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Transcriptional Activation of the Testis-Specific Histone *H1t* Gene by RFX2 May Require Both Proximal Promoter X-Box Elements

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Abstract The rat testis-specific linker histone *H1t* gene is transcribed in pachytene primary spermatocytes during spermatogenesis. Our previous work using transgenic mice demonstrated that spermatocyte-specific transcription of the *H1t* gene is dependent upon a proximal promoter element designated the TE element. TE is composed of two adjacent and inverted imperfect repeat sequences designated TE1 and TE2 and both of these palindromic elements are similar in sequence to the X-box, a DNA consensus sequence that binds regulatory factor X (RFX). RFX2 is the major enriched protein derived from rat testis nuclear extracts when using the TE1 element as an affinity chromatography probe. Co-expression of RFX2 together with an H1t promoted reporter vector in transient expression assays activates the H1t promoter in the GC-2spd germinal cell line, and mutation of either X-box significantly represses activity. However, RFX2 partially reactivates the promoter when either of the X-box elements is independently mutated. In order to totally block reactivation by RFX2, it is necessary to mutate both X-boxes simultaneously. Therefore, RFX2 appears to be able to bind to either X-box independently to partially activate the promoter of the testis-specific histone *H1t* gene, but simultaneous binding of RFX2 to both X-box elements may be required for maximal promoter activation. J. Cell. Biochem. 94: 317–326, 2005.
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Key words: histone *H1t* gene; RFX2; tissue-specific transcription; gene regulation; DNA binding proteins

Several consensus sequence elements within the H1t promoter bind transcriptional activators or repressors to regulate testis-specific *H1t* gene transcription [Drabent and Doenecke, 1997; vanWert et al., 1998; Wolfe and Grimes, 1999; Wilkerson et al., 2002a; Horvath et al., 2004; Wolfe et al., 2004]. Promoter elements found in most linker histone genes including the *H1t* gene include the TATA-box, the CCAAT-box, a GC-box, and an AC-box [Osley, 1991; Grimes et al., 2003]. These common elements may be needed for transcription of the *H1t* gene, but it seems unlikely that they are responsible for the remarkable tissue-specific transcription of the gene seen in pachytene primary spermatocytes during maturation of testis germinal cells.

A sequence element located between the AC-box and CCAAT-box that is unique to the H1t promoter, designated the TE element, is a candidate for binding a tissue-specific transcription factor [Grimes et al., 1992a,b; vanWert et al., 1998]. TE contains two imperfect inverted repeat sequences designated TE1 and TE2 and both appear to bind the same protein [Wolfe et al., 1995; vanWert et al., 1995, 1998; Wilkerson et al., 2002a; Grimes et al., 2003]. Protein binding to TE appears to be dependent upon the palindromic sequence CCTAGG, which is in the core or the TE1 subelement [Grimes et al., 1992a; Wolfe et al., 1995]. The element binds to nuclear proteins from primary spermatocytes to generate a low mobility complex in electrophoretic mobility shift assays

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(EMSA) [Grimes et al., 1992a; Wolfe et al., 1995] and nuclear proteins from other tissues or cell types fail to produce this low mobility complex [Grimes et al., 1992a; Wolfe et al., 1995]. Furthermore, EMSA competition assays show that the excess TE1 probe competes binding of spermatocyte nuclear proteins to the TE2 probe and vice versa [Wolfe et al., 1995].

It has been difficult to study the role of the TE1 and TE2 elements in testis-specific transcription in part because of an overlapping GC-box sequence located between the two elements (Figs. 1 and 5). Furthermore, mutation of the GC-box partially inactivates the promoter in transient expression assays in non-germinal cell types [Wilkerson et al., 2002a,b, 2003]. In addition, the GC-box of the H1t promoter can bind Sp1 and Sp3, cognate transcription factors that are present in testis [Wilkerson et al., 2002b]. Even though Sp1 and Sp3 levels are low in primary spermatocytes, co-expression of either factor together with an H1t promoted reporter leads to activation of the H1t promoter in transient expression assays in a number of cell lines [Wilkerson et al., 2002a,b].

Our laboratory demonstrated that the larger TE element is essential for pachytene spermatocyte specific transcription of the rat *H1t* gene in transgenic mice [vanWert et al., 1995, 1998]. The rat wild-type promoter provides spermatocyte-specific transcription in mice, and replacement of the element with a heterologous DNA fragment inactivates the promoter. However, this study failed to rule out a possible role for the GC-box in transcriptional activation in spermatocytes.

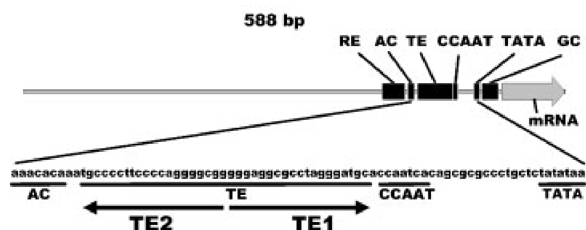

Fig. 1. A model of the testis-specific histone H1t promoter and TE element. The wild type H1t promoter region depicted here has been cloned into the pGL3 expression vector and designated Wt TE. The promoter contains consensus sequence elements that are important for regulating transcription of the *H1t* gene [Grimes et al., 2003; Grimes, 2004]. These include a GC-rich region [Clare et al., 1997] downstream from the TATA box, a TATA box, a CCAAT box, a TE element, an AC box, and an RE element. The TE element is shown in the sequence extending from the TATA box to the AC box. The divergent palindromic TE1 and TE2 subelements are indicated by arrows.

We reported that TE1 is similar in sequence to the X-box consensus element that serves as the binding site for regulatory factor X (RFX) transcription factor family members [Wilkerson et al., 2003]. We also discovered that several RFX family members are present in rat primary spermatocytes and that RFX2 is the major protein that binds the TE1 sequence [Wolfe et al., 2004]. We found that RFX2 is most abundant in pachytene primary spermatocytes and early spermatids during maturation of germinal cells with relatively low levels found in other germinal and non-germinal testis cell types. Furthermore, RFX2 activates the H1t promoter in transient expression assays in the GC-2spd germinal cell line [Wolfe et al., 2004]. Subsequent to these reports, another laboratory confirmed the binding of RFX2 to the H1t promoter and its presence in primary spermatocytes and early spermatids [Horvath et al., 2004].

RFX family members bind to the X-box sequences of many genes [Gajiwala et al., 2000] and binding to the X-box of histocompatibility complex class II (MHCII) promoters has been studied in detail [Masternak and Reith, 2002]. More importantly, RFX genes or RFX-related genes are found in most eukaryotic organisms including yeast [Emery et al., 1996], fungi [Schmitt and Kuck, 2000], nematodes [Swoboda et al., 2000], fruit flies [Durand et al., 2000], and vertebrates [Doyle et al., 1996]. Human RFX5 is a key regulator of the activities of immune response regulating MHCII promoters [Moreno et al., 1997] and RFX 1,2, and 3, regulate activity of the IL5Rα promoter [Iwama et al., 1999], but several RFX genes are expressed at very high levels in testis [Reith et al., 1994]. In spite of this progress, only a few target genes for RFX2, RFX3, and RFX4 have been identified thus far [Iwama et al., 1999] and no testis target genes for any of the RFX family members had been reported before our report that RFX2 binds to and activates the testis-specific H1t promoter [Wolfe et al., 2004].

In this study we confirm that TE1 and TE2 are X-box elements within the proximal promoter of the testis-specific histone *H1t* gene. RFX2 is the major rat testis nuclear protein that binds to a double-stranded TE1 DNA element used as an affinity chromatography probe. When either X-box is mutated individually,

activity of the promoter is reduced significantly in transient expression assays, but RFX2 partially reactivates these mutant promoters. A double mutant, that simultaneously alters both X-boxes, is required to totally inhibit H1t promoter activity and eliminate reactivation by RFX2.

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. 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 germinal cells were grown at 32°C as described [Hofmann et al., 1994]. The GC-2spd germinal cell line was established by co-transfecting 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 experiments in this study, excess p53 neutralizes the proliferative effect of the viral protein.

Nuclear Extracts and Protein Purification

Nuclear extracts were prepared from testis of adult rats [Dignam et al., 1983]. A DNA binding method with a double-stranded TE1 element as an affinity probe was used to enrich the DNA binding proteins. To prepare the probe, the forward strand of a DNA affinity probe was synthesized and biotin labeled at the 5′ end [Genosys (The Woodlands, TX) now Sigma-Genosys in alliance with Fisher Scientific (http://www.fisheroligos.com)] with the sequence Biotin-5′-GCGGTGCCTAGGT-GATGCA-3′. The complementary strand with the sequence 5′-TGCATCACCTAGGCACCGC-3′ was annealed to the forward strand to form the double-stranded DNA fragment. This probe was used in a binding assay with 13 ml of rat testis nuclear extract representing 260 mg of protein with binding conditions similar to those used in our EMSA [Wolfe et al., 2004]. Streptavidin coated MagneSphere Paramagnetic Particles (Promega, Madison, WI) were added to the nuclear extracts, so that they could bind to the complex of biotinylated probe with bound proteins. The beads were washed with fresh binding buffer to remove loosely bound proteins and the tight binding proteins were eluted with binding buffer containing 1M KCl. When eluted proteins were dialyzed and analyzed by SDS–PAGE, a major enriched Coomassie blue stained electrophoretic band of approximately 90 kDa was observed (Fig. 3). This gel band was excised, proteins were eluted, and tryptic peptides were analyzed by microsequence analysis by Harvard Microchem. The major protein component in the gel slice was RFX2.

Transient Transfection Assays

Transient transfection assays were performed as described previously [Wolfe et al., 1999]. Cells grown in 60-mm dishes were transfected using 2 μg of plasmid DNA and 20 μg of Lipofectamine per dish. Triplicate transfection assays were performed to correct for variations in cell number and transfection efficiency. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI) and nuclear extract protein concentrations were determined spectrophotometrically [Warburg and Christian, 1942]. Counts per pg protein were calculated and plotted using Sigma Plot software (SPSS Inc., Chicago, IL). The RFX2 expression vector was ordered from ATCC (item number MGC-6105 with description mouse 3497910 pCMV-SPORT6). The vector contained mouse cDNA for RFX2 cloned into vector pCMV-SPORT6. Luciferase expression vectors were the pGL3 series from Promega, Madison, WI. The constructs pGL3Basic (no promoter—basic), pGL3-1866 (wild type H1t promoter—Wt H1t), MutTE1 (Wt H1t with a mutation in TE1) and MutTE2 (Wt H1t with a mutation in TE2), Mut Pvu + 12 (Wt H1t with a large TE mutation) that were used in this study (Fig. 5) have been described [Wilkerson et al., 2003; Wolfe et al., 2004].

A TE double mutant designated TEDM was prepared as follows: a PCR amplification reaction was conducted using the pGL3-MutTE2 plasmid as a template. The PROMEGA pGL3 forward sequencing primer (RVprimer3) 5′-CT-AGCAAAATAGGCTGTCC-3′ and the reverse primer 5′-GGGCGCGCTGTGATTGGTGCATC **GAATTC**CGCCTCCC-3′ beginning at nucleotide −37 from the H1t mRNA start site were used as PCR primers. The bold and underlined region in the reverse primer represents the TE1 mutation and the Mut TE2 template already had the TE2 mutation. The PCR product with the TE1 and TE2 double mutation was cut with Pst I and BssH II and the Pst I–BssH II fragment was purified and ligated into the wild type (WT) pGL3-1866 vector that had been cut with Pst I and BssH II to excise the normal H1t promoter. Expression vectors used in transfection assays were prepared from *E. coli* strain HB101.

RESULTS

TE Element of the Testis-Specific Histone H1t Promoter Is Composed of Two Adjacent and Inverted X-Box Consensus DNA Sequences

The TE promoter element of the testis-specific histone *H1t* gene is essential for transcription of the gene (Fig. 1). The TE element is composed of two subelements designated TE1 and TE2 that are imperfect inverted repeats arranged tail to tail, as shown by the arrows in the figure. TE1 and TE2 serve as binding sites specifically for nuclear proteins from primary spermatocytes that produce a low mobility complex in EMSA [Grimes et al., 1992a,b; Wolfe et al., 1995]. The proteins that bind to either element to form the low mobility complex are not found in nuclear proteins from other cell types or tissues and the proteins are composed of factors that may lead to transcriptional activation of the *H1t* gene in primary spermatocytes in vivo [vanWert et al., 1998].

The DNA sequence of TE1 is similar to the complementary sequence of TE2 as shown in Figure 2. When the sequences of these diverging DNA promoter elements are compared to the X-box consensus element and to the X-box from the IL-5Rα enhancer, all of the sequences can be seen to be similar (Fig. 2). It is interesting that there is also an X-box within the testis-specific Testisin promoter as shown in the same figure [Hooper et al., 2000].

Both TE 1 and TE2 are X-boxes

Consensus X-box	GTNRCCNNRGYAAC
IL-5Rα enhancer	GTTGCCTAGGAGAC
Rat TE1	GGCGCCTAGGGATG
Rat TE2r	CGCCCCTGGGGAAG
Testisin	CGCCCCTAGGGGCT

Fig. 2. The testis histone H1t TE1 and TE2 promoter elements are X-box elements. The sequences of the forward strand of the rat TE1 subelement and the complement strand of the TE2 subelement are similar. Both sequences are similar to the X-box consensus sequence and to the X-box from the IL-5Rα enhancer. An X-box sequence present in the testis-specific Testisin promoter [Hooper et al., 2000] is also shown.

Regulatory Factor 2 (RFX2) Binds to the Testis-Specific Histone H1t X-Box Sequence

We initiated a study to identify proteins that can bind to the histone H1t X-box element. To identify testis proteins that bind to the X-box, we used a biotinylated, double-stranded TE1 DNA fragment as an affinity probe to enrich nuclear proteins that bind tightly. Testis proteins that bind with highest affinity to the biotinylated probe were eluted with buffer containing 1M KCl and analyzed by SDS–

Purification of TE1 binding proteins

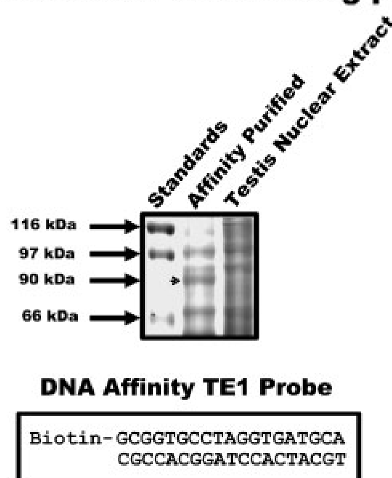

Fig. 3. RFX2 binds to the histone H1t X-box. The **center lane** in the top part of this figure presents stained nuclear protein bands separated by SDS–PAGE that were eluted from the double-stranded histone H1t X-box affinity probe as described in the Materials and Methods. Note that the major enriched peptide marked with an arrow has an apparent molecular weight of 90 kDa. Protein molecular weight standards are in the **left lane** and the original nuclear extract is in the **right lane**. The sequence of the biotin-labeled double-stranded histone H1t X-box probe (TE1 probe) used in the affinity purification is shown in the lower part of the figure.

PAGE as shown in Figure 3. Several stained bands were enriched in this one affinity purification step compared to the original nuclear protein mixture, but one Coomassie stained band with an apparent molecular weight of approximately 90 kDa was enriched to the greatest extent (Fig. 3, center lane). Analysis of tryptic peptides prepared from the protein extracted from this excised gel band revealed that the major protein was RFX2. The DNA binding domain of RFX transcription factor family members bind to X-box DNA sequences, which is consistent with our finding that TE1 and TE2 sequences are X-boxes. We conclude from this study that RFX2 is present in rat testis nuclear extracts and is the DNA binding protein that binds with high affinity to the TE1 probe.

Mutagenesis of Both Histone H1t X-Box Sequences Is Required to Block Promoter Activity

One way to examine the role of a specific promoter sequence element in regulating expression of a gene is to mutate the sequence and examine the effects of the mutation on promoter activity in transient expression assays or in transgenic mice. In our previous studies of the activity of the rat H1t promoter in transgenic mice [vanWert et al., 1998], we replaced the entire TE element with a stuffer DNA fragment as shown in Figure 4. This TE mutation blocked transcription of the rat histone H1t transgene in the testis of transgenic mice [vanWert et al., 1998] and significantly reduced activity in transient expression assays.

However, a GC-rich sequence containing a perfect GC-box is located between TE1 and TE2 and partially overlaps both of the X-box sequences as shown in Figure 5. Therefore, there is a possibility that the GC-box was responsible for H1t promoter activity in primary spermatocytes and that the mutation of the GC-box was responsible for blocking promoter activity in the transgenic animals. In addition, the length of the stuffer DNA between the AC-box and the CCAAT-box used in the mutant transgene was a little shorter than the original sequence. Therefore, we prepared individual mutations within each X-box, designated Mut TE2 and Mut TE1, and we also prepared a larger TE mutant, designated Pvu + 12, which lengthened the stuffer DNA that had been used in the original mutant transgene (compare sequences in Figs. 4 and 5). Finally, we prepared a double mutant, designated TEDM that simultaneously mutated both X-boxes while leaving the GC-box intact (Fig. 5).

We used these WT and mutant H1t promoter expression vectors in transient expression assays together with an RFX2 expression vector to test the effect of the transcription factor RFX2 on H1t promoter activity. A ratio of 2 μg of RFX2 expression vector to 0.5 μg of WT H1t expression vector led to activation of the H1t promoter in transient expression titration assays in the GC-2spd germinal cell line. Therefore, we used

TE mutation blocks transcription

WILD TYPE H1t PROMOTER

EcoNI *AvrII*

AAACACAAATGCCCCTT/ CCCCAGGGGCGGGGGAGGCGC/CTAGGGATGCACCAATCACA

H1/AC BOX TE ELEMENT CCAAT BOX

MUTANT H1t PROMOTER

AAACACAAATGCCCCTT/ CCTTTAAGCTTTCGTTCT/ ACCAATCACAGCGCGCCCT

H1/AC BOX STUFFER DNA CCAAT BOX

Fig. 4. A TE mutation that blocks transcription of the rat gene in vivo. This figure compares the wild type H1t promoter sequence in the **upper panel** to an H1t promoter sequence with a mutant TE element in the **lower panel**. This TE mutation eliminates both X-box elements as well as the GC-box and effectively blocked transcription of the rat *H1t* gene in vivo in a transgenic mouse study [vanWert et al., 1998]. Notice that the DNA that replaces the wild type TE element is shorter than the wild type sequence.

TE Mutations

Wild Type and Mutant H1t promoter sequences

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Wt TE    TGCCCCTTCCCCAGGGGCGGGGGAGGCGCCTAGGGATGCAC
Mut TE2  TGCCCCTGAATTCGGGGCGGGGGAGGCGCCTAGGGATGCAC
Mut TE1  TGCCCCTTCCCCAGGGGCGGGGGAGGCGGAATTCGATGCAC
Pvu+12   TGCCCCTTCCTTTAAGCTTTCGTTCTGTTCTAGG-ATGCAC
TEDM     TGCCCCTGAATTCGGGGCGGGGGAGGCGGAATTCGATGCAC
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TE2 GC-box TE1

Fig. 5. TE Mutants used in transient expression assays. This figure presents the wild type H1t promoter sequence and four sequences with mutations in either one or both X-box elements. The GAATTC sequence was used to replace wild type sequence in the core of the X-box elements in Mut TE2, Mut TE1, and in the double-mutant TEDM. Note that the GC-box is intact in TEDM. A mutant, designated Pvu + 12, represents a replacement of both X-boxes as well as the GC-box, and the length of this mutant is closer to the length of the wild type sequence than the mutant used in the transgenic mouse experiment (see Fig. 4 for comparison).

the same ratio of RFX2 with each of the expression vectors in the current experiment.

All four mutants significantly reduced activity of the H1t promoter compared to the WT sequence, but the double mutant (TEDM) reduced the activity almost to the background level (Fig. 6). The large TE mutant (Pvu + 12) was almost as effective in blocking promoter activity in this assay (Fig. 6).

When an RFX2 expression vector was co-transfected with the WT and mutant H1t expression vectors, all of the mutants except TEDM were reactivated to some degree. Reactivation of the single X-box mutants (Mut TE1 and Mut TE2) may occur because the H1t promoter uses both X-box elements to activate transcription of the gene. When only one of the X-box elements is mutated, RFX2 can bind to the other element and to activate transcription. In the case of Puv + 12, the X-box core sequences in TE1 and TE2 are not completely mutated and thus may allow RFX2 to bind weakly to partially activate the promoter. Nevertheless, the reactivation in Puv + 12 is weak compared to reactivation in Mut TE1 or Mut TE2. The lack of reactivation of the TE double mutant shows that activation requires both X-boxes; the GC-box in this mutant is still intact. If the GC-box was involved in reactivation in this assay, we should have seen partial reactivation of it with this TE double mutant.

A model depicting the potential independent binding of RFX2 to the two H1t promoter X-boxes in pachytene primary spermatocytes is shown in Figure 7. NFY, which can bind to the CCATT-box sequence, is also shown. It is not known whether a co-activator participates with RFX2 in activation of the H1t promoter in primary spermatocytes, so the potential co-activator is shown with a question mark. The lower panel of this figure shows a hypothetical model of nuclear protein binding in cells where the *H1t* gene is not expressed such as spermatogonia.

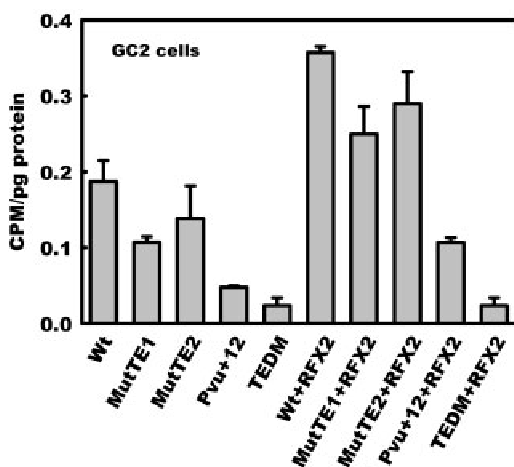

Fig. 6. Mutation of both X-boxes totally represses H1t promoter activity. To examine the effect of RFX2 on H1t promoter activity we conducted transient expression assays. Wild type (Wt H1t) and mutant (Mut TE1, Mut TE2, Pvu + 12, and TEDM) expression vectors were used to transfect the GC-2spd germinal 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 wild type and mutant H1t promoters. The RFX2 expression vector (2 µg) was co-transfected along with the H1t promoter vector constructs in some assay samples to determine the effect of RFX2 on H1t promoter activity. RFX2 significantly increased activity of the wild type H1t promoter (WtH1t). TE1 (Mut TE1), TE2 (Mut TE2), and Pvu + 12 mutations repressed activity compared to wild type H1t but expression of RFX2 partially reactivated these promoters. Only the TEDM double X-box mutant prevented reactivation by RFX2.

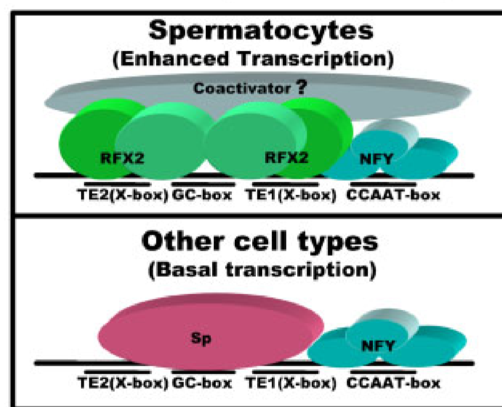

Fig. 7. RFX2 binding to the H1t promoter in spermatocytes and other cell types. The **upper panel** shows a hypothetical model of the active H1t promoter in primary spermatocytes with RFX2 bound to the two X-box elements and with NFY bound to the downstream CCAAT-box. Proteins that bind the upstream AC box and downstream TATA box are not shown. It is not known whether a co-activator, such as CIITA, associates with RFX2 to form a transcription complex as in other genes that contain a functional X-box, but CIITA can interact with chromatin remodeling factors and components of TFIID to form a transcription initiation complex [Masternak and Reith, 2002]. The **lower panel** shows a model of an inactive H1t promoter in a non-expressing germinal cell type such as in spermatogonia. In this basal state in non-expressing cells, Sp may bind to the GC-box of the promoter between the X-boxes leading to repressed transcription [Wilkerson et al., 2002b]. In non-germinal cell types hypermethylation may also play an important role in silencing the *H1t* gene [Singal et al., 2000]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

The testis-specific linker histone *H1t* gene is transcribed only in germinal cells during spermatogenesis and only in pachytene primary spermatocytes [Grimes et al., 1990]. Transgenic mouse studies demonstrated that the rat WT H1t promoter was fully active in pachytene primary spermatocytes and expression of the rat gene was testis specific [vanWert et al., 1995]. An experiment designed to test the importance of the TE promoter element within the H1t promoter by replacement of the TE element with stuffer DNA showed that the TE element is essential for promoter activity [vanWert et al., 1998]. This unique H1 promoter element is composed of two imperfect inverted repeat sequences designated TE1 and TE2 and comparison of these elements with an X-box element reveals that both are X-box elements (Fig. 2).

A study of the role of these two X-box elements in the regulation of transcription of the histone *H1t* gene has been difficult for two major reasons. One difficultly is that there is currently no germinal cell line that faithfully expresses the genes found in primary spermatocytes that can be used for transient transfection assays to examine transcriptional activity of the H1t promoter. Furthermore, the lack of a good cell line has hindered efforts to purify the nuclear proteins that bind to this element. However, some progress has been made to produce such cell lines and we have used one of these cell lines in the current study [Hofmann et al., 1994]. A second difficulty in the study of the X-box elements is that both elements overlap a GC-rich sequence that forms a perfect GC-box that is located between the two X-boxes (Fig. 5).

In this study, we address both of these problems. Concerning the first major problem, we were able to begin a study to identity nuclear proteins that bind to the histone H1t X-box by using a biotinylated double-stranded DNA probe with a sequence identical to the H1t X-box in an affinity purification method to partially enrich the testis nuclear proteins that bind tightly to the sequence. The major testis nuclear binding protein enriched by this method generates a stained band on SDS–PAGE of approximately 90 kDa. Sequence analysis of tryptic peptides extracted from this stained band indicated that the major protein was the transcription factor RFX2 (Fig. 3). RFX transcription factors are the cognate DNA binding proteins for the X-box sequence [Gajiwala et al., 2000] and the discovery that RFX2 binds to TE1 was consistent with our discovery that the sequences of TE1 and TE2 are X-box sequences.

RFX2 has been reported to be present in testis [Reith et al., 1994], but this is the first report of a testis gene that serves as a target of RFX2 or any other RFX family member. We have not detected binding of other RFX family members to the H1t promoter. RFX family members can bind as homodimers or heterodimers with other RFX family members to the X-box [Morotomi-Yano et al., 2002]. Therefore, RFX2 may bind as a homodimer to each of the divergent H1t X-boxes within the H1t promoter as shown in the model in Figure 7 or it may bind as a homodimer to only one of the X-boxes. 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 to form a transcription initiation complex as shown in Figure 7. From sequence alignment studies conducted thus far, it seems likely that many testis-specific genes serve as the targets of RFX2. RFX2 and possibly other RFX family members play a very important role in transcriptional activation of many genes in the primary spermatocytes and early spermatids.

Concerning the known roles of RFX transcription factors, they are involved in several diseases and in cancer. Altered expression of MHC class II genes are involved with autoimmunity, tumor growth, and an inadequate immune response [Moreno et al., 1997]. Altered RFX binding is involved in MHCII deficiency also known as bare lymphocyte syndrome [Moreno et al., 1997]. Absence of MHCII expression results from defects in RFX transcription factors. Suppressed levels of RFX-B protein are found in human colorectal adenocarcinomas and lower RFX-B protein levels in macrophages are found in colorectal cancerous tissue compared to non-cancerous tissue [Dimberg et al., 2002]. RFX4 was identified in human breast cancer as a partial cDNA encoding a short RFX-type DNA-binding domain fused to the estrogen receptor [Dotzlaw et al., 1992].

It is clear that the RFX genes encode important regulatory transcription factors involved in tissue-specific gene expression and DNA repair [Huang et al., 1998] and the expression of these genes is altered in some cancers and they

contribute to cancer in many cases. However, very little is known about the transcriptional regulation of the genes encoding these essential RFX factors and the signal transduction pathways that are involved. Significant gaps exist in our current knowledge of the targets of RFX, the possible roles of RFX in DNA repair, and the molecular mechanisms used by RFX in activating testis-specific genes.

In this study, we also address the second major problem in the study of the H1t X-boxes. This problem relates to the confusion in dissecting the relative regulatory roles played by the X-boxes and the GC-box in regulating testis-specific transcription of the *H1t* gene. The confusion is caused by the partial overlap of the X-boxes with the GC-box (Fig. 5). We addressed this problem by testing independent mutations of the two X-boxes as well as a double mutant that simultaneously abolished both X-boxes but left the GC-box intact.

Although this study does not rule out a possible role for the GC-box in the testis-specific regulation of transcription of the *H1t* gene, it does show that RFX2 most likely binds to both X-boxes in primary spermatocytes to activate transcription of the gene. If either X-box is mutated individually, RFX2 is still able to partially reactivate the promoter when co-expressed with the H1t promoter–reporter vector. This is probably due to the binding of RFX2 to the other unmodified X-box and this binding to one of the X-boxes appears to be sufficient to partially reactivate the promoter. However, if both X-boxes are mutated, RFX2 is not able to reactivate the promoter. In this case, RFX2 cannot bind to the promoter and, thus, binding appears to be absolutely essential for activating the H1t promoter. In any case, RFX2 binding to the H1t promoter appears to be essential for maximal transcriptional activity.

It should be mentioned that we have shown previously that Sp transcription factors can bind to the H1t/GC-box [Wilkerson et al., 2002a,b], which leaves open the possibility that the GC-box may have an important role in silencing transcription of the *H1t* gene in cells where the gene is not expressed. Sp1 and Sp3 levels are low in primary spermatocytes, while the level of RFX2 is relatively high. These relative abundance levels may be reversed in other cell types. This is the case, for example, in spermatogonia where the level of Sp transcription factors appears to be elevated but the level of RFX2 is low. Therefore, Sp transcription factors could compete with RFX2 for binding in this germinal cell type where the histone *H1t* gene is silenced, as depicted in the model of the testis histone H1t promoter in the lower part of Figure 7. In this case Sp may compete with RFX2 to repress H1t promoter activity. The transcription factor Sp3 has been reported to be able to serve as a transcriptional repressor in some promoters and it could serve this role with the H1t promoter [Hagen et al., 1994]. During the maturation of spermatogonia into spermatocytes during spermatogenesis, the levels of Sp1 and Sp3 drop and the level of RFX2 increases and *H1t* gene transcription is activated.

This simplified model of *H1t* gene repression in spermatogonia may be important in some non-expressing cell types, but it is probably not involved in all cell types. We have shown, for example, that the seven CpG dinucleotides within the H1t promoter are unmethylated in testis primary spermatocytes but all seven are methylated in rat liver [Singal et al., 2000]. DNA hypermethylation coupled with histone deacetylation has been correlated to gene silencing for many genes [Razin, 1998; Wade et al., 1999], and this may be an important regulatory mechanism for the *H1t* gene in some cell types. Therefore, a number of complex mechanisms may be important in repressing transcription of the *H1t* gene depending upon the cell type.

In summary, in this study we have shown that the DNA promoter elements TE1 and TE2 are X-boxes and that testis 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 either X-box greatly represses activity of the promoter. However, a double mutation of both X-boxes is required to prevent reactivation by RFX2. This leads us to conclude that RFX2 can bind to both X-boxes. Furthermore, binding to both X-boxes may be required to fully activate transcription of the *H1t* gene.

REFERENCES

Clare SE, Fantz DA, Kistler WS, Kistler MK. 1997. The testis-specific histone *H1t* gene is strongly repressed by a G/C-rich region just downstream of the TATA box. J Biol Chem 272:33028–33036.

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.

Dimberg J, Hugander A, Hall-Karlsson BM, Sirsjo A. 2002. RFX-B, a MHC class II transcription factor, suppressed in human colorectal adenocarcinomas. Int J Mol Med 9:213–216.

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

Doyle J, Hoffman S, Ucla C, Reith W, Mach B, Stubbs L. 1996. Locations of human and mouse genes encoding the RFX1 and RFX2 transcription factor proteins. Genomics 35:227–230.

Drabent B, Doenecke D. 1997. Testis-specific expression of the mouse histone gene *H1t* is regulated by several promoter elements. Biol Reprod 57:1375–1383.

Durand B, Vandaele C, Spencer D, Pantalacci S, Couble P. 2000. Cloning and characterization of dRFX, the *Drosophila* member of the RFX family of transcription factors. Gene 246:285–293.

Emery P, Durand B, Mach B, Reith W. 1996. RFX proteins, a novel family of DNA binding proteins conserved in the eukaryotic kingdom. Nucleic Acids Res 24:803–807.

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

Grimes SR. 2004. Testis-specific transcriptional control. Gene (in press).

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.

Hagen G, Muller S, Beato M, Suske G. 1994. Sp1-mediated transcriptional activation is repressed by Sp3. Embo J 13:3843–3851.

Hofmann MC, Hess RA, Goldberg E, Millan JL. 1994. Immortalized germ cells undergo meiosis in vitro. Proc Natl Acad Sci USA 91:5533–5537.

Hooper JD, Bowen N, Marshall H, Cullen LM, Sood R, Daniels R, Stuttgen MA, Normyle JF, Higgs DR, Kastner DL, Ogbourne SM, Pera MF, Jazwinska EC, Antalis TM. 2000. Localization, expression and genomic structure of the gene encoding the human serine protease testisin. Biochim Biophys Acta 1492:63–71.

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 (in press).

Huang M, Zhou Z, Elledge SJ. 1998. The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell 94:595–605.

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.

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

Moreno CS, Rogers EM, Brown JA, Boss JM. 1997. Regulatory factor X, a bare lymphocyte syndrome transcription factor, is a multimeric phosphoprotein complex. J Immunol 158:5841–5848.

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

Osley MA. 1991. The regulation of histone synthesis in the cell cycle. Ann Rev Biochem 60:827–861.

Razin A. 1998. CpG methylation, chromatin structure and gene silencing—a three-way connection. Embo J 17:4905–4908.

Reith W, Ucla C, Barras E, Gaud A, Durand B, Herrero-Sanchez C, Kobr M, Mach B. 1994. RFX1, a transactivator of hepatitis B virus enhancer I, belongs to a novel family of homodimeric and heterodimeric DNA-binding proteins. Mol Cell Biol 14:1230–1244.

Schmitt EK, Kuck U. 2000. The fungal CPCR1 protein, which binds specifically to beta-lactam biosynthesis genes, is related to human regulatory factor X transcription factors. J Biol Chem 275:9348–9357.

Singal R, vanWert J, Bashambu M, Wolfe SA, Wilkerson DC, Grimes SR. 2000. Testis-specific histone *H1t* gene is hypermethylated in nongerminal cells in the mouse. Biol Reprod 63:1237–1244.

Swoboda P, Adler HT, Thomas JH. 2000. The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in *C. elegans*. Mol Cell 5:411–421.

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 primary spermatocytes. Biol Reprod:704–710.

Wade PA, Gegonne A, Jones PL, Ballestar E, Aubry F, Wolffe AP. 1999. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 23:62–66.

Warburg O, Christian W. 1942. Isolierung und Kristallisation des Garungsferments Enolase. Biochem Z 310:384–414.

Wilkerson DC, Wolfe SA, Grimes SR. 2002a. H1t/GC-box and H1t/TE1 element are essential for promoter activity of the testis-specific histone *H1t* gene. Biol Reprod 67:1157–1164.

Wilkerson DC, Wolfe SA, Grimes SR. 2002b. Sp1 and Sp3 activate the testis-specific histone H1t promoter through the H1t/GC-box. J Cell Biochem 86:716–725.

Wilkerson DC, Wolfe SA, Grimes SR. 2003. Functional differences in the TE1 and TE2 subelements of the testis-specific histone H1t promoter. J Cellular Biochemistry 88:1177–1187.

Wolfe SA, Grimes SR. 1999. Binding of nuclear proteins to an upstream element involved in transcriptional regulation of the testis-specific histone *H1t* gene. J Cell Biochem 75:555–565.

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.