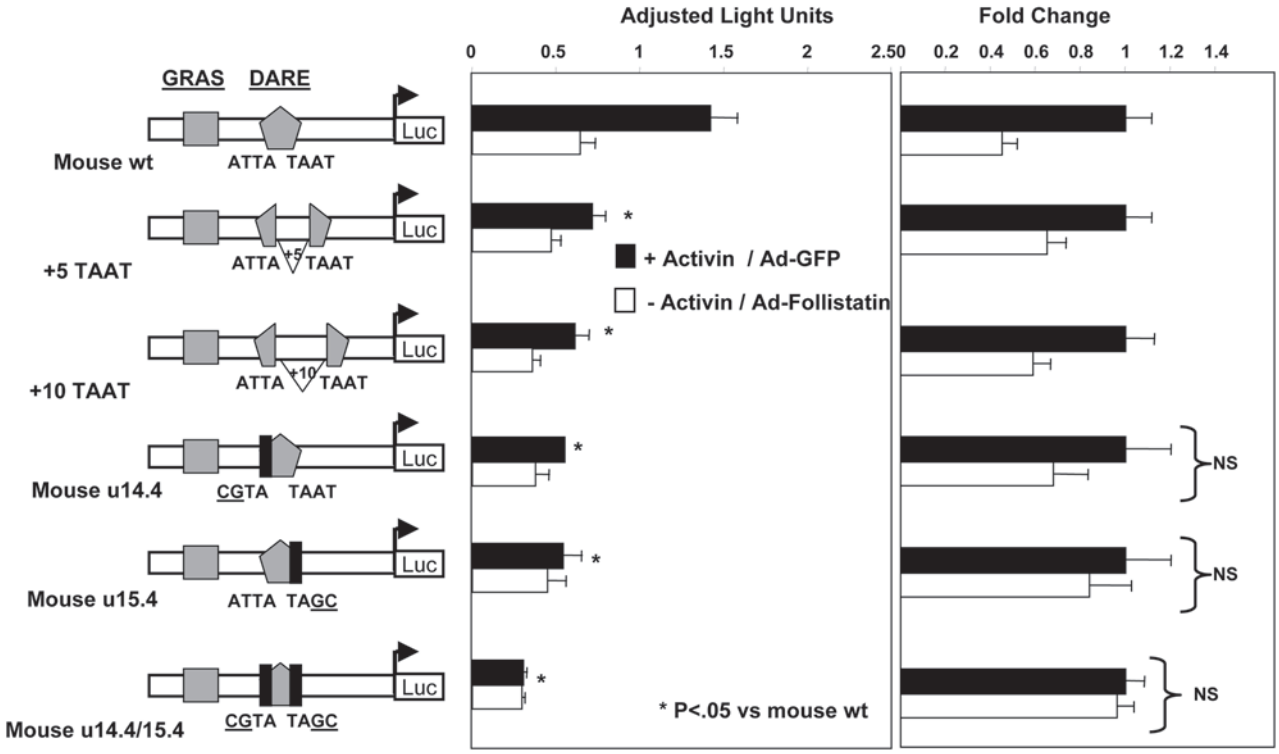

Fig. 4. Separation of the TAAT repeats within DARE attenuates activin regulation of the promoter constructs. Constructs containing the insertions of 5 or 10 nucleotides within DARE and constructs containing mutations in the TAAT repeats were transiently transfected into αT3-1 cells. After 3 h, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 h, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. *Represents values different ($p < 0.05$) from mouse wild-type Ad-GFP infected construct.

moter. Only with mutation of both TAAT motifs (μ14.4/μ15.4) was follistatin regulation completely abrogated.

Separation of GRAS and DARE by Half-Turns of the Helix Attenuates Transcriptional Activity of the Mouse GnRHR

To investigate the spatial relationship between GRAS and DARE a series of promoters were constructed that reduced the separation by 5, 10, or 15 bp or increased the separation by 5, 10, 15, or 20 bp. Our choice of separating the two elements by multiples of 5 bp was to determine if there is a functional consequence of separation by half- or full turns of the DNA helix. Each of the promoter constructs were co-transfected with RSV-LacZ into αT3-1 cells. At 3 h post-transfection, transfection reagent and media were replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin *(11)*. Media containing the adenovirus constructs remained on the cells for 48 h after which cells were harvested in lysis buffer *(11)*. Cellular lysates were analyzed for luciferase activity and adjusted for β-galactosidase activity as a control for transfection efficiency. Any mutation that altered the spatial arrangement between GRAS and DARE by half-turns of the helix reduced the transcriptional activity of the promoter in the presence of activin (Fig. 5). Thus, the activity of the –5, –15, +5, and +15 promoter constructs was lower than the –600 wild-type promoter in Ad-GFP infected cells. In contrast, there was no effect of altering the spatial orientation of GRAS and DARE by full turns of the helix (–10, +10, and +20 constructs). Thus, a critical spatial relationship exists between GRAS and DARE such that any half-turn that presumably disrupts the sidedness of the elements reduced the effective functional contribution of the GRAS–DARE enhanceosome to transcriptional activity in the presence of activin. Interestingly, however, while activin stimulated promoter activity is reduced by half-turn separations, a significant attenuation in promoter activity in the presence of follistatin suggests that activin responsiveness was at least partially retained in all of the constructs. Thus, altering the spatial relationship between GRAS and DARE reduces but does not eliminate the activin responsive properties of this complex enhanceosome. As would be predicted there was no impact of the spacer mutants on promoter activity in the absence of activin (Ad-follistatin–infected cells).

Discussion

Approximately 10 yr have passed since the initial description of a transcriptionally active promoter fragment from the murine *GnRHR* gene *(3,4)*. During this period much progress has been made in constructing a functional map of the key regulatory elements that mediate both cell-specific regu-

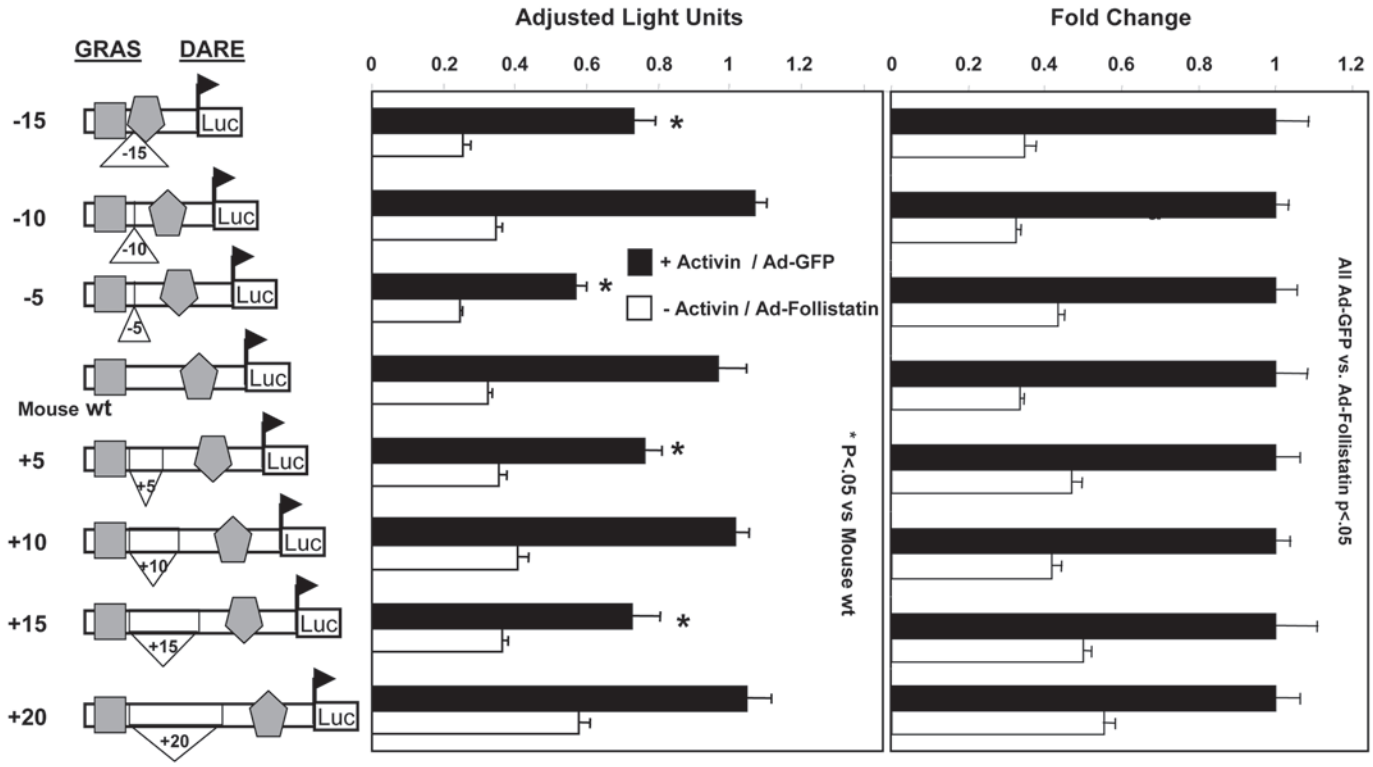

Fig. 5. Activin regulation of the mouse *GnRHR* promoter is disrupted by the insertion of half turns in the DNA helix. Deletions of 5, 10, or 15 and insertions of 5, 10, 15, or 20 nucleotides were transiently transfected into αT3-1 cells. After 3 h, transfection mixture was replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin. After an additional 48 h, cells were harvested and assayed for luciferase activity. Values represent mean ± the standard error of the mean of triplicate samples. *Represents values different ($p <$ 0.05) from mouse wild-type Ad-GFP infected construct.

lation and endocrine responsiveness of this promoter. Our understanding of the *GnRHR* gene promoter suggests a hierarchy of *cis*-acting regulatory elements with both distinct and overlapping roles affecting transcription. For example, AP-1 and GRAS make unique contributions in mediating either GnRH or activin responsiveness of the *GnRHR* gene; however, the combination of both regulatory elements yields a synergistic response to combined GnRH and activin treatment that is greater than the individual treatments *(5,21)*. Thus, as with many eukaryotic promoters, the complex functional phenotypes of the *GnRHR* gene requires specific combinations of individual regulatory elements.

Certainly the combination of GRAS and DARE reflects the emergence of a functional property (i.e., activin responsiveness). In fact, there is no partial phenotype—when either element is mutated, the activin response is lost. Thus, the combination of GRAS and DARE defines a unique activin responsive unit (ARU). Presumably, this functional relationship reflects some level of protein–protein interaction either directly via interactions of transcription factors or presentation of a stereospecific face for binding of co-activator(s). If correct, then the specific architecture of the activin responsive unit may be important. The positioning of GRAS and DARE exactly two helical turns apart places the binding components on the same side of the helical backbone in the wild-type promoter. We hypothesized that insertion or deletion of full turns in the DNA helix should not alter GRAS and DARE "sidedness" and have little affect on the activin response of the mouse *GnRHR* promoter. In contrast, we predicted that insertion or deletion of half-turns in the DNA helix would eliminate or attenuate activin responsiveness of the *GnRHR* promoter. Consistent with this hypothesis, we find that separation of GRAS and DARE by half- but not full helical turns attenuates the transcriptional activity of the *GnRHR* promoter in the presence of activin. These half-helical turns would, presumably, place GRAS and DARE on opposite sides of the DNA helix resulting in either a loss of protein–protein contact between the key transcription factors or an inappropriate architecture for co-activator interaction with the ARU. Thus, helical orientation of GRAS and DARE is important for the full functional activity of the ARU in the murine *GnRHR* gene promoter. Because eliminating activin negates the contribution of the GRAS–DARE ARU to promoter activity, we were not surprised that, in the absence of activin (follistatin overexpression), spatial separation of GRAS and DARE had essentially no effect on promoter activity.

Another important spatial relationship appears to exist within the DARE component of the ARU. Previous work with homeodomain proteins has shown that spacing between

tandem TAAT motifs is a critical determinant of function. Members of the paired-homeodomain family are found in P2 or P3 spacing arrangements, with two or three intervening bases, respectively *(25)*. LHX3 has been shown to bind to a consensus sequence that consists of abutting TAAT motifs *(26,27)*, again suggesting an important role for spacing. While the four-base spacing differs from these characterized patterns, we still find it critical to full function. Specifically, spacing of the core TAAT motifs in DARE is critical for the functional contribution of this element to the ARU as a whole such that separation of the TAAT repeats by either 5 or 10 bp leads to a significant decrease in activin-stimulated transcriptional activity of the *GnRHR* promoter. It is interesting that this decrease in promoter function associated with the TAAT separations is comparable to the loss of activin-regulated activity of constructs containing mutations in either one of the two TAAT repeats. Thus, spatial separation of the TAAT sites in DARE is functionally equivalent to mutating one of the motifs reflecting the contribution, presumably, of the single remaining homeodomain binding site. Interestingly, a single TAAT site within DARE appears to at least partially retain activin responsiveness of the ARU. That is, activin responsiveness is attenuated with spatial separation or mutation of the distal or proximal TAAT motifs (μ14.4 or μ15.4) but is lost only when both TAAT sites are eliminated (μ14.4/μ15.4). Finally, we should underscore that the effect of spatial separation of the distal and proximal TAAT sites in DARE would not appear to be due to disruption of a critical sequence since mutation of the four intervening nucleotides did not affect the functional activity of this element *(11)*.

While the binding components at the distal member of the ARU (GRAS) have been studied extensively *(22,23)*, it is clear that one of the key questions regarding DARE is the identity of the protein(s) that mediate the functional contribution of this element to activin responsiveness. Although the wide array of homeodomain proteins expressed in αT3-1 cells presents multiple candidates, we were particularly intrigued with a recent study in which LHX3 was identified as a potential binding protein at DARE *(9)*. The potential contribution of this protein to activin regulation of the mouse *GnRHR* gene promoter was not, however, specifically addressed. Similar to McGillivray et al. *(9)*, we find that overexpression of LHX3 enhances transcriptional activity of the wild-type murine *GnRHR* promoter. Furthermore, both a single copy of DARE (1X DARE) or multimerized elements (3X DARE) were capable of conferring LHX3 responsiveness on the rat prolactin minimal promoter. Given the composite nature of the ARU, it was not particularly surprising that activin responsiveness was not evident for either the 1X or 3X DARE construct. Thus, to explore the potential role of LHX3 to the functional attributes of the ARU, we utilized a dominant repressor of LHX3 in which the Kruppel domain of human KOX1 is fused to the first 230 amino acids of human LHX3a (LHX3a–KRAB) *(24)*. Consistent with the ability of LHX3 to stimulate *GnRHR* promoter activity, overexpression of LHX3a–KRAB reduced transcriptional activity of the mouse wild-type *GnRHR* gene promoter. The impact of the KRAB fusion protein was attenuated with mutation of either the distal or proximal TAAT motifs and lost when both sites were mutated. Importantly, however, the LHX3a–KRAB fusion protein did not affect activin responsiveness of the *GnRHR* promoter. Thus, LHX3 appears to be capable of regulating transcriptional activity of the murine *GnRHR* promoter through DARE. This is curious given that the four-base spacing between the tandem TAAT motifs differ from the abutting TAAT motifs previously characterized for LHX3 *(26,27)*. However, these data do not support the notion that LHX3 is key to the contribution of DARE to the activin-responsive properties of the ARU. If correct, then Oct1 may represent another distinct possibility for mediating the functional contribution of DARE. Also, a homeodomain protein, Oct1, has been shown to interact with the CCAAT box-binding factor NF-Y at the proximal end of DARE *(10)*.

It has become increasingly clear that complex functional responses of promoters such as hormonal responsiveness are often dependent not on a single regulatory element but rather specific combinations of multiple elements that cooperate to form a hormone responsive unit. Consistent with this concept, activin responsiveness of the *GnRHR* gene promoter requires spatially distinct elements termed GRAS and DARE. Furthermore, it is not simply the presence of these elements that yield an ARU but a specific helical orientation. This spatial arrangement presumably facilitates protein–protein contacts that are necessary for the functional attributes of the *GnRHR* ARU; however, the specifics of this mechanism await a clearer understanding of the key activin responsive binding components at DARE. In this regard, while LHX3 is capable of activating the *GnRHR* promoter through DARE, our data do not support the notion that this protein accounts for the functional role of this element as part of the ARU.

Materials and Methods

Materials

αT3-1 cells were generously provided by Dr. Pamela Mellon (University of California San Diego). Dr. Wylie Vale (Salk Institute, La Jolla, CA) provided the adenoviral follistatin (AdCAFS288, hereafter referred to as Ad-follistatin) and GFP (Ad-GFP) constructs *(28)*. LHX3a and LHX3a–KRAB expression vectors were a gift from Dr. Simon Rhodes (Indiana University, Indianapolis, IN) *(24)*. Oligonucleotides were obtained from Invitrogen (Carlsbad, CA). DNA sequencing was conducted by Davis Sequencing, LLC (Davis, CA). Restriction enzymes and DNA-modifying enzymes were obtained from Fermentas (Hanover, MD) and New England Biolabs (Beverly, MA). The amplification and purification of Ad-CAFS288 and Ad-GFP were performed as

previously described *(29)*. DMEM tissue culture media was purchased from Mediatech, Inc. (Herndon, VA), while serum for tissue culture was obtained from Gemini Bioproducts (Woodland, CA). SuperFect was purchased from Qiagen Sciences (Valencia, CA).

Cell Culture and Transient Transfections

All cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. αT3-1 cells were maintained in high glucose DMEM containing 2 m*M* glutamine 5% fetal bovine serum, 5% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate.

Transient transfections and overexpression assays were performed according to a Qiagen SuperFect protocol. Briefly, αT3-1 cells were seeded 1 d prior to transfection at 80,000 cells per well. Eight hundred ng (.8μg) of promoter vectors and .2μg of RSV-β-galactosidase as a control for transfection efficiency were transfected per well. Cells were incubated with transfection mix for 3 h at which time mix was replaced with αT3-1 media containing Ad-follistatin or Ad-GFP. In the case of overexpression experiments, .5μg of promoter reporter constructs, .5μg of expression vectors, and .2μg of RSV-β-galactosidase were all co-transfected. According to Qiagen transient transfection protocol, cells were transfected for 3 h at which time transfection media was removed and replaced with media containing 1000 MOI of either Ad-GFP or Ad-follistatin.

Forty-eight hours post-infection, cells were harvested and assayed for luciferase activity *(5,23)*. Luciferase activity was normalized for transfection efficiency by dividing the luciferase activity by β-galactosidase activity. All transfections were repeated at least three times using two or three different plasmid preparations. Values are presented as the mean ± SEM.

Vector Construction

The pMGR-600Luc, 3XGRAS-Luc, 2 bp mutants μ14.4 and μ15.4 and the double TAAT motif mutant μ14.4/μ15.4 were previously described *(4,8,11)*. The plasmids containing 5, 10, 15, or 20 nucleotide insertions between GRAS and DARE were constructed in a multistep process. The sequence between the *Not*I site of the BR13 mutant *(6)* and the GRAS element was replaced with oligonucleotides containing the additional sequence. Oligonucleotides were ligated to an upstream fragment (from a *Hpa*I half site immediately downstream of GRAS to –600/*Sac*I) previously described *(11)* and then ligated into the BR13 mutant digested with *Sac*I and *Nco*I.

The constructs removing 5, 10, or 15 nucleotides between GRAS and DARE were created using PCR. The downstream fragments with deletion of nucleotides in the BR13 region were created by PCR using oligonucleotides containing deletions in the BR13 region and the GL2 primer. Resultant products were blunted with Klenow, digested with *Nco*I, and ligated with the upstream *Sac*I to *Hpa* fragment described above into pGL3.

Plus five (+5) and +10 TAAT spacer constructs were created by PCR. Upstream and downstream PCR fragments incorporated a *Hind*III site as part of the 5 or 10 bp insert between the TAAT motifs. The upstream fragment was digested with *Kpn*I/*Hind*III and the downstream fragment with *Hind* III/*Pst*I. These two fragments were then ligated into *Kpn*I/*Pst*I digested plasmid (pGL3).

Minimal promoter constructs containing one or three copies of the DARE element (1XDARE–Luc and 3XDARE–Luc) fused to the rat prolactin minimal promoter were constructed using oligonucleotides with sticky 5' *Cla*I and 3' *Hind*III ends. Oligonucleotides were annealed and ligated into pBSK cut with *Cla*I/*Hind*III. The oligonucleotides were released from pBSK by *Kpn*I/*Eco*RV digestion and ligated into a plasmid containing the rat prolactin minimal promoter fused upstream of the cDNA encoding luiferase *(5)*.

Statistical Analysis

In every transfection, each treatment and vector was analyzed in triplicate and the experiments replicated three times using different plasmid preparations. Data are expressed as means ± the standard error of the mean. Student's *t*-test was used to compare the difference between cells infected with Ad-GFP and Ad-follistatin within a vector. Student–Newman–Keuls (SNK) test was used to compare the activity of the mouse wild-type promoter to other vectors.

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