Journal of Cellular Biochemistry 104:1087–1101 (2008)

Binding of RFX2 and NF-Y to the Testis-Specific Histone H1t Promoter May be Required for Transcriptional Activation in Primary Spermatocytes

Jane M. vanWert,[1,2] Steven A. Wolfe,[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 testis-specific linker histone H1t is transcribed exclusively in pachytene spermatocytes during spermatogenesis. The H1t promoter contains two imperfect inverted repeats which together comprise the X-box motif that is known to bind the transcription factor regulatory factor X (RFX). Out of all the histone H1 family promoters this motif appears only in the H1t promoter and may contribute to H1t tissue-specific expression. We show by Western blotting, EMSA, ChIP analysis, and real-time RT-PCR that the rat H1t X-box is bound by RFX2 in vivo in spermatocytes. We demonstrate that transcription factor NF-Y binds to the CCAAT-box motif that is located downstream and adjacent to the X-box and that testis NF-Y interacts either directly or indirectly with RFX2. Furthermore, we show that both the X-box and CCAAT-box are required for promoter activity and that co-expression of RFX2 greatly enhances testis histone H1t promoter activity in the GC-1spg germinal cell line. J. Cell. Biochem. 104: 1087–1101, 2008.

Key words: testis; histone H1t; spermatocytes; transcription; RFX2; NF-Y

The X-box is a relatively abundant gene promoter regulatory motif with the DNA sequence 5′-GTNRCC(0-3N)RGYAAC-3′ (where N is any nucleotide, R is a purine, and Y is a pyrimidine). Two half sites of the X-box form an imperfect inverted repeat. Crystallization studies show that two Regulatory Factor X (RFX) proteins, as a homo- or heterodimer, bind to a single X-box element in opposite orientations, contacting both half sites [Gajiwala et al., 2000]. A recent survey found the X-box motif appeared 698 times within a population of mammalian conserved non-coding promoter elements. In addition the X-box motif appears more than 30 times in yeast, is predicted to appear over 700 times in *C. elegans* [Xie et al., 2007], and is found in Drosophila [Otsuki et al., 2004].

The X-box is the binding site for the RFX family of proteins, winged helix DNA binding proteins with a highly conserved 76-residue DNA binding domain (DBD). The crystal structure of the DBD is very similar to that of the globular domains of linker histones [Gajiwala et al., 2000]. Based on the DBD sequence, so far five members of the RFX family have been identified, beginning with RF-X (now RFX5) [Reith et al., 1988]. Given the prevalence of the X-box motif in mammalian genomes it is likely the binding proteins will be found to serve many functions in addition to those so far described [Iwama et al., 1999; Masternak and Reith, 2002; Araki et al., 2004; Horvath et al., 2004; Wolfe et al., 2004; Kim et al., 2006; Ma et al., 2006; Xu et al., 2006; Zhang et al., 2006; Ait-Lounis et al., 2007].

The *cis*-acting CCAAT motif occurs in 30% of eukaryotic promoters, including those of histones, and is usually located approximately 60–100 nucleotides upstream of the CAP site [Zhong et al., 1983; van Wijnen et al., 1988; Mantovani, 1999]. A novel CCAAT binding

Grant sponsor: Merit Review Grant to SRG from the Department of Veterans Affairs.

*Correspondence to: 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 11 December 2007; Accepted 14 December 2007
DOI 10.1002/jcb.21694

protein from HeLa cell extracts was found to bind to the essential human H1t promoter sequence, and was named H1TF2 [Gallinari et al., 1989]. One subunit, H1TF2A, was cloned and sequenced [Martinelli and Heintz, 1994] and the published sequence matches that of NF-YC (GenBank # NM_014223). The NF-Y CCAAT binding factor (CBF) complex was found to bind to the CCAAT-box sequences of MHC class II genes and to be distinct from the known CBF-box, CBP and CTF/NF-1 [Hooft van Huijsduijnen et al., 1987]. The NF-Y complex is known to require three subunits for binding activity [Maity et al., 1992].

Binding sites for NF-Y (CCAAT) and RFX (X-box) are adjacent on MHC class II genes, and these genes are regulated by cooperative binding of NF-Y and RFX [Reith et al., 1994; Guerra et al., 2007], mediated through the RFX5 C-terminus [Villard et al., 2000]. The NF-Y complex binds to the CCAAT-box in H1 promoters [van Wijnen et al., 1988] and in the H1t promoter just downstream of the X-box [Gallinari et al., 1989]. NF-YA, B, and C share a high degree of homology with the yeast Hap-2, 3, and 5 factors [Serra et al., 1998]. Sequences of the core regions of NF-YB and NF-YC show homology to H2A and H2B and interact through histone fold motifs in a head-to-tail fashion [Bellorini et al., 1997; Guerra et al., 2007]. NF-YA binds the NF-YB/NF-YC dimer; the completed trimer binds the CCAAT box sequence with high affinity (K_d between 10^{-10} and 10^{-11} M) through the NF-YA core domain [Guerra et al., 2007]. Rat NF-YA and B protein subunits are about 36 and 23 kDa, respectively (rat genome database RGD #70976 and #3172 in EMBL webpages http://rat.embl.de/rat/p185/p18576.htm and http://rat.embl.de/rat/p631/p63140.htm), and NF-YC is 40 kDa [Maity et al., 1992].

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 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 in only one germinal cell type, pachytene primary spermatocytes, making it an excellent system in which to study tissue-specific gene expression [Grimes et al., 2003]. In our experiments this large tetraploid germinal cell type is highly enriched by centrifugal elutriation in fraction 5 (Fig. 1B, inset). During this meiotic phase of spermatogenesis, histone H1t has weak chromatin condensing activity [Nayernia et al., 2005]; this may be due to a K52Q substitution in the H1t globular domain with reference to the sequence of H1d which has stronger condensing activity [Ramesh et al., 2006]. 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. A single knock-out of the H1t gene has no obvious short-term effect on fertility in the laboratory, but a knock-out in combination with the sperm mitochondrian-associated cysteine-rich protein gene and either the transition protein 2 gene or proacrosin gene dramatically reduces fertility [Nayernia et al., 2005].

Unlike other histone promoters, the H1t promoter contains an imperfect inverted repeat called the testis element (TE) shown to contain the two elements of the X-box initially called TE1 and TE2, now called X-box 1 and X-box 2 [Grimes et al., 2003; Wilkerson et al., 2003]. Studies in transgenic mice showed that mutation of X-box 1 and X-box 2 in the rat H1t promoter abolished expression of the transgene [vanWert et al., 1998]. 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].

In this article we expand our studies of the functional interactions between the transcription factor RFX2 and the H1t promoter X-box.

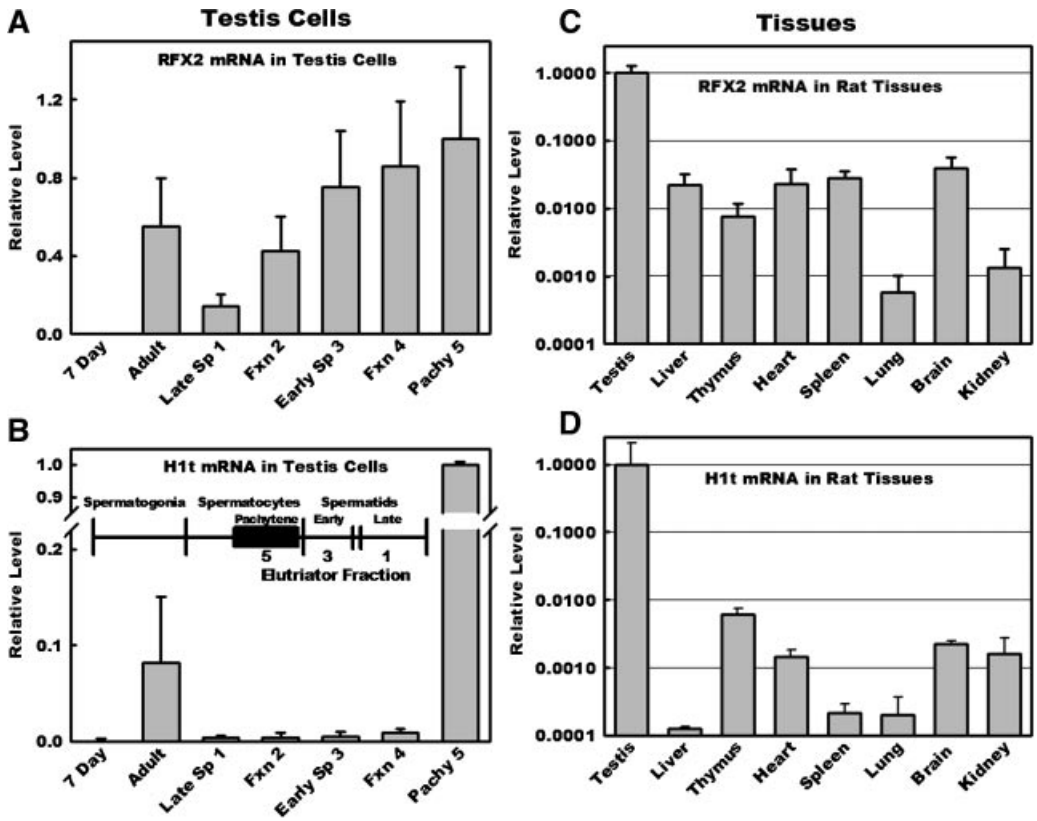

Fig. 1. H1t and RFX2 are most actively expressed in pachytene primary spermatocytes. Samples of mRNA from tissues and testis fractions were subjected to quantitative real-time RT-PCR. **A**: The steady-state RFX2 mRNA level is maximal in pachytene spermatocytes, it is present at lower levels in other stages of spermatogenesis and in adult rat testis, but little is found in testes from sexually immature 7-day-old rats. Columns labeled Late Sp, Early Sp and Pachy represent populations of germinal cells prepared by the technique of centrifugal elutriation and enriched in late spermatids, early spermatids and pachytene primary spermatocytes respectively. Fxn 1-Fxn 5 represents the five populations of germinal cells enriched by centrifugal elutriation (see inset panel B). **B**: H1t expression is almost exclusively limited to pachytene spermatocytes, which are found only in testis from sexually mature adult rats. The inset shows the 60-day time line of germinal cell development in rats and the three numbers represent the most enriched populations of germinal cell types prepared by centrifugation (one-late spermatids, three-early spermatids, and five-pachytene spermatocytes). H1t mRNA is seen only in tetraploid pachytene spermatocytes (elutriator fraction 5) marked by the heavy bar in the time line. Testis H1t histone and core histones are synthesized at a high rate in spermatocytes and are maintained through two meiotic cell divisions to generate haploid early spermatids with spherical nuclei (elutriator fraction 3). Histones have been replaced in large part by protamines in haploid late spermatids with highly condensed nuclei (elutriator fraction 1). **C**: RFX2 expression is more than an order of magnitude higher in testis than in other tissues tested. **D**: H1t expression is more than two orders of magnitude higher in testis than in other tissues tested.

We include in vivo binding experiments and expression studies and we investigate binding of the NF-Y complex with the H1t CCAAT-box element and the possible association of RFX2 and NF-Y upon binding.

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 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.

Preparation of Chromatin

Chromatin was prepared from rat tissues following the protocol of Fujii et al. [2006] with modifications. Briefly, 500 mg of tissue was minced, washed in PBS, and crosslinked with 1% formaldehyde 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 0.1mM 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 10-fold and with the addition of a second preclearing step with normal goat IgG, Protein A/G Plus Agarose beads (Santa Cruz) and sonicated salmon sperm DNA. Antibody to RFX2 (C-15) and the control anti-goat antibody were purchased from Santa Cruz. In addition a no-antibody control and a positive histone H1t gene control (plasmid pPS3 [Grimes et al., 1987]) were included. Sonicated chromatin representing 50 μg of DNA was used in each ChIP reaction. Primers used for amplification, which covered the H1t promoter, were F5 5′-TGTGTCATAACCTGAGCGATTC-3′, and R5 5′-GCTGTGATTGGTGCATCCC-3′.

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 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: RFX2 RT F2 5′-GTCTTCCCAGAGTTCCCAGCAC-3′, RFX2 RT R2 5′-AGTTCCAGAAGGACAACCACAGC-3′, RFX2 RT F3 5′-TCCAGTTTCAGTACATTGAGAAGC-3′, RFX2 RT R3 5′-CACACTTACACAGAGAGATGAGC-3′, H1t RT F3 5′-TCTTGACCAGCTCTTGACTATG-3′, H1t RT R3 5′-CCATGCCAGGCTTCTTCC-3′, NF-YA RT F1 5′-GTGGTCAACTCAGGAGGAATGG-3′, and NF-YA RT R1 5′-AGGATGCGGTGATACTGTTTGG-3′. Standard curves for each primer pair used in real-time PCR were generated using a 10-fold dilution series of plasmids containing their respective templates (for H1t, pPS3 [Grimes et al., 1987] and for RFX2, MGC-6105 (ATCC, Manassas, VA)). For H1t ChIP primers the standard curve was generated using a twofold dilution series of sonicated rat testis genomic DNA.

Mammalian Cell Lines, Expression Vectors and Reporter Vectors

The mouse GC-1spg spermatogonial cell line, purchased from ATCC (CRL-2053), was grown in Dulbecco's Modified Eagle's medium (DMEM) containing 10% FBS at 32°C in 5% CO_2. The mouse RFX2 expression vector was purchased from ATCC (MGC-6105); RFX2 cDNA was expressed in pCMV-SPORT6 and grown in. *E. coli* strain XL-Blue (Stratagene, La Jolla, CA). The reporter vectors were described previously [Wolfe et al., 1999]. H1t promoter fragments designated 1866 (−520 bp from the transcription start site) and *Pst* (−141) were used as designed and with mutations in X-box 1 [Grimes et al., 2005] and CCAAT (Fig. 3), respectively.

Transient Transfection Assays

Cells were transfected using Lipofectamine Reagent (Invitrogen) as described in the supplier's protocol. GC-1spg cells were grown in 35 mm dishes to a density of about 2×10^5 cells per dish. Transfections were performed in triplicate using a 4:1 ratio of expression vector to reporter plasmid in serum free medium and grown for 5 h before adding an equal volume of

DMEM with 20% FBS. Equivalent amounts of filler DNA and reporter vector were transfected into cells as a control. Cells were harvested after 48 h.

Cell lysates were prepared and assayed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol. Assays were performed in a Beckman LS6500 liquid scintillation counter with the coincidence counter inactivated. Protein quantities were determined using a Bio-Rad Protein Assay (500-0006) following the supplier's protocol and a Beckman DU 800 spectrophotometer.

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 essentially as described previously [Grimes et al., 1990; Wolfe et al., 2004] using X-box and CCAAT-box probes and DNA competitors to analyze protein binding. Probes were made by annealing two complimentary oligonucleotides (Sigma-Genosys) and end-labeling the product with T4 polynucleotide kinase (New England Biolabs) and (γ-^{32}P)ATP (Perkin-Elmer). Probe and competitor sequences are shown in the Figures. EMSA supershift antibodies were purchased from Santa Cruz: RFX2 (A-18x), NF-YA (CBF-B H-209x), NF-YB (CBF-A FL-207x), NF-YC (CBF-C H-120x), Sp1 (Pep2x), and TFIID (SI-1x).

Western Blot Analysis

Western blots were performed as described [Grimes et al., 1990; Wolfe et al., 2004] using whole cell extracts from various tissues and testis cell types and anti-RFX2 antibodies from Santa Cruz. Proteins were electrophoresed on Bio-Rad Tris–HCl Ready Gels using the Bio-Rad mini-PROTEAN gel apparatus, along with Precision Plus pre-stained 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 antibody, HRP conjugated donkey anti-goat, was purchased from Santa Cruz. Westerns blots were developed using the SuperSignal West Dura Kit from Pierce for chemiluminescent detection and analyzed using a Bio-Rad Versa-Doc imaging system. Band areas were determined using Sigma Gel and plotted using Sigma Plot, both by SYSTAT Software, Inc.

Affinity Purification of NF-Y

A double stranded biotinylated probe (Sigma-Genosys) corresponding to the rat H1t promoter CCAAT-box sequence was used to affinity purify DNA binding proteins from pachytene spermatocyte nuclear extracts. Offset oligonucleotides were annealed and the recessed ends were filled using the Klenow fragment of DNA polymerase I (NEB). The oligonucleotides were Biotin-5′-GGATGCACCAATCACAGCGCG-3′ and 5′-GAGCAGGGCGCGCTGTGATTGGT-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 Laemmli Sample buffer (Bio-Rad #161-0737) to release the proteins. Equal loads of eluted proteins were then identified by Western blotting using anti RFX (C-15) and Anti NFY-B (CBF-A Fl-207) from Santa Cruz.

RESULTS

RFX2 and NF-Y Are Abundant in Testis and Most Highly Enriched in Spermatocytes Where the Testis-Specific Histone H1t Gene Is Expressed

Since the H1t promoter contains two X-box sequence elements that can bind RFX, we compared relative levels of RFX2 mRNA and protein in different rat germinal cell types and levels in testis compared to levels in other tissues. Using primer pairs that avoided amplification of the conserved RFX DBD shared among RFX family members RFX1, RFX2, RFX3, RFX4 and RFX5, we examined RFX2 levels at different stages of germinal cell development. Assays were performed using mRNA from enriched populations of germinal cells prepared by centrifugal elutriation and

from unfractionated testis cells from sexually immature (7-day-old) and adult rats.

RFX2 mRNA level is highest in a testis cell population enriched in pachytene spermatocytes (elutriator fraction 5) where DNA replication is absent but where the testis histone H1t gene is expressed maximally compared to other testis cell types (Fig. 1A, column 7 designated Pachy 5). The inset in Figure 1B shows a 60 day time line of rat testis germ cell development and the numbers 1, 3, and 5 represent the three testis germinal cell types that are most highly enriched by centrifugal elutriation. The level of H1t mRNA, found in unfractionated adult testis (Fig. 1B,D), is also most abundant in pachytene spermatocytes where this testis linker histone is synthesized (Fig. 1B, elutriator fraction 5). Significant levels of H1t mRNA were not detected in sexually immature 7-day-old rat testes that are enriched in spermatogonia stem cells, type A spermatogonia, intermediate spermatogonia, and type B spermatogonia but lacking more mature germinal cells. A very low level of H1t mRNA was present in early spermatids with spherical nuclei that contain histones (Fig. 1B, column 5 designated Early Sp 3) and late spermatids with elongated nuclei where histones have been replaced by protamines (column 3 designated Late Sp 1). These haploid spermatids are formed by two meiotic cell divisions of tetraploid spermatocytes (Fig. 1B).

The steady-state mRNA level of RFX2 is almost two orders of magnitude higher in testis than in other tissues surveyed (Fig. 1C). The H1t mRNA level is over two orders of magnitude higher in testis than in other tissues (Fig. 1D). Note that relative RFX2 and H1t mRNA levels in panels C and D of Figure 1 are plotted on log scales in order to depict the high level in testis compared to other tissues.

RFX2 protein level is also highest in germinal cells enriched in spermatocytes. This is shown by blots of proteins in nuclear extracts from unfractionated testis cells and from enriched testis cell populations (Fig. 2A). RFX2 is present at a lower level in cells enriched in early spermatids and at the lowest level in cells enriched in late spermatids (Fig. 2A). As seen for RFX2 mRNA, RFX2 protein is most abundant in testis compared to other rat tissues (Fig. 2B).

An NF-Y trimer (NF-YA, NF-YB, and NF-YC) binds to the CCAAT-box present in many gene promoters regulating transcription of those genes. The H1t promoter contains a canonical CCAAT-box located between X-box1 and the TATA box important for H1t gene expression (Figs. 3 and 10). Since NF-Y binds the CCAAT-box located downstream and adjacent to an X-box in the MHCII promoter and since NF-Y might bind the H1t CCAAT-box, we examined relative levels of NF-Y in testis and various other tissues as well as the relative levels in enriched germinal cell types. The pattern of NF-YA mRNA levels reflects those of RFX2 mRNA and H1t mRNA. NF-YA, RFX2, and H1t mRNAs are present at relatively high levels in testis compared to other tissues (compare Figs. 2D, 1C,D). An exception is the NF-YA level in kidney which appears to be similar to the level in testis. Furthermore, relative levels of these mRNAs are highest in spermatocytes where the H1t gene is transcribed (compare Figs. 2C, 1A,B).

Evidence That RFX2 and NF-Y Bind the H1t Promoter

EMSA revealed nuclear protein binding to various H1t promoter elements. Previous studies with an H1t X-box element showed a testis-specific pattern of nuclear protein binding. In this study, it was surprising that a larger element spanning a region containing the X-box1, CCAAT-box and TATA box yielded a similar binding pattern (Fig. 3). There is strong protein binding in nuclear extracts from cell populations enriched in pachytene spermatocytes (elutriator fraction 5) and in early spermatids (elutriator fraction 3) (Fig. 3, left panel). However, spermatocyte proteins produce a double band pattern while those from early spermatids produce a single band co-migrating with the lower mobility band of the spermatocyte doublet (compare lanes 4 and 6).

These two bands appear to be due in large part to NF-Y binding. In the right panel a labeled probe with a CCAAT-box mutation fails to produce the spermatocyte doublet compared to the wild type sequence (Fig. 3, right panel, lane 3 vs. lane 2). Likewise the mutant probe fails to form the single early spermatid band compared to the wild type probe (Fig. 3, right panel, lane 5 vs. lane 4). A much less distinct and lower mobility band is present in both mutant lanes 3 and 5 labeled RFX2 based in part upon assays shown in later figures.

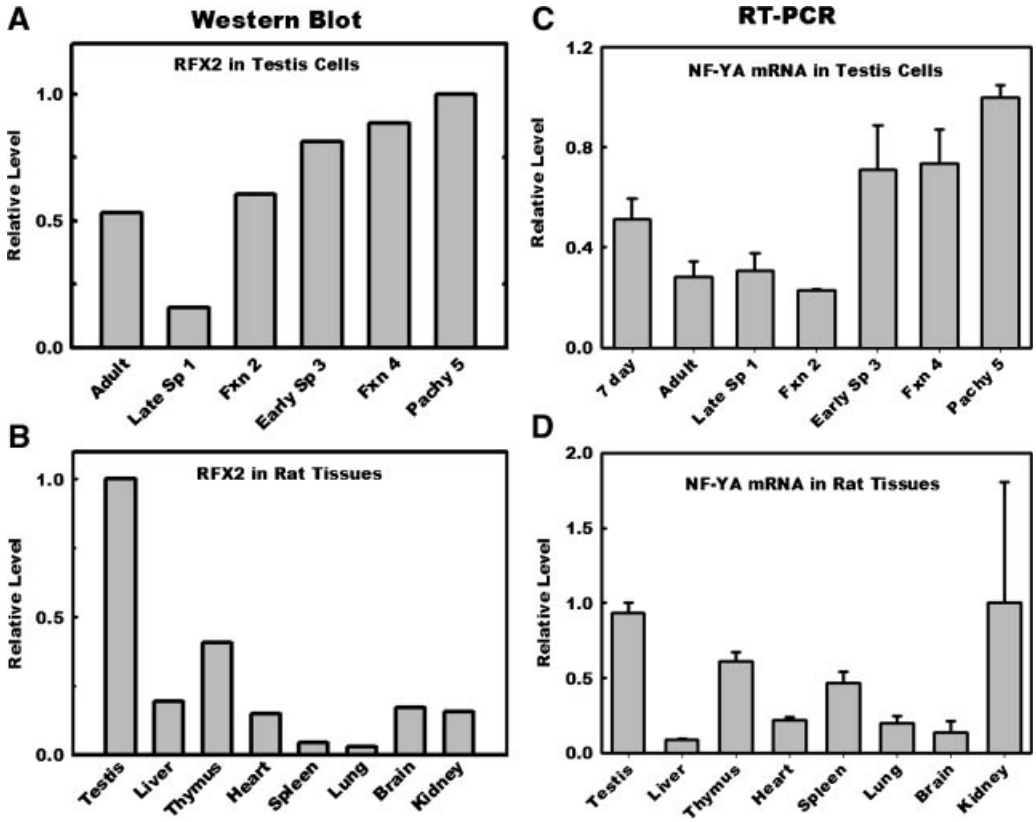

Fig. 2. RFX2 protein and NF-YA mRNA are most abundant in pachytene spermatocytes. Whole cell extracts from rat tissues and nuclear extracts from testis cell fractions were subjected to Western blotting. **A**: Polyclonal antibodies against RFX2 revealed the highest level of RFX2 protein in elutriator fraction 5 enriched in pachytene spermatocytes. **B**: The highest level of RFX2 protein among tissues examined was found in testis. **C**: Real-time RT-PCR using RNA extracted from whole testis of immature and mature rats and from populations of cells enriched by centrifugal elutriation shows the highest steady-state level of NF-YA transcript is in pachytene spermatocytes (elutriator fraction 5). **D**: Analysis of RNA prepared from a variety of rat tissues shows the steady-state level of NF-YA mRNA to be present to a very high degree in testis.

To help determine the sequences responsible for the banding patterns, we conducted EMSA competition assays using the same wild type probe used in Figure 3. We see a greatly reduced NF-Y doublet band with pachytene spermatocytes extracts when competed with the CCAAT-box sequence purchased from Santa Cruz (Fig. 4, left panel, lane 3 vs. lane 2). The competition is not complete because sequences surrounding the Santa Cruz CCAAT element are not identical to the H1t sequence. Furthermore, there may be a testis-specific NF-Y that specifically recognizes the H1t CCAAT-box. Unexpectedly, the sequence spanning the region between the H1t CCAAT-box and TATA box is a good competitor of the doublet (Fig. 4, left panel, lane 4) and likely reflects the importance of this region for binding of the testis NF-Y. As expected, consensus TFIIDsc and SP1sc sequences from Santa Cruz are poor competitors of the shifted bands (Fig. 4, left panel, lanes 5 and 6). These competitors lacked the X-box sequence and thus are poor competitors of the faint band labeled RFX2.

Probe 2, a competitor spanning a region containing the X-box1 and the sequence down to but lacking the TATA box effectively competed for binding of proteins that produce the NF-Y doublet band as well as the faint RFX2 band (Fig. 4, right panel, lane 3). A similar competitor but one that also lacks the X-box1 sequence effectively competed the NF-Y bands but it was not effective in competing the RFX2 band (Fig. 4, right panel, lane 4). When the Santa Cruz CCAATsc sequence was used as a labeled probe, a doublet banding pattern again was obtained with nuclear extracts from pachytene spermatocytes (elutriator fraction 5) and a single band with extracts from early spermatids (elutriator fraction 3) that resembled the bands produced with our Probe 1 (spanning X-box 1 and the TATA box) (Fig. 4, right panel, lanes 6

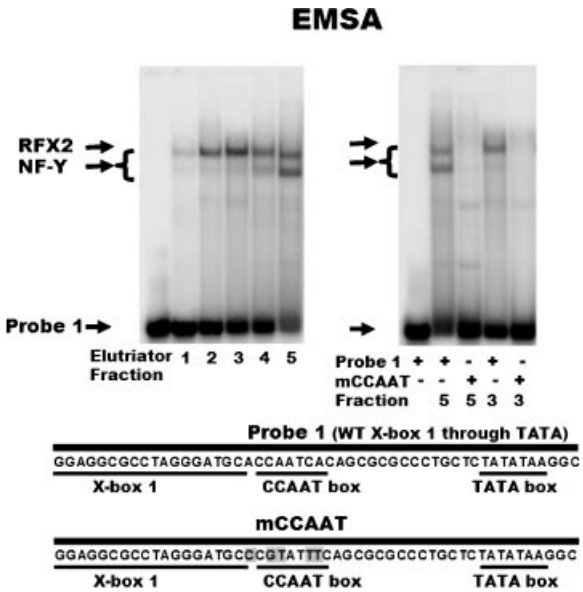

Fig. 3. Patterns of germinal cell nuclear protein binding to the H1t promoter change during spermatogenesis. EMSA were conducted using nuclear extracts from the five enriched populations of germinal cells prepared by centrifugal elutriation and a large double-stranded DNA probe (Probe 1) that includes X-box1 and extends through the TATA box of the rat H1t promoter (**left panel**). Nuclear extracts from germinal cell populations 1, 3 and 5 are enriched in late spermatids, early spermatids, and pachytene primary spermatocytes respectively (see inset in Fig. 1B). Note that the two major bands in spermatocytes (**lane 6**—fraction 5) and the single band in early spermatids (lane 4—fraction 3) are designated NF-Y. A less distinct band designated RFX2 with a slightly lower mobility than NF-Y is present in fractions 2–5. If the CCAAT-box is mutated (mCCAAT probe), the double band does not form with spermatocyte extracts (compare **lanes 2** and **3**) or with early spermatid extracts (compare **lanes 4** and **5**) but the faint RFX2 band is still present.

and 7). This probe is shorter, and thus the free probe migrates faster, but since it lacks the X-box, it does not form the RFX2 band.

If these NF-Y and RFX2 band designations are correct, it should be possible to confirm them in EMSA supershift assays with antibodies raised against the specific transcription factors. Therefore, we conducted an assay using Probe 2 spanning the H1t promoter region containing X-box 1, the CCAAT-box and the sequence down to but not including the TATA box (see the probe in Fig. 4). Spermatocyte nuclear extracts generate a doublet band and early spermatid nuclear extracts generate a singlet band with this Probe 2 similar to the bands seen with Probe 1 (Fig. 5, left panel, lanes 2 and 4). Confirmatory supershifts designated SS are generated with antibodies against RFX2 using nuclear extracts from spermatocytes (lane 3—elutriator fraction 5) and early spermatids (lane 5—elutriator fraction 3) (Fig. 5, left panel, lanes 3 and 5). The spermatocyte NF-Y doublet and the early spermatid NF-Y singlet are only slightly diminished in the lanes representing the supershifts. However, the RFX2 band is diminished in intensity when RFX2 is shifted (lanes 3 and 5). When the mCCAAT probe is used the NF-Y bands are absent and the RFX2 band is more visible as seen in Figure 3. When RFX2 supershift antibodies are used with this probe, an RFX2 supershifted band designated SS is generated (Fig. 5, right panel, lane 4). It is interesting that a supershifted band also is formed when TFIID supershift antibodies are used with this mCCAAT probe that contains a normal X-box1 and TATA box but a non-functional CCAAT-box (Fig. 5, right panel, lane 3).

We conducted another supershift assay to confirm binding of NF-Y using Probe 1 and antibodies against NF-YA, NF-YB, and NF-YC. This polyacrylamide gel was electrophoresed for a longer time period to better separate the NF-Y and RFX2 bands but consequently free probe electrophoresed off the gel. With spermatocyte nuclear extracts we see strong supershifted bands using anti-NF-YB and NF-YA (Fig. 6, left panel, lanes 2 and 3) but a weaker and slightly higher mobility supershifted band using anti-NF-YC. With early spermatid nuclear extracts we see supershifted bands that are less prominent (Fig. 6, right panel). Anti-RFX2 antibody was used as a positive control and anti-SP1 was used as a negative control with each extract.

To determine whether RFX2 binds to the H1t promoter in vivo in spermatocytes, chromatin from a cell population enriched in pachytene spermatocytes was used in a ChIP assay in which chromatin was immunoprecipitated with the C-15 antibody to RFX2 (Santa Cruz) and the precipitated DNA was amplified with a primer pair covering the H1t promoter. It is clear that the DNA that was precipitated with anti-RFX2 yielded a PCR product of the expected size, 125 bp as compared to the positive control (Fig. 7), suggesting that RFX2 binds to the H1t promoter in vivo in spermatocytes.

Evidence That Both RFX2 and NF-Y Function to Enhance Histone H1t Promoter Activity

Previous studies revealed that both X-box1 and X-box2 are important for transcription of the H1t gene in vivo and in reporter assays in

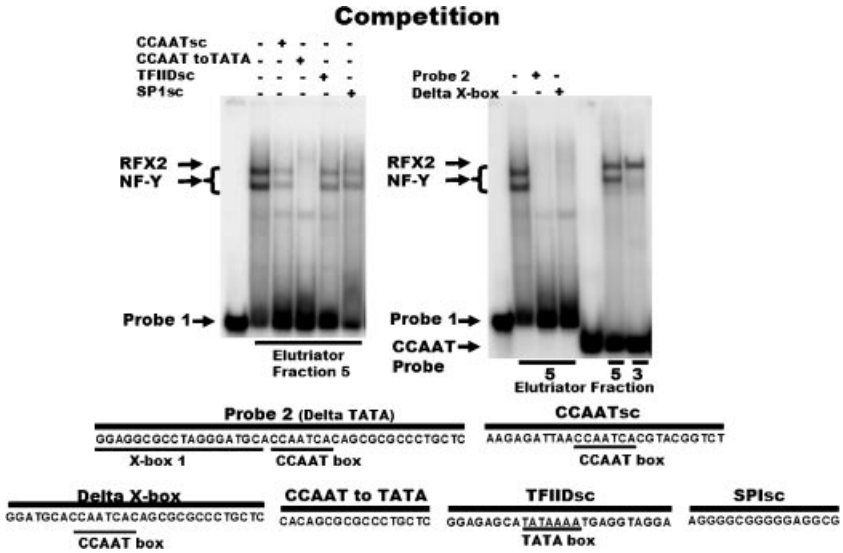

Fig. 4. EMSA competition assays reveal specific protein binding to the X-box and CCAAT-box. Double-stranded competitor DNA sequences helped establish identity of the EMSA bands. A consensus CCAAT-box from Santa Cruz (CCAATsc) successfully competes the two major bands formed with Probe 1 and nuclear extracts from pachytene spermatocytes (**left panel**, compare **lanes 2** and **3**). The rat testis CCAAT competitor (CCAAT to TATA) completely competes the two major spermatocyte bands but not the faint lower mobility RFX2 band (**left panel**, **lane 4**). On the other hand the negative control sequences (TFIIDsc and SP1sc) failed to compete the NF-Y and RFX2 bands (**left panel**, **lanes 5** and **6**). The Probe 2 sequence almost completely eliminated all shifted bands (**right panel**, compare **lanes 2** and **3**). A sequence similar to Probe 2 but lacking the X-box 1 (Delta X-box) successfully competes the two spermatocyte major bands but not the RFX2 band (**right panel**, **lane 4**). Although the CCAATsc probe differs in sequence from the rat CCAAT sequence, it produces the two major spermatocyte bands and one major early spermatid band but it fails to generate the RFX2 band, since it lacks the X-box (**right panel**, **lanes 6** and **7**).

the testis GC-2spd (ts) cell line (ATCC CR L-2196). We observed a twofold activation of the wild type H1t promoter upon co-expression of RFX2 in the GC-2spd cell line. We now see an approximate 10-fold activation upon RFX2 co-expression in another testis cell line designated GC-1spg using a short promoter extending 141 nucleotides upstream from the transcription initiation site to the *Pst*I restriction site (compare columns 1 and 2 of Fig. 8) as well as using a longer promoter extending 520 nucleotides from the initiation site (compare columns 5 and 6). Mutagenesis of the CCAAT-box in the shorter promoter resulted in an approximate 10-fold inactivation of the promoter (compare columns 1 and 3 showing −141 mCCAAT and wt −141) while co-expression of RFX2 restored activity of the mCCAAT mutant to that of the −141 wild type level (compare columns 3 and 4). Mutagenesis of X-box1 (mXbox1) reduced promoter activity of the longer promoter about 50% (compare columns 5 and 7) while RFX2 co-expression increased activity of both the wild type and X-box1 mutant (compare columns 5 and 6 and columns 7 and 8). The activated level of the X-box1 mutant is almost 80% of the activated wild type promoter (compare columns 6 and 8), a result similar to our observation using GC-2spd cells.

Evidence That Spermatocyte RFX2 May Associate With NF-Y in Binding to the Histone H1t Promoter

To test the hypothesis that NF-Y may associate with RFX2 in binding to the H1t promoter, proteins were affinity purified from pachytene spermatocyte nuclear extract using a biotinylated CCAAT-box probe that is known to bind NF-Y. The CCAAT probe used in this experiment lacks X-box1 and is designated Delta X-box in Figure 9. The affinity recovered proteins were assayed by Western blots. NF-YB is clearly present in this affinity purified material as shown in the left panel of Figure 9. The concentration of NF-YB was very low in the spermatocyte extract before affinity purification (Fig. 9, left panel, lane 2), but the protein was enriched in the affinity-purified material (Fig. 9, left panel, lane 3). Antibodies against NF-YA and C produced similar results (data not shown). The concentration of RFX2 was high in the spermatocyte nuclear extract before affinity

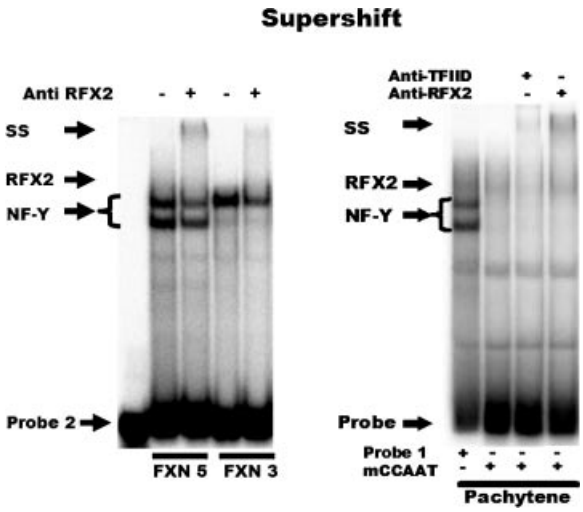

Fig. 5. EMSA supershift assays confirm RFX2 binding to the H1t X-box. Nuclear extracts from pachytene spermatocytes (**lanes 2** and **3**) and early spermatids (**lanes 4** and **5**) were probed with Probe 2 that contains X-Box 1 and extends to just above the TATA box (Fig. 4). Polyclonal antibodies against RFX2 generated a supershifted complex with both extracts (**left panel**, **lanes 3** and **5**). The mutant CCAAT probe (mCCAAT) does not form the two major pachytene spermatocyte bands but it does form the RFX2 band (**right panel**, **lane 3**). However, antibodies against RFX2 generated a supershifted complex and the intensity of the RFX2 band is diminished (**right panel**, **lane 4**). Since the mCCAAT probe has a normal TATA box, it is not surprising that antibodies against TFIID generated a supershifted band.

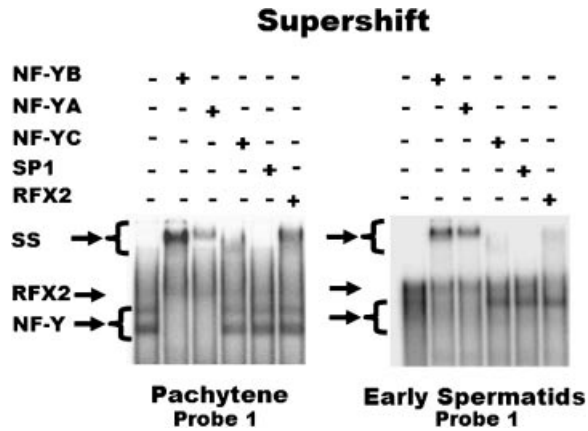

Fig. 6. EMSA supershift assays confirm NF-Y binding to the H1t CCAAT-box. Nuclear extracts from pachytene spermatocytes (**left panel**) and early spermatids (**right panel**) were probed with Probe 1 containing X-Box 1 and extending through the TATA box (Fig. 3). The gel was electrophoresed for an extended time period so that free probe migrated off the gel in order to better resolve the NF-Y and RFX2 bands. Antibodies against NF-YA, NF-YB and NF-YC generated supershifted bands with extracts from pachytene spermatocytes (**left panel**, **lanes 2–4**) and early spermatids (**right panel**, **lanes 2–4**), although early spermatid extracts generated fainter supershifted bands. Antibodies against RFX2 generated supershifted bands with both extracts (lane 6 of both panels) and diminished the intensity of the band designated RFX2. On the other hand, antibodies against SP1 did not produce supershifted bands with Probe 1.

purification (Fig. 9, right panel, lane 2), but RFX2 was recovered efficiently by this probe that lacks an X-box in the affinity purification (right panel, lane 3). This evidence supports our hypothesis that RFX2 associates with NF-Y; RFX2 may bind to NF-Y before NF-Y binds to DNA or it may bind during or after NF-Y binding to DNA. RFX2 must be bound tightly to NF-Y in this experiment, since the bound complex was washed extensively before Western blot analysis of the bound proteins.

The model in Figure 10 shows binding of RFX2 dimers to X-box 2 and X-box 1 of the testis-specific histone H1t promoter in pachytene spermatocytes where the gene is transcribed. It also shows association of RFX2 with an NF-Y trimer that is bound to the adjacent CCAAT-box.

DISCUSSION

H1t is a testis-specific linker histone expressed maximally in pachytene spermatocytes during meiosis I of spermatogenesis where DNA synthesis is absent. Histone expression in spermatocytes is remarkable, since most linker and core histones are expressed only during S-phase of the mitotic cell cycle. Along with the AC box, GC box, CCAAT-box, and TATAA box that are conserved among H1 family members, the H1t promoter contains unique elements that are important to its tissue and cell-stage specific expression [vanWert et al., 1995]. An element surrounding and including the rat GC

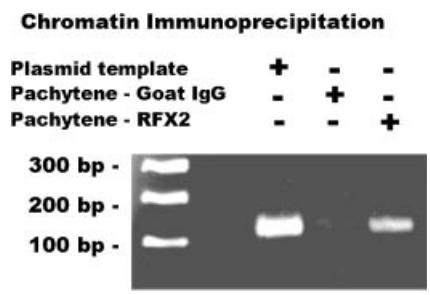

Fig. 7. RFX2 binds to the H1t promoter in primary spermatocytes in vivo. DNA from a ChIP reaction was amplified by real-time PCR using primers that cover the H1t promoter. Purified DNA derived from chromatin from pachytene spermatocytes that had been precipitated with antibodies against RFX2 yielded a PCR product of the expected size (125 bp) compared to chromatin precipitated using control goat IgG antibodies (compare **lanes 4** and **5**).

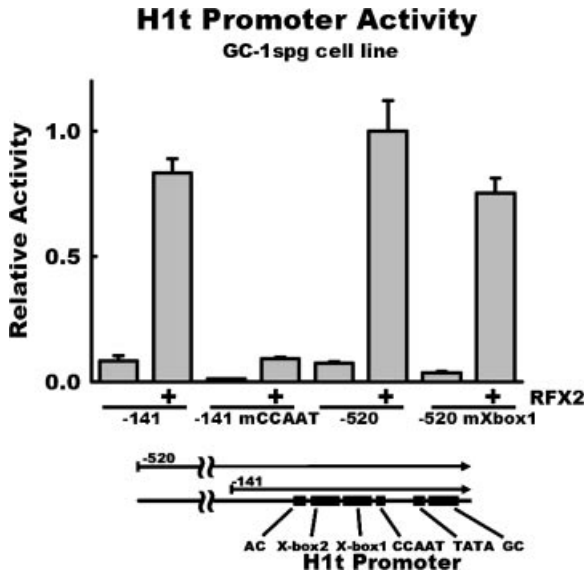

Fig. 8. The H1t promoter is activated by RFX2 but activity is inhibited by mutations within either the X-box1 or CCAAT-box. A germinal cell line designated GC-1spg was transfected with a Luciferase expression vector promoted by a short wild type H1t promoter (−141) or a longer wild type H1t promoter (−520). We also tested a CCAAT mutation in the short promoter (−141 mCCAAT) and an X-box1 mutation in the longer promoter (−520 mXbox1). Each cell sample was transfected with either an RFX2 overexpression vector (pSPORT-RFX2; even numbered lanes) or plasmid control DNA to balance the DNA concentration (odd numbered lanes). Both the CCAAT-box mutant (**lane 3**) and X-box1 mutant (lane 7) inactivated the H1t promoter showing the essential nature of both promoter elements to transcription. RFX2 greatly enhanced the short and longer wild type probes and the X-box1 mutant (compare **lanes 1–2**, **lanes 5–6**, and **lanes 7–8**). It also activated the mutant CCAAT promoter but only up to the level of the wild type promoter without RFX2 (compare lanes 1 and 4).

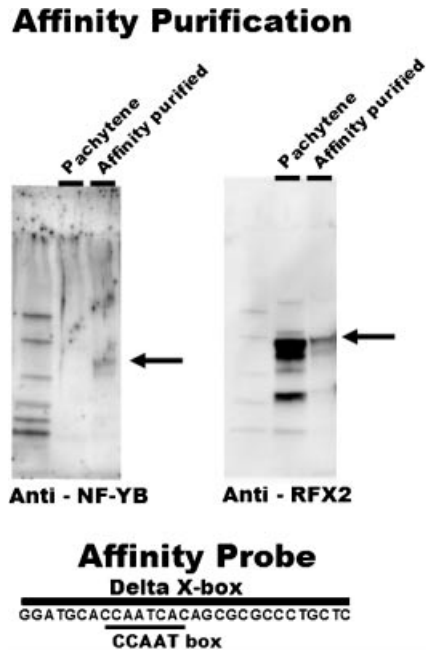

Fig. 9. Transcriptional factors RFX2 and NF-Y are associated in pachytene spermatocytes. Proteins in nuclear extracts from pachytene spermatocytes were affinity purified with a biotinylated rat H1t CCAAT probe that lacks X-box1 (designated Delta X-box). The original nuclear extract and the affinity purified proteins were analyzed by Western blotting. Polyclonal antibodies against NF-YB revealed very low concentrations of NF-YB in the original extract (**left panel**, **lane 2**) but NF-YB was concentrated in the affinity purified sample (**left panel**, **lane 3**). The concentration of RFX2 was elevated in the original extract (**right panel**, **lane 2**) and it was present at a high level in the affinity purified sample. This was unexpected because the probe did not contain an intact X-box. These data support our hypothesis that RFX2 may associate with NF-Y in binding to the H1t promoter in pachytene spermatocytes where the H1t gene is actively transcribed.

box was found to be essential for tissue-specific expression in transgenic mice, and was named the TE [Grimes et al., 1990, 1992a,b; vanWert et al., 1995, 1998]. The element was subsequently found to consist of two half sites that were imperfect inverted repeats designated TE1 and TE2 [Wolfe et al., 1995]. The sequences of TE1 and TE2 are similar to the X-box element found in abundance throughout the genome in the promoters of many other genes and thus these elements are now designated X-box1 and X-box2 [Xie et al., 2007]. The X-box element also appears as imperfect inverted repeats, and is bound by RFX family members, which include RFX1, RFX2, RFX3, RFX4, and RFX5.

RFX proteins are transcription factors that regulate activity of a variety of genes, and given the number of promoters containing X-box elements there are no doubt many more regulatory pathways involving RFX factors to be elucidated. They can bind X-box elements as homodimers or heterodimers, in a head-to-tail fashion [Gajiwala et al., 2000]. Their ability to bind as homodimers or heterodimers provides a possible mechanism of regulating RFX activity to enhance or represses promoter activity. In the testis of rats and mice the H1t X-box element is recognized and bound by RFX2 [Horvath et al., 2004; Wolfe et al., 2004]. We have shown that the steady-state level of RFX2 is high in whole testis samples and in enriched testis germinal cell fractions with the highest level in pachytene spermatocytes where the H1t gene is transcribed. We have shown previously by EMSA that a nuclear protein complex that binds to the H1t X-box does so to a significant

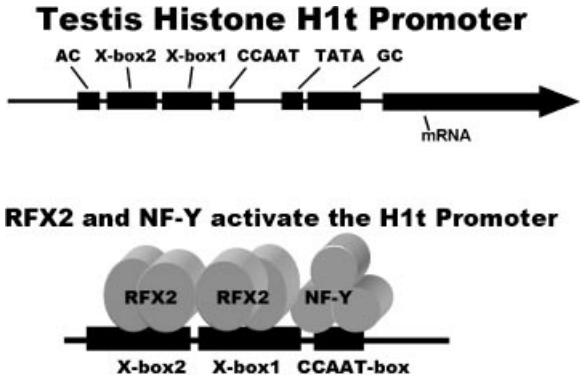

Fig. 10. Model of the testis histone H1t promoter and a representation RFX2 and NF-Y binding to the H1t X-box and CCAAT-box, respectively. The consensus sequence element of the testis-specific histone H1t promoter is shown in the upper diagram. The focus of this article is binding of RFX2 to the X-box and NF-Y to the adjacent CCAAT-box as shown in the model in the lower diagram. Both X-box1 and X-box2 bind RFX2 dimers and the CCAAT-box binds an NF-Y trimer. It is likely that RFX2 and NF-Y associate in formation of a transcription initiation complex in the H1t promoter in pachytene primary spermatocytes where the H1t gene is actively expressed.

level only in testis [Wolfe et al., 1995]. RFX2 is known to bind to the IL-5 receptor α promoter as a homodimer or as a heterodimer with RFX1 or RFX3 [Iwama et al., 1999] and is likely to be involved in the regulation of other X-box containing genes in other tissues.

We used Western blots to show that the RFX2 protein is present at a high level in testis compared to other tissues examined with the highest relative level seen in pachytene spermatocytes. We confirmed RFX2 binding to the H1t promoter in pachytene spermatocytes in vitro by EMSA supershift and in vivo by chromatin immunoprecipitation. We also demonstrated the functional importance of RFX2 binding by showing that RFX2 enhances H1t promoted Luciferase expression 10-fold over basal expression levels in the testis GC-1spg cell line. This observation is interesting because we previously measured only a twofold enhancement over basal levels using the same expression vectors in the testis GC-2spd cell line [Grimes et al., 2005]. The new results showing an enhanced level of H1t-promoter activity in GC-1spg cells upon overexpression of RFX2 lead us to speculate that the endogenous level of RFX2 might be high in GC-2spd cells. We have observed endogenous RFX2 in both GC-1spg and GC-2spd cell lines and preliminary experiments show that the relative level of RFX2 is higher in GC-2spd than in GC-1spg cells. A low endogenous level of RFX2 in GC1-spg cells may leads to a relatively low basal level of H1t promoter activity and thus, overexpression of RFX2 may lead to a relatively high level of enhancement of H1t promoter activity. Of course there are other possible explanations for the different enhancement levels seen in these two cells lines, but thus far this seems to be the simplest explanation.

It is not yet clear how RFX2 enhances H1t promoter activity in pachytene primary spermatocytes where the gene is expressed but not in early spermatids where the H1t gene is silenced. We observe significant levels of RFX2 in both germinal cells types, although the relative level appears to be higher in spermatocytes. RFX2 may enhance promoter activity in a tissue-specific or cell type specific way, activating the H1t promoter in primary spermatocytes but repressing it in haploid spermatids and nongerminal cell types. Alternatively, RFX2 may bind to the H1t promoter in spermatocytes but another RFX such as RFX1 may bind the H1t promoter preferentially in spermatids or in other cell types to silence the promoter [Kim et al., 2006]. A third possibility and one consistent with our preliminary studies is that RFX2 binds as a homodimer to enhance H1t promoter activity in spermatocytes but it may bind as a heterodimer possibly along with RFX4 to repress H1t promoter activity in early spermatids. Furthermore, it is possible that the H1t promoter is active in both spermatocyte and spermatid stages of spermatogenesis, but H1t mRNA may be stable only in spermatocytes. Further experiments are needed to test these and other possibilities.

The H1t CCAAT-box and its cognate transcription factor NF-Y also appear to be important for regulating expression of the H1t gene. In MHC class II genes a CCAAT-box sequence present just downstream of the X-box is bound by a trimeric NF-Y complex. This NF-Y complex binds cooperatively with the RFX complex through interaction with the C-terminal tail of RFX5 [Villard et al., 2000]. In this study we used pachytene nuclear extracts to perform an EMSA supershift using antibodies to NF-YA, B, and C, and RFX2. Each antibody shifted the bound complex, indicating that all of those proteins are present, and may interact. To test the possibility of RFX2 and NF-Y interaction, a biotinylated oligonucleotide corresponding to

the CCAAT-box sequence was used to purify binding proteins from pachytene nuclear extracts. When the affinity purified proteins were examined by Western blotting both NF-Y subunits and RFX2 proteins were detected.

It is quite interesting that we observed significant binding of RFX2 to this CCAAT-box affinity purification probe, since an X-box binding motif was not present. These experimental results suggests that NF-Y and RFX2 may bind to each other in pachytene nuclear extracts. We have not yet demonstrated NF-Y binding to the H1t promoter in vivo, but this could be a function of the antibodies used. Formation of an NF-Y trimer may mask the epitopes, or there may be testis-specific isoforms of the NF-Y proteins not recognized by the commercial antibodies used.

The H1t CCAAT-box has been shown in previous studies to be critical for H1t promoter activity [Gallinari et al., 1989]. It is interesting that we find significant levels of NF-Y in both pachytene spermatocytes where the H1t gene is expressed and in early spermatids where the H1t gene is repressed. This is similar to the situation of RFX2 where it is present during both stages of spermatogenesis but it is most abundant in spermatocytes where the H1t gene is expressed. It is possible that transcriptional factors such as p53 or PCAF that can interact with and regulate activity of NF-Y in some other promoters [Di Agostino et al., 2006; Peng et al., 2007] may contribute to regulation of H1t promoter activity in spermatocytes and early spermatids. These factors, either by direct or indirect interaction with histone-modifying HDAC, may modulate H1t promoter activity. Further work is needed to test this possibility.

Additional mechanisms of spermatogenic gene regulation are certainly possible. Recent work has identified the *piwi* family of genes that is important in stem cell division, gametogenesis, and RNA silencing [Cox et al., 1998]. The *piwi* family of genes is essential in spermatogenesis [Deng and Lin, 2002]. A testis-specific gene designated *miwi*, the murine homolog of *piwi*, encodes a regulatory protein in spermatocytes and early spermatids [Deng and Lin, 2002]. Mice lacking *miwi* display spermatogenesis arrest in spermatid development, a phenotype similar to *CREM* mutants. There is a concomitant down-regulation of the mRNAs of *ACT* (activator of CREM in testis) and CREM target genes with this arrest [Fimia et al., 1999] and MIWI forms a complex with these mRNAs. MIWI associates with translational machinery and small non-coding PIWI-interacting RNAs (piRNAs) in regulating spermatogeneis [Grivna et al., 2006] and there may be a gene silencing role for piRNA complex (piRC) in mammals [Lau et al., 2006]. Involvement of *piwi* family genes in regulating expression of histone H1t or testis core histone genes at the transcriptional or translational level during spermatogenesis is unknown.

The present study reveals that RFX2 binds to the H1t promoter in spermatocytes during spermatogenesis and that this binding is functionally important for activating the H1t promoter. We observe significant enhancement of H1t promoter activity in transient expression assays in testis GC-1spg cells when RFX2 is co-expressed. We show that all three NF-Y protein trimer subunits bind tightly to the H1t CCAAT-box motif and to RFX2 in spermatocytes in vitro. RFX2 certainly is also present in early spermatids and in most non-germinal cell types examined. However, further work is needed to determine the nature of the binding of RFX2 or whether RFX2 binds at all to the H1t promoter in early spermatids or in other tissues in vivo where the H1t gene is silenced. Further work is also needed to elucidate the nature of the testis NF-Y protein components, to determine whether the NF-Y complex binds the H1t CCAAT-box in spermatocytes in vivo, and to determine the functional relevance of NF-Y binding to the CCAAT-box and its interaction with RFX2 which is bound to the adjacent X-box.

ACKNOWLEDGMENTS

This research was supported by a Merit Review grant from the Veterans Administration to SRG.

REFERENCES

Ait-Lounis A, Baas D, Barras E, Benadiba C, Charollais A, Nlend Nlend R, Liegeois D, Meda P, Durand B, Reith W. 2007. Novel function of the ciliogenic transcription factor RFX3 in development of the endocrine pancreas. Diabetes 56:950–959.

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.

Bellorini M, Zemzoumi K, Farina A, Berthelsen J, Piaggio G, Mantovani R. 1997. Cloning and expression of human NF-YC. Gene 193:119–125.

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

Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H. 1998. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev 12:3715–3727.

Deng W, Lin H. 2002. miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. Dev Cell 2:819–830.

Di Agostino S, Strano S, Emiliozzi V, Zerbini V, Mottolese M, Sacchi A, Blandino G, Piaggio G. 2006. Gain of function of mutant p53: The mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell 10:191–202.

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.

Fimia GM, De Cesare D, Sassone-Corsi P. 1999. CBP-independent activation of CREM and CREB by the LIM-only protein ACT. Nature 398:165–169.

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.

Gallinari P, La Bella F, Heintz N. 1989. Characterization and purification of H1TF2, a novel CCAAT-binding protein that interacts with a histone H1 subtype-specific consensus element. Mol Cell Biol 9:1566–1575.

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.

Grimes SR, Wolfe SA, Anderson JV, Stein GS, Stein JL. 1990. Structural and functional analysis of the rat testis-specific histone H1t gene. J Cell Biochem 44:1–17.

Grimes SR, Wolfe SA, Koppel DA. 1992a. Temporal correlation between the appearance of testis-specific DNA-binding proteins and the onset of transcription of the testis-specific histone H1t gene. Exp Cell Res 201: 216–224.

Grimes SR, Wolfe SA, Koppel DA. 1992b. Tissue-specific binding of testis nuclear proteins to a sequence element within the promoter of the testis-specific histone H1t gene. Arch Biochem Biophys 296:402–409.

Grimes SR, Wilkerson DC, Noss KR, Wolfe SA. 2003. Transcriptional control of the testis-specific histone H1t gene. Gene 304:13–21.

Grimes SR, Prado S, Wolfe SA. 2005. Transcriptional activation of the testis-specific histone H1t gene by RFX2 may require both proximal promoter X-box elements. J Cell Biochem 94:317–326.

Grivna ST, Pyhtila B, Lin H. 2006. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. Proc Natl Acad Sci USA 103:13415–13420.

Guerra RF, Imperadori L, Mantovani R, Dunalp DD, Finzi L. 2007. DNA compaction by the nuclear factor Y (NF-Y). Biophys J 93:176–182.

Hooft van Huijsduijnen RA, Bollekens J, Dorn A, Benoist C, Mathis D. 1987. Properties of a CCAAT-box-binding protein. Nucleic Acids Res 15:7265–7282.

Horvath GC, Kistler WS, Kistler MK. 2004. RFX2 is a potential transcriptional regulatory factor for histone H1t and other genes expressed during the meiotic phase of spermatogenesis. Biol Reprod 71:1551–1559.

Iwama A, Pan J, Zhang P, Reith W, Mach B, Tenen DG, Sun Z. 1999. Dimeric RFX proteins contribute to the activity and lineage specificity of the interleukin-5 receptor alpha promoter through activation and repression domains. Mol Cell Biol 19:3940–3950.

Kim M, Li D, Cui Y, Mueller K, Chears WC, DeJong J. 2006. Regulatory factor interactions and somatic silencing of the germ cell-specific ALF gene. J Biol Chem 281:34288–34298.

Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE. 2006. Characterization of the piRNA complex from rat testes. Science 313:363–367.

Ma K, Zheng S, Zuo Z. 2006. The transcription factor regulatory factor X1 increases the expression of neuronal glutamate transporter type 3. J Biol Chem 281:21250–21255.

Maity SN, Sinha S, Ruteshouser EC, de Crombrugghe B. 1992. Three different polypeptides are necessary for DNA binding of the mammalian heteromeric CCAAT binding factor. J Biol Chem 267:16574–16580.

Mantovani R. 1999. The molecular biology of the CCAAT-binding factor NF-Y. Gene 239:15–27.

Martinelli R, Heintz N. 1994. H1TF2A, the large subunit of a heterodimeric, glutamine-rich CCAAT-binding transcription factor involved in histone H1 cell cycle regulation. Mol Cell Biol 14:8322–8332.

Masternak K, Reith W. 2002. Promoter-specific functions of CIITA and the MHC class II enhanceosome in transcriptional activation. EMBO J 21:1379–1388.

Nayernia K, Drabent B, Meinhardt A, Adham IM, Schwandt I, Muller C, Sancken U, Kleene KC, Engel W. 2005. Triple knockouts reveal gene interactions affecting fertility of male mice. Mol Reprod Dev 70: 406–416.

Otsuki K, Hayashi Y, Kato M, Yoshida H, Yamaguchi M. 2004. Characterization of dRFX2, a novel RFX family protein in Drosophila. Nucleic Acids Res 32:5636–5648.

Peng Y, Stewart D, Li W, Hawkins M, Kulak S, Ballermann B, Jahroudi N. 2007. Irradiation modulates association of NF-Y with histone-modifying cofactors PCAF and HDAC. Oncogene 26:7576–7583.

Ramesh S, Bharath MM, Chandra NR, Rao MR. 2006. A K52Q substitution in the globular domain of histone H1t modulates its nucleosome binding properties. FEBS Lett 580:5999–6006.

Reith W, Satola S, Sanchez CH, Amaldi I, Lisowska-Grospierre B, Griscelli C, Hadam MR, Mach B. 1988. Congenital immunodeficiency with a regulatory defect in MHC class II gene expression lacks a specific HLA-DR promoter binding protein, RF-X. Cell 53:897–906.

Reith W, Siegrist CA, Durand B, Barras E, Mach B. 1994. Function of major histocompatibility complex class II promoters requires cooperative binding between factors RFX and NF-Y. Proc Natl Acad Sci USA 91:554–558.

Serra E, Zemzoumi K, di Silvio A, Mantovani R, Lardans V, Dissous C. 1998. Conservation and divergence of NF-Y

transcriptional activation function. Nucleic Acids Res 26: 3800–3805.

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

van Wijnen AJ, Wright KL, Massung RF, Gerretsen M, Stein JL, Stein GS. 1988. Two target sites for protein binding in the promoter region of a cell cycle regulated human H1 histone gene. Nucleic Acids Res 16:571–592.

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, Panek HR, Wolfe SA, Grimes SR. 1998. The TE promoter element of the histone H1t gene is essential for transcription in transgenic mouse primary spermatocytes. Biol Reprod 59:704–710.

Villard J, Peretti M, Masternak K, Barras E, Caretti G, Mantovani R, Reith W. 2000. A functionally essential domain of RFX5 mediates activation of major histocompatibility complex class II promoters by promoting cooperative binding between RFX and NF-Y. Mol Cell Biol 20:3364–3376.

Wilkerson DC, Wolfe SA, Grimes SR. 2003. TE2 and TE1 sub-elements of the testis-specific histone H1t promoter are functionally different. J Cell Biochem 88:1177–1187.

Wolfe SA, van Wert JM, Grimes SR. 1995. Expression of the testis-specific histone H1t gene: Evidence for involvement of multiple cis-acting promoter elements. Biochemistry 34:12461–12469.

Wolfe SA, Mottram PJ, vanWert JM, Grimes SR. 1999. Localization of upstream elements involved in transcriptional regulation of the rat testis-specific histone H1t gene in somatic cells. Biol Reprod 61:1005–1011.

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.

Xie X, Mikkelsen TS, Gnirke A, Lindblad-Toh K, Kellis M, Lander ES. 2007. Systematic discovery of regulatory motifs in conserved regions of the human genome, including thousands of CTCF insulator sites. Proc Natl Acad Sci USA 104:7145–7150.

Xu Y, Sengupta PK, Seto E, Smith BD. 2006. Regulatory factor for X-box family proteins differentially interact with histone deacetylases to repress collagen alpha2(I) gene (COL1A2) expression. J Biol Chem 281:9260–9270.

Zhang D, Stumpo DJ, Graves JP, DeGraff LM, Grissom SF, Collins JB, Li L, Zeldin DC, Blackshear PJ. 2006. Identification of potential target genes for RFX4_v3, a transcription factor critical for brain development. J Neurochem 98:860–875.

Zhong R, Roeder RG, Heintz N. 1983. The primary structure and expression of four cloned human histone genes. Nucleic Acids Res 11:7409–7425.