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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

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Abstract Previous work in our laboratory revealed upregulated transcription of the testis-specific linker histone *H1t* gene in pachytene primary spermatocytes during spermatogenesis. Using the H1t X-box as an affinity chromatography probe, we identified Regulatory Factor X2 (RFX2), a member of the RFX family of transcription factors, as a nuclear protein that binds the probe. We also showed that RFX2 activated the H1t promoter in transient expression assays. However, other RFX family members have the same DNA-binding domain and they also may regulate *H1t* gene expression. Therefore, in this study we examined the distribution of RFX2 and other RFX family members in rat testis germinal cells and in several tissues. Among tissues examined, RFX2 is most abundant in testis. Testis RFX2 is most abundant in spermatocytes where transcription of the *H1t* gene is upregulated and the steady-state H1t mRNA level is high. RFX2 levels decrease but RFX1 levels increase in early spermatids where *H1t* gene transcription is downregulated. Antibodies against RFX2 generate a shifted band in electrophoretic mobility shift assays (EMSA) using H1t or testisin X-box DNA probes with nuclear proteins from spermatocytes. These data support the hypothesis that RFX2 expression is upregulated in spermatocytes where it participates in activating transcription of the *H1t* gene and other testis genes. These data also support the possibility that other RFX family members may bind to the H1t promoter in other testis germinal cell types and in nongerminal cells to downregulate *H1t* gene transcription. J. Cell. Biochem. 99: 735–746, 2006.

Key words: RFX2; testis; transcription factor; transcription regulation; histone H1t

The mammalian testis-specific linker histone *H1t* gene has been used as a model for the study of tissue-specific gene transcription [Cole et al., 1986; Grimes et al., 1987, 1990; Wolfe and Grimes, 1993]. The gene is excellent for this type of study. It appears to be relatively simple with a short proximal promoter and short coding region lacking introns and producing an mRNA that is not polyadenylated. A high steady-state level of H1t mRNA is present in testis spermatocytes but very low levels, if any, are present in other germinal cell types such as spermatogonia [Drabent et al., 1998], early spermatids, and late spermatids, and in nongerminal cells [Grimes et al., 1990]. Furthermore, populations of testis cells enriched in spermatocytes, early spermatids, and late spermatids can be prepared by centrifugal elutriation for molecular biology studies [Meistrich et al., 1981]. Although histone gene transcription and histone synthesis typically occur during S-phase of the cell cycle when DNA replication occurs, the *H1t* gene is transcribed and histone H1t is synthesized in spermatocytes at a time that is uncoupled from DNA replication [Grimes et al., 1990].

The functional significance of histone H1t has been questioned, because homozygous knockouts of the gene failed to exhibit defects in

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fertility [Drabent et al., 2000; Lin et al., 2000; Fantz et al., 2001]. Redundancy of the linker histones and compensation of other linker histones for H1t may obscure detection of defects upon loss of H1t. However, other testis genes that also failed to exhibit fertility defects when inactivated included histone H1.1 (*H1.1*) [Rabini et al., 2000], transition protein 2 (*TNP2*) [Adham et al., 2001], proacrosin (*Acr*) [Baba et al., 1994], and sperm mitochondria-associated cysteine-rich protein (*Smcp*)[Nayernia et al., 2002]. On the other hand, two triple knockout lines, *Acr*/*H1t*/*Smcp* and *Tnp2*/*H1t*/*Smcp*, exhibited dramatically reduced fertility on mixed genetic backgrounds [Nayernia et al., 2005]. The experiments showed that male fertility involves synergistic interactions of several genes (including the *H1t* gene) that ultimately affect sperm motility and sperm-egg adhesion during fertilization [Nayernia et al., 2005].

A promoter element designated the testis element (TE) is found only in *H1t* of all the mammalian *H1* genes identified (Fig. 2) [Grimes et al., 1990, 1992a,b]. TE contains adjacent inverted repeat sequences that were originally designated TE1 and TE2 but are now designated X-box1 and X-box2 (Fig. 2) [Wolfe et al., 1995]. Alignment of H1t X-box1 and X-box2 with the canonical X-box shows their similarity (Fig. 2) [Wilkerson et al., 2003; Grimes, 2004; Grimes et al., 2005]. Nuclear proteins from unfractionated testis cells or from cell populations enriched in spermatocytes or early spermatids bind to these elements to generate a low mobility complex in EMSA [Grimes et al., 1992a,b; Wolfe et al., 1995]. Nuclear proteins from other tissues fail to form this low mobility complex [Wolfe et al., 1995; vanWert et al., 1996]. TE is essential for transcription of the *H1t* gene as shown in studies of transgenic mice, where replacement of TE with a stuffer DNA sequence eliminated transcription of the rat H1t transgene [vanWert et al., 1996, 1998]. When we used an X-box1 DNA sequence as an affinity purification probe to purify the testis nuclear-binding proteins, RFX2 was the major binding protein identified [Wolfe et al., 2004]. Polyclonal antibodies against RFX2, when added to a DNA-binding reaction using testis nuclear extracts and an H1t X-box probe, generated a shifted complex [Horvath et al., 2004; Wolfe et al., 2004]. Therefore, RFX2 is a major component of the testis nuclear protein–DNA complex formed with the H1t X-box [Wolfe et al., 2004].

These experiments showed that RFX2 can bind the histone H1t X-box where it may be essential for upregulation of transcription of the testis-specific *H1t* gene in spermatocytes [Wolfe et al., 2004; Grimes et al., 2005]. However, RFX2 and other RFX family members contain similar DNA-binding domains [Gajiwala et al., 2000; Morotomi-Yano et al., 2002]. RFX is critical for transcriptional regulation of *MHCII* genes [Iwama et al., 1999; Gajiwala et al., 2000; Masternak and Reith, 2002; Morotomi-Yano et al., 2002] and RFX family members in other tissues may be important for silencing transcription of the *H1t* gene [Reith et al., 1994; Iwama et al., 1999; Morotomi-Yano et al., 2002]. For example, RFX1 was found to be present in mouse testis early spermatids and it can bind to H1t X-box [Horvath et al., 2004]. Therefore, a better understanding of transcriptional regulation of the *H1t* gene by RFX requires an examination of the distribution of RFX2 and other RFX family members in different testis cell types and among various organs.

In this study, we examined distribution of RFX family members among enriched testis cell populations and among various rat tissues. RFX2 was most abundant in testis and within the testis it was most abundant in spermatocytes where it can bind to the X-box of the actively transcribed testis-specific histone *H1t* and *testisin* genes. However, RFX2 was also present in early spermatids and in nongerminal tissues where *H1t* gene transcription is inhibited. Therefore, other RFX family members such as RFX1 may bind the H1t X-box and inactivate transcription of this gene in these cells.

MATERIALS AND METHODS

Materials

Deoxynucleotides and Ampli-Taq for polymerase chain reaction (PCR) were purchased from Perkin-Elmer, Foster City, CA. Oligonucleotides were purchased from Fisher Scientific (http://www.fisheroligos.com). The Klenow fragment of DNA polymerase was purchased from New England Biolabs, Inc., Beverly, MA. Radiolabeled [α-^{32}P]-dCTP was purchased from Perkin Elmer Life and Analytical Sciences, Boston, MA.

Animal Use and Cell Culture

Rats were purchased from Harland Sprague–Dawley, Madison, WI. We conducted these animal studies in an AAALAC, Inc., accredited facility in accordance with the Guiding Principles for the Care and Use of Research Animals.

Single cell suspension of rat testis cells from adult 250 gm rats were prepared by trypsinization and enriched populations of testis cells were prepared by centrifugal elutriation of the single cell suspension [Meistrich et al., 1981]. Other tissues were obtained from adult male 250 gm rats and testes were obtained from sexually immature 9-day-old rats.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assays (EMSA)

Nuclear protein extracts [Dignam et al., 1983] were prepared from testes of adult rats and from enriched populations of testis cells prepared by centrifugal elutriation. Protein concentrations in these extracts were determined using the Bio-Rad Experion automated electrophoresis system and using Bio-Rad protein dye reagent reading absorbance of light at 595 nm.

EMSAs were conducted essentially as described before [Grimes et al., 1990] to examine binding of nuclear proteins to the X-box DNA sequences found in the promoters of the *H1t* or *Testisin* genes. Rat H1t and human Testisin X-box sequences used as DNA-binding probes in this study were 5′-GAGGCGCCTAGGGATGCAC-3′ and 5′-GACCCTCCCCTAGGGGCTG-3′, respectively. Probes were prepared by annealing overlapping single-stranded oligonucleotides, filling the recessed ends using the Klenow fragment of DNA polymerase (New England Biolabs) with a mixture of dATP, dGTP, dTTP, and [α-^{32}P] dCTP to introduce radioactivity, and polyacrylamide gel purification of the radioactive double-stranded DNA [Sambrook et al., 1989; Grimes et al., 1992a,b]. Oligonucleotides used for the H1t X-box were 5′-GAGGCGCCTAGGG-3′ and 5′-GTGCATCCCTAGGC-3′ and those used for the Testisin X-box were 5′-GACCCGCCCCTAG-3′ and 5′-CAGCCCCTAGGGGC-3′.

Preparation of Samples for Western Blot Analysis

Total cellular extracts. Whole tissue samples were homogenized directly in an equal volume of 2 × SDS–PAGE sample loading buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue) [Laemmli, 1970]. After boiling, proteins were quantitated using a Bio-Rad Experion automated electrophoresis system.

Nuclei and cytoplasm. Samples of testis or testis cell populations enriched by centrifugal elutriation were homogenized gently in an equal volume of ice cold Buffer A (10 mM HEPES pH 7.9,1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol) containing 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 10 μl per ml protease inhibitor (Sigma Protease Inhibitor Cocktail (Catalog number P8340)) as for preparation of nuclear extracts [Dignam et al., 1983; Grimes et al., 1992b]. Samples were centrifuged at 2000 × g for 10 min at 4°C to obtain nuclear pellets and cytoplasmic supernatants were removed and centrifuged again at 5000 × g to remove any contaminating nuclei. Cytoplasmic supernatants were homogenized in an equal volume of 2 × SDS–PAGE sample loading buffer, a small portion was removed for protein analysis and the remainder was frozen at −80°C. The original nuclear pellets were washed twice in 20 volume of Buffer A centrifuging 2000 × g for 10 min each time. Washed nuclei were homogenized with an equal volume of 2 × SDS sample loading buffer, a portion was removed for protein analysis and the remainder was frozen at −80°C.

Western Blot Analysis

Samples were analyzed by Western blotting essentially as described [Wilkerson et al., 2002]. Sample proteins were separated by SDS–PAGE using a Bio-Rad Mini-PROTEAN cell or Criterion electrophoresis cell with pre-cast Tris-HCl polyacrylamide gels, and blotted to Immuno-Blot PVDF membranes. Cruz Markers protein molecular weight standards (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Precision Plus prestained protein molecular weight markers for monitoring progress of electrophoresis and blotting to PVDF membranes (Bio-Rad) were also electrophoresed.

Blotted membranes were blocked for 1 h in a solution of 5% Powdered Milk diluted in 1 × PBS (137 mM NaCl, 2.7 mM KCl, and 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 pH 7.4). Blots were then incubated for 1 h with primary antibody (1:1,000 dilution in 1 × PBS), rinsed with 1 × PBS containing 0.05% Tween 20, incubated for 1 h with secondary antibody (donkey anti-goat IgG HRP-conjugated), and

rinsed with PBS containing 0.05% Tween 20. Chemiluminescence was developed using a SuperSignal West Dura Kit from Pierce and images were captured using a Bio-Rad Versa-Doc imaging system. Band areas were determined using Sigma Gel (SYSTAT Software, Inc.) and data were plotted using Sigma Plot (SYSTAT Software, Inc.)

Primary polyclonal antibodies purchased from Santa Cruz were prepared against peptides from RFX1 (peptide EI-16, catalog number sc-10651x), RFX2 (A-18, sc-10659x), RFX3 (T-17, sc-10662x), RFX5 (E-17, sc-10667x), and RFXB (C-18, sc-9821x). Donkey anti-goat and anti-rabbit IgG HRP-conjugated secondary antibodies were purchased from Santa Cruz (catalog numbers sc-2033 and sc2317).

RNA Isolation and Reverse Transcriptase Real Time PCR

Total cellular RNA was extracted from whole testis and from enriched testis cell populations using RNA Stat-60 (Tel-Test Inc., Friendswood, TX) by the manufacturer's protocol as described previously [vanWert et al., 1996]. These RNA samples were analyzed and quantitated using the Experion automated electrophoresis system and used as templates for Real Time PCR using Bio-Rad's iScript One-Step RT-PCR kit with SYBR Green. RT-PCR was conducted using a MyiQ Real Time PCR Detection System (Bio-Rad). The following PCR primer pair was used for histone H1t cDNA amplification: 5′-CCCCAAGAGTAGTAAGACCAAGG-3′ (forward) and 5′-GCATGACCATCTTTGACTTC-CC-3′ (reverse). Plasmid pPS3 that contains the cloned rat histone *H1t* gene was used as a template to prepare a standard curve to determine copy number of the template versus the Ct value of amplified product.

RESULTS

H1t Gene Transcription Is Upregulated in Testis

Histone H1t mRNA has been detected in whole testis and at a very high level in testis primary spermatocytes by Northern blot analysis [Grimes et al., 1987, 1990; vanWert et al., 1996]. However, this method was not sufficiently sensitive to reveal H1t mRNA in other cell types. Therefore, we examined steady-state mRNA levels in testes and other rat tissues using the method of Real Time RT-PCR, which has been used to detect basal levels of H1t mRNA in mouse spermatogonia [Drabent et al., 1998].

Equal amounts of RNA were used as templates in RT-PCR. The estimated copy number of H1t mRNA in each sample was calculated based upon analysis of a known copy number of plasmid DNA containing the H1t template. As expected, the steady-state H1t mRNA level is very high in testes of adult rats (Fig. 1). We did see basal levels of H1t mRNA within thymus, kidney, brain, and heart (over 100-fold lower than in testis), but very low levels were found in lung, spleen, and liver.

RFX2 from Primary Spermatocytes Binds to the *H1t* Promoter X-Box

Our laboratory and another laboratory reported detecting RFX2 in spermatocytes where the *H1t* gene is transcribed but it was also seen in early spermatids [Horvath et al., 2004; Wolfe et al., 2004; Grimes et al., 2005] where the steady-state H1t mRNA level is very low. RFX binds to a promoter sequence designated the X-box [Gajiwala et al., 2000; Morotomi-Yano et al., 2002] and the H1t promoter contains two adjacent X-boxes (Fig. 2). In fact, transcriptional activation of the *H1t* gene may require testis RFX2-binding to both X-boxes [Grimes et al., 2005]. The H1t and Testisin

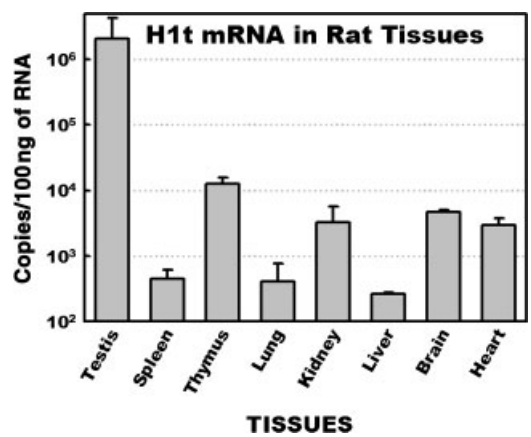

Fig. 1. Steady-state levels of H1t mRNA in rat tissues. Total cellular RNA samples from several tissues were quantitated and 100 ng of each sample was used for a reverse transcription reaction. The H1t mRNA steady-state levels are plotted on the Y-axis and error bars represent standard error of the mean. Notice that the level in testes is over 100-fold higher than in any other tissue.

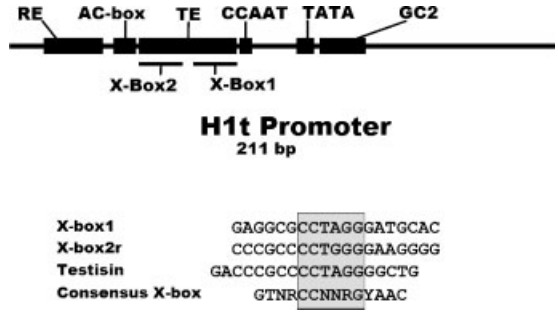

Fig. 2. H1t promoter diagram and alignment of H1t, Testisin, and Consensus X-boxes. The upper portion of the figure is a diagram of a 211 bp portion of the H1t promoter extending downstream to the ATG start codon showing the location of the TE element that contains the two X-boxes. The lower portion of the figure presents an alignment of the H1t X-box1, H1t X-box2, Testisin X-box, and consensus X-box sequences. N represents any nucleotide, R represents A or G and Y represents C or T. Note that the palindromic sequence ''CCTAGG'' (the shaded region in the alignment) is highly conserved.

[Hooper et al., 2000] X-Box probes used in the EMSAs in this study are aligned with a consensus X-box sequence in Figure 2. Notice that the central region of these X-box sequences is a conserved palindrome (CCTAGG) (Fig. 2).

Although the H1t and consensus X-box sequences differ slightly, EMSAs revealed tight binding of testis RFX2 to the H1t X-box. Nuclear proteins derived from rat testis and from populations of testis cells enriched by centrifugal elutriation including primary spermatocyte (fraction 5) and early spermatid (fraction 3) bind to produce a low mobility complex designated the TE complex (Fig. 3, left panel). Proteins from late spermatids (LS) bind very weakly and proteins from 9-day-old whole testis (9d) bind but do not form the typical TE complex. Polyclonal antibodies against RFX2, when added to binding reactions with nuclear proteins from adult whole testis or cell populations enriched in spermatocytes or early spermatids, shifted this complex to a lower mobility (Fig. 4, left panel). When the Testisin X-box probe was used, a low mobility complex also formed (Fig. 3, right panel) and antibodies against RFX2 also shifted this complex (Fig. 4, right panel). However, the amount of RFX2 in the Testisin complex appeared to be lower based upon the density of the supershifted band.

RFX2 Level Is High in Primary Spermatocytes Compared to Other Germinal Cell Types

The previous experiments showed that nuclear RFX2 samples from spermatocytes

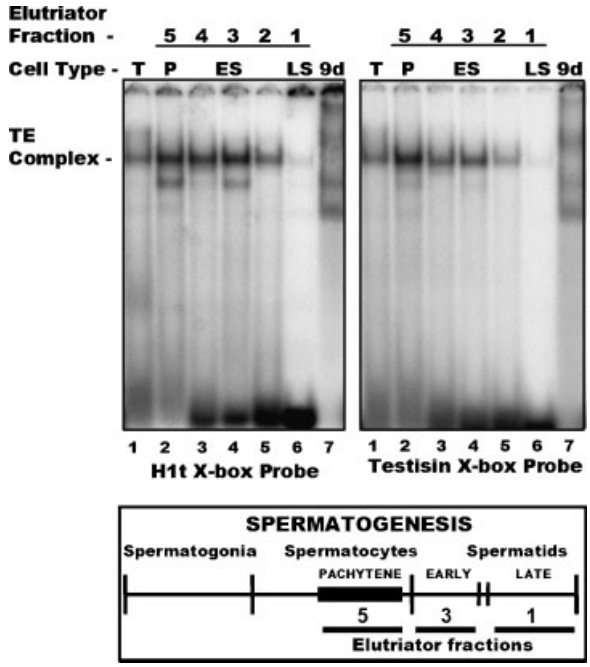

Fig. 3. EMSAs using testis nuclear extracts and H1t and testisin X-box probes and a Time line for spermatogenesis in the rat. The upper panels are EMSAs conducted to examine binding of nuclear proteins to X-box probes from the H1t (left panel) and Testisin (right panel) promoters. Equal quantities of nuclear protein extracts [Dignam et al., 1983] from testes of adult and 9-day-old rats and from enriched populations of testis cells (elutriator fractions 1–5) were used. Proteins from unfractionated testis (T) and from cell populations enriched in pachytene spermatocytes (P), early spermatids (ES), late spermatids (LS), and testes from 9-day-old rats were electrophoresed in Lanes 1, 2, 4, 6, and 7 in each panel. The major low mobility complex is designated TE. The lower panel presents a 60-day time line for spermatogenesis in the rat. Cell populations enriched by centrifugal elutriation [Meistrich et al., 1981] include pachytene spermatocytes (fraction 5), early spermatids (fraction 3), and late spermatids (fraction 1) as indicated.

and early spermatids were functional in binding to the X-box. However, the relative distribution of RFX2 and other RFX family member in the cytoplasm and nucleus of testis germinal cells has not been reported. Therefore, we examined RFX family members that are present in germinal cells at different stages of spermatogenesis.

Nuclei and cytoplasm were obtained from testes of adult rats and from enriched germinal cell populations for detection of RFX family members. Nuclear proteins were analyzed by Western blot analysis as described in the Methods and an example blot using antibodies against RFX2 is shown in Figure 5 where the major 87 kDa band represents RFX2. Fivefold more cytoplasmic protein was analyzed to

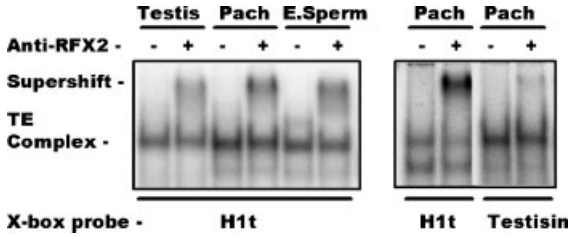

Fig. 4. EMSA supershifts with antibodies against RFX2. Polyclonal antibodies against RFX2 generate a supershifted EMSA band (+ sign in the figure) with the H1t X-box probe and binding proteins derived from unfractionated testis, pachytene primary spermatocytes, and early spermatids (left panel). The supershifted band produced with the Testisin X-box probe is less abundant than the one with the H1t X-box probe when using nuclear proteins from pachytene spermatocytes (right panel).

facilitate detection of cytoplasmic RFX and this protein overload increased the mobilities of cytoplasmic RFX bands slightly compared to the nuclear RFX bands (Fig. 5). RFX2 appears to be most abundant in nuclei from elutriator fraction 5 the fraction enriched in pachytene primary spermatocytes.

Areas of nuclear and cytoplasmic bands representing RFX2 were plotted to reveal more clearly the relative levels of RFX2 (Fig. 6B). RFX2 was more abundant in nuclei of primary spermatocytes (Elutriator fraction 5), than in nuclei of early spermatids (fraction 3) or any other cell population. RFX2 was very low in the cytoplasm of every sample examined.

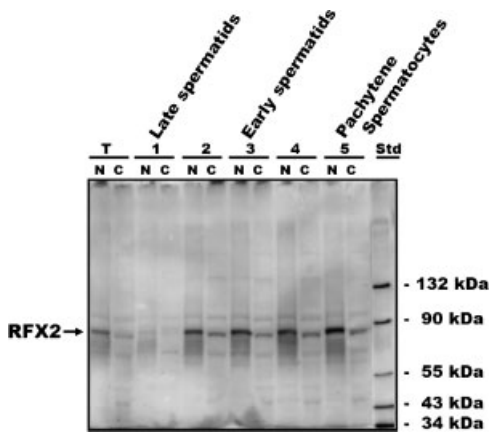

Fig. 5. Nuclear and cytoplasmic RFX2 in populations of testis cells. Western blots were conducted as described in the Methods. Nuclear proteins (12.5 μg per lane) and cytoplasmic proteins (62.5 μg per lane) were separated by SDS–PAGE and blotted to a PVDF membrane. After blocking, membranes were incubated with goat polyclonal primary antibody (RFX2 in this example) and then with donkey anti-goat IgG secondary antibody (HRP-conjugated). Samples from testis (T) and from testis cell populations (elutriator fractions 1–5) are marked at the top and nuclear (N) and cytoplasmic (C) fractions are indicated. The area of the major band in each lane (migrating at about 87 kDa in this example of RFX2) was measured and relative band areas, corrected for the fivefold difference in nuclear and cytoplasmic protein, were plotted in Figure 6B. Notice that the RFX2 level is highest in spermatocytes (fraction 5) but it is also abundant in early spermatids (fraction 3).

It has been reported that mouse testis RFX1 can bind as a heterodimer to the H1t X-box probe and it was reported that only early spermatids stain for RFX1 by immuno-staining of mouse testis sections [Horvath et al., 2004]. Therefore, we examined levels of other RFX family members including RFX1, RFX3, RFX5, and RFXB in these testis cells in the same way (Fig. 6, panels A,C,D,E). All RFX family members, like RFX2, were concentrated in nuclei. RFX1 was present in all testis nuclear and cytoplasmic samples tested, but unlike RFX2, the nuclear level of RFX1 was higher in early spermatids than in pachytene primary spermatocytes (a distribution opposite that of RFX2). RFX5 like RFX2 was more abundant in spermatocytes than early spermatids, but levels of RFX3 and RFX-B were almost equal in these two cell populations.

RFX2 Level Is High in Testis Compared to Other Tissues

The level of RFX2 was highest in testis compared to other tissues that we examined and the next highest level was in thymus (Fig. 7B). RFX2 was detected at moderate levels in stomach, kidney, liver, brain, and heart, but the levels were low in spleen and lung. We also examined the abundance of other RFX family member in these tissues. RFX1 was abundant in the testis, and like RFX2, the levels of both RFX1 and RFX3 were highest in testis and thymus (Fig. 7A,C). However, unlike RFX2, the levels of RFX1, RFX5, and RFXB were high in spleen (Fig. 7A,D,E). It should be mentioned that RFXB levels were so low that they were difficult to detect with the antibody used.

DISCUSSION

The results of experiments presented in this report represent an extension of our work on the protein complex that binds to the critical TE promoter element of the testis-specific histone *H1t* gene [Wolfe et al., 2004; Grimes et al., 2005]. This element contains two X-box sequences and previous experiments from our laboratory indicated that testis RFX2 binds to both of these

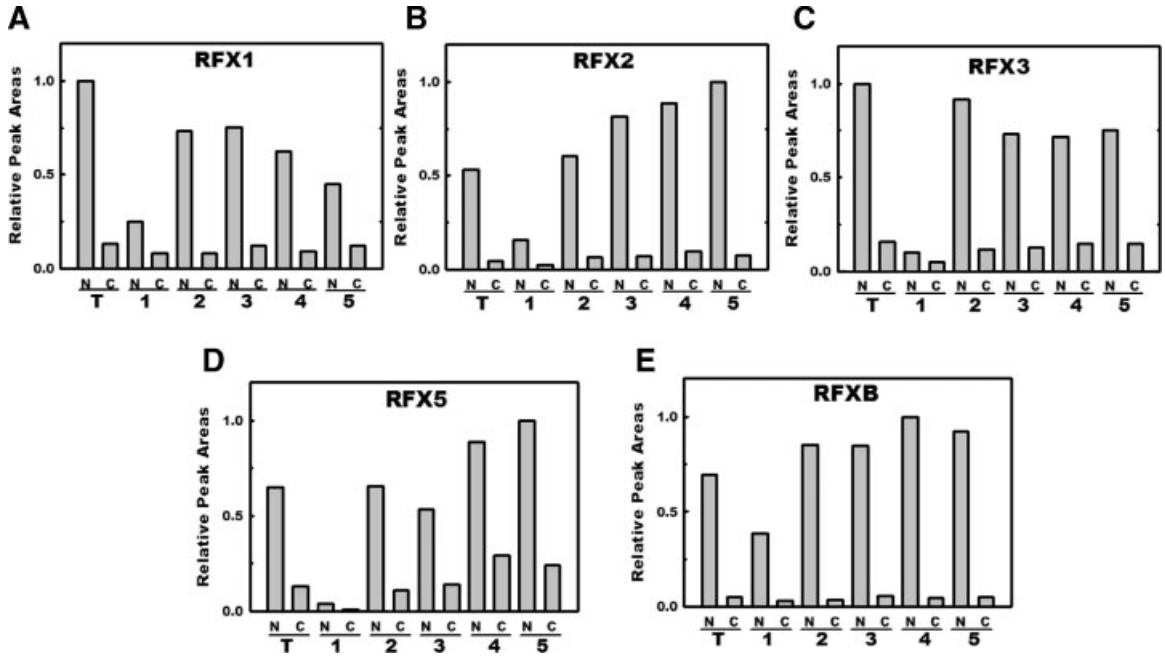

Fig. 6. Distribution of RFX family members in nuclear and cytoplasmic fractions of rat testis cell populations. Western blot analyses were conducted with the same protein samples shown in Figure 5 using primary antibodies against RFX family members. The panels (A–E) represent the data for RFX1, RFX2, RFX3, RFX5, and RFX-B, respectively. Areas of the bands were measured and relative peak areas were plotted as described for Figure 5. The levels of all RFX family members were high in primary spermatocytes. Notice that RFX2 is slightly higher in primary spermatocytes (fraction 5) than in early spermatids (fraction 3), while RFX1 is slightly higher in early spermatids than in spermatocytes.

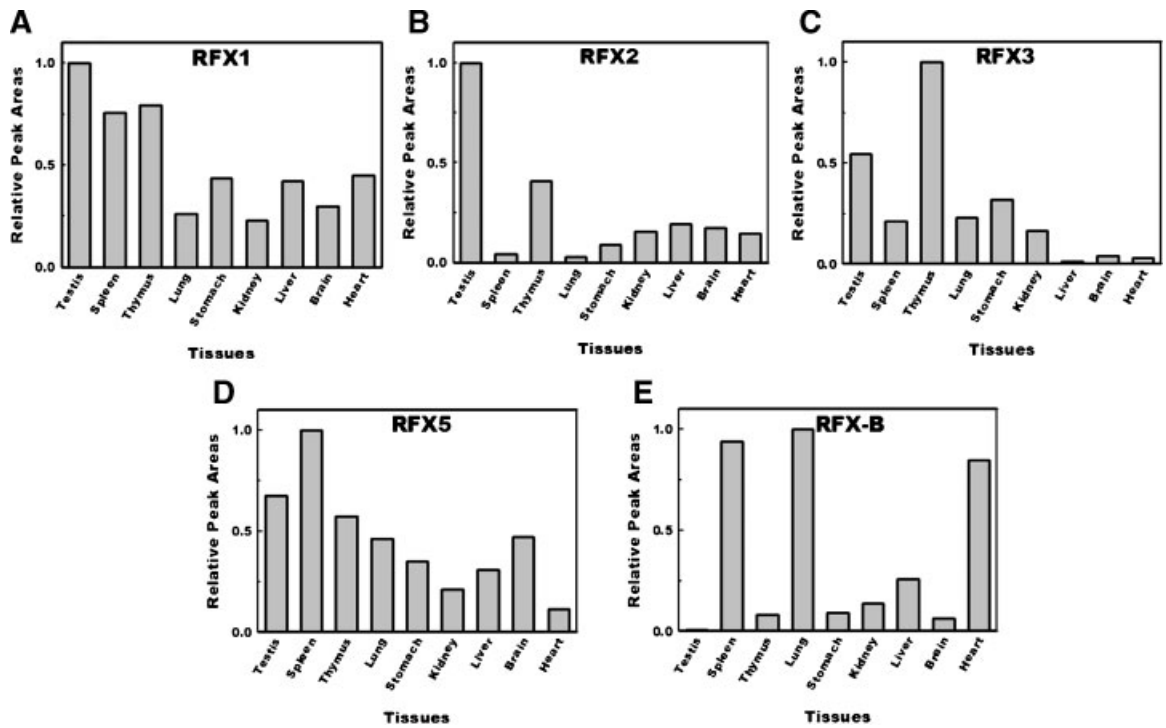

Fig. 7. Distribution of RFX family members in rat tissues. Western blots were conducted as described before but using 40 μg of protein extract from each rat tissue. Protein samples from left to right in each plot are testis, spleen, thymus, lung, stomach, kidney, liver, brain, and heart. Areas of the major bands were measured and the relative peak areas were plotted as described for Figure 6. All RFX family members except RFX-B are present at a relatively high level in testis and thymus.

sites to upregulate transcription of the histone *H1t* gene in primary spermatocytes [Wolfe et al., 2004; Grimes et al., 2005]. RFX2, a member of the family of transcription factors that contain an X-box DNA-binding domain [Gajiwala et al., 2000], can bind the X-box as a homodimer or as a heterodimer with other RFX family members such as RFX1. Mouse RFX1 from testis and somatic organs may bind the H1t X-box as a heterodimer, but RFX1 was detected only in early spermatids in that study [Horvath et al., 2004]. RFX family members other than RFX2 certainly are present in testis and at least one, RFX1, can bind the H1t X-box and may be involved in regulating transcription of the *H1t* gene during spermatogenesis. Therefore, in experiments presented in this study, we examined the testes of adult rats for the presence of RFX family members, we determined relative distribution of these RFX variants among specific testis germinal cell populations, and we examined the relative RFX distribution among several rat organs.

The steady-state H1t mRNA level was known to be high in rat testes [Grimes et al., 1987], but Northern blot analyses used for those studies lacked the sensitivity to detect low basal levels of H1t mRNA in other cell types. Therefore, we examined levels in several rat organs using Real Time RT-PCR. These studies show that the level is very high in testes of adult rats (Fig. 1). However, low levels of H1t mRNA are present in other organs examined with the highest levels in thymus (about 100-fold less abundant than in testis) brain and heart and with the lowest levels in liver, spleen and lung. These data reflect the transcriptional upregulation of the gene and the high level of stability of the mRNA in testis.

A low mobility electrophoretic band is produced in EMSA representing a protein complex that forms when adult testis nuclear proteins bind to the H1t X-box sequence. A similar quantity of nuclear protein from a cell population enriched in spermatocytes generates more of this band revealing formation of more of the complex (Fig. 3, left panel). RFX2 is a major component of this low mobility protein complex as shown by shifting this band to lower mobility with polyclonal antibodies against RFX2. Since the low mobility protein complex appears to be more abundant in primary spermatocytes compared to those from whole testis, RFX2 appears to be most abundant in primary spermatocytes. Western blot analyses (Fig. 6B) confirm a relatively high level of RFX2 in primary spermatocytes. In addition, binding activity of RFX2 in primary spermatocytes may be enhanced in some way. When EMSA's were conducted with testis nuclear extracts using antibodies against RFX1, RFX3, RFX5, and RFXB under the same experimental conditions, they did not shift the TE complex [Wolfe et al., 2004]. However, under altered conditions using early spermatid extracts, we have observed a weak shift with antibodies against RFX1 (data not shown).

The X-box sequence is found in promoters of many genes that are transcribed specifically in testis. For example, the promoter of the *testisin* gene contains the CCTAGG region of an X-box (Fig. 2) [Hooper et al., 2000]. When we used the testisin X-box sequence as an EMSA probe with spermatocyte nuclear protein, a low mobility shifted band is formed (Fig. 3, right panel) and RFX2 is detectable in the complex with polyclonal antibodies against RFX2 (Fig. 4, right panel). Evidence now exists for binding of RFX2 to the X-box of several testis genes including the *H1t* [Wolfe et al., 2004] and *Testisin* genes [Hooper et al., 2000] (data in this study) and the *lamin C2* [Nakajima and Abe, 1995] and *Sgy* [Kaneko and DePamphilis, 2000] genes (data in [Horvath et al., 2004]). Furthermore, *C-MOS* [Zhao et al., 1991; van der Hoorn et al., 1991], *RT7* [van der Hoorn and Tarnasky, 1992], *Protamine* [Johnson et al., 1988], and *Pdha2* [Iannello et al., 1993] are testis-specific genes with promoters containing an X-box that may require RFX for enhanced transcription. However, RFX2 binding to the X-box of the *C-MOS* and *Pdha2* genes was not detected [Horvath et al., 2004]. Perhaps as many as ten percent of testis genes contain Sox 17 and RFX transcription-binding sites [Nelander et al., 2005], and it is likely that many of these binding sites are functional in regulating transcription of the respective genes at various stages during spermatogenesis.

To determine whether a high level of nuclear RFX2 in primary spermatocytes corresponded to the increased RFX2-binding activity seen with nuclear proteins from these cells, we examined relative levels of RFX in enriched populations of germinal cells. Nuclear and cytoplasmic fractions were prepared and proteins were analyzed by Western blot analysis. RFX2 was most abundant in the nucleus but low

levels were present in the cytoplasm of every cell population except late spermatids. Perhaps one way to control testis RFX2 activity is to control relative nuclear and cytoplasmic levels of the factor. The level of RFX2 is maximal in primary spermatocytes where *H1t* gene transcription and H1t steady state mRNA levels are maximal and the level is lowest in late spermatids where transcription of most genes is repressed.

Previous experiments indicated that nuclear proteins derived from both primary spermatocytes and early spermatids can bind to the H1t X-box probe to form a low mobility EMSA complex. Therefore, we were not surprised to see RFX2 in early spermatids. Thus, the relative binding activities of RFX2 from enriched germinal cell populations detected in EMSAs correspond to the relative abundance of nuclear RFX2 determined by Western blotting. Nevertheless, it is paradoxical that the RFX2 level is high in early spermatids, since the steady state level of H1t mRNA is very low in early spermatids [vanWert et al., 1996]. Although there is some cross-contamination of cell types in the elutriated cell populations, this does not invalidate the observation of higher levels of RFX2 in spermatocytes (fraction 5) compared to early spermatids (fraction 3) (Fig. 6A,B) nor does it invalidate the observation of higher levels of RFX1 in early spermatids compared to spermatocytes (Fig. 6A,B). Fraction 5 contains 60–80% pachytene primary spermatocytes (9% early spermatids, 3% secondary spermatocytes or cells in meiotic division) while fraction 3 contains 60–75% early spermatids (6% late spermatids and 10% cytoplasts) [Meistrich et al., 1981]. Furthermore, H1t mRNA was detected in spermatocytes (fraction 5) but not in fraction 3, while transition protein 1 (TP1) mRNA was detected in early spermatids (fraction 3) and not in spermatocytes fraction 5 confirming the soundness of using these cell populations for this type of comparison. [Grimes et al., 1992a,b].

If a high level of RFX2 is important for maximal transcription of the *H1t* gene in spermatocytes, other mechanisms must be responsible for downregulating expression of the *H1t* gene in early spermatids. It has been suggested that the particular form of RFX that binds to the H1t X-box may play a role in downregulating transcription of the *H1t* gene expression in early spermatids [Horvath et al., 2004]. The RFX complex bound in early spermatids may differ from the one bound in primary spermatocytes. For example, RFX2 may bind the H1t X-box as a homodimer in primary spermatocytes to activate transcription, but it may bind as an RFX2-RFX1 heterodimer in early spermatids to repress transcription [Horvath et al., 2004]. Data presented in this study indicate that RFX1 levels are higher in early spermatids than in primary spermatocytes and that RFX2 levels are higher in spermatocytes than in early spermatids (Fig. 6A,B). Nevertheless, RFX2 is relatively abundant in early spermatids and RFX1 is relatively abundant in spermatocytes, so this simple explanation for regulating *H1t* gene expression is not completely satisfying.

Previous experiments conducted to test the binding of nuclear proteins from various tissues and cells lines to the H1t X-box1 revealed that testis nuclear proteins bind with the highest affinity [Grimes et al., 1992a,b; Wolfe et al., 1995]. When the binding proteins were cross-linked with UV light, the testis nuclear protein complex had an estimated molecular weight of approximately 180 kDa on SDS–PAGE [Wolfe et al., 1995]. Since the molecular weight of RFX2 is approximately 87 kDa, these data are consistent with the binding of an RFX2 homodimer to form the low mobility nuclear complex.

Because RFX family members, other than RFX2, are present and may play an important role in transcriptional regulation in the various germinal cell populations, we examined the relative abundance of RFX1, RFX3, RFX5, and RFXB. In these experiments, we measured the relative level of each RFX variant in each enriched cell population. RFX2 is more abundant in primary spermatocytes than in early spermatids, while RFX1 is slightly more abundant in early spermatids than in primary spermatocytes. RFX3 and RFXB levels in these two populations are almost equal. As expected, levels of all RFX family members are very low in late spermatids. It should be mentioned that we could not determine which particular RFX family member was most abundant in each cell population with this method. For example, it is not clear that RFX2 was more abundant than other RFX family members in spermatocytes.

To compare relative levels of RFX family members in different tissues, protein extracts from testis, brain, heart, kidney, liver, lung, spleen, stomach, and thymus were analyzed.

Levels of all RFX variants except RFXB were high in testis. RFX1 and RFX2 were most abundant in testis but RFX3 and RFX5 were also moderately abundant. Levels of RFX2 roughly reflected the steady-state levels of histone H1t mRNA in these organs (compare Figs. 1 and 7B) with the highest RFX2 and H1t mRNA levels in testis and the next highest levels in thymus. The correlation between abundance of RFX2 and H1t mRNA is consistent with an important role for RFX2 in testis-specific expression of the *H1t* gene.

In addition to RFX2 which binds the H1t X-box, other transcription factors bind to the TATA-box, CCAAT-box, AC-box, and GC-box. For example, Sp may bind the GC-box located between the two X-boxes [Wilkerson et al., 2002]. In another example, either HiNF-B which is also called NF-Y [van Wijnen et al., 1988; Mantovani, 1999] or HiNF-D which contains the homeodomain protein CDP-cut as its DNA-binding subunit [van den Ent et al., 1994; van Wijnen et al., 1996; Nepveu, 2001] may bind to the CCAAT-box. Interactions of RFX2 homodimers with these other transcription factors may be critical for upregulation of *H1t* gene transcription in primary spermatocytes. Therefore, in future studies it will be important to identify the factors that bind to the H1t promoter in different cell types and to identify interactions among these factors. In this study, we found that RFX2 is abundant in primary spermatocytes and early spermatids, but *H1t* gene transcription is upregulated in primary spermatocytes and downregulated in early spermatids. Therefore, in future studies it will be important to examine the possible binding of RFX family members such as RFX1 to the H1t X-box in early spermatids and in nongerminal cells. A change in binding from an RFX2 homodimer in primary spermatocytes to binding as a heterodimer with family members such as RFX1 in early spermatids may serve as a switch to downregulate *H1t* gene transcription. A similar mechanism may contribute to transcriptional repression of the *H1t* gene in other tissues [Clare et al., 1997; Wolfe et al., 1999; Singal et al., 2000; Wolfe and Grimes, 2003a,b]. Changes in the proteins that bind, changes in the binding affinities of the proteins, and changes in the interactions of these nuclear regulatory proteins with each other and with the H1t promoter in different cell types are currently being investigated to understand the regulation of transcription of this important testis-specific linker histone gene.

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