Sequence-specific promoter elements regulate temporal-specific changes in chromatin required for testis-specific activation of the *Pgk2* gene

Zhangsheng Yang[†], Hirotaka Yoshioka[‡] and John R McCarrey

Department of Biology, The University of Texas at San Antonio, 1 UTSA Circle, San Antonio, Texas 78249, USA

Correspondence should be addressed to J R McCarrey; Email: john.mccarrey@utsa.edu

[†]Z Yang is now at Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229, USA

[‡]H Yoshioka is now at Department of Calcified Tissue Biology, Institute of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

Abstract

The phosphoglycerate kinase-2 (*Pgk2*) gene is regulated in a tissue-, cell type-, and developmental stage-specific manner during spermatogenesis and is required for normal sperm motility and fertility in mammals. Activation of *Pgk2* transcription is regulated by testis-specific demethylation of DNA and binding of testis-specific transcription factors to enhancer and core promoter elements. Here, we show that chromatin remodeling including reconfiguration of nucleosomes and changes in histone modifications is also associated with transcriptional activation of the *Pgk2* gene during spermatogenesis. Developmental studies indicate that the order of events involved in transcriptional activation of the *Pgk2* gene includes demethylation of DNA in T₁- and T₂-prospermatogonia, binding of a factor to the CAAT box in type A and B spermatogonia, followed by recruitment of chromatin remodeling factors, displacement of a nucleosome from the *Pgk2* promoter region, binding of factors to the *Pgk2* core promoter and enhancer regions, and, finally, initiation of transcription in primary spermatocytes. Transgene studies show that *Pgk2* core promoter elements are required to direct demethylation of DNA and reconfiguration of nucleosomes, whereas both enhancer and core promoter elements are required to direct changes in histone modifications and initiation of transcription. These results provide novel insight into the developmental order of molecular events required to activate tissue-specific transcription of the *Pgk2* gene, the distinct elements in the 5'-regulatory region of the *Pgk2* gene that regulate each of these events, and the relationship among these events in that each step in this process appears to be a necessary prerequisite for the subsequent step.

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Introduction

Spermatogenesis is a highly ordered process by which the germ cell lineage gives rise to functional gametes in the male. As with other specific lineages in multicellular organisms, the characteristic differentiation of the male germ line proceeds as a result of expression of unique combinations of genes (Eddy 2002, Shima *et al.* 2004). Among the many germ cell type-specific genes expressed during spermatogenesis in mammals is a set encoding testis-specific glycolytic isozymes. These include the *Ldhc*, *Gapdhs*, *Hk1s*, *Pgm2*, *Pdha2*, and *Pgk2* genes, each of which encodes sperm-specific isozymes of glycolytic enzymes (Fitzgerald *et al.* 1992, Mori *et al.* 1993, Eddy 2002, Boussouar & Benahmed 2004, Tang *et al.* 2008).

Phosphoglycerate kinase (PGK) is a key enzyme involved in the metabolism of glucose via glycolysis.

In most mammals, PGK is encoded by two genes, *Pgk1* and *Pgk2* (VandeBerg *et al.* 1973, VandeBerg 1985, McCarrey & Thomas 1987). *Pgk1* is an X-linked gene that is ubiquitously expressed in all somatic cells, oogenic cells, and premeiotic spermatogenic cells (McCarrey *et al.* 1992). The autosomal *Pgk2* gene is expressed in a tissue-, cell type-, and developmental stage-specific manner during spermatogenesis and is required for normal sperm motility and fertility (VandeBerg *et al.* 1973, McCarrey *et al.* 1992, Danshina *et al.* 2010). Because the spermatogenic cell lineage is particularly well characterized and accessible in mammals, it provides an excellent system in which to chronicle events leading up to activation of tissue-specific gene expression (McCarrey 1998).

Pgk2 transcription is first seen in primary spermatocytes, with transcript levels increasing throughout the

first meiotic prophase and on into postmeiotic round spermatids (McCarrey *et al.* 1992). Transcription of this gene continues until midway through the process of spermiogenesis when the spermatid nucleus becomes so condensed that all transcription ceases (Kumari *et al.* 1996). We have previously demonstrated that a fragment carrying as little as 230 bp of the *Pgk2* 5'-regulatory region is sufficient to direct proper tissue-, cell type-, and developmental stage-specific transcription of a reporter gene in transgenic mice (Robinson *et al.* 1989, Zhang *et al.* 1999), suggesting that this region contains sequence elements sufficient to direct all molecular changes required for properly regulated transcriptional activation of this gene. In addition, we characterized protein–DNA interactions associated with transcriptional activation of the *Pgk2* gene and showed that the Sp3 factor is bound to a GC box in the *Pgk2* core promoter and that PBX4, along with its co-activator, PREP1, is bound to the E3–E4 enhancer region immediately upstream of the *Pgk2* core promoter in spermatogenic cells in which this gene is actively expressed (Yoshioka *et al.* 2007). Surprisingly, however, we found that a CAAT box, which is also present in the *Pgk2* core promoter, is unbound in these same expressing cells, although we did observe an *in vivo* footprint at this element in earlier spermatogenic cell types (spermatogonia) prior to the onset of *Pgk2* transcription (Yoshioka *et al.* 2007).

We have also examined changes in DNA methylation at the *Pgk2* locus and found that a domain of demethylation begins to develop over the *Pgk2* promoter region in T₁-prospermatogonia (McCarrey 2013) and becomes complete by the primitive type A spermatogonium stage (Geyer *et al.* 2004). This demethylation precedes transcription factor binding at the *Pgk2* promoter/enhancer by several days (Geyer *et al.* 2004). Changes in DNA methylation are often associated with changes in chromatin structure (Robertson 2002), and at least two classes of chromatin modifications have been shown to contribute to regulation of transcription. One is ATP-dependent chromatin remodeling, which mobilizes nucleosomes by weakening the contacts between histone octamers and DNA, including transient unwrapping of the DNA from core histones or displacement of nucleosomes relative to specific landmarks in the promoter regions of genes, both of which enhance accessibility of key promoter elements to transcription factors (Becker & Horz 2002, Flaus & Owen-Hughes 2004, Smith & Peterson 2005, Cairns 2009).

A second predominant type of chromatin modification involves post-translational modifications of histones, which include a variety of different types of modifications occurring on different amino acids in various positions within the core histones (Sterner & Berger 2000, Jenuwein & Allis 2001). Such modifications are particularly prominent on the N-terminal tails of histones H3 and H4 and include lysine acetylation, lysine or

arginine methylation, and serine or threonine phosphorylation among others (Berger 2002, Lachner *et al.* 2003, Kurdistani *et al.* 2004). These modifications can directly lead to altered structures of chromatin and/or can provide binding sites for regulatory proteins that further influence chromatin structure and transcription (Cairns 2009, Ho & Crabtree 2010). In general, chromatin structure and nucleosome positioning have been found to be distinctly different in a tissue-specific gene in cell types in which the gene is actively expressed or terminally repressed respectively (Narlikar *et al.* 2002, Yuan *et al.* 2005, Mavrich *et al.* 2008, Schones *et al.* 2008), suggesting that these parameters are related to mechanisms regulating transcription.

The objectives of the study reported here were to i) examine histone modifications and nucleosome reconfiguration in the *Pgk2* gene to identify features of chromatin structure that are unique to the actively expressed state of this gene, ii) determine the timing of appearance of these features relative to the timing of initiation of transcription of the *Pgk2* gene during spermatogenesis and the timing of other molecular changes associated with activation of this gene, and iii) identify elements in the 5'-regulatory region of the *Pgk2* gene that are involved in signaling or directing these chromatin changes. Our results indicate that there is an ordered series of molecular events that lead to activation of transcription of the *Pgk2* gene in spermatogenic cells and that these events are signaled by distinct elements in the *Pgk2* promoter/enhancer region. In addition, our data suggest that the occurrence of each of these events is largely dependent on the occurrence of the preceding event.

Materials and methods

Generation of transgenic mice

515*Pgk2*-CAT/WT and 188*Pgk2*-CAT transgenic mice (*Mus musculus*) were prepared for previous studies as described (Robinson *et al.* 1989). Three new lines of mice carrying a 515*Pgk2*-CAT/CAAT-minus transgene were generated for this study using similar procedures. The transgene used to generate these new lines was identical to that used for the 515*Pgk2*-CAT/WT lines except that the CAAT box in the *Pgk2* core promoter was mutated to ATCA by *in vitro* site-directed mutagenesis using recombinant PCR as described (Higuchi 1990, Barik 1996). The cesium chloride-purified, linearized transgene construct was introduced into early BDF1 (C57Bl6×DBA2 F1 hybrid) embryos by pronuclear injection at the University of Texas Health Science Center at San Antonio transgenic mouse core as described previously (Nagy *et al.* 2003). Tail snips from pups were initially screened for transgene integration by PCR with specific primers shown in Supplementary Table 1, see section on supplementary data given at the end of this article, as described previously (Walter *et al.* 1989). PCR-positive mice were further confirmed as founders by Southern blot hybridization to genomic DNA from tail tissue (data not shown).

Three transgenic founders (#9, #10, and #20) were identified and used to establish separate lines for the study described here.

Preparation of cells

Somatic spleen cells and spermatogenic germ cells for micrococcal nuclease (MNase) sensitivity and chromatin immunoprecipitation (ChIP) assays were recovered from either the transgenic mouse lines described earlier or from WT CD-1 mice purchased from Charles River Laboratories (Wilmington, MA, USA). All procedures involving live animals were approved in advance by the University of Texas at San Antonio Institutional Animal Care and Use Committee.

For isolation of spleen cells, spleens were dissected from freshly killed mice and placed in PBS and then mechanically teased open with hypodermic needles. The resulting cell suspension was filtered through 100 μ m nylon mesh (Millipore, Billerica, MA, USA), then treated with hypotonic buffer to lyse any contaminating erythrocytes, and pelleted by centrifugation at 175 *g* for 10 min at 4 °C.

For isolation of germ cells, testes were dissected from freshly killed prepuberal (6–8 days *post partum* (dpp)), puberal (18 dpp), or adult (≥ 60 dpp) CD-1 WT or BDF1 transgenic mice; decapsulated; and transferred into KREBS buffer (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 1.3 mM CaCl₂, 11.1 mM glucose, 1 mM glutamine, 10 ml/l of essential amino acids, 10 ml/l of nonessential amino acids, 100 μ g/ml streptomycin, and 100 U/ml penicillin G (K⁺ salt)) containing 0.5 mg/ml collagenase type 2 (Worthington) and incubated in a water bath for 15 min at 33 °C with vigorous shaking. Cells from prepuberal or puberal testes were then collected by centrifugation at 175 *g* for 10 min at 4 °C, while seminiferous tubule segments were collected from adult testes by pouring off the collagenase-containing KREBS buffer as described (Bellve 1993). The recovered cells or tubule segments were then washed with fresh KREBS buffer and incubated for another 15 min at 33 °C with vigorous swirling in KREBS buffer containing 0.5 mg/ml trypsin (Sigma). At the end of this incubation, 1 μ g/ml DNase I was added and the cells were pipetted up and down repeatedly to dissociate the cells, and then filtered through 80 μ m nylon mesh to yield a single cell suspension. This yielded a 'mixed germ cell' population representative of the spermatogenic cell types present in testes at each age of male mice utilized.

To obtain purified populations of specific spermatogenic cell types, the mixed germ cell population derived from testes at each age was loaded onto a 2–4% BSA 'StaPut' gradient and the cells were allowed to sediment through the gradient at unit gravity for 4 h at 4 °C as described previously (McCarrey *et al.* 1992, Bellve 1993, Yoshioka *et al.* 2007). Primitive type A spermatogonia were isolated from testes of male mice at 6 dpp. Type A and type B spermatogonia were isolated from testes of 8 dpp male mice. Preleptotene spermatocytes, leptotene plus zygotene spermatocytes, and early (juvenile) pachytene spermatocytes were isolated from the testes of 18 dpp male mice. Pachytene spermatocytes, round spermatids, and elongated spermatids plus residual cytoplasmic bodies were isolated from the testes of adult (≥ 60 dpp) male mice.

Testicular spermatozoa were isolated from the testes of adult mice by dissociation of tissue and sonication to lyse all other cells. The purity of each cell population was determined by morphological examination under phase optics. Purities of spermatogonial cell types and juvenile spermatocytes were $\geq 85\%$, while those of adult spermatocytes, spermatids, and spermatozoa were $\geq 95\%$.

Standard RT-PCR and real-time qRT-PCR

For standard RT-PCR and real-time qRT-PCR assays, total cellular RNA was extracted from each cell population using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The resulting RNA was quantified by Nanodrop (Thermo Scientific, Waltham, MA, USA). Before RT, 1 μ g total RNA was treated with 1 unit of RQ1 RNase-free DNase (Fisher Scientific, Hampton, NH, USA) for 30 min at 37 °C to remove any DNA contamination. The RNA was then used as a template to synthesize first-strand cDNA using the iScript cDNA synthesis Kit (Bio-Rad) according to the manufacturer's instructions. To further control for the possibility of genomic DNA contamination, an additional first-strand cDNA synthesis reaction was conducted without RT and exposed to PCR amplification as a no-RT control.

RT-PCR was performed to detect the CAT transgene or the coding sequence of the constitutively expressed house-keeping gene, *Gapdh*. These target sequences were amplified using the RT-PCR primers shown in [Supplementary Table 1](#). PCR products were resolved by electrophoresis through 1.5% agarose gels and visualized by ethidium bromide staining and detection on a VersaDoc imaging system (Bio-Rad).

For real-time qRT-PCR, first-strand cDNAs were amplified using SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio, Inc. Otsu, Shiga, Japan) on a Chromo4 Continuous Fluorescence Detector (Bio-Rad) using the primers listed in [Supplementary Table 1](#). Relative expression of target mRNAs and 18s rRNAs expressed as copy number was determined using a standard curve generated from DNA samples of known concentration.

Bisulfite genomic sequencing of DNA

To assess the status of DNA methylation at the *Pgk2* promoter, we used bisulfite conversion followed by sequencing as described previously (Frommer *et al.* 1992, Clark *et al.* 1994). Total genomic DNA was extracted from germ cells or spleen cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen). After quantification by Nanodrop (Thermo Scientific), 500 ng genomic DNA were treated with 130 μ l CT conversion reagent from the EZ DNA methylation-Gold Kit according to the manufacturer's instructions (Zymo Research Corporation, Irvine, CA, USA). Converted DNA was then purified using the QIAquick Gel Extraction Kit (Qiagen) and amplified using primers delineating the promoter or coding regions of the *Pgk2* gene ([Supplementary Table 1](#)). Anticipated bands were excised from the 1.0% agarose gel and DNA was recovered using the QIAquick Gel Extraction Kit (Qiagen). The products were cloned into the TOPO TA cloning vector (Invitrogen) according to the manufacturer's instructions. Positive (white) colonies were selected and prepared for

sequencing by the Nucleic Acids Core Facility at the University of Texas at San Antonio Health Science Center. The methylation status of each CpG dinucleotide was determined by comparison of sequences from amplicons of bisulfite-converted and untreated DNA respectively.

MNase sensitivity assay

An MNase sensitivity assay was used to map nucleosome positioning as previously described with minor modifications (Sekinger *et al.* 2005, Shim *et al.* 2007, Lam *et al.* 2008). Briefly, spleen or germ cells were isolated from transgenic (BDF1) or WT (CD-1) mice, washed three times with ice-cold PBS buffer, and resuspended in Tris-buffered saline prior to permeabilization with Tween 20 plus a proteinase inhibitor cocktail and phenylmethanesulfonyl fluoride (PMSF). Cells were then homogenized in a Dounce homogenizer using several strokes to release the nuclei, which were then pelleted and resuspended in digestion buffer (0.32 M sucrose, 50 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 1 mM CaCl₂, 0.1 mM PMSF, and 5 mM Na-butyrate) and incubated with the appropriate concentration of MNase (Sigma, N5386) at 37 °C. The reaction was stopped by adding SDS lysis buffer and proteinase K and incubating at 55 °C overnight, followed by extraction of the DNA with phenol/chloroform and precipitation in ethanol. Subsequently, the DNA was resuspended in TE buffer and RNase A was added and incubated for 30 min at 37 °C, followed by electrophoresis of the resulting mononucleosome DNA fragments through 1% agarose gel. The fragments were then recovered using a QIAquick Gel Extraction Kit (Qiagen) and redissolved in PCR grade water in preparation for real-time qPCR.

For analysis of MNase sensitivity in the endogenous *Pgk2* gene and at *Pgk2* transgenes, we used real-time qPCR with a series of primer pairs that delineated amplicons spanning the 5'-half of the *Pgk2* gene, with each amplicon of 100±8 bp representing regions of the gene located 40±10 bp away from each other (Supplementary Table 1). The relative enrichment of target sequences was determined on the basis of copy numbers, which were calculated using a standard curve generated from DNA of known concentrations as described earlier.

ChIP assay

ChIP assays were performed using an immunoprecipitation assay kit (#ab500; Abcam, Cambridge, UK) according to the manufacturer's instructions, with minor modifications. After isolation from mice, spleen and germ cells were fixed in a final concentration of 1% formaldehyde for 10 min at 37 °C and then the reaction was stopped by adding 125 mM glycine. After cells were washed once with PBS, the cells were lysed, the lysate was sonicated using a Branson Digital Sonifier (Branson Ultrasonics, Danbury, CT, USA) for 6 min with alternating 20 s on and 20 s off intervals at 45% amplitude, and with a longer pause every 2 min to cool down the samples. This sheared the chromatin to a fragment size range of 100–1000 bp. The sample was centrifuged to remove cell debris and immunoprecipitations were performed with antibodies against acetyl-histone H3, acetyl-histone H4, and tri-methylation of lysine 4

of histone H3 (H3k4me3) using antibodies from Active Motif. Antibody to rabbit IgG (Abcam) was used as a non-specific, negative control. After samples were incubated with antibody overnight at 4 °C, protein G beads were added to pull down immunoprecipitated complexes, which were then washed with ChIP buffer (provided in the Abcam Kit), followed by DNA purification using the DNA purifying slurry (provided in the Abcam Kit) plus 1 µl of proteinase K (provided in the Abcam Kit) to digest proteins following the kit instructions.

The resulting DNA was then analyzed by real-time qPCR on a Chromo4 real-time PCR detection system (Bio-Rad) using SYBR Premix Ex Taq (Perfect Real time, Takara Bio, Inc.). Relative amounts of DNA in the input and bound fractions were determined on the basis of copy numbers, using a standard curve generated from DNA samples of known concentration. Values for enrichment were calculated as the average of three independent samples.

Results

Transgenic mice examined in this study

Three different transgenes were used in this study (Fig. 1B). These included a construct carrying 515 bp of 5'-flanking sequence from the human *Pgk2* gene including the *Pgk2* core promoter plus the E1/E2 and E3/E4 enhancer regions (515*Pgk2*-CAT/WT). We have previously shown that this transgene is expressed in the same tissue-, cell type-, and developmental stage-specific manner as the endogenous mouse *Pgk2* gene, indicating that all regulatory information required to direct this transcriptional pattern is included in this fragment of the *Pgk2* gene (Robinson *et al.* 1989, Zhang *et al.* 1999). This reflects the fact that both the human and mouse *Pgk2* gene promoters contain highly conserved regulatory elements including a GC box, a CAAT box, and an enhancer region just upstream from the CAAT box (McCarrey 1987, 1990). A second transgene (188*Pgk2*-CAT/WT) carried the human *Pgk2* core promoter but not the upstream E1/E2 + E3/E4 enhancer region. This transgene is not expressed in any tissue in transgenic mice, including spermatogenic cells (Robinson *et al.* 1989, Zhang *et al.* 1999). To investigate the function of the CAAT box in the *Pgk2* core promoter, we produced three new lines of transgenic mice carrying a transgene identical to the 515*Pgk2*-CAT/WT transgene except that the CAAT box was mutated from CAAT to ATCA (515*Pgk2*-CAT/CAAT-minus). Because this transgene carries all other sequence information normally required to direct properly regulated transcription of the *Pgk2* gene, this construct allowed us to focus on the function of the CAAT box in this process. Importantly, as noted in our previous studies (Robinson *et al.* 1989, Zhang *et al.* 1999), expression of the promoter-reporter transgene, which encodes the prokaryotic chloramphenicol acetyl transferase enzyme, imposes no measurable phenotypic effects on testis or systemic functions or characteristics in the transgenic mice.

Figure 1 Effects of disruption of the *Pgk2* CAAT box on transcription and demethylation of the *Pgk2* gene. (A) A map of the 5' regulatory region of the *Pgk2* gene is shown. A 188-bp core promoter includes the transcription start site (TSS) at the +1 position, a GC box at -30 to -22, and a CAAT box at -105 to -101. A 44-bp enhancer region lies immediately upstream from the core promoter and includes two subregions, E1/E2 and E3/E4. In cells actively expressing the *Pgk2* gene, the Sp3 factor is bound to the GC box in the core promoter and the PBX4 transcriptional activator and its co-activator, PREP1, are bound to the E1/E2 portion of the enhancer region. Prior to initiation of transcription (but not during transcription), an unknown factor (?) is bound to the CAAT box. (B) Three different transgenes were used for this study: *515Pgk2*-CAT/WT – a transgene that includes the WT core promoter and enhancer region sequences, *515Pgk2*-CAT/CAAT-minus – a transgene identical to the *515Pgk2*-CAT/WT transgene except that the CAAT box has been mutated to ATCA, and *188Pgk2*-CAT – a transgene that includes the WT *Pgk2* core promoter sequence but lacks the enhancer region. Mice carrying the *515Pgk2*-CAT/WT and *188Pgk2*-CAT transgenes were generated for a previous study (Robinson *et al.* 1989), whereas three lines carrying the *515Pgk2*-CAT/CAAT-minus transgene were generated for this study. All transgenes were composed of human *Pgk2* promoter fragments ligated to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene plus the simian virus 40 (SV40) 3'-UTR poly(A) addition sequences. The *515Pgk2*-CAT/WT transgene showed strong expression in mouse testis (WT); however, the *515Pgk2*-CAT/CAAT-minus showed no detectable expression in testis tissue from any of the three lines carrying this transgene (#9, #10, and #20) as measured by either standard RT-PCR (C) or qRT-PCR (D). A map of the 5' half of the *Pgk2* gene (E) shows the locations of 42 CpG dinucleotides and the four amplicons, I, II, III, and IV, used to analyze DNA methylation at these sites by bisulfite genomic sequencing analysis of testis tissue (F), or somatic spleen tissue (G) from each line. Unmethylated CpGs are represented as open circles, whereas methylated CpGs are represented as filled circles. Each row of circles represents analysis of a different original DNA strand. The *515Pgk2*-CAT/WT line and all three *515Pgk2*-CAT/CAAT-minus lines showed complete or partial demethylation in testis tissue but remained hypermethylated in spleen tissue. GC, GC box; CAAT, CAAT box; ATCA, mutant sequence substituted for the CAAT box in the *515Pgk2*-CAT/CAAT-minus transgene.

The CAAT box in the *Pgk2* core promoter is required for initiation of transcription

We used RT-PCR to examine expression of the *515Pgk2*-CAT/WT and *515Pgk2*-CAT/CAAT-minus transgenes in testis RNA (Fig. 1C). As expected, we confirmed

expression of the *515Pgk2*-CAT/WT transgene in testis tissue. However, the *515Pgk2*-CAT/CAAT-minus transgene was not expressed in testis tissue recovered from males of any of the three different lines we generated carrying this transgene (#9, #10, and #20). This indicates

that activation of transcription cannot occur in the absence of an intact CAAT box in the *Pgk2* core promoter. To confirm these results, we also performed real-time qRT-PCR to detect the expression of these same transgenes, and this also showed that the 515*Pgk2*-CAT/WT transgene was expressed in testis tissue whereas the 515*Pgk2*-CAT/CAAT-minus transgene was not (Fig. 1D).

The CAAT box in the *Pgk2* core promoter is not required for tissue-specific demethylation of DNA in the *Pgk2* gene

We used bisulfite genomic sequencing to determine whether the absence of an intact CAAT box would inhibit the demethylation process that normally occurs over the 5'-half of the *Pgk2* gene prior to activation of *Pgk2* transcription (Geyer *et al.* 2004). We previously showed that both the 515*Pgk2*-CAT/WT and 188*Pgk2*-CAT transgenes undergo tissue-, cell type-, and developmental stage-specific demethylation of DNA in early spermatogenic cells (T₁- and T₂-prospermatogonia and type A spermatogonia) in a manner analogous to that which the endogenous *Pgk2* gene undergoes prior to initiation of transcription in spermatogenic cells *in vivo* (Ariel *et al.* 1994, Geyer *et al.* 2004). Importantly, both of those constructs carried an intact CAAT box. Here, we examined DNA methylation of the 515*Pgk2*-CAT/CAAT-minus transgene in testicular cells from the three different lines of mice carrying this transgene (Fig. 1F). While the 515*Pgk2*-CAT/CAAT-minus transgene in line #20 showed only partial demethylation of the 5'-portion of the transgene, lines #9 and #10 showed demethylation to a similar extent as that seen in the 515*Pgk2*-CAT/WT transgene. Further, in each case, the observed demethylation event was testis specific as indicated by the hypermethylated status of both the 515*Pgk2*-CAT/WT and 515*Pgk2*-CAT/CAAT-minus transgenes in spleen tissue from the same mice in which methylation of the transgenes in germ cells was assessed (Fig. 1G). This indicates that the CAAT box alone is not the signal directing tissue-specific demethylation of the *Pgk2* gene.

Testis-specific reconfiguration of nucleosomes in the *Pgk2* promoter accompanies active *Pgk2* transcription

Most genomic DNA in eukaryotes is incorporated into regularly, albeit randomly spaced nucleosomes to facilitate packaging and various processes associated with the DNA (Rando & Chang 2009). However, regions of DNA closely associated with nucleosome core histones are typically inaccessible for binding by regulatory factors and/or the transcriptional preinitiation complex (Jenuwein & Allis 2001). This conflict is normally resolved by reconfiguration of nucleosome presence and/or positioning relative to specific promoter sites required for protein–DNA interactions that facilitate activation of transcription (Cairns 2009). Thus, multiple

studies have revealed differences in nucleosome positioning associated with the same gene in cells where the gene is actively expressed or terminally repressed respectively (Sekinger *et al.* 2005, Lam *et al.* 2008, Leimgruber *et al.* 2009). In addition, genome-level methodologies such as ChIP-Chip or ChIP-Seq have revealed tissue- or cell type-specific patterns of nucleosomal reconfiguration in multiple species including *Saccharomyces cerevisiae* (Yuan *et al.* 2005, Lee *et al.* 2007), *Drosophila melanogaster* (Mavrich *et al.* 2008), and *Caenorhabditis elegans* (Valouev *et al.* 2008), as well as in promoter (Ozsolak *et al.* 2007, Schones *et al.* 2008) and enhancer (He *et al.* 2010) regions in the human genome. These studies reveal a characteristic pattern of nucleosome reconfiguration leading to unobstructed access facilitating binding of factors to enhancer, core promoter, and transcription start site regions in tissue-specific genes.

We examined nucleosome positioning at the *Pgk2* gene promoter and enhancer region to determine whether similar tissue-specific differences in nucleosome positioning are associated with different transcriptional states of the *Pgk2* gene. We isolated somatic spleen cells in which the *Pgk2* gene is terminally repressed, and spermatogenic cells at stages prior to and during the time when the *Pgk2* gene is actively transcribed, and used the MNase sensitivity assay to map nucleosome positioning in the 5'-flanking region of the *Pgk2* gene in each cell type. Consistent with similar studies of other tissue-specific genes, we found that nucleosome positioning in the *Pgk2* gene is distinctly different in non-expressing spleen cells and expressing spermatogenic cells. Thus, in spleen cells, we found occupancy at the –1 nucleosome position that includes the *Pgk2* promoter/enhancer region plus the nearby transcription start site (Fig. 2B), while in a mixed population of pachytene spermatocytes and round spermatids in which the *Pgk2* gene is actively expressed, the –1 nucleosome position was unoccupied (Fig. 2C). At the same time, the +1 nucleosome position remained occupied in both non-expressing spleen cells (Fig. 2B) and actively expressing germ cells (Fig. 2C), indicating a location-specific reconfiguration of nucleosomes in the *Pgk2* 5'-regulatory region.

To determine the timing of the reconfiguration of nucleosomes normally associated with activation of the *Pgk2* gene in spermatogenic cells, we examined nucleosome positioning in the 5' portion of the *Pgk2* gene in premeiotic spermatogonia – a stage at which transcription of the *Pgk2* gene has not yet begun (Gebara & McCarrey 1992, McCarrey *et al.* 1992, 2005), and in pachytene spermatocytes and round spermatids – meiotic and postmeiotic spermatogenic cell types in which active expression of the *Pgk2* gene is ongoing (McCarrey *et al.* 1992, 2005). We found that the –1 nucleosome position in the *Pgk2* promoter was still occupied in premeiotic spermatogonia (Fig. 2D) but then became unoccupied in

Figure 2 Nucleosome positioning in the endogenous *Pgk2* gene in somatic and spermatogenic cells in the mouse. The MNase sensitivity assay was used to examine nucleosome positioning in the endogenous mouse *Pgk2* gene in somatic cells in which the *Pgk2* gene is terminally repressed, premeiotic spermatogonia in which the *Pgk2* gene is undergoing potentiation prior to initiation of expression, and meiotic spermatocytes and postmeiotic spermatids in which the *Pgk2* gene is actively expressed. Regions containing elevated amplicon levels represent the site of a nucleosome that inhibited access of the MNase to the underlying DNA, whereas regions of low amplicon levels represent more accessible sites devoid of nucleosomes. (A) Maps of the 5' half of the *Pgk2* gene provide context for each set of MNase assays. (B) Somatic spleen cells show the presence of nucleosomes (dark gray ovals) at the -1 region, which covers the *Pgk2* enhancer and core promoter regions and at the $+1$ position within the *Pgk2* coding sequence downstream of the transcription start site. (C) A mixed population of spermatogenic cells consisting primarily of *Pgk2*-expressing spermatocytes and spermatids shows the absence of a nucleosome at the -1 position in these cells (dashed oval), but the continued presence of a nucleosome at the $+1$ position. (D) A purified population of primitive type A spermatogonia in which the *Pgk2* gene is not yet expressed shows the presence of nucleosomes at both the -1 and $+1$ positions, whereas in pachytene spermatocytes (E), the -1 nucleosome is completely missing, and in round spermatids (F), the -1 nucleosome presence is reduced (light gray dashed oval), while the $+1$ nucleosome persists in all these cell types.

pachytene spermatocytes (Fig. 2E) and was still partially, although not as completely, unoccupied in round spermatids (Fig. 2F). Note that transcription of the *Pgk2* gene ceases immediately after the round spermatid stage in elongating spermatids (McCarrey *et al.* 1992). Importantly, although we observed some variation among the different cell types investigated in the overall extent of post-MNase digestion signal intensity detected for each gene region, the differences were consistent within each gene region and were consistently distinct between those cell types in which the corresponding gene was or was not expressed. The post-MNase digestion signals do not reach 100 or 0% in any case because i) the purities of the enriched cell populations investigated were 85–95% and ii) reconfiguration of nucleosomes on any promoter is likely to reflect an equilibrium state that never reaches 100 or 0% simultaneously in all cells examined, even within a particular cell type.

The CAAT box is required for reconfiguration of nucleosomes in the *Pgk2* promoter

The tissue- and location-specific reconfiguration of nucleosomes at the -1 position in the 5'-region of the *Pgk2* gene implies a highly regulated process. Because we had previously observed an *in vivo* footprint over the CAAT box in spermatogenic cells before but not during the time of active transcription of the *Pgk2* gene, we next examined the extent to which normal nucleosome reconfiguration occurred in *515Pgk2*-CAT transgenes lacking an intact CAAT box (i.e., in *515Pgk2*-CAT/CAAT-minus transgenes). We first confirmed that the *515Pgk2*-CAT/WT transgene carrying an intact CAAT box undergoes a tissue-specific reconfiguration of nucleosomes at the -1 position in a manner similar to that we observed in the endogenous *Pgk2* gene (Fig. 3A). Interestingly, in addition to nucleosome occupancy at the -1 position in this transgene, we also observed occupancy at a site upstream from the -1 position in both spleen and germ cells that we termed the -2 position (Fig. 3A). This was not observed in the endogenous *Pgk2* gene, presumably because the sequence differs in the regions upstream of the endogenous *Pgk2* gene and any integrated *Pgk2* transgenes respectively. We also did not observe consistent nucleosome occupancy at the $+1$ position in these transgenes, which we suspect also reflects sequence differences between the transgenes (carrying the CAT reporter gene downstream of the *Pgk2* promoter) and the endogenous *Pgk2* gene. However, the key observation was that when nucleosome mapping was performed on the 5'-portion of the *515Pgk2*-CAT/WT transgene in non-expressing spleen cells and in expressing spermatocytes or spermatids, respectively, only occupancy at the -1 position changed, becoming unoccupied in expressing germ cells consistent with the result we observed for the endogenous *Pgk2* gene.

We next examined nucleosome positioning in the *515Pgk2*-CAT/CAAT-minus transgenes in these same cell types. In all three lines carrying the CAAT-minus transgene, there was no difference in nucleosome occupancy in spleen and germ cells respectively (Fig. 3B), such that the -1 position remained occupied even in spermatogenic cell types in which the equivalent position in the endogenous *Pgk2* gene or in the *515Pgk2*-CAT/WT transgene became unoccupied. This correlated with the lack of expression of this transgene in testis tissue from any of these lines described earlier. Thus, it appears that an intact CAAT box is required for both reconfiguration of nucleosomes over the *Pgk2* promoter and tissue-specific initiation of transcription from this promoter.

Testis-specific modifications of histones accompany activation of *Pgk2* transcription

A common molecular event that accompanies physical remodeling of chromatin and activation of tissue-specific transcription is modification of core histones (Jenuwein & Allis 2001). Both histone tails and globular domains are subject to a vast array of posttranslational modifications sometimes referred to as the histone code (Berger 2002, Barth & Imhof 2010). These modifications include acetylation, methylation, ubiquitination, ADP-ribosylation, and sumoylation of lysine residues, methylation of arginine residues, and other modifications (Kouzarides 2007). Commonly, acetylation of histone H3 and histone H4 (acetyl-H3 and acetyl-H4), and tri-methylation of lysine 4 of histone H3 (H3K4me3), is associated with a euchromatic chromatin structure and active transcription. Therefore, we used the ChIP assay to investigate these three modifications in different regions of the *Pgk2* gene in somatic spleen cells in which the *Pgk2* gene is terminally repressed, premeiotic spermatogonia in which the *Pgk2* gene is not yet expressed, and spermatocytes and spermatids in which the *Pgk2* gene is actively expressed (Fig. 4). We also investigated similar modifications in two control genes in the same cell populations – the β -actin gene that is constitutively expressed in spermatogenic and spleen cells and the *Cryaa* gene, which is terminally repressed in these same cell types.

All three modifications were elevated in the β -actin gene relative to the *Cryaa* gene in both somatic spleen cells and pachytene spermatocytes, confirming the association of these modifications with actively transcribed genes (Fig. 4B). Similarly, these modifications were generally low in the *Pgk2* gene in spleen cells and in premeiotic spermatogonia, similar to levels seen for the repressed *Cryaa* gene in both somatic and germ cells. However, beginning in primary spermatocytes in which active transcription of the *Pgk2* gene is initiated (McCarrey *et al.* 1992), we observed a gradual increase in all three modifications that reached a peak in either pachytene spermatocytes or round spermatids (Fig. 4B).

Figure 3 Nucleosome positioning in *Pgk2* transgenes with or without intact CAAT boxes. (A) The MNase sensitivity assay was used to examine nucleosome positioning in the *515Pgk2-CAT/WT* transgene in spleen cells and in a mixed population of male germ cells (pachytene spermatocytes and round spermatids). A nucleosome (gray oval) was present at the -1 position over the *Pgk2* enhancer and core promoter regions in non-expressing spleen cells but was absent from this position in expressing germ cells. An additional nucleosome was persistently present further upstream in what has been termed the -2 position, but no persistent nucleosome was detected at the $+1$ position in these transgenes. Note that the DNA sequence in the -2 and $+1$ regions of these transgenes differs from that in the equivalent positions of the endogenous *Pgk2* gene. (B) The nucleosome at the -1 position persisted in the *515Pgk2-CAT/CAAT-minus* transgene in both spleen cells and male germ cells in all three lines investigated.

in a pattern that directly mimicked the gradual increase in expression levels of this gene in the same cell types (McCarrey *et al.* 1992, Yoshioka *et al.* 2007). Levels of all three modifications associated with the *Pgk2* gene in pachytene spermatocytes and round spermatids were similar to those seen for the same modifications in the constitutively expressed β -actin gene, indicating that these modifications are associated with a state of active expression of the *Pgk2* gene. The gradually increasing pattern of these modifications in progressively later spermatogenic cell types was similar for all three modifications investigated (Fig. 4B) and correlates with

previously published results describing ongoing transcription of the *Pgk2* gene in primary spermatocytes and round spermatids (Kumari *et al.* 1996). In addition, the increase in these modifications was seen in both the promoter and coding regions of the *Pgk2* gene, with the increase being greatest in the coding region.

The *Pgk2* enhancer region plus the CAAT box are required to direct activating histone modifications

As with reconfiguration of nucleosomes, the tissue specificity and developmental stage specificity of these

Figure 4 ChIP analysis of activating histone modifications in the endogenous *Pgk2* gene. (A) A map of the intronless *Pgk2* gene showing the positions of amplicons analyzed for histone modifications by ChIP. One amplicon (filled black bars in the graphs) was located within the *Pgk2* promoter and a second amplicon (open bars in the graphs) was located in the *Pgk2* coding sequence. (B) ChIP analysis of three different activating histone modifications, (a) acetyl-H3, (b) acetyl-H4, and (c) H3k4me3, in the *Pgk2* gene in different cell types, and in two control genes (*β-actin* or *Cryaa*) in spleen cells (Spleen) and pachytene spermatocytes (Pachs). The *β-actin* gene is ubiquitously active in all cell types and showed consistent enrichment for all three activating histone modifications in both spleen cells and spermatocytes, while the *Cryaa* gene is repressed in both spleen cells and spermatocytes and showed an absence of activating histone modifications in both cell types. The *Pgk2* gene also showed an absence of activating histone modifications in spleen cells where this gene is terminally repressed, and in premeiotic type A and type B spermatogonial cells in which the *Pgk2* gene has not yet undergone initiation of transcription. However, all three activating histone modifications showed enrichment in the *Pgk2* gene in cells actively expressing this gene, including successive types of primary spermatocytes – preleptotene spermatocytes (Prelep), leptotene plus zygotene spermatocytes (Lep/Zyg), early pachytene spermatocytes (Ea Pachys), and fully mature pachytene spermatocytes (Pachs), as well as in round spermatids (Round). Enrichment for these histone modifications was generally greater in the *Pgk2* coding sequence than in the *Pgk2* promoter in cells actively expressing this gene.

histone modifications suggest a highly regulated process on a gene-specific level. To determine which part of the *Pgk2* gene signals these modifications, we examined transgenes carrying the WT *Pgk2* core promoter alone (= the *188Pgk2*-CAT transgene) or the WT *Pgk2* core promoter plus enhancer regions (= the *515Pgk2*-CAT/WT transgene), as well as the transgene carrying the *Pgk2* enhancer plus core promoter regions but with a mutated CAAT box (= the *515Pgk2*-CAT/CAAT-minus transgene) (Fig. 5). The intact *515Pgk2*-CAT/WT transgene showed germ cell-specific enrichments of acetyl-H3 and acetyl-H4 (especially in spermatocytes and spermatids) that were diminished in the *188Pgk2*-CAT transgene (Fig. 5B). This indicated that at least part of the signal that directs tissue-specific acquisition of acetylation of histones H3 and H4 in the *Pgk2* promoter appears to reside in the upstream enhancer region. However, the *515Pgk2*-CAT/CAAT-minus transgene also failed to acquire tissue-specific acetylation of H3 and H4, suggesting that the complete gene-specific signal that directs this modification may extend from the *Pgk2* enhancer region through the 5'-portion of the *Pgk2* core promoter where the CAAT box resides.

Summary of *Pgk2* promoter regions required to signal each molecular event associated with transcriptional activation of the *Pgk2* gene

Table 1 summarizes the occurrence of four different molecular events in the presence of different regions of the *Pgk2* promoter+enhancer regions in transgenes. Thus, from a transgene carrying an intact *Pgk2* enhancer plus core promoter region, transcription is initiated with normal tissue specificity and developmental stage specificity (Robinson *et al.* 1989, Zhang *et al.* 1999). However, in the absence of the enhancer region, or of an intact CAAT box in the core promoter region even when the enhancer region is present, transcription is not initiated from this promoter. Acquisition of activating histone modifications (acetyl H3, acetyl H4, and H3k4me3) requires an intact CAAT box in the *Pgk2* core promoter along with the presence of the *Pgk2* enhancer region. Reconfiguration of nucleosomes also requires an intact CAAT box in the core promoter. Whether or not the *Pgk2* enhancer region is required for nucleosome reconfiguration could not be determined because the *188Pgk2*-CAT transgene that lacks the enhancer region also lacks a significant portion of the upstream sequence that normally makes up the –1 nucleosome position. Finally, the signal for cell type- and developmental stage-specific demethylation of DNA in the 5' half of the *Pgk2* gene appears to reside in the *Pgk2* core promoter and does not require the presence of the enhancer region. However, an intact CAAT box in the core promoter does not appear to be a specific requirement for this signaling function.

Figure 5 ChIP analysis of histone acetylation in *Pgk2* transgenes. (A) A map of the *Pgk2*-CAT transgenes shows the position of amplicons analyzed for acetyl-H3 or acetyl-H4 in *Pgk2*-CAT transgenes. (B) Relative enrichment of acetylated H4 (graphs on the left) or acetylated H3 (graphs on the right) is shown for each transgene as well as for the two control genes, β -actin and *Cryaa* in spleen cells or mixed male germ (spermatogenic) cells. The *515Pgk2*-CAT/WT transgene, which is actively expressed in spermatogenic cells, shows significant enrichment for both acetyl-H3 and acetyl-H4 in male germ cells but not in spleen cells where this transgene is not expressed. The *515Pgk2*-CAT/CAAT-minus transgene, which contains all regulatory sequences normally required to direct testis-specific expression except for an intact CAAT box in the core promoter, and which is not expressed in either spleen cells or spermatogenic cells, shows no enrichment of acetyl-H3 or acetyl-H4 in either cell type. Similarly, the *188Pgk2*-CAT transgene, which contains only the *Pgk2* core promoter and no enhancer region and is not expressed in either spleen or spermatogenic cells, shows no enrichment for either histone modification in either cell type.

Table 1 Regulation of molecular events associated with transcriptional activation of the *Pgk2* gene.

Molecular event	Specific <i>Pgk2</i> promoter regions		
	Intact enhancer + intact core promoter	Intact core promoter only	Intact enhancer + core promoter lacking CAAT
Demethylation of DNA	+	+	+
Nucleosome reconfiguration	+	NA ^a	—
Histone modifications	+	—	—
Initiation of transcription	+	—	—

^aNote that in the absence of the enhancer region, the sequence over which the —1 position at which a nucleosome is normally found in non-expressing cells is missing.

Discussion

Spermatogenesis is a highly dynamic process of cellular differentiation that has been correlated with a highly dynamic series of changes in gene expression (Almstrup *et al.* 2004, Shima *et al.* 2004, Roy Choudhury *et al.* 2010). These changes include the activation of numerous testis-specific genes including several tissue-specific members of gene families that encode testis-specific isozymes (McCarrey & Thomas 1987, Mori *et al.* 1993, Boussouar & Benahmed 2004, Tang *et al.* 2008). One example is the testis-specific *Pgk2* gene, transcriptional activation of which occurs in primary spermatocytes coincident with the onset of meiotic prophase. Interestingly, this is also the time at which the X-linked *Pgk1* gene, which encodes the form of the glycolytic enzyme phosphoglycerate kinase (PGK-A) that is expressed in all somatic or germ cell types carrying at least one transcriptionally active X chromosome, undergoes silencing due to the process of meiotic sex chromosome inactivation (MSCI; McCarrey & Thomas 1987, McCarrey *et al.* 1992, Wang *et al.* 2005, Turner 2007). Thus, meiotic and postmeiotic spermatogenic cells are unique in the absence of at least one active X chromosome, and this presents a dilemma because these cells, especially spermatozoa, require PGK to carry out glycolysis. It appears that a gene duplication event that occurred early during mammalian evolution has solved this dilemma in mammals by providing an autosomal copy of the *Pgk* gene (*Pgk2*), which encodes a testis-specific isozyme of PGK-A (PGK-B) that is not subject to MSCI, and that undergoes tissue-specific transcriptional activation at the same time that the *Pgk1* gene becomes silenced, such that a source of PGK protein is maintained throughout spermatogenesis (McCarrey *et al.* 1992, 1996).

Because the development and differentiation of the male germ line in mammals has been well characterized, and because many of the different cell types that make up this lineage can be recovered in relatively pure populations, spermatogenesis provides an excellent opportunity to study the developmental process of transcriptional activation of tissue-specific genes

(Bellve 1993, McCarrey 1998). This has allowed us to chronicle molecular events that are associated with transcriptional activation of the spermatogenesis-specific *Pgk2* gene (McCarrey *et al.* 1992, 1996, Geyer *et al.* 2004, Yoshioka *et al.* 2007). We previously showed that activation of the *Pgk2* gene is associated with binding of ubiquitous transcription factors to the *Pgk2* core promoter (Gebara & McCarrey 1992, Berg *et al.* 1993, Yoshioka *et al.* 2007) and testis-specific factors to the *Pgk2* enhancer region (Robinson *et al.* 1989, Gebara & McCarrey 1992, Yoshioka *et al.* 2007). We also showed that there is a general increase in nuclease (DNase I) sensitivity over the entire *Pgk2* gene (Kramer *et al.* 1998), as well as the development of a DNase I hypersensitive site at the *Pgk2* promoter (Kumari *et al.* 1996) in cells that express this gene. Finally, we showed that activation of the *Pgk2* gene is preceded by demethylation of DNA over the 5'-portion of the gene (Ariel *et al.* 1994, Zhang *et al.* 1998, Geyer *et al.* 2004).

In the study described here, we have shown that two additional molecular events associated with chromatin remodeling – reconfiguration of nucleosomes and acquisition of activating histone modifications – also occur during the process of transcriptional activation of the *Pgk2* gene. Thus, a nucleosome at the —1 position that corresponds to a region spanning the enhancer and core promoter region of the *Pgk2* gene is present in non-expressing cells but is absent in cells in which the *Pgk2* gene is actively transcribed. Interestingly, an adjacent nucleosome at the +1 position in the 5' portion of the coding sequence remains present in both expressing and non-expressing cell types. This indicates that reconfiguration and/or displacement of the nucleosome at the —1 position is specifically correlated with events ongoing in the promoter region of the *Pgk2* gene associated with transcriptional activation of this gene.

The acquisition of histone modifications including acetyl H3, acetyl H4, and H3k4me3 also shows a direct correlation with active expression of this gene. However, these modifications occur in both the *Pgk2* promoter and coding sequence regions. Indeed, these modifications are most pronounced in the *Pgk2* coding sequence in expressing cell types. This likely reflects the fact that nucleosome reconfiguration significantly reduces the overall representation of histones in the *Pgk2* promoter region and that these histone modifications facilitate the elongation as well as initiation phases of transcription (Berger 2002). Acetylation of lysine residues is known to contribute to changes in nucleosome/chromatin structure that facilitate recruitment of other transcription factors to promoter regions and/or more efficient processivity of RNA polymerase during the elongation phase of transcription (Brownell *et al.* 1996, Marmorstein & Roth 2001, Allis *et al.* 2007, Marmorstein & Trievel 2009). Our findings that reconfiguration of nucleosomes and modifications of histones accompany activation of transcription of the *Pgk2* gene are consistent with those

previously reported for several other genes (Kouskouti & Talianidis 2005, Villagra *et al.* 2006, Plachetka *et al.* 2008) and are therefore not novel in that respect. However, our results do provide novel information regarding the order of these and other molecular events involved in transcriptional activation of the *Pgk2* gene in particular and of tissue-specific genes in general.

Thus, based on the data presented here, as well as those from our previously published studies including bisulfite sequencing analysis of demethylation of the *Pgk2* gene (Zhang *et al.* 1998, Geyer *et al.* 2004), *in vivo* footprinting of the *Pgk2* 5'-regulatory region (Yoshioka *et al.* 2007), general nuclease sensitivity studies (Kramer *et al.* 1998), analysis of DNase I hypersensitive site formation (Kumari *et al.* 1996), and ChIP assays to detect binding of specific factors to the *Pgk2* enhancer and core promoter regions *in vivo* (Yoshioka *et al.* 2007), we can now propose a developmental order of molecular regulatory events involved in activation of transcription of the *Pgk2* gene (Fig. 6). These include two events that clearly precede initiation of transcription of the *Pgk2* gene – step 1) demethylation of DNA in the 5' half of the *Pgk2* gene beginning in T₁-prospermatogonia at 18 days postcoitum (dpc) and completed by the primitive type A spermatogonial stage at 6 dpp, and step 2) binding of one or more factors (possibly CCAAT/enhancer binding protein (C/EBPβ), see below) to a region in the *Pgk2* core promoter that extends from the CAAT box to just upstream of the GC box in premeiotic spermatogonia (Geyer *et al.* 2004), plus recruitment of chromatin remodeling factors or complexes in early primary spermatocytes. These events are followed by a series of events that occur just prior to, or coincident with initiation of *Pgk2* transcription in primary spermatocytes, beginning with step 3) reconfiguration of the –1 nucleosome covering the *Pgk2* core promoter and enhancer regions as shown in this study, formation of a DNase I hypersensitive site as shown in a previous study (Kumari *et al.* 1996), binding of testis-specific factors (PBX4 and PREP1) to the E3/E4 portion of the *Pgk2* enhancer region, and Sp3 to the GC box in the *Pgk2* core promoter as shown in another previous study (Yoshioka *et al.* 2007), changes in histone modifications as shown in this study, and an increase in general nuclease sensitivity throughout the *Pgk2* locus as shown in yet another previous study (Kramer *et al.* 1998). Finally, step 4) formation of a complete preinitiation complex is completed by recruitment of general transcription factors plus RNA polymerase II and transcription is initiated. This scheme is depicted in Fig. 6.

In this study, we have also shown that an intact CAAT box is required for both the acquisition of activating histone modifications and nucleosome reconfiguration. The CAAT box is an element found in the core promoters or enhancers of many ubiquitously expressed and tissue-specific genes (Mantovani 1999, Villagra *et al.* 2006, Plachetka *et al.* 2008). It is known to serve as a binding site for multiple ubiquitous transcription factors including

Figure 6 Model of events leading up to transcriptional activation of the *Pgk2* gene during spermatogenesis. A series of molecular events involved in transcriptional activation of the *Pgk2* gene during spermatogenesis is proposed based on observations reported in this and previous studies. Where possible, these events are shown in developmental chronological order and include Step 1 – demethylation of DNA in the 5'-half of the *Pgk2* gene, which begins in T₁-prospermatogonia and is completed by the primitive type A spermatogonia stage; Step 2 – binding of one or more factors (possibly C/EBPβ) to the CAAT box and surrounding sequences in the *Pgk2* core promoter to attract a chromatin remodeling complex; Step 3 – displacement of the nucleosome at the –1 position, binding of factors to the enhancer and core promoter regions, and recruitment of histone-modifying enzymes; and Step 4 – formation of the complete preinitiation complex including recruitment of RNA polymerase II and initiation of transcription. CH3, methyl groups on CpG dinucleotides; Enh, enhancer region; CAAT, CAAT box; GC, GC box; Ac, histone acetylation; Me3, histone tri-methylation.

CCAAT transcription factor/nuclear factor 1 (Nagata *et al.* 1982, Santoro *et al.* 1988), C/EBP (Chang *et al.* 1990, Roman *et al.* 1990), and nuclear factor Y/Hem activation protein (Li *et al.* 1992, McNabb *et al.* 1995), among others (Nepveu 2001). Thus, while it is not surprising to find that the CAAT box in the *Pgk2* promoter is important for initiation of transcription, we were surprised to observe in our ChIP studies of protein–DNA interactions associated with the *Pgk2* gene in spermatocytes or spermatids (Yoshioka *et al.* 2007) that none of the common ubiquitous factors known to bind to CAAT boxes appear to be bound to this sequence in the actively expressed *Pgk2* gene. This was confirmed by our *in vivo* footprinting studies (Yoshioka *et al.* 2007) that showed this site remains unoccupied in these same cells. Interestingly, we did observe *in vivo* footprints over the

CAAT box and nearby regions of the *Pgk2* core promoter during the spermatogonial stage, prior to initiation of *Pgk2* transcription in primary spermatocytes. This has led us to speculate that an intact CAAT box is required for chromatin remodeling events that immediately precede initiation of *Pgk2* transcription. Specifically, we suggested that the CAAT box and nearby sequences serve as signals to direct chromatin remodeling by binding factors or chromatin remodeling complexes in a gene-, cell type-, and developmental stage-specific manner. Our observation that these events fail to occur in a transgene lacking an intact CAAT box but possessing all other regulatory regions normally required for tissue-specific activation of transcription from the *Pgk2* promoter suggests that the CAAT box does indeed play a role in signaling chromatin remodeling, which is, in turn, a mandatory prerequisite for subsequent molecular events required for transcription from the *Pgk2* promoter, including binding of some or all of the transcription factors that are normally required for initiation of *Pgk2* transcription.

There have been previous reports of factors binding to CAAT boxes to initiate chromatin remodeling in conjunction with tissue-specific activation of transcription. Tissue-specific activation of the bone-specific *OC* gene requires chromatin remodeling by the SWI/SNF complex recruited to the proximal promoter by C/EBP β , which binds to a CAAT box-like sequence (Villagra *et al.* 2006). Similarly, C/EBP β has been shown to bind to an upstream enhancer site in the tissue-specific chicken *mim-1* gene to induce decondensation of chromatin prior to activation of transcription (Plachetka *et al.* 2008). Both these studies showed that the C/EBP β factor is required for chromatin remodeling associated with, and required for, activation of tissue-specific transcription. Our study shows that an intact CAAT box is specifically required for this process in the testis-specific *Pgk2* gene and suggests this element may function by recruiting C/EBP β and chromatin remodeling complexes to promote acquisition of activating histone modifications, decondensation of chromatin, and reconfiguration of nucleosomes, although we have yet to directly demonstrate this.

A *Pgk2* transgene lacking an intact CAAT box but possessing all other 5'-regulatory sequences normally required for tissue-specific activation of *Pgk2* transcription failed to acquire activating histone modifications or initiate transcription, indicating that chromatin remodeling directed by the *Pgk2* CAAT box is a mandatory prerequisite for activation of transcription from the *Pgk2* promoter. Our observation of *in vivo* footprints over the CAAT box and nearby regions in spermatogonia, but not in spermatocytes or spermatids (Yoshioka *et al.* 2007), indicates that these chromatin remodeling steps normally occur prior to the initiation of transcription and do not need to be continually reinforced during active transcription. Thus, the model we propose in Fig. 6 suggests that a single chromatin remodeling event is required to facilitate access for most or all of the

transcription factors that regulate ongoing expression of the *Pgk2* gene to bind to their cognate sites in the *Pgk2* core promoter and enhancer regions. We further observed that a transgene carrying the *Pgk2* core promoter but not the upstream enhancer region failed to undergo activating histone modifications, suggesting that the enhancer region is also required to direct acquisition of these modifications.

We previously showed that the PBX4 transcription factor and its co-activator, PREP1, become bound to the *Pgk2* E3/E4 enhancer region beginning in primary spermatocytes (Yoshioka *et al.* 2007), which is the same time at which we now show activating histone modifications appear. A previous report showed that the PBX4 factor can interact with histone lysine acetyltransferases to direct acetylation of chromatin (Choe *et al.* 2009). Taken together, our results are consistent with a model in which the CAAT box in the *Pgk2* core promoter is required to direct chromatin remodeling (potentially through binding of C/EBP β), and the E3/E4 enhancer region is required to direct acquisition of activating histone modifications through binding of the PBX4 activator and its co-activator, PREP1. Because a transgene carrying an intact *Pgk2* enhancer region but a disrupted CAAT box failed to undergo the histone modifications normally associated with transcriptional activation, we would further suggest that the chromatin remodeling step directed by the CAAT box is a prerequisite for the histone modification step directed by the enhancer region in the *Pgk2* gene.

Our previous studies have also shown that demethylation of DNA in the 5' half of the *Pgk2* gene precedes the chromatin remodeling events, as well as binding of transcription factors to the *Pgk2* promoter and initiation of transcription. We previously showed that the *Pgk2* enhancer region is not required to direct demethylation of the *Pgk2* gene (Zhang *et al.* 1998, Geyer *et al.* 2004), and in this study, we showed that an intact CAAT box is also not required for this demethylation event. Thus, tissue-specific demethylation appears to be regulated independently from chromatin remodeling and activating histone modifications in the *Pgk2* gene. How the demethylation step is signaled in a gene-, cell type-, and developmental stage-specific manner and whether or not that event is a mandatory prerequisite for the subsequent molecular events associated with activation of the *Pgk2* transcription remain to be determined.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0311>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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