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Regulatory Factor X2 (RFX2) Binds to the H1t/TE1 Promoter Element and Activates Transcription of the Testis-Specific Histone *H1t* Gene

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Abstract Transcription of the mammalian testis-specific linker histone *H1t* gene occurs only in pachytene primary spermatocytes during spermatogenesis. Studies of the wild type (Wt) and mutant H1t promoters in transgenic mice show that transcription of the *H1t* gene is dependent upon the TE promoter element. We purified an 85 kDa protein from rat testis nuclear extracts using the TE1 subelement as an affinity chromatography probe and analysis revealed that the protein was RFX2. The TE1 element is essentially an X-box DNA consensus element and regulatory factor X (RFX) binds specifically to this element. Polyclonal antibodies directed against RFX2 supershift the low mobility testis nuclear protein complex formed in electrophoretic mobility shift assays (EMSA). RFX2 derived from primary spermatocytes, where the transcription factor is relatively abundant, binds with high affinity to the TE1 element. Coexpression of RFX2 together with an H1t promoter/reporter vector activates the H1t promoter in a cultured GC-2spd germinal cell line, but mutation of either the TE1 subelement or the TE2 subelements represses activity. These observations lead us to conclude that the TE1 and TE2 subelements of the testis-specific histone H1t promoter are targets of the transcription factor RFX2 and that this factor plays a key role in activating transcription of the *H1t* gene in primary spermatocytes. J. Cell. Biochem. 91: 375–383, 2004. Published 2003 Wiley-Liss, Inc.†

Key words: histone *H1t* gene; RFX2; tissue-specific transcription; gene regulation; DNA binding proteins

Several laboratories have identified promoter elements that bind transcriptional activators or repressors that regulate *H1t* gene transcription [Drabent and Doenecke, 1997; vanWert et al., 1998; Wolfe and Grimes, 1999; Wilkerson et al., 2002a]. The proximal promoter of most linker histone genes contain a TATA-box, a CCAAT-box, a GC-rich region, and an AC-box [Osley, 1991; Grimes et al., 2003]. Our laboratory identified a palindromic promoter element designated the TE element that is essential for transcription of the *H1t* gene in transgenic mice [vanWert et al., 1996, 1998]. This element contains three subelements designated TE1, GC-box, and TE2 (Fig. 1). The TE1 and TE2 subelements are imperfect inverted repeats [Wolfe and Grimes, 1993; vanWert et al., 1998; Wilkerson et al., 2003] and both appear to bind similar proteins. TE1 serves as a binding site for a transcriptional activator [Wilkerson et al., 2003] and the element binds to nuclear proteins from primary spermatocytes to give a low mobility complex in electrophoretic mobility shift assays (EMSA) [Grimes et al., 1992a,b; Wolfe et al., 1995; vanWert et al., 1998]. No other tissue or cell type produces the low mobility TE complex that is seen with this probe. TE1 and TE2 have similar sequences and EMSA competition assays show that the TE1 probe competes binding of spermatocyte nuclear proteins to the TE2 probe and vice versa [Wolfe et al., 1995], but there appear to be functional differences in

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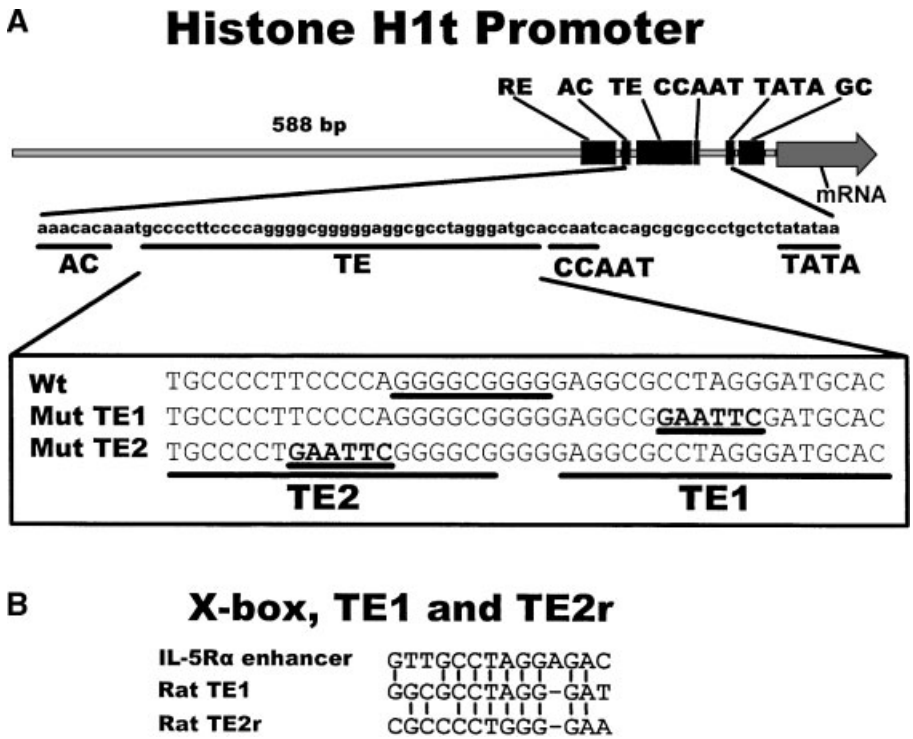

Fig. 1. Model of the testis histone H1t promoter and TE element. **A**: The H1t promoter region cloned into the pGL3 expression vector. The H1t promoter, described in earlier studies, contains consensus sequence elements that are important for regulating transcription of the gene. These include a GC-rich region downstream from the TATA box, a TATA box, CCAAT box, TE element, AC box, and an RE element. The core sequence extending from the TATA box to the AC box and an expanded view of the TE element are shown below the promoter diagram. TE1 and TE2 subelements are indicated and the GC box can be seen between the two elements in the wild type (Wt) sequence. Sequences of the TE1 mutant (Mut TE1) and the TE2 mutant (Mut TE2) used in this study are shown in bold type. **B**: The H1t/TE1 and H1t/TE2 subelements are essentially X-boxes. The sequences of the forward strand of the rat TE1 subelement and the complement strand of the TE2 subelement are similar. Both of these sequences are similar to the X-box consensus sequence as shown by the alignment with the X-box from the IL-5Rα enhancer. Vertical bars indicate identical bases.

these two sites [Wilkerson et al., 2003]. The wild type (Wt) H1t promoter provides spermatocyte-specific transcription in transgenic mice [vanWert et al., 1995, 1998; Bartell et al., 1996]. Mutagenesis of the TE element, accomplished by replacing the entire element with a heterologous DNA fragment, leads to inactivation of the promoter [vanWert et al., 1998].

The TE1 element is essentially an X-box consensus element that is known to bind RFX transcription factors in some genes [Gajiwala et al., 2000]. The RFX transcription factors were identified as proteins that bind to the X-box of histocompatibility complex class II (MHCII) promoters [Masternak and Reith, 2002]. RFX has a highly conserved DNA-binding domain that is characterized by a unique winged-helix structure composed of 76 amino acids [Gajiwala et al., 2000]. RFX family members are highly conserved in evolution and are present in a broad range of eukaryotic organisms, including yeast [Emery et al., 1996], fungi [Schmitt and Kuck, 2000], nematode [Swoboda et al., 2000], fruit fly [Durand et al., 2000], and vertebrates [Doyle et al., 1996]. In humans, several RFX family members have been identified (RFX1–RFX5, RFXANK, RFXAP, and RFX-B) [Emery et al., 1996; Morotomi-Yano et al., 2002]. RFX5 is a key regulator of the immune response regulating MHCII promoters [Moreno et al., 1997] and RFX1, 2, and 3 have been shown to bind the IL5Rα promoter [Iwama et al., 1999]. At least five *RFX* genes are expressed at high levels in testis [Reith et al., 1994] and RFX4 is testis-specific. Human RFX4 was isolated from breast cancer as a partial cDNA encoding a short RFX-type DNA-binding domain fused to the estrogen receptor [Dotzlaw et al., 1992]. RFX4 expression is normally testis-specific and it can physically interact with itself to form a homodimer or it can form heterodimers with RFX2 and RFX3 but not with RFX1 [Morotomi-Yano et al., 2002].

However, only a few target genes for RFX2, RFX3, and RFX4 have been identified [Iwama et al., 1999] and no testis gene targets have been identified.

Several pieces of evidence point to the possible biological functions of some RFX family members. In *S. cerevisiae*, Crt1, which is encoded by an RFX-related gene, functions as a transcriptional repressor in DNA damage and replication block checkpoint pathways [Huang et al., 1998]. With DNA damage, Crt1 is phosphorylated, leading to transcriptional derepression. This is important in relation to our work because the testis *H1t* gene is a target of RFX2 and the testis histones are important for DNA repair mechanisms [Celeste et al., 2002]. In *D. melanogaster*, dRFX, which is encoded by an RFX-related gene, regulates formation of cilia and is expressed in brain and spermatids [Durand et al., 2000; Vandaele et al., 2001]. In *C. elegans*, DAF-19 (encoded by an RFX-related gene) plays an important role in development of ciliated sensory neurons [Swoboda et al., 2000]. Microtubule-associated protein MAP1A is expressed in mature neurons and is necessary for maintenance of neuronal morphology and localization of some molecules in association with the microtubule-based cytoskeleton [Nakayama et al., 2003]. RFX1 down-regulates the MAP1A promoter activity in non-neuronal cells. Neither RFX1 nor RFX3 down-regulate promoter activity in neuronal cells.

In this article, we show that RFX2 binds to the TE1 promoter element of the testis-specific histone *H1t* gene. RFX2 is relatively abundant in primary spermatocytes. Coexpression of an RFX2 expression vector along with an H1t promoter/reporter expression vector leads to activation of the Wt H1t promoter in a germinal cell line.

MATERIALS AND METHODS

Materials

Radiochemicals were purchased from New England Nuclear, Boston, MA. Deoxynucleotides and Ampli-Taq for polymerase chain reaction (PCR) were purchased from Perkin-Elmer (Foster City, CA). Polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Synthetic oligonucleotides and 5′ biotin modified oligonucleotides were purchased from Genosys (The Woodlands, TX) now Sigma-Genosys in alliance with Fisher Scientific (http://www.fisheroligos.com).

Animals and Cell Culture

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

GC-2spd cells were grown at 32°C as described [Hofmann et al., 1994]. The GC-2spd germinal cell line was established by cotransfecting testis cells with the simian virus 40 large tumor antigen (LTAg) and a temperature-sensitive mutant of the mouse *p53* gene [Hofmann et al., 1994]. The p53 protein is inactive at 39°C, allowing the LTAg to retain its immortalizing ability. However, at the permissive temperature of 32°C used in experiment in this article, excess p53 neutralizes the proliferative effect of the viral protein.

Protein Purification and Identification

We employed a DNA binding method using a double-stranded TE1 element as an affinity probe. The forward strand of the DNA affinity probe was synthesized with biotin at the 5′ end and had the sequence biotin-5′-GCGGTGCCTAGGTGATGCA-3′. The unbiotinylated complement strand with the sequence 5′-TGCATCACCTAGGCACCGC-3′ was annealed to the forward strand to form the double-stranded DNA fragment. This biotinylated double-stranded TE1 probe was used in an affinity binding assay with 13 ml (260 mg protein) of rat testis nuclear extract. The binding conditions were similar to those used in our EMSA experiments and all steps including the binding and wash steps were conducted on ice [Dignam et al., 1983]. Streptavidin coated MagneSphere Paramagnetic Particles (Promega, Madison, WI) were added to bind to the complex of biotinylated probe with bound proteins. The beads were washed with binding buffer and tight binding proteins were eluted with binding buffer containing 1 M KCl. When eluted proteins were dialyzed and analyzed by SDS–PAGE, a major Coomassie stained electrophoretic band of approximately 85–90 kDa was observed. The band was excised, tryptic peptides were analyzed by microsequence analysis by Harvard Microchem. The protein was identified as RFX2 from the peptide analysis.

Nuclear Extracts and Western Blots

Nuclear extracts were prepared by the Dignam procedure [Dignam et al., 1983] from testis of 9 day old and adult rats, from enriched germinal cell populations prepared by centrifugal elutriation of single cell suspensions derived from adult rat testis cells [Grimes et al., 1990], and from cultured cells. Western blotting of proteins in the nuclear extracts was performed as described previously [Wilkerson et al., 2002a]. Chemiluminescence was generated using a SuperSignal West Dura Kit from Pierce (Pierce, Rockford, IL) and visualized using a BioRad VersaDoc imaging system (Bio-Rad Laboratories, Inc., Hercules, CA).

EMSAs

Binding of proteins in nuclear extracts to labeled DNA probes for EMSA was performed on ice as described previously [Grimes et al., 1992a,b] in a total volume of 20 μl with 400 ng of the non-specific competitor poly (dG-dC) added to each assay. To prepare TE1 probes for EMSA, the following upper (U) and lower (L) oligonucleotides were annealed: (U) 5′-GAGGCGCCTAGGG-3′; (L) 5′-GTGCATCCCTAGGC-3′. The product was filled with dATP, dGTP, dTTP, and [α-^{32}P] dCTP using the Klenow fragment of DNA polymerase as described previously [Grimes et al., 1992a,b]. When polyclonal antibodies were used in supershift assays, they were added after the initial nuclear protein binding period and incubated for an additional hour. Following binding, samples were electrophoresed through 4% polyacrylamide gels (60:1, acrylamide:bisacrylamide) using low ionic strength buffer containing 6 mM Tris-HCl, pH 7.9, 3.3 mM sodium acetate, and 1 mM EDTA with buffer recirculation. Gels were dried after electrophoresis, exposed to a Phospher Imaging Screen and examined using a Perkin Elmer Packard Cyclone Storage Phosphor System.

Transient Transfection Assays

Transient transfections were performed as described previously [Wolfe et al., 1999]. Cells were grown in 60 mm dishes and transfected using 0.5 or 2.5 μg of plasmid DNA and 20 μg of lipofectamine per dish. Transfections were performed in triplicate to correct for variations in cell number and transfection efficiency. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega Corp.) and protein concentrations in nuclear extracts were determined spectrophotometrically as described previously [Warburg and Christian, 1942]. The counts per picogram protein for each sample were calculated and plotted using Sigma Plot software (SPSS, Inc., Chicago, IL). The expression vector for RFX2 was obtained from ATCC (item number MGC-6105 with description mouse 3497910 pCMV-SPORT6). This vector contained the mouse cDNA for RFX2 cloned into the expression vector pCMV-SPORT6 and grown in *E. coli* strain XL-1 Blue (Stratagene, La Jolla, CA.). Luciferase expression vectors were the pGL3 series from Promega. The constructs pGL3Basic (no promoter-Basic), pGL3-1866 (Wt H1t promoter), pMutTE1 (Wt H1t with a mutation in TE1), and pMutTE2 (Wt H1t with a mutation in TE2) that were used in this study have been described [Wolfe et al., 1999; Wilkerson et al., 2002a,b]. All of these expression vectors were prepared from *E. coli* strain HB101.

RESULTS

TE1 Element of the Testis-Specific Histone *H1t* Gene Is an X-box That Binds RFX2

In previous studies, our laboratory identified a palindromic promoter element designated TE that is essential for transcription of the *H1t* gene [Grimes et al., 1992a,b; vanWert et al., 1998] (Fig. 1A). TE1 and TE2 subelements, located within the larger TE element, are imperfect inverted repeats that serve as binding sites for nuclear proteins from primary spermatocytes that produce a low mobility complex in EMSA [Grimes et al., 1992a,b; vanWert et al., 1996]. The proteins within this complex are most likely factors that lead to transcriptional activation of the *H1t* gene in primary spermatocytes. Therefore, we used a biotinylated, double-stranded TE1 DNA fragment as an affinity probe to purify nuclear proteins with high affinity for this DNA sequence as described in "Materials and Methods." SDS–PAGE analysis of the affinity-purified proteins revealed a Coomassie stained electrophoretic band of approximately 85–90 kDa. Analysis of tryptic peptides prepared from the excised band revealed that the protein was RFX2. We conclude that RFX2 is present in rat testis nuclear extracts and that it binds with high affinity to the TE1 probe.

The sequences of TE1 and TE2 (subelements of TE) are similar to each other. When these

DNA promoter elements are compared to an X-box consensus element from the IL-5Rα enhancer that is known to bind RFX transcription factors, the sequences are similar but not identical. Alignment of the forward strand of TE1, the complement strand of TE2, and the forward strand of the X-box from the IL-5Rα enhancer element is presented in Figure 1B with identical nucleotides marked with vertical bars.

Testis RFX2 Binds to the TE1 Probe in EMSA

One way to show that a specific protein can bind to a DNA fragment is by using EMSA. For the following experiments, we radiolabeled a double-stranded TE1 probe for use in binding nuclear proteins from rat testis. When we conducted EMSA using testis nuclear extracts, we saw formation of the typical low mobility TE complex [Grimes et al., 1992a,b; vanWert et al., 1996] (Fig. 2A). We also tested a panel of polyclonal antibodies formed against various RFX family members in a supershift assay in the same figure. Antibodies against transcription factor RFX2 produced a significant shift.

We conducted an additional supershift experiment using a higher RFX2 antibody concentration as shown in Figure 2B. Almost the entire low mobility TE complex was shifted to the lower mobility band. We did not see a supershift (or reduced binding to the TE1 probe) using higher antibody concentration or higher nuclear protein concentration with any antibodies against any other RFX family member.

Primary Spermatocytes Have a Relatively High Level of RFX2

Although we had seen evidence for the presence of RFX2 in testis nuclear extracts, we did not know if it was present in germinal cells. If this factor activates the *H1t* gene, it should be present at a significant level in pachytene primary spermatocytes where transcription of the gene occurs. Therefore, we conducted an assay to detect RFX2 in nuclear extracts derived from testis from sexually immature 9 day old rats, from testis from sexually mature adult

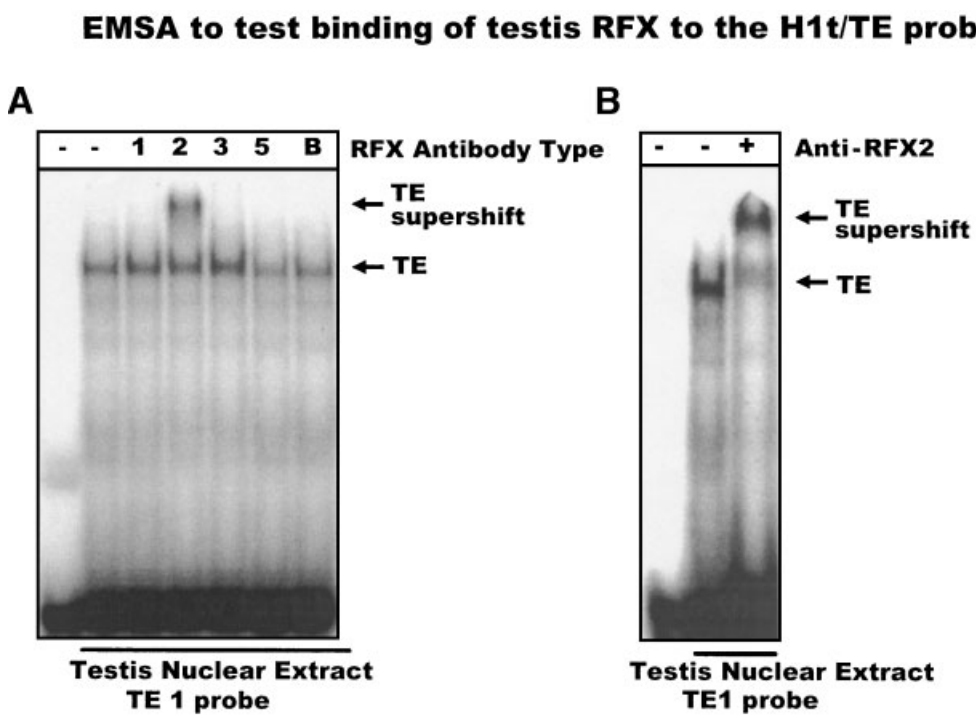

Fig. 2. RFX2 binds to the H1t/TE1 element. **A**: Nuclear protein binding to the H1t/TE1 probe was examined by EMSA. Probe alone is shown in the first lane. A low mobility protein complex forms when testis nuclear proteins are mixed with the TE1 probe as shown in the second lane. Binding of individual RFX family members was tested by adding polyclonal antibodies against the specific family members to the binding reaction as indicated at the top. Only antibodies against RFX2 produced a supershift as indicated by the arrow on the right. **B**: RFX2 binding to the TE1 element. The experiment was repeated with a higher concentration of antibodies against RFX2 but using the same TE1 probe and testis nuclear extract. The majority of the TE complex band was shifted to the lower mobility band with the addition of a higher concentration of antibodies.

rats, and from enriched germinal cell populations prepared by centrifugal elutriation of cells from adult rat testis. Nuclear proteins were separated on SDS–PAGE and blotted to NYTRAN. RFX2 was detected by Western blotting using the same antibodies used for the EMSA supershifts.

The relative level of RFX2 was very low in extracts from testes of 9 day old rats where the *H1t* gene is repressed but it was high in the population of cells enriched in pachytene primary spermatocytes (Fig. 3). The relative level in spermatocytes was higher than the level in total testis. The relative level of RFX2 in early spermatids was also high, but H1t mRNA is not present in early spermatids indicating possible repression of the gene in spermatids.

We also wanted to test the binding affinity of the RFX2 from the different germinal cell types. To do this we conducted an EMSA and found that RFX2 from pachytene primary spermatocytes binds as tightly or more tightly than RFX2 from total testis (Fig. 4). Although Western blots showed that RFX2 was present in early spermatids at a relatively high level (Fig. 3), it did not appear to bind with high affinity to the TE1 probe in EMSA supershifts (Fig. 4). Proteins from early spermatids produced a shifted TE complex, as we have seen before, but antibodies against RFX2 failed to produce a strong supershift of this TE complex as seen for total testis and pachytene primary spermatocytes.

RFX2 Activates the H1t Promoter in Transient Expression Assays

In previous studies, we showed that the Wt H1t promoter was active in the germinal cell line GC-2spd and that mutagenesis of the TE1 and TE2 promoter elements within the H1t promoter (Mut TE1 and Mut TE2) reduced promoter activity significantly [Wilkerson et al., 2002a,b]. In this study, we used the same expression vectors to test the effect of the transcription factor RFX2 on H1t promoter activity. In preliminary experiments, we found that a ratio of 2 μg of RFX2 plus 0.5 μg of Wt H1t led to activation of the H1t promoter (data not shown). Therefore, in the current study we used this same ratio of expression vectors (RFX2–Wt H1t, RFX2–Mut TE1, and RFX2–Mut TE2).

The data presented in Figure 5, reveal that RFX2 activates transcription of the H1t promoter about five fold. Mutagenesis of either TE1 or TE2 significantly reduced activity of the H1t promoter below the level of Wt H1t as we have seen before. Coexpression of RFX2 with Mut TE1 or with Mut TE2 increased activity of the mutants but not to the same high level seen with Wt H1t. We conclude that RFX2 greatly enhances activity of the H1t promoter and that a mutation of either the TE1 or TE2 binding sites for RFX2 reduces its ability to activate transcription.

DISCUSSION

The *H1t* gene is transcribed maximally during spermatogenesis in pachytene primary spermatocytes. The promoter of this gene has been cloned and transgenic mouse studies revealed that the promoter was active in pachytene primary spermatocytes and that the TE promoter element was essential for activity [vanWert et al., 1995, 1998]. The TE element

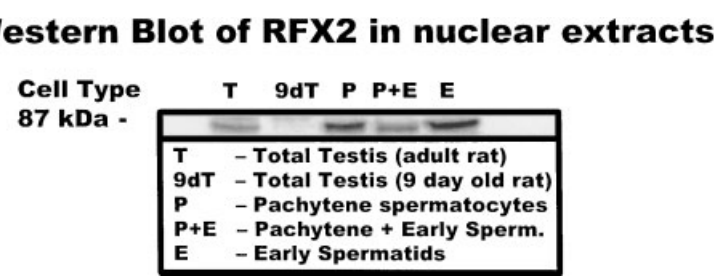

Fig. 3. RFX2 is present in different rat testis samples and germinal cell populations. Western blots were conducted to detect RFX2 in the nuclear extract from adult testis (T) used for the EMSAs shown in Figure 2. We also used nuclear extracts from testes of 9 day old rats (9dT) that lack spermatocytes and from cell populations prepared by centrifugal elutriation and enriched in pachytene primary spermatocytes (elutriator fraction 5-P), early spermatids (elutriator fraction 3-E), and a mixture of these two cell populations (elutriator faction 4-P + E). The polyclonal antibody against RFX2 used in EMSAs in Figure 2 was used in this Western blot assay as described in the ''Materials and Methods.'' The adult testis sample produces a strong signal with an electrophoretic band at about 87 kDa as expected for RFX2, but there is a higher level of RFX2 in pachytene primary spermatocytes and early spermatids.

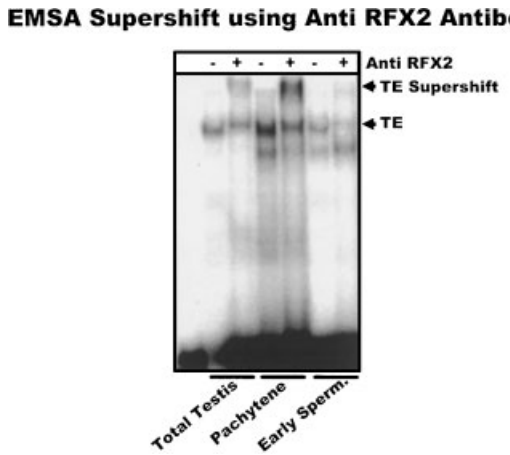

Fig. 4. EMSAs confirm the presence of RFX2 in pachytene primary spermatocytes. Nuclear extracts used in the Western blot in Figure 3 were used in an EMSA to confirm that RFX2 detected in primary spermatocytes could bind to the TE1 probe. Total testis was used as a control binding reaction. Antibodies against RFX2 were added to the binding reaction as indicated by the plus sign at the top. RFX2, present in pachytene primary spermatocytes, binds with high affinity and produces a stronger supershift signal than total testis nuclear extracts. This reflects the relative abundance seen in the Western blot in Figure 3. RFX2 was present in early spermatids as measured in the Western blot but the protein appeared to have a relatively weak binding activity in this EMSA.

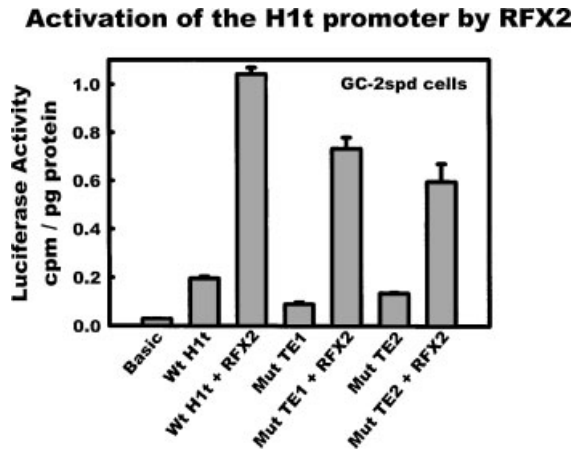

Fig. 5. RFX2 expression increases H1t promoter activity but TE1 and TE2 mutations repress activity. To examine the effect of RFX2 on H1t promoter activity we conducted transient expression assays. Wt H1t and mutant (Mut TE1 and Mut TE2) expression vectors used in previous studies were used to transfect the GC-2spd germinal cell line using 0.5 μg of each per assay. The promoterless pGL3 expression vector (Basic) served as a control and was also used for cloning the Wt and mutant H1t promoters. The RFX2 expression vector (2 μg) was cotransfected along with the H1t promoter vector constructs in some samples to determine the effect on H1t promoter activity. RFX2 significantly increased activity of the Wt H1t promoter. TE1 (Mut TE1) and TE2 (Mut TE2) promoter mutations repressed activity compared to Wt H1t and coexpression of RFX2 increased activity of the mutants but not to the same level high level as Wt plus RFX2.

is composed of two imperfect inverted repeats designated TE1, TE2 with a GC-box located between these two subelements. Previous studies revealed that the GC-box is essential for maximal H1t promoter activity in transient expression assays in the GC-2spd cell line [Wilkerson et al., 2002a]. Previous studies showed that mutagenesis of either TE1 or the GC-box reduced activity of the H1t promoter [Wilkerson et al., 2002a]. Although the TE1 subelement is required for maximal activity of the H1t promoter, we did not know the identity of the factors that bind to this element to activate transcription. In this study, we used an affinity purification method to identify testis proteins with high affinity for the TE1 sequence. The major testis binding protein was found to be the transcription factor RFX2. Furthermore, the sequence of the TE1 element is essentially an X-box, a canonical binding site for RFX family members. In this study, we show that RFX2 is present in rat testis and that testis RFX2 binds to the H1t/TE1 promoter element in EMSA to form the low mobility TE protein complex. When we used a high concentration of the RFX2 antibodies, most of the low mobility TE complex shifts to form a lower mobility complex. Western blots showed that the level of RFX2 is relatively high in primary spermatocytes where the *H1t* gene is transcribed but that the level of RFX2 is relatively low in testis from 9 day old rats that lack spermatocytes. RFX2 [Reith et al., 1994] has been reported to be present in testis, but this is the first report of a testis gene that serves as a target of RFX2 or any RFX family member.

The RFX transcription factors are involved in several diseases and in cancer. Aberrant expression of MHCII genes is associated with autoimmunity, tumor growth and failure to mount an immune response [Moreno et al., 1997]. RFX is involved in MHCII deficiency also known as bare lymphocyte syndrome [Moreno et al., 1997]. There is an absence of MHCII expression in this disease resulting from defects in RFX transcription factors. There are four different groups of patients (complementation A, B, C, and D) corresponding to four distinct MHCII regulatory genes. The genes affected in complementation groups B, C, and D encode the three subunits (RFXANK, RFX5, and RFXAP) of RFX, the DNA-binding protein that binds all

MHCII promoters [Masternak and Reith, 2002]. RFX-B protein levels are suppressed in human colorectal adenocarcinomas and RFX-B protein levels in macrophages were lower in colorectal cancerous tissue compared to non-cancerous tissue [Dimberg et al., 2002].

It is clear that the *RFX* genes encode important regulatory transcription factors involved in tissue-specific gene expression and DNA repair and that the expression of these genes is altered in some cancers and that they contribute to cancer in many cases. However, little is known about the transcriptional regulation of these essential *RFX* genes and the signal transduction pathways that are involved. There are large gaps in knowledge of the targets of RFX, the role of RFX in DNA repair, and the molecular mechanism used by RFX in activating testis-specific genes.

Many questions remain concerning the binding of RFX2 to the H1t promoter and the role of RFX2 in regulating *H1t* gene expression. RFX family members typically bind as homodimers or heterodimers with other RFX family members to the X-box [Morotomi-Yano et al., 2002]. RFX2 possibly binds as a homodimer to either TE1 or TE2, or to both subelements within the H1t promoter. The sequences of TE1 and TE2 are similar and both subelements probably serve as binding sites for similar proteins. Mutation of either TE1 or TE2 reduces H1t promoter activity significantly below the Wt activity. However, coexpression of RFX2 with either mutant produces activity higher than Wt alone but lower than Wt plus RFX2. It is possible that when only one of the subelements is mutated the other can function and may still bind RFX2 to activate transcription. We plan to test this idea by mutating both TE1 and TE2 in the same expression vector. Although other RFX family members are present in testis cells, we have not been able to detect binding activity of RFX family members other than RFX2 in EMSA supershift assays using the TE1 probe.

It is interesting that Sp1 and Sp3 can bind to the GC-box located between and partially overlapping TE1 and TE2 subelements [Wilkerson et al., 2002a,b]. The binding may be mutually exclusive so that RFX2 binds to the TE element in spermatocytes activating transcription but Sp1 (or Sp3) binds to the GC-box in some other cell types repressing transcription. However, it is also possible that RFX2 and Sp bind at the same time and interact to activate transcription. We have no data to support either of these speculations. It is also possible that RFX2 interacts with factors bound to the H1t/CCAAT element located just downstream from the TE1 element and with other factors in the promoter to form a transcription initiation complex.

In summary, we have shown that RFX2 binds to the H1t/TE1 promoter element with high affinity. The relative level of RFX2 is high in primary spermatocytes where the *H1t* gene is transcribed. In addition, expression of RFX2 leads to activation of the H1t promoter in transient expression assays and mutation of the TE1 and TE2 promoter elements greatly represses activity of the promoter.

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