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Distal regulatory elements are required for *Fshr* expression, *in vivo*

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Abstract

The gonadotropin follicle-stimulating hormone (FSH) is required for initiation and maintenance of normal gametogenesis and acts through a specific, cell-surface receptor (Fshr) present only on Sertoli and granulosa cells in the gonads. Despite extensive examination of the transcriptional mechanisms regulating *Fshr*, the sequences directing its expression to these cells remain unidentified. To establish the minimal region necessary for *Fshr* expression, we generated transgenic mice carrying a yeast artificial chromosome (YAC) that contained 413 kilobases (kb) of the rat *Fshr* locus (YAC60). Transgene expression, as determined by RT-PCR, was absent from immature testis and Sertoli cells, limited to germ cells of the adult testis, and never observed in the ovary. While the data is limited to only one transgenic line, it suggests that the 413 kb region does not specify the normal spatiotemporal expression pattern of *Fshr*. Comparative genomics was used to identify potential distal regulatory elements, revealing seven regions of high evolutionary conservation (>80% identity over 100 bp or more), six of which were absent from the transgene. Functional examination of the evolutionary conserved regions (ECRs) by transient transfection revealed that all of the ECRs had modest transcriptional activity in Sertoli or myoid cells with two, ECR4 and ECR5, showing differential effects in expressing and non-expressing cells. These data reveal that distal regulatory regions (outside the 413 kb in YAC60) are required for appropriate temporal and spatial *Fshr* expression and implicate the identified ECRs in transcriptional regulation of *Fshr*.

Keywords: Fshr; Sertoli cell; Yeast artificial chromosome; Comparative genomics; Transgenics

1. Introduction

Mammalian gonad function is regulated by a hierarchical axis of hormones that modulate gametogenesis and steroidogenesis. Pulsatile secretion of gonadotropin releasing hormone by a population of neurons in the hypothalamus dominates control of the reproductive endocrine axis. This hormone stimulates anterior pituitary gonadotropes to produce the gonadotropin hormones, lutenizing hormone (LH) and FSH, which, in turn, stimulate somatic cells in the gonads via their specific G-protein coupled receptors. Both FSH and LH, along with their receptors, are requisite for normal gonad function and fertility in males and females (reviewed in Themmen and Huhtaniemi, 2000). The receptor for FSH, Fshr, is found only on testicular Sertoli cells and ovarian granulosa cells, and therefore restricts FSH action to these two cell types and defines the specificity of the hormone (Camp et al., 1991; Tilly et al., 1992; Rannikki et al., 1995; Tisdall et al., 1995). In the rodent testis and ovary, *Fshr* is expressed late in embryonic development and remains restricted to Sertoli and granulosa cells throughout postnatal life (reviewed in Simoni et al., 1997). Thus, transcriptional activation of the gene encoding Fshr is exquisitely controlled to limit its expression to only two cell types, providing an excellent model for examining cell-specific gene regulation in granulosa and Sertoli cells and the underlying control of FSH signaling.

The transcriptional mechanisms regulating *Fshr* expression have been examined in a number of species, including human, mouse, rat, and sheep, with the majority of these studies utilizing transient transfection and protein-DNA binding experiments to identify regulatory elements within the promoter region (Huhtaniemi et al., 1992; Gromoll et al., 1994; Sairam and Subbarayan, 1997; Heckert et al., 1998; Heckert and Griswold, 2002). Importantly, these studies highlighted several conserved features of the *Fshr* promoter. In particular, the minimal region required for maximal promoter activity generally resides within the first few hundred base pairs of 5′ flanking sequence (Gromoll et al., 1994; Linder et al., 1994; Heckert et al., 1998; Xing and Sairam, 2001). This promoter region contains a conserved E-box

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(5′-CACRTG-3′) in all four species, AP-1 (5′-TTARTCA-3′) and inverted GATA elements in the rodent promoters, and an E2F site (5′-TTTTCGCTGC-3′) in the mouse, rat, and human promoters (reviewed in Heckert, 2005). Principle among these elements is the E-box, which is responsible for the majority of *Fshr* promoter activity and is required for binding and transcriptional induction of *Fshr* by the ubiquitous basic helix–loop–helix transcription factors Usfl and Usf2 (Goetz et al., 1996; Heckert et al., 1998, 2000). Additionally, the orphan nuclear receptor steroidogenic factor 1 (SF-1) activates the *Fshr* promoters from mouse, rat, and sheep (Heckert, 2001; Levallet et al., 2001; Xing et al., 2002). Since Sertoli and granulosa cells are a subset of SF-1 expressing cells, SF-1 may participate in activating cell-specific *Fshr* expression (Val et al., 2003). In the rat, *Fshr* activation by SF-1 requires active Usf1 and Usf2 proteins as well as an intact E-box, suggesting that the E-box coordinates transcriptional regulation of the gene (Heckert, 2001). While informative, data from the above studies were largely generated by *in vitro* and cell culture analyses, and thus, a significant limitation of these studies was their inability to accurately evaluate transcriptional features associated with cell-specific gene expression. Therefore, to examine the promoter's ability to direct transcription to Sertoli and granulosa cells, animal models were employed.

To evaluate *Fshr* promoter function, *in vivo*, studies employed transgenic mice using promoter sequences to drive expression of marker genes (Linder et al., 1994; Heckert et al., 2000; Nordhoff et al., 2003). Two of these studies concluded that sequences outside the promoter region were needed to properly direct cell-specific expression (Heckert et al., 2000; Nordhoff et al., 2003). One examined 16 lines of transgenic mice that contained transgenes with either 5000 bp or 198 bp of promoter sequence, and found that cell-specific expression is not controlled through these promoter regions (Heckert et al., 2000). While each promoter fragment directed some transgene expression in the testis and ovary, there was also extensive expression in *Fshr*-negative tissues, demonstrating inappropriate or ectopic activation of the transgenes (Heckert et al., 2000). Most importantly, transgene expression in the testis originated primarily from the germ cell population, suggesting that the promoters did not recapitulate *Fshr* expression. Similar findings with respect to inaccurate temporal and germ cell expression were observed in a study employing a transgene with 1500 bp of the human *FSHR* promoter (Nordhoff et al., 2003). Thus, the evidence indicates that regulatory elements located outside of the promoter region (5000 bp and 1500 bp of 5′ flanking sequence in the rat and human genes, respectively) must play a key role in establishing proper cell-specific and temporal regulation of *Fshr*.

In support of the above conclusion, studies evaluating sequence conservation and DNase I hypersensitivity, two hallmarks of transcriptonal regulatory regions, also revealed that important elements for *Fshr* expression are located outside its promoter region (Hermann and Heckert, 2005). Numerous highly conserved, non-coding sequences, which serve as candidate regulatory elements, were shown to extend across *Fshr* and some of these colocalized with DNase I hypersensitive sites (Hermann and Heckert, 2005). However, given the large number of these conserved sites and the potential for them to act at large distances from the gene's coding region, it is clear that additional criteria are needed to help select those sites most likely to be functional. In the current study, we examined expression of a 413 kb yeast artificial chromosome in one line of transgenic mice in order to help define the sequences required for appropriate *Fshr* expression, and thus, restrict analyses of potential regulatory elements to those residing within a transcriptionally competent region. We also employed more advanced comparative sequence analysis to refine our search to only the most highly conserved sequences and thus ones most likely to be functional. The findings indicate that regulatory elements required for proper *Fshr* expression are located at a significant distance from the coding region and point to seven highly conserved regions as important factors in *Fshr* cell-specificity.

2. Materials and methods

2.1. Yeast strain and propagation

Original YACs purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA) were propagated in the host *Saccharomyces cerevisiae* strain J57D (*MAT*a, ψ^+, *ura3-52 trp1 ade2-101 his3-2,15 can1-100 leu2-3. 112*) (Zhong et al., 1998) and grown in complete medium lacking uracil and tryptophan. Modified YACs containing the rat *Fshr* gene were grown in complete media lacking at least one selective nutrient required for YAC maintenance. All media were prepared as described elsewhere and all yeast cell cultures were carried out overnight at 30 °C under vigorous agitation (Green et al., 1997; Riethman et al., 1997). Transformation of spheroplasted yeast cells was performed as described except yeast cells were treated with lyticase for approximately 15 min at 37 °C (Riethman et al., 1997; Karpova et al., 2005).

2.2. Rat Fshr YAC isolation and manipulation

DNA pools of a rat YAC library (constructed by the Whitehead Institute for Biomedical Research and MIT Center for Genome Research; average insert size of 830 kb) were purchased from Invitrogen Life Technologies and screened for rat *Fshr* using the polymerase chain reaction (PCR) and oligodeoxynucleotide primers directed against exon 1 (5′*Fshr*, Table 1) and exon 10 (3′*Fshr*, Table 1). Six *Fshr*-containing YACs, 124A9, 135C2, 197B11, 341C7, 368E4, and 392G6 were identified and purchased from Invitrogen. Rat *Fshr* YAC clone 135C2 was used for all further manipulation. High-molecular weight DNA was analyzed and mapped by pulsed-field gel electrophoresis (PFGE) and Southern blot analysis as described (Riethman et al., 1997; Karpova et al., 2005). Hybridization probes for Southern blot analysis were as follows: 5′*Fshr* (*Fshr* exon 1), 3′*Fshr* (*Fshr* exon 10), HIS3 (to detect the acentric arm of YAC r*Fshr*-HIS3), TRP1 (to detect the centric pRMLl vector arm), and URA3 (to detect the acentric pRML2 vector arm of the original YACs). DNA for Southern blot probes were PCR-amplified from yeast chromosomal or rat genomic DNA using the primers noted in Table 1 and radiolabeled with a random primer labeling system according to the vendor's recommendations (New England Nuclear, Boston, MA).

YAC r*Fshr*-HIS3 was generated by retrofitting 135C2 with a pRML2-HIS3 vector, resulting in the replacement of the URA3 marker with a HIS3 marker. pRML2-HIS3 was generated by removing the URA3 cassette from pRML2 by *Hin*dIII restriction digestion, blunting the overhangs with Klenow fragment (Roche Diagnostics Corporation, Indianapolis, IN), and inserting a 1.2 kb blunt-end HIS3 cassette generated by PCR (Table 1). All restriction enzymes were purchased from either Roche Diagnostics Corporation, New England Biolabs (Ipswich, MA) or Invitrogen Life Technologies. Yeast containing the 135C2 YAC were transformed with a 4.5-kb *Sca*I fragment of pRLM2-HIS3 and selection for growth on minimal medium lacking histidine and tryptophan. Positive colonies were screened by PFGE and Southern blot analysis utilizing a HIS3 probe (described above). Insertion of the bacteriophage P1 Cre recombinase gene was performed by two-step replacement using a yeast integration