ARTICLE
Journal of Cellular Biochemistry 105:61–69 (2008)

Journal of Cellular Biochemistry

Transcription Factor RFX4 Binding to the Testis-Specific Histone H1t Promoter in Spermatocytes may be Important for Regulation of H1t Gene Transcription During Spermatogenesis

Steven A. Wolfe,[1,2] Jane M. vanWert,[1,2] and Sidney R. Grimes[1,2*]

[1]*Research Service (151), Overton Brooks Veterans Administration Medical Center, Shreveport, Louisiana 71101-4295*

[2]*Department of Biochemistry and Molecular Biology, LSU Health Sciences Center, Shreveport, Louisiana 71130-3932*

ABSTRACT

The X-box binding protein RFX4 is highly expressed in testis in contrast with other tissues, but its function there is unknown. Another family member abundant in testis, RFX2, has been shown to bind to the X-Box elements in the promoter of the testis specific histone H1t, which is expressed only in pachytene spermatocytes. RFX proteins are known to dimerize, and there is the possibility that the abundant testis RFX4, which is also expressed in pachytene spermatocytes as shown by RT-PCR and Western blotting, may interact with RFX2 in these cells. In EMSA anti-RFX2 polyclonal antibodies produce a supershifted complex with testis extracts and an X-Box probe. On the other hand, RFX4 polyclonal antibodies do not supershift the complex but appear to enhance formation of the complex. RFX4 appears to co-precipitate with RFX2 in immunoprecipitation, and to co-purify with RFX2 in an affinity purification using a biotinylated X-box affinity probe. In ChIP assays RFX4 also binds to the H1t promoter in vivo. These data suggest a possible regulatory role for RFX4 in transcription of the histone H1t gene during spermatogenesis. J. Cell. Biochem. 105: 61–69, 2008.

KEY WORDS: TESTIS; HISTONE H1t; SPERMATOCYTES; TRANSCRIPTION; RFX4; RFX2

RFX4 is one member of a subfamily of helix-turn-helix (winged-helix) transcription factors that bind to symmetrical X-box DNA elements of the sequence 5′-GTNRCC(0-3N)RGYAAC-3′ (where N is any nucleotide, R is a purine, and Y is a pyrimidine) [Gajiwala et al., 2000]. The RFX proteins, RFX 1, 2, 3, 4, and 5, share a DNA binding domain (DBD) of 76 conserved residues. The crystal structure of the DBD is very similar to that of the globular domains of linker histones [Gajiwala et al., 2000]. RFX proteins also share other conserved elements including a glutamine rich domain, a proline- and glutamine-rich domain, and a dimerization domain [Morotomi-Yano et al., 2002].

RFX4 was first discovered as part of a cDNA isolated from human breast cancer, encoding a partial DNA binding Domain (DBD) fused to the estrogen receptor [Dotzlaw et al., 1992]. The complete protein consists of several of the conserved RFX domains, including a dimerization domain as well as the DBD, but lacks a transcriptional activation domain [Morotomi-Yano et al., 2002]. RFX4 has been shown to dimerize with RFX2 and RFX3 as well as itself, but not RFX1. The C-terminal domain is a possible transcriptional repressor domain [Morotomi-Yano et al., 2002].

Several alternatively spliced isoforms have been identified; RFX4 A,B&C [Matsushita et al., 2005] found in testis, D in brain superchiasmatic nucleus [Araki et al., 2004], and E&F which are overexpressed in some gliomas [Matsushita et al., 2005]. The level of expression of the brain isoform D appears to be about 1% that of the testis isoforms [Matsushita et al., 2005].

We reported that testis RFX2 binds to the testis-specific linker histone H1t promoter and it appears to activate transcription of the H1t gene in pachytene primary spermatocytes during spermatogenesis. Histones are highly conserved basic proteins that bind and compact DNA and regulate gene expression through a system referred to as the histone code [Strahl and Allis, 2000]. The term "histone code" refers to dynamic post-translational modifications including acetylation, phosphorylation, methylation, ubiquitylation, sumoylation, and ADP-ribosylation [Strahl and Allis, 2000; Berger, 2007]. H1 histones, also called linker histones, bind to

Grant sponsor: Department of Veterans Affairs (Merit Review Grant to SRG).

*Correspondence to: Dr. Sidney R. Grimes, PhD, Research Service (151), Overton Brooks VA Medical Center, 510 E. Stoner Ave., Shreveport, LA 71101-4295. E-mail: sidgrimes@comcast.net

Received 24 March 2008; Accepted 26 March 2008 • DOI 10.1002/jcb.21793 • 2008 Wiley-Liss, Inc.

Published online 5 May 2008 in Wiley InterScience (www.interscience.wiley.com).

DNA between nucleosomes, aiding the transition from the loose "beads-on-a-string" conformation to the more condensed solenoid conformation.

The testis H1t linker histone is expressed only in pachytene primary spermatocytes, making it an excellent system in which to study tissue-specific gene expression [Wolfe and Grimes, 2003]. See Figure 2 for a time line of spermatogenesis and major germinal cell types. In our experiments this large tetraploid germinal cell type is highly enriched by centrifugal elutriation in fraction 5. Histone H1t is probably important for some of the dramatic changes in gene expression seen in germinal cells and it may be important for critical DNA repair and crossing over events that occur in spermatocytes. After completion of two meiotic cell divisions, H1t transcription ceases in haploid early spermatids (elutriator fraction 3). Later during spermiogenesis (late spermatids, elutriator fraction 1) all histones are replaced by protamines as the chromosomes are highly condensed.

Unlike other histone promoters which lack an X-box, the H1t promoter contains the two X-box elements initially called TE1 and TE2, now called X-box1 and X-box 2 [Wolfe and Grimes, 2003; Wolfe et al., 2004]. See Figure 7 for a diagram of the testis-specific Histone H1t promoter. Studies in transgenic mice showed that mutation of the rat H1t X-boxes abrogated expression of the rat transgene [vanWert et al., 1995, 2008]. When the X-box sequence was used as an affinity probe to purify binding proteins from testis nuclear extracts, the major protein identified was RFX2 [Wolfe et al., 2004], a protein of 692 amino acids (GenBank #XM_23677) with a mass of approximately 87 kDa. Electrophoretic Mobility Shift Assays (EMSA) using the H1t X-box as a probe produced a complex that could be shifted using a polyclonal antibody to RFX2 [Wolfe et al., 2004]. Chromatin Immunoprecipitation (ChIP) assays verified that RFX2 is bound to the H1t promoter in pachytene primary spermatocytes in vivo [vanWert et al., 2008].

RFX4 is also highly expressed in testis, although its function there is unknown [Morotomi-Yano et al., 2002; Matsushita et al., 2005], and is known to be able to heterodimerize with RFX2 [Morotomi-Yano et al., 2002]. This article examines the possibility that RFX4 is involved in regulation of expression of H1t in developing germinal cells.

MATERIALS AND METHODS

ISOLATION OF POPULATIONS OF HIGHLY ENRICHED GERMINAL CELL TYPES

Rats were purchased from Harland Sprague-Dawley (Madison, WI). Animal studies were performed in an AAALAC accredited facility in accordance with the *Guide for Care and Use of Laboratory Animals* in a protocol approved by our institution. Seminiferous epithelium from rat testes was minced and trypsinized to create a single cell suspension. Cells were then separated by centrifugal elutriation to produce fractions enriched in specific cell types (Fig. 2) as described previously [Wolfe et al., 2006].

ISOLATION OF RNA

RNA from adult and 7-day-old rat tissues and from populations of germinal cells enriched by centrifugal elutriation was isolated using RNA Stat-60 (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. Purified RNA was resuspended in nuclease free water (Promega, Madison, WI) and quantitated using an Experion system (Bio-Rad, Hercules, CA) with RNA StdSens chips. Each RT-PCR reaction used 100 ng of RNA.

RFX4 CLONING AND ANTISERUM PRODUCTION

RFX4 was initially amplified from rat testis cDNA using the RFX4 primer pair 5′-GATGTGATGTACTCAAAGAAAGG-3′ (plus strand) and 5′-CTATGAAAGCAGTCACTTAGCCC-3′ (minus strand). The resulting 1.8 kb PCR product was isolated, cloned into pCR2.1 TOPO (Invitrogen) and sequenced. The RFX4 insert was used as a template to amplify a fragment using the same minus strand primer and a modified forward primer, 5′-CGGGAATTCCGACGATGACGATAA-GATGTACTCAAAGAAAGG-3′, introducing an *Eco*RI site and permitting an in-frame fusion with the protein A gene sequence contained in pRIT2T. The amplified fragment was digested with *Eco*RI and inserted into the corresponding site in pRIT2T. Expression of the protein A-RFX4 fusion protein was induced in Escherichia coli following the pRIT2T supplier's protocol (Pharmacia LKB Biotechnology, Inc.). The recombinant protein was purified using an IgG Sepharose 6 fast flow column (GE Healthcare) and used for production of antiserum in a rabbit by the Animal Resources facility at LSUHSC (Shreveport, LA). Anti-RFX4 IgG was isolated using an antibody affinity chromatography kit (Sigma, PURE-1A). The RFX4 clone is depicted in the middle diagram and the region used for the fusion protein is depicted in the lower diagram of Figure 3.

Another RFX4 clone, pRFX4, was obtained using RNA from adult rat testis to make cDNA using Promega's Reverse Transcription kit and random primers. A fragment comprising most of the rat RFX4 transcript was amplified by PCR using forward primer 5′-ATAACAGAGCGTCCAAGCC-3′ and reverse primer 5′-CCCGATT-CACTGACGAAAGTATC-3′. These primers extend from rat RFX4 cDNA nucleotides 553 through 2627 with reference to GenBank #XM-576205.2. The PCR product was ligated into Invitrogen's pCR 2.1 TOPO cloning vector and transformed into One Shot competent cells (Invitrogen). This vector, containing a longer form of rat RFX4 cDNA that includes the DNA Binding Domain, was used as a quantitation control template in RT-PCR.

PREPARATION OF CHROMATIN

Chromatin was prepared from rat tissues following the protocol of Fujii et al. [2006] and Zeng et al. [2006] with modifications. Briefly, 500 mg of tissue was minced, washed in PBS, crosslinked with 1.5 mM Ethylene glycolbis-(succinimidylsuccinate) (EGS) in PBS at room temperature for 30 min, then with 1% formaldehyde (final) in PBS for 10 min at room temperature, followed by inactivation with 1 M glycine. Populations of germinal cell types enriched by centrifugal elutriation were crosslinked in PBS in tissue culture flasks in the same manner. Cells and tissues were recovered by centrifugation, rinsed with PBS, homogenized in Dignam Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol) using a Dounce homogenizer and centrifuged [Dignam et al., 1983]. The cells were resuspended in Buffer A containing 0.1 mM PMSF and a protease inhibitor cocktail (Sigma #P8340), homogenized, and incubated on ice. After centrifugation

the nuclear pellets were resuspended in lysis buffer (from Upstate's ChIP kit) with protease inhibitors, incubated on ice, and sonicated to yield chromatin consisting of 500–1,000 bp DNA fragments. Chromatin was stored at −80°C.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAYS

ChIP assays were performed using the ChIP kit from Upstate following the manufacturer's protocol, except that reactions were scaled down ten fold and with the addition of a second pre-clearing step with normal rabbit IgG (Santa Cruz) and Protein A Agarose beads with sonicated salmon sperm DNA (Upstate). One microliter of a 1:40 dilution of RFX4 antiserum stock solution amounting to approximately 500 ng of protein was used in the Immunoprecipitation. The control anti-rabbit antibody was purchased from Santa Cruz. In addition a no-antibody control and a positive histone H1t gene control (500 ng sonicated genomic DNA from rat testis) were included. Sonicated chromatin representing 50 μg of DNA was used in each ChIP reaction. Primers used for amplification, which covered the rat H1t promoter, were F5 5′-TGTGTCATAACCTGAGCGATTC-3′ and R5 5′-GCTGTGATTGGTG-CATCCC-3′ producing a 125 bp DNA fragment (nucleotides 2,217–2,341 based upon GenBank sequence # M28409).

REAL-TIME PCR AND RT-PCR

Quantitative real-time PCR and RT-PCR were performed on a MyiQ Real-Time PCR Detection System from Bio-Rad using their iQ SYBR Green Supermix for PCR with cDNA made from Bio-Rad's iScript cDNA synthesis kit, or their iScript One-Step RT-PCR kit with SYBR Green for mRNA surveys following the protocols supplied with the reagents. Optimal annealing temperatures were determined using temperature gradients performed on the MyiQ. Primers used for real-time RT-PCR were:

5′-AGTCTGGCAGCAATGTCC-3′, RFX4 RT R2;
5′-TGTGATGTACTCAAAGAAAGG-3′, RFX4 RT F2;
5′-TCCCTTCATGGCTCGCATCAG-3′, RFX4 R;
5′-CGCAGACTCATCATCCAGTTG-3′, RFX4 F.

Primer pair F2-R2 amplifies a 115 bp fragment just downstream of the DBD (nucleotides 833–948), primer pair F-R amplifies a 338 bp fragment beginning in the dimerization domain and extending downstream (1,527–1,865).

Standard curves for each primer pair used in real-time PCR were generated using a 10-fold dilution series of plasmid pRFX4 spanning the mRNA sequence from +553 to +2,629 (with reference to GenBank sequence XM-576205). For H1t ChIP primers the standard curve was generated using a twofold dilution series of sonicated rat testis genomic DNA.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

Nuclear extracts from enriched testis cell populations isolated by centrifugal elutriation were prepared by the Dignam method as described previously [Dignam et al., 1983; Wolfe et al., 2004]. Purified extracts were stored in Buffer D (20 mM HEPES pH 8.0, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol) at −80°C. Whole cell extracts from rat tissues were prepared using the RIPA Lysis Buffer kit (sc-24948) from Santa Cruz following the supplier's protocol. Protein concentrations were determined using the Bio-Rad Experion system or by Bradford assay using the Bio-Rad Protein Assay dye reagent.

EMSAs were performed using nuclear extracts from tissues and testis cell types as described previously [Grimes et al., 1987; Wolfe et al., 2004] using X-box (5′-GGAGGCGCCTAGGGATGC-3′) and IL-5Rα (5′-AGTGTGCCTAGGAGACAGA-3′) double-stranded probes. The probes were made by annealing complimentary oligonucleotides (Sigma–Genosys) and end-labeling the product with T4 polynucleotide kinase (New England Biolabs) and (γ-^{32}P)-ATP (Perkin Elmer). EMSA anti-RFX2 (A-18x) supershift antibody was purchased from Santa Cruz; RFX4 antiserum preparation was described earlier.

WESTERN BLOT ANALYSIS

Western blots were performed as described [Grimes et al., 1987; Wolfe et al., 2004] using whole cell extracts from various tissues and testis cell types and RFX4 antiserum. Proteins were electrophoresed on Bio-Rad Tris–HCl Ready Gels using the Bio-Rad mini-PROTEAN gel apparatus, along with Precision Plus prestained molecular weight standards from Bio-Rad and Cruz Markers protein molecular weight standards from Santa Cruz, and blotted to a PVDF membrane (Bio-Rad). Secondary antibodies, HRP conjugated donkey anti-rabbit (used for RFX4) and HRP conjugated donkey anti-goat (used for RFX2), were purchased from Santa Cruz. Western blots were developed using the SuperSignal West Dura Kit from Pierce for chemiluminescent detection and analyzed using a Bio-Rad Versa-Doc imaging system.

IMMUNOPRECIPITATION

Testis nuclear extracts brought to a volume of 500 μl with Dignam Buffer D were precleared with Protein AG Plus agarose (Santa Cruz) at 4°C for 30 min. Beads were centrifuged for 30 s and the supernatants transferred to fresh tubes. Antibody was added to the supernatants and incubated for 1 h at 4°C with rotation. Protein A/G Plus agarose beads (20 μl) were added and incubated for 1–3 h at 4°C with mixing. Beads were collected by centrifugation and washed 3 times with PBS containing PMSF and protease inhibitor cocktail (Sigma). After the final wash the beads were resuspended in 50 μl 2× SDS electrophoresis sample buffer, boiled for 5 min and loaded on a 10% SDS–polyacrylamide gel.

AFFINITY PURIFICATION OF RFX4

A double-stranded biotinylated probe (Sigma–Genosys) corresponding to the rat H1t promoter X-box sequence was used to affinity purify DNA binding proteins from elutriator fraction 4 nuclear extracts. To form this DNA probe offset oligonucleotides were annealed and the recessed ends were filled using the Klenow fragment of DNA polymerase I (NEB). The oligonucleotides were Biotin-5′-GAGGCGCCTAGGGATGCAC-3′ and 5′-GTGCATCCC-TAGGCGCCTC-3′. Bound probe was recovered with streptavidin magnetic beads (Promega), rinsed three times with binding buffer to remove non-specific binding, and the beads were boiled in SDS–PAGE Sample buffer (Bio-Rad #161-0737) to release the proteins. Equal loads of eluted proteins were then identified by Western blotting using RFX4 antiserum.

RESULTS

RFX4 mRNA LEVELS ARE HIGH IN TESTIS AND MOST ABUNDANT IN SPERMATOCYTES AND EARLY SPERMATIDS

In previous studies we reported that the histone H1t proximal promoter contained two adjacent X-box sequences located just upstream from a CCAAT-box. We found that Regulatory Factor RFX2 binds to these two histone H1t X-box sequences in vitro and in vivo in rat testis pachytene primary spermatocytes and that the two X-boxes and their cognate RFX2 transcription factor are critical for transcription of the histone H1t gene in spermatocytes. Reports from other laboratories indicate that RFX4 is present in testis at a high level and originally RFX4 was designated the testis-specific RFX. Therefore, we wanted to confirm the presence of RFX4 in testis and to determine which testis cell types expressed RFX4. We also wanted to test the possibility that RFX4 may bind to the X-boxes of the testis-specific histone H1t during spermatogenesis, since it contains a highly conserved DNA binding domain.

When we examined total cellular RNA from different adult rat tissues by RT-PCR, we found that the steady-state level of RFX4 mRNA was much more abundant in testis that in any other cell type examined confirming earlier literature reports (Fig. 1). To examine expression of RFX4 in various testis cell types, we prepared single cell suspensions of adult testis cells that were separated into enriched populations of cell types by centrifugal elutriation. RT-PCR of total cellular RNA from these enriched populations of cells revealed that the steady-state level RFX4 mRNA was highest in pachytene primary spermatocytes (elutriator fraction 5) and in early spermatids (elutriator fraction 3) (Fig. 2). RFX4 mRNA levels were very low in late spermatids as well as in testes from 7-day-old rats. The testes from these sexually immature 7-day-old rats lack both spermatocytes and late spermatids but are enriched in spermatogonial stem cells (Fig. 2).

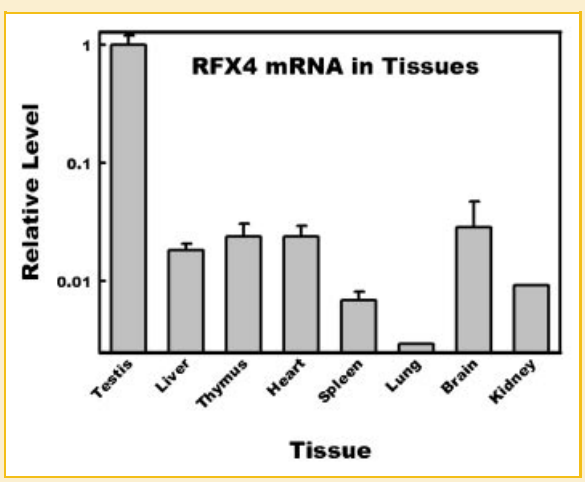

Fig. 1. RFX4 is most actively expressed in testis. Samples of mRNA from rat tissues were subjected to quantitative real-time RT-PCR. The testis steady-state RFX4 mRNA level is an order of magnitude higher than levels in liver, thymus, heart, and brain and more than two orders of magnitude higher in testis than in spleen, lung, and kidney. Relative mRNA levels are plotted on a log scale.

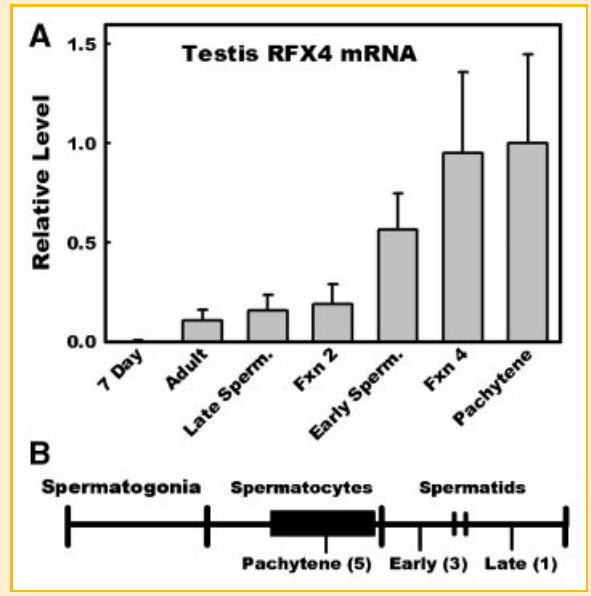

Fig. 2. RFX4 is most actively expressed in pachytene primary spermatocytes. The steady-state RFX4 mRNA level is maximal in pachytene spermatocytes, it is in lower levels in other stages of spermatogenesis and adult rat testis, and little is found in testes from sexually immature 7-day-old rats. Fxn 1–Fxn 5 represent 5 populations of germinal cells enriched by centrifugal elutriation with Fxns 1, 3, and 5 highly enriched in late spermatids (late sperm), early spermatids (early sperm), and pachytene primary spermatocytes (pachytene) respectively. Relative mRNA levels are plotted on a linear scale.

TESTIS RFX4 PROTEIN IS ABUNDANT IN SPERMATOCYTES, EARLY SPERMATIDS AND LATE SPERMATIDS

Commercial antibodies were available for Western blot analysis of RFX1, RFX2, RFX3, and RFX5 but not for analysis of rat RFX4. Therefore, to prepare polyclonal antibodies against rat testis RFX4, we prepared cDNA from rat testis, cloned and screened this cDNA for RFX4 sequences as described in Materials and Methods Section. We amplified a region of one RFX4 cDNA clone starting just downstream from the highly conserved DNA binding domain (DBD) and covering nucleotides 836–2,534 based upon the numbering system of the rat cDNA sequence (XM_576205) as shown in the diagram in Figure 3. We cloned this DNA region into the pRIT2T expression vector. A fusion protein composed of protein A and RFX4 was expressed in E. coli, affinity purified using Sepharose IgG beads, and used as an antigen in rabbits. IgG was prepared from the rabbit antiserum using protein A/G beads and the purified antibodies were used in our Western blot analyses.

We conducted Western blots to detect RFX4 in testis cell populations and to determine whether the RFX4 protein levels reflected RFX4 mRNA levels seen in germinal cell types. We were surprised to find a major band of about 67 kDa that was abundant in all cell populations enriched by centrifugal elutriation including spermatocytes (elutriator fraction 5), early spermatids (elutriator fraction 3) and late spermatids (elutriator fraction1), since the steady-state mRNA level was highest in primary spermatocytes (Figs. 2 and 4). An additional stained band of about 34 kDa was also present in all germinal cell types (Fig. 4). In this blot, the 34 kDa band

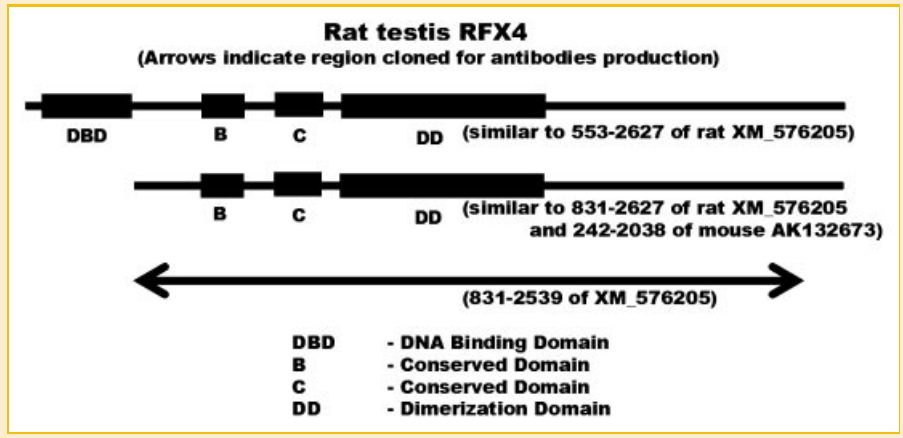

Fig. 3. Map of our rat testis RFX4 gene showing conserved regions. The sequence of our longer rat testis RFX4 clone is similar to the rat GenBank clone XM_576205. The sequence of our shorter testis RFX4, lacking the DBD, is almost identical in sequence and size to the mouse testis RFX4 sequence (GenBank #AK132673). The highly conserved DNA Binding Domain (DBD), conserved domains (B) and (C), and a Dimerization Domain (DD) are indicated. A line with arrow heads represents the region subcloned into the expression vector pRIT2T for protein A–RFX4 fusion protein expression. This fusion protein was used as antigen to produce antisera against rat testis RFX4.

appeared to be more intense in late spermatids (LS—elutriator fraction 1) and less intense in pachytene spermatocytes (P—elutriator fraction 5), but in repeated blots, the 34 kDa band was more equally distributed in the different cell populations so that the apparent relative level was not appreciably lower in spermatocytes than in other cell populations. The relative levels of the 67 and 34 kDa bands were fairly constant in the various cell populations. Both 67 and 34 kDa bands appeared to be doublets when examined in blots of gels of various polyacrylamide concentrations. The doublets are apparent in the late spermatids (LS—elutriator fraction 1) (Fig. 4).

ANTIBODIES AGAINST TESTIS RFX4 APPEAR TO ENHANCE BINDING OF RFX2 TO THE X-BOX IN EMSA

In previous studies we were able to demonstrate in vitro binding of RFX2 to the H1t X-box sequence in EMSA and EMSA supershift assays. To determine whether our polyclonal antibodies against testis RFX4 could detect RFX4 binding in this way, we conducted EMSAs with a canonical X-box probe. As we have reported previously, when the radiolabeled X-box probe was mixed with testis nuclear extracts, a shifted complex was formed as shown in lane 1 of Figure 5. When commercial polyclonal antibodies directed against RFX2 were added to the testis nuclear extract plus probe mixture, a supershifted band was formed as seen before (lane 2 of Fig. 5). When polyclonal antibodies against RFX4 were added to the testis nuclear extract plus probe, a supershifted band did not form (lane 3 of Fig. 5). It is possible that RFX4 does not bind significantly to the X-box or that our polyclonal antibody preparation against RFX4 interferes with binding of RFX4 to the probe.

Also notice that when anti-RFX4 antibodies were added, the intensity of the band representing the shifted complex was increased and intensity of the free probe band decreased (compare intensities of the shifted complex in lanes 3 and 1). When the anti-RFX4 antibody was added to the extract plus probe mixture and then anti-RFX2 antibody was added, the supershifted RFX2 band formed, but the intensity of the supershifted band was higher and the intensity of the free probe band was much lower as shown in lane 4 of Figure 5 (compare lanes 4 and 2).

In these experiments it appears that anti-RFX4 antibody actually enhanced binding of RFX2 to the X-box. To test this possibility, we added increasing amounts of anti-RFX4 as shown in lanes 5–7 of Figure 5. As more anti-RFX4 was added the intensity of the shifted complex increased and the intensity of the free probe decreased. It

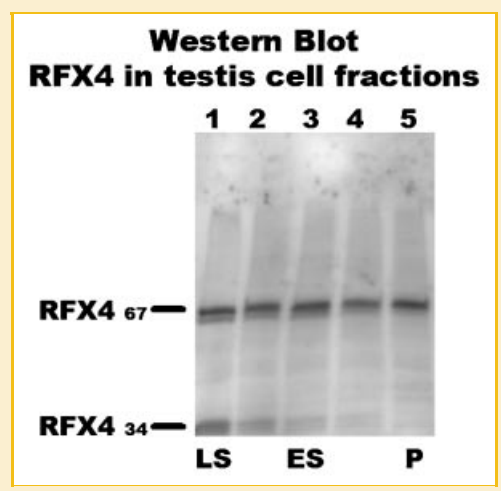

Fig. 4. RFX4 protein is abundant in nuclei from all testis germinal cell types. Proteins for use in Western Blots were extracted from nuclei derived from the five testis germinal cell populations enriched by the technique of centrifugal elutriation. Blots were probed using anti-RFX4 IgG prepared as described in Materials and Methods Section. Two major RFX4 bands of 67 and 34 kDa were detected. The 67 kDa band is the predicted size of the peptide from the mouse testis RFX4 sequence (GenBank #AK132673). While relative levels of the 67 kDa band were equal in all five populations of germinal cells, the level of the 34 kDa band appeared higher in late spermatids. However, in repeats of SDS–PAGE and Western blots this higher mobility band was more equally distributed in all five cellular populations. Lanes 1, 3, and 5 (Fractions 1, 3, and 5) represent nuclear proteins from late spermatids (LS), early spermatids (ES), and pachytene primary spermatocytes (P).

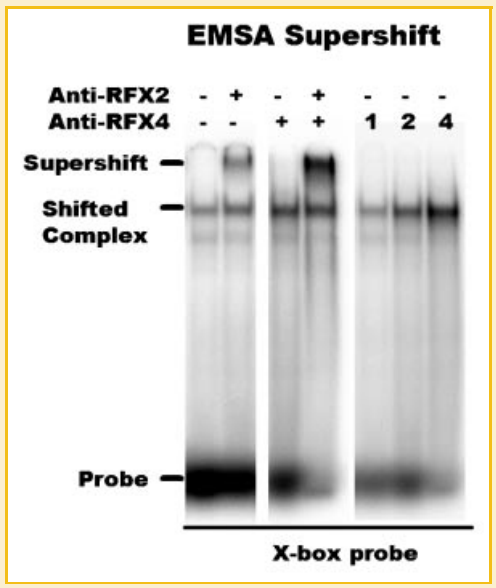

Fig. 5. Rat testis RFX4 antibodies appear to enhance RFX2 binding to the X-box. Nuclear extracts from total testis and a double-stranded consensus IL-5Rα X-box probe (Materials and Methods Section) were used in each lane (not shown are similar results obtained with the H1t X-box probe). Lane 1 shows the typical shifted complex seen with binding of testis nuclear extracts to this probe and lane 2 shows a supershifted band that forms when anti-RFX2 antibodies are added to the binding reaction. Purified rabbit anti-RFX4 IgG enhanced the intensity of the shifted complex (compare lanes 1 and 3). Anti-RFX4 also enhanced the supershifted band produced by anti-RFX2 (compare lanes 2 and 4). Adding increasing amounts of anti-RFX4 increased the enhanced shifted complex (lanes 5–7).

appears that adding anti-RFX4 to the testis nuclear extract allowed more binding of RFX2 to the X-box probe. Normal rabbit IgG and other commercial antibody preparations were tested as controls and no other antibody preparation enhanced formation of the band representing the shifted complex or the supershifted RFX2 complex (data not shown).

RFX4 BINDS WEAKLY TO THE HISTONE H1t X-BOX USING AN X-BOX DNA AFFINITY PROBE

In a previous study we showed that RFX2 could bind to the X-box sequence in a DNA affinity experiment where we used a double-stranded DNA probe labeled with biotin on one end as the affinity probe. We detected RFX2 in the affinity purified protein in those experiments by Western Blot analysis using commercial antibodies against RFX2. To determine whether RFX4 might bind in the same way to this X-box DNA affinity probe, we conducted the experiment again, but the Western Blot was analyzed using the polyclonal antibodies against RFX4. In this case, the anti-RFX4 antibodies would not interfere with binding of RFX4 to the DNA. When testis nuclear proteins were eluted from the affinity beads, the two RFX4 bands of 67 and 34 kDa were observed in Western Blot analysis but the bands were very faint as shown in Figure 6. In this experiment it is possible that RFX4 may bind directly to the DNA as a homodimer, or it may bind directly to the DNA as an RFX4-RFX2 heterodimer or as a heterodimer between RFX4 and another RFX family member.

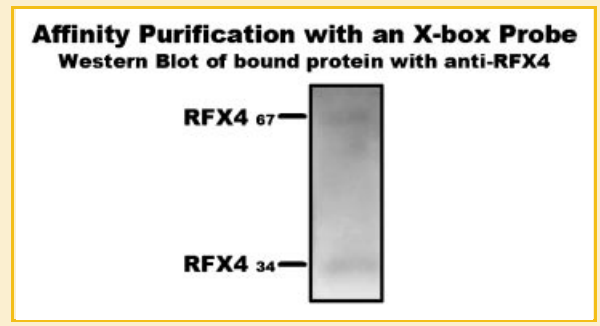

Fig. 6. Transcriptional factor RFX4 appears to bind to an X-box affinity probe. Proteins in testis nuclear extracts were affinity purified with a biotinylated rat H1t X-box probe and were analyzed by Western blot analysis. Polyclonal antibodies against rat testis RFX4 revealed the same 67 and the 34 kDa bands seen in extracts of nuclei from enriched populations of germinal cells (Fig. 4). The bands were faint possibly indicating weak direct binding to the DNA probe or indirect binding to another factor that binds directly to the X-box sequence.

Alternatively, RFX4 may not bind directly to the DNA but may associate with a protein complex that binds the X-box affinity probe.

RFX4 BINDS THE X-BOX OF THE HISTONE H1t PROMOTER IN PACHYTENE SPERMATOCYTES IN VIVO

Previously, we demonstrated that RFX2 is bound to the histone H1t promoter in vivo in a population of cells enriched in pachytene primary spermatocytes using chromatin immunoprecipitation assays. To determine whether RFX4 was bound to the H1t promoter in primary spermatocytes we conducted ChIP using our polyclonal antibodies against RFX4. As shown in Figure 7, RFX4 was bound to the H1t promoter in pachytene spermatocytes. As a positive control we used rat genomic DNA and as a negative control we used a normal rabbit IgG preparation. To compare RFX4 and RFX2 binding we precipitated another sample using antibodies against RFX2. RFX2 and RFX4 both clearly bind to the promoter in pachytene primary spermatocytes.

When we repeated the experiment using a cell population enriched in early spermatids, there was no significant binding of either RFX2 or RFX4 to the H1t promoter in early spermatids in vivo (data not shown).

RFX4 MAY ASSOCIATE WITH RFX2 IN SPERMATOCYTES AND EARLY SPERMATIDS

Data presented in Figure 6 reveal that RFX4 can bind to the H1t promoter X-box sequence in DNA affinity binding assays. RFX4 appears to bind the histone H1t promoter in pachytene primary spermatocytes in vivo (Fig. 7). RFX2 also binds to the H1t promoter in spermatocytes in vivo as confirmed in Figure 7. It is possible that RFX2 homodimers and RFX4 homodimers can bind the histone H1t X-box sequence independently. However, it is possible that RFX2 and RFX4 may bind the X-box sequence as RFX2-RFX4 heterodimers. Alternatively, only RFX2 homodimers may bind directly to the DNA sequence and RFX4 dimers may associate

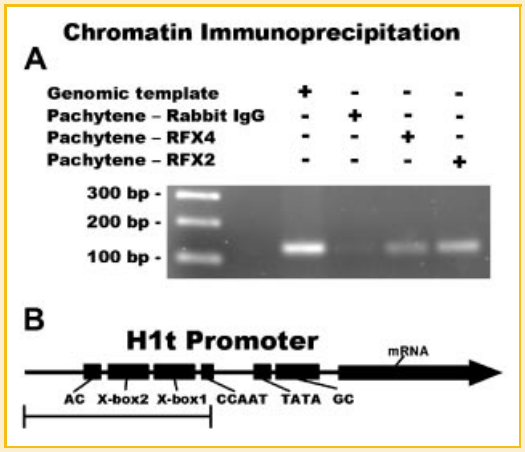

Fig. 7. Both RFX4 and RFX2 bind to the H1t promoter in pachytene spermatocytes in vivo. DNA from ChIP reactions was amplified by real-time PCR using primers that cover the H1t promoter as shown in the diagram. DNA derived from pachytene spermatocyte chromatin that had been precipitated with antibodies against RFX2 yielded a PCR product of the expected 125 bp size (last lane) compared to chromatin precipitated using control rabbit IgG antibodies. DNA from the same pachytene chromatin but precipitated with antibodies against RFX4 also produced a PCR product of the expected size (next to last lane).

directly with RFX2 homodimers or indirectly with accessory factors that are also associated with RFX2.

To test the possibility that RFX2 and RFX4 may associate with each other in testis, we conducted immunoprecipitation assays as described in Materials and Methods Section. Western blot analysis (using anti-RFX4 IgG) of proteins immunoprecipitated with anti-RFX4 revealed precipitation of abundant RFX4 in the positive control shown in lane 4 of the left panel of Figure 8. However, RFX4 was also present in RFX2 immunoprecipitates of proteins from nuclear extracts derived from cells enriched in early spermatids, pachytene spermatocytes, and total testis as shown in lanes 1–3, respectively.

When the same blot was stripped and re-probed with anti-RFX2 IgG (or a new blot was probed directly with anti-RFX4), RFX2 was clearly present where proteins were immunoprecipitated with anti-RFX2 (lanes 1–3 of the right panel of Fig. 8), but RFX2 was also present where proteins were immunoprecipitated with anti-RFX4 (lane 4 of the right panel of Fig. 8). These experiments support the hypothesis that testis RFX4 and RFX2 may associate with each other in primary spermatocytes and early spermatids.

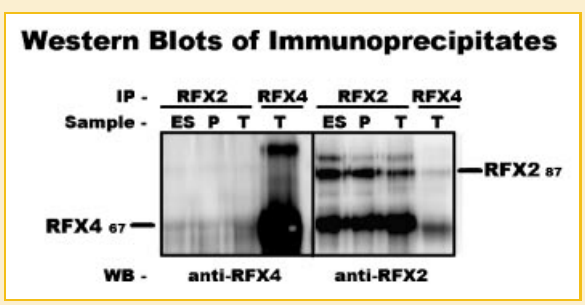

Fig. 8. RFX4 may associate with RFX2 in spermatocytes and early spermatids. Immunoprecipitation assays were conducted as described in Materials and Methods Section. Western blot analysis of proteins immunoprecipitated with anti-RFX4 polyclonal antibodies revealed precipitation of RFX4 when blots were developed using anti-RFX4 (positive control in lane 4 of the left panel). RFX4 was also present in anti-RFX2 immunoprecipitates of proteins from nuclear extracts from early spermatids, pachytene spermatocytes, and total testis (lanes 1–3, respectively). When this blot was stripped and re-probed with anti-RFX2 antibodies, RFX2 was present in lanes 1–3 where proteins were immunoprecipitated with anti-RFX2. RFX2 was present in lane 4 where proteins were immunoprecipitated with anti-RFX4 antibodies.

DISCUSSION

The mammalian testis-specific linker histone H1t is expressed in spermatocytes during spermatogenesis [Grimes et al., 1987]. The H1t promoter contains a TATA-box, a CCAAT-box, and an AC-box that are conserved in most mammalian H1 family members [vanWert et al., 1995]. However, only the H1t promoter contains X-box sequences [Wolfe and Grimes, 2003; Wolfe et al., 2004]. Two X-box sequences that are located between the TATA-box and AC-box are critical for activation of the H1t promoter. When the rat H1t promoter region containing the two X-boxes was deleted in transgenic mouse experiments, the rat transgene was no longer expressed in mouse spermatocytes [vanWert et al., 1995]. Nuclear proteins extracted from rat pachytene primary spermatocytes and early spermatids bind tightly in these X-box sequences in EMSA [Wolfe et al., 2004]. Regulatory Factor 2, RFX2, is a transcription factor that was found to be a major protein component of the complex that binds to the H1t X-box in EMSA [Wolfe et al., 2004]. The transcription factor NF-Y binds to the CCAAT-box adjacent and downstream from the X-box. RFX2 and NF-Y may bind to their cognate DNA sequences on the H1t promoter and they also may associate with each other in forming a transcription initiation complex in spermatocytes [vanWert et al., 2008].

RFX2 is one member of a family of related transcription factors that include RFX1, RFX2, RFX3, RFX3, RFX4, and RFX5. These family members contain a conserved 76-amino acid winged-helix DNA binding domain (DBD) and may contain activation domains and a dimerization domain. RFX as a homo- or heterodimer binds to the X-box sequence. RFX2 binding to the H1t promoter may activate the promoter in primary spermatocytes [Wolfe et al., 2004]. In co-transfection assays of immortalized mouse germinal cells, over-expression of RFX2 increases activity of the rat H1t promoter in luciferase expression assays [Wolfe et al., 2004]. Therefore, the H1t X-box is essential for expression of the H1t gene in spermatocytes and RFX2 binds to the H1t X-box and activates the H1t promoter. RFX2 also binds to the H1t promoter in pachytene primary spermatocytes in vivo as shown in ChIP assays [vanWert et al., 2008]. In our EMSA supershift assays RFX2 binding was evident, but it is not yet clear in EMSA whether RFX members RFX1, RFX3, or RFX5 bind the H1t promoter. Some antibodies that work well in Western Blots do not work well in EMSA supershifts, so it remains a possibility that RFX1, RFX3, and RFX5 also bind the H1t X-box.

Work from other laboratories showed that RFX4 mRNA was abundant in testis and we wanted to know whether RFX4 mRNA was abundant in pachytene primary spermatocytes and to test the hypothesis that testis RFX4 may bind the H1t promoter. RT-PCR confirmed that RFX4 mRNA was most abundant in testis of all tissues examined including brain. It also revealed that the mRNA was most abundant in a cell population enriched in pachytene primary spermatocytes.

Our previous studies with RFX2 were facilitated by the availability of antibodies against RFX1, RFX2, RFX3, and RFX5. However, commercial antibodies were not available for rat RFX4. Therefore, we prepared cDNA from rat testis total cellular RNA and cloned and sequenced the rat testis RFX4 cDNA. Sequences of our clones were practically identical to the rat RFX4 sequence published in GeneBank (XM_576205). We subcloned a region downstream from the DBD representing 566 amino acids including the conserved domains B and C and the dimerization domain (DD) as shown in Figure 3. A peptide from this region of RFX4 was overexpressed in E. coli as a fusion peptide between RFX4 and protein A. The fusion peptide was purified and used as an antigen in rabbits. Anti-RFX4 IgG was prepared from serum from the immunized rabbit and used in our studies.

The antibody raised against RFX4 worked well in Western blots of proteins from rat testis germinal cell populations revealing two major peptides of 67 and 34 kDa in all enriched germinal cells fractions including pachytene primary spermatocytes where the H1t gene is expressed (Fig. 4). The 67 and 34 kDa peptides formed bands that appeared as doublets on SDS–PAGE and it is possible that each peptide is modified by phosphorylation, glycosylation or other posttranslational modification.

Using the polyclonal antibody preparation against RFX4, we were not able to demonstrate RFX4 binding to the X-box sequence in EMSA supershift assays. In preparation of the rat testis RFX4 antigen, we avoided the DNA binding domain, but it is possible that antibody binding to the RFX4 conserved domains, dimerization domain, or other regions interferes with RFX4 dimerization or the ability of RFX4 to bind to DNA or to other associated nuclear proteins. However, it was surprising to find that RFX4 antibodies enhanced binding of nuclear proteins to form the shifted X-box complex and they enhanced binding of RFX2 in supershift assays (Fig. 5). One possible explanation for this phenomenon is that RFX4 may normally bind to RFX2 as a heterodimer in our germinal cell populations. Addition of RFX4 antibodies to our nuclear extracts and binding of the antibodies to RFX4 may prevent dimerization of RFX4 with itself or with RFX2 in effect making more RFX2 free for homodimerization and binding to the X-box in our EMSA supershift assays. Inhibition of RFX4 homo- or heterodimerization may also explain why we do not see a supershift with the anti-RFX4 antibodies.

In previous work we showed the RFX2 in testis nuclear extracts could bind to a biotin labeled DNA X-box affinity probe. RFX2 in the affinity purified proteins was detected in Western blot analysis. In our current experiments, we conducted the same type of X-box DNA affinity analysis but developed the blots with our anti-RFX4 antibodies. In this experiment both the 67 and 34 kDa RFX4 bands were seen but the bands were faint compared to the more intense 87 kDa RFX2 band seen previously with commercial antibodies. In these experiments the antibodies do not interfere with direct or indirect protein binding to the DNA probe and are only used to develop the Western blots. However, it is possible that our polyclonal antibodies against RFX4 are weaker than the commercial RFX2 antibodies. It is also possible that testis RFX4 binds more weakly than testis RFX2 to the X-box probe.

To examine in more detail the possibility that testis RFX4 binds the H1t X-box, we conducted chromatin immunoprecipitation assays (ChIP) using our polyclonal antibodies against RFX4. We showed in early work that RFX2 was bound to the H1t promoter in vivo in pachytene spermatocytes where the H1t gene was expressed. We conducted these assays again but this time using the RFX4 antibodies for immunoprecipitation of sheared chromatin from spermatocytes that had been treated to crosslink proteins and DNA. We found in vivo binding of RFX4 to the H1t promoter in spermatocytes and confirmed binding of RFX2. Presumably, the anti-RFX4 antibodies were able to bind to the crosslinked nuclear proteins and DNA. Crosslinked protein did not interfere with anti-RFX4 binding and anti-RFX4 could not interfere with the cross-linked RFX4 as in EMSAs.

In similar experiments with chromatin from early spermatids we could not detect binding of either RFX2 or RFX4. Therefore, although RFX2 and RFX4 are abundant in both pachytene spermatocytes and early spermatids, they were both found to bind to the H1t promoter only in spermatocytes.

If both RFX2 and RFX4 bind to the H1t promoter in spermatocytes, it is possibly that they bind as RFX2 or RFX4 homodimers or alternatively as RFX2-RFX4 heterodimers. If these transcription factors bind as heterodimers, we speculated that immunoprecipitation of RFX4 from nuclear extracts, would co-precipitate RFX2. Conversely, immunoprecipitation of RFX2 also should co-precipitate RFX4. This is indeed what we found as presented in Figure 8. When anti-RFX2 was used for IP, we detected not only RFX2 but also RFX4 in the precipitates by Western blotting. When anti-RFX4 was used for IP, we detected RFX4 as well as RFX2 in the precipitates. It should be noted that the higher mobility band in lanes 1–3 of the right panel were apparent only in anti-RFX2 immunoprecipitates detected by Western blotting with anti-RFX4. Although they co-migrate with the 67 kDa RFX4 band in the left panel, they are not visible in Western blots of the original nuclear extracts; they are only apparent in anti-RFX2 immunoprecipitates of these extracts that are detected by anti-RFX4.

In these IP experiments, it is possible that RFX4 monomers associate directly with RFX2 monomers in nuclear extracts to form RFX2-RFX4 heterodimers. If this is the case, then RFX4 may serve a regulatory role in limiting the availability of RFX2 for binding as RFX2 homodimers to the H1t promoter. RFX2-RFX4 heterodimers may bind weakly or not at all to the H1t X-box sequences in spermatocytes. However, it is also possible that RFX4 monomers or homodimers bind indirectly to RFX2 monomers or homodimers through associated proteins. If this occurs, it is possible that RFX2 binds directly to the X-box sequences of the H1t promoter and that RFX4 binds indirectly through accessory proteins. Experiments in this study do not shed light on the exact nature of RFX2 or RFX4 binding to the X-box of the H1t promoter. However, our experi-

ments show that RFX4 is abundant in spermatocytes and in early spermatids and that both RFX4 and RFX2 bind to the H1t promoter in spermatocytes where the H1t gene is expressed.

It is interesting to speculate that RFX2 homodimers bind to the two H1t X-box sequences to activate the H1t promoter for transcription of the H1t gene in spermatocytes. RFX4 which is also abundant in spermatocytes and early spermatids may bind RFX2 to form heterodimers and in this way sequester RFX2 so that it is not available to form RFX2 homodimers. In this way RFX4 may be able to regulate binding affinity of RFX2 and thus regulate the activity of the histone H1t promoter. In turn the affinity of RFX4 for RFX2 may be regulated by modification of RFX4 or RFX2 by phosphorylation or other post-synthetic modification. Changes in binding affinity of RFX4 may alter the availability of RFX2 in spermatocytes where the H1t gene is transcribed and in early spermatids where the H1t gene is silent.

ACKNOWLEDGMENTS

This research was supported by a Merit Review grant from the Department of Veterans Affairs to SRG.

REFERENCES

Araki R, Takahashi H, Fukumura R, Sun F, Umeda N, Sujino M, Inouye ST, Saito T, Abe M. 2004. Restricted expression and photic induction of a novel mouse regulatory factor X4 transcript in the suprachiasmatic nucleus. J Biol Chem 279:10237–10242.

Berger SL. 2007. The complex language of chromatin regulation during transcription. Nature 447:407–412.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489.

Dotzlaw H, Alkhalaf M, Murphy LC. 1992. Characterization of estrogen receptor variant mRNAs from human breast cancers. Mol Endocrinol 6:773–785.

Fujii G, Nakamura Y, Tsukamoto D, Ito M, Shiba T, Takamatsu N. 2006. CpG methylation at the USF-binding site is important for the liver-specific transcription of the chipmunk HP-27 gene. Biochem J 395:203–209.

Gajiwala KS, Chen H, Cornille F, Roques BP, Reith W, Mach B, Burley SK. 2000. Structure of the winged-helix protein hRFX1 reveals a new mode of DNA binding. Nature 403:916–921.

Grimes S, Weisz-Carrington P, Daum H III, Smith J, Green L, Wright K, Stein G, Stein J. 1987. A rat histone H4 gene closely associated with the testis-specific H1t gene. Exp Cell Res 173:534–545.

Matsushita H, Uenaka A, Ono T, Hasegawa K, Sato S, Koizumi F, Nakagawa K, Toda M, Shingo T, Ichikawa T, Noguchi Y, Tamiya T, Furuta T, Kawase T, Date I, Nakayama E. 2005. Identification of glioma-specific RFX4-E and -F isoforms and humoral immune response in patients. Cancer Sci 96:801–809.

Morotomi-Yano K, Yano K, Saito H, Sun Z, Iwama A, Miki Y. 2002. Human regulatory factor X 4 (RFX4) is a testis-specific dimeric DNA-binding protein that cooperates with other human RFX members. J Biol Chem 277:836–842.

Strahl BD, Allis CD. 2000. The language of covalent histone modifications. Nature 403:41–45.

vanWert JM, Wolfe SA, Grimes SR. 1995. Testis-specific expression of the rat histone Hlt gene in transgenic mice. Biochemistry 34:8733–8743.

vanWert JM, Wolfe SA, Grimes SR. 2008. Binding of RFX2 and NF-Y to the testis-specific histone H1t promoter may be required for transcriptional activation in primary spermatocytes. J Cell Biochem DOI10.1002/jcb.21694

Wolfe SA, Grimes SR. 2003. Transcriptional repression of the testis-specific histone H1t gene mediated by an element upstream of the H1/AC box. Gene 308:129–138.

Wolfe SA, Wilkerson DC, Prado S, Grimes SR. 2004. Regulatory factor X2 (RFX2) binds to the H1t/TE1 promoter element and activates transcription of the testis-specific histone H1t gene. J Cell Biochem 91:375–383.

Wolfe SA, van Wert J, Grimes SR. 2006. Transcription factor RFX2 is abundant in rat testis and enriched in nuclei of primary spermatocytes where it appears to be required for transcription of the testis-specific histone H1t gene. J Cell Biochem 99:735–746.

Zeng PY, Vakoc CR, Chen ZC, Blobel GA, Berger SL. 2006. In vivo dual cross-linking for identification of indirect DNA-associated proteins by chromatin immunoprecipitation. Biotechniques 41:694, 696, 698.