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RNA Polymerase II interacts with the Hspa1b Promoter in Mouse Epididymal Spermatozoa

Donald C. Wilkerson and **Kevin D. Sarge**
Department of Molecular and Cellular Biochemistry, University of Kentucky, 741 S. Limestone Street, Lexington, KY

Abstract

The *Hspa1b* (*Hsp70.1*) gene is one of the first genes expressed after fertilization, with expression occurring during the minor zygotic genome activation in the absence of stress. This expression can take place in the male pronucleus as early as the one-cell stage of embryogenesis. The importance of HSPA1B for embryonic viability during times of stress is supported by studies showing that depletion of this protein results in a significant reduction in embryos developing to the blastocyte stage. Recently we began addressing the mechanism responsible for allowing expression of *Hspa1b* during the minor ZGA and found that HSF1 and HSF2 bind the *Hspa1b* promoter during late spermatogenesis. In this report, we have extended those studies using western blots and chromatin immunoprecipitation assays and found that RNA Polymerase II is present in epididymal spermatozoa and bound to the *Hspa1b* promoter. These present results, in addition to our previous results, support a model in which the binding of HSF1, HSF2, Sp1, and RNA Polymerase II to the promoter of *Hspa1b* would allow the rapid formation of a transcription-competent state during the minor ZGA, thereby allowing *Hspa1b* expression.

INTRODUCTION

The zygotic genome activation (ZGA) is a process where maternal control of development is shifted to the zygote (reviewed in (Minami *et al.* 2007)). This transition can be divided into a minor ZGA where a small subset of genes including *Hspa1b* are expressed as early as the one-cell stage, and the major ZGA, which occurs during the two-cell stage and is characterized by a significant burst in both transcription and translation (Bellier *et al.* 1997; Flach *et al.* 1982; Howlett *et al.* 1985; Latham *et al.* 1991; Nothias *et al.* 1995; Taylor & Piko 1987) with more stringent transcriptional regulation (Majumder *et al.* 1993; Martinez-Salas *et al.* 1989; Rothstein *et al.* 1992; Wiekowski *et al.* 1991). During the minor ZGA, transcription in the one-cell embryo appears to be relatively promiscuous and opportunistic (Choo *et al.* 2002; Ma *et al.* 2001) with the majority of transcription occurring in the male pronucleus (Aoki *et al.* 1997; Worrad *et al.* 1994).

The *Hspa1b* gene encodes one of two known 70 kDa stress-inducible heat shock proteins (HSP70) and is one of the first genes expressed following fertilization, with spontaneous expression taking place as early as the one-cell stage of embryogenesis in the absence of stress

Correspondence should be addressed to Kevin Sarge; E-mail: E-mail: kdsarge@uky.edu.

Declaration of Interest
There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

(Bensaude *et al.* 1983; Christians *et al.* 1995; Fiorenza *et al.* 2004). Studies using inducible *Hsp70* promoter driven reporter genes confirms this embryonic pattern of expression (Thompson *et al.* 1995). Spontaneous expression of *Hspa1b* mRNA increases to peak levels at the 2 cell stage and then decreases until becoming stress-inducible by the blastocyte stage (Morange *et al.* 1984). In bovine embryos, HSP70 appears to be stress-inducible at the 2 cell stage (Edwards & Hansen 1996).

The importance of stress-inducible HSP70 proteins for embryonic viability is supported by a number of studies. In one study HSP70 proteins were immunodepleted (Matwee *et al.* 2001) while another study used antisense oligonucleotides to inhibit stress-induced HSP70 expression in (Dix *et al.* 1998). In both studies there was a significant reduction in embryos developing to the blastocyte stage, indicating that expression of inducible HSP70 proteins is important for preimplantation embryogenesis. Although HSPA1B is important for embryonic viability, the mechanism responsible for expression of the *Hspa1b* gene during the minor ZGA is not known.

In somatic cells, the promoters of a number of genes, including those of the *Hspa1b* and *C-myc* genes, remain uncompacted and accessible during mitosis (Christova & Oelgeschlager 2002; John & Workman 1998; Martinez-Balbas *et al.* 1995; Michelotti *et al.* 1997; Sarge & Park-Sarge 2005). The lack of compaction of promoter regions in mitotic cells is referred to as “bookmarking” and is believed to function to permit genes that existed in a transcription-competent state prior to entry into mitosis to be maintained in a form that can be rapidly reassembled into the active state in G1. Previous research in our laboratory found that the *Hspa1b* promoter was bookmarked during mitosis by the binding of heat shock transcription factor 2 (HSF2) to the heat shock element (HSE) of the *Hspa1b* promoter (Xing *et al.* 2005). Bookmarking *Hspa1b* during mitosis allows the rapid expression of this cytoprotective gene in early G1 if the cell encounters stress. Recently we extended those studies from tissue culture to spermatogenic cells and found that HSF1, HSF2, and SP1 are present in mature spermatozoa and bound to the *Hspa1b* promoter (Wilkerson *et al.* 2008). Other reports have found that in mice HSF1 and HSF2 transcripts are present in preimplantation embryos as early as the one cell stage, while HSF1 and HSF2 proteins have been identified in 2 cell embryos (Christians *et al.* 1997). Interestingly, a heat-inducible DNA binding activity has been observed in one and two cell embryos (Mezger *et al.* 1994). Furthermore, the HSE promoter element, which is a promoter binding site for HSF1 and HSF2, plays a role in expression of *Hspa1b* during the early stages of embryogenesis (Christians *et al.* 1997).

In this report we show that RNA Polymerase II is present in mature spermatozoa and bound to the *Hspa1b* promoter. This observation, in addition to our previous report showing that HSF1, HSF2, and SP1 are also bound to the *Hspa1b* promoter in mature spermatozoa clearly supports a unique scenario where the promoter of this particular gene is maintained in a form that is ready for rapid activation in an environment where most transcription has ceased (Monesi 1964; Monesi *et al.* 1978; Zheng *et al.* 2008), chromatin has been reorganized and highly compacted (Ward & Coffey 1991), and numerous basal transcription factors, transcriptional regulators, and architectural factors are displaced from chromatin (Zheng *et al.* 2008). These results suggest a mechanism by which *Hspa1b* could be expressed during the earliest stages of embryogenesis including expression in the male pronucleus of the one-cell embryo.

RESULTS

RNA Polymerase II is Present in Mouse Sperm Nuclei

Our previous observations showed that HSF1, HSF2, and SP1 are bound to the *Hspa1b* gene in mature sperm. These results suggested the intriguing possibility that RNA Polymerase II may also remain associated with this promoter in mature spermatozoa. As a first test of this

hypothesis, we sought to determine if RNA Polymerase II was present in mature spermatozoa using western blot analysis. We isolated and purified mature spermatozoa from the caudal epididymus of adult CD-1 mice. To minimize any extranuclear protein contamination, the purified sperm were treated with the detergent CTAB in conjunction with a 3–5 second sonication on ice to further disrupt the sperm membrane and facilitate tail removal. Light microscopy was used to confirm the removal of sperm tails and purity of the samples which was determined to be greater than 98% (data not shown). Protein extracts were prepared from sperm nuclei and a mouse C2C12 cell line (as a control) and assayed by western blot using monoclonal antibodies against the largest subunit (Rpb1) of RNA Polymerase II (Figure 1). The images presented in Figure 1 are from the same gel, only separated by 2 empty lanes to eliminate the possibility of cross-contamination. The results are representative of the banding pattern observed from western blot analysis of two independent mouse sperm protein isolations, and indicate that RNA Polymerase II is present in mature spermatozoa. In both the sperm lane (left image) and C2C12 lane (right image) we observed one predominant band migrating at approximately 220kDa that is consistent with the size of RNA Polymerase II detected in previous studies (Luthi-Carter *et al.* 2002;Wilkerson *et al.* 2007).

Chromatin Immunoprecipitation Analysis of RNA Polymerase II in Mature Sperm

In light of our western blot results shown in Figure 1, we hypothesized that RNA Polymerase II, a factor necessary for transcription, could also be bound to the *Hspa1b* promoter in mature spermatozoa as part of a mechanism for allowing the preferential expression of *Hspa1b* in the male pronucleus of the one-cell embryo. To test this hypothesis, we performed chromatin immunoprecipitation assays on spermatozoa obtained from the caudal region of mouse epididymides using monoclonal antibodies against RNA Polymerase II and mouse IgG as a negative control antibody (Figure 2). We assayed the binding of RNA Polymerase II to the promoter of the *Hspa1b* gene as well as the *β-globin (Hbb-b1)* promoter, a negative control gene that is not expressed in spermatogenic cells and lacks a recognizable HSE promoter element. To ensure that the observed binding was not due to residual occupancy on genes that were expressed earlier in spermatogenesis, we assayed the binding of RNA Polymerase II to the promoters of histone H4 (*Hist1h4b)*, transition protein 2 (*Tnp2*), and testis-specific histone H1 (*Hist1h1t)*. DNA fragments precipitated by the indicated antibodies were assayed by quantitative real time PCR. The results indicate that binding of RNA Polymerase II to the *Hspa1b* promoter in mature spermatozoa is significantly higher than to the promoters of *Hbb-b1, Hist1h4b, Tnp2, and Hist1h1t.* There was no statistical difference ($P>0.05$) in the binding of RNA Polymerase II and the promoters of *Hbb-b1, Hist1h4b, Tnp2, and Hist1h1t*.

DISCUSSION

A wide range of species utilize members of the HSP70 family to promote cell survival through expression of stress-induced HSPs including HSPA1A and HSPA1B. Due to the potential role of HSPA1B in promoting embryonic viability during times of stress, we initiated studies to better understand the mechanisms regulating transcription of this gene. In this report we show that RNA Polymerase II is present in mature spermatozoa and bound to the *Hspa1b* promoter.

Recently, we found that that HSF1, HSF2, and SP1 are bound to the *Hspa1b* promoter in mature spermatozoa (Wilkerson *et al.* 2008). The presence of these transcription factors on the *Hspa1b* promoter in sperm chromatin is surprising given the fact that transcription has ceased and the chromatin has been reorganized and compacted to a level that is approximately 6-fold more compact than chromatin found in mitotic cells (Ward & Coffey 1991). It is generally thought that transcriptional silence in mature sperm is due to the high level of sperm chromatin compaction which likely excludes most sequence-specific transcription factors from binding. Due to this high level of compaction, it is unlikely that HSF1, HSF2, and SP1 are actively

binding to the *Hspa1b* promoter at the later stages of spermiogenesis. Our hypothesis is that these factors are binding prior to, or during the condensation phase of spermatogenesis and remains bound to the *Hspa1b* promoter even after fertilization. The presence of RNA Polymerase II on the *Hspa1b* promoter provides an interesting mechanism by which the *Hspa1b* gene could be rapidly and preferentially expressed during the earliest stages of embryogenesis including the male pronucleus. With the opportunistic and essentially unregulated transcription in the one-cell embryo (Choo *et al.* 2002; Ma *et al.* 2001), the presence of HSF1, HSF2, SP1, and RNA Polymerase II on the *Hspa1b* promoter would allow the rapid transcription of this gene versus other genes that are not bound by transcription factors. In support of this hypothesis, a previous study showed that when first round replication is blocked by aphidicolin there was an increase in expression of a select number of genes including *Hspa1b* (Sonehara *et al.* 2008). It is thought that the first round of replication disrupts the compacted chromatin and allows transcription factors to assemble on promoter regions of genes (Wolffe 1991; Wolffe 1991). The transcriptional activity of *Hspa1b* prior to the first round of replication supports our hypothesis that the *Hspa1b* promoter is already poised for transcription. The observed drop in expression of *Hspa1b* following the first round of replication (Sonehara *et al.* 2008) could be due to the removal of transcription factors that were present prior to replication.

The observation that RNA Polymerase II, a factor essential for transcription, can remain bound to specific regions of DNA after compaction is unique, although not exclusive to mature spermatozoa. Previously, we observed in Jurkat cells blocked in mitosis that the binding of RNA Polymerase II to a number of HSE containing promoters was reduced, but not completely eliminated (Wilkerson *et al.* 2007). There was clear and reproducible binding of RNA Polymerase II to the promoters of *Hsp90aa1 (Hsp90), fos, and Hspb1 (Hsp27)* in mitotic cells.

Our findings, shown schematically in Figure 3, indicate that in mature mouse spermatozoa the *Hspa1b* promoter is bound by a number of transcription factors important for expression of this gene. The regulatory region of the inducible HSP70 is 85% homologous between human and mouse sequences. The two heat shock elements (HSEs) are known binding sites for HSF1 and HSF2. Although HSF1 and HSF2 bind DNA as trimers and are capable of forming heterotrimers (Ostling *et al.* 2007;Sandqvist *et al.* 2009), it is not clear if these factors bind the *Hspa1b* promoter in mature spermatozoa as homotrimers or heterotrimers. HSF1 and HSF2 are highly homologous with their DNA binding domains being 72% identical (Schuetz *et al.* 1991). There is also a report suggesting an interplay between HSF1 and HSF2 (Ostling *et al.* 2007). The GC box is a consensus binding site for SP1, a transcription factor that is necessary for transcription of the *Hspa1b* gene. Our previous result showing that SP1 is bound to this GC box in mature spermatozoa suggests that SP1 has a role in transcription of *Hspa1b* in the one-cell embryo. Interestingly, a previous report has shown that SP1 is required for transcription of *Hspa1b* in the two-cell embryo (Bevilacqua *et al.* 2000). The mouse *Hspa1b* promoter contains a TATA box located at −30 which helps serve as the site for assembly of the pre-initiation complex (PIC) which most likely occurs prior to, or during the condensation phase of spermatogenesis. Based on the location of the primers used for the ChIP assays, our results suggest that RNA Polymerase II is binding at, or near the transcription start site. The culmination of our findings suggests that *Hspa1b* is in a state of transcriptional readiness in mature spermatozoa and ultimately the male pronucleus.

In mice there are two known heat inducible HSP70 proteins, HSP70.1 (HSPA1A) and HSP70.3 (HSPA1B), with HSPA1B the focus of the studies presented here. The *Hspa1a* and *Hspa1b* genes are found in a tandem array on mouse chromosome 17 (Hunt *et al.* 1993) and the resulting proteins differ at only two residues, rendering them nearly indistinguishable at the protein level. However, due to differences between these two genes at the nucleic acid level, they can be readily distinguished. The amino acid and gene sequences, and expression pattern of *Hspa1a*

and *Hspa1b* are highly conserved between rodents and humans (Milner & Campbell 1990). Studies using antisense oligonucleotides showed that reduced levels of *Hspa1a* and *Hspa1b* disrupted preimplantation embryogenesis and increased embryonic sensitivity to arsenic (Dix *et al.* 1998). Another study, using knockout mice lacking both *Hspa1a* and *Hspa1b* (*Hsp70.1/3*$^{-/-}$), suggested that these factors were not necessary for embryogenesis (Hunt *et al.* 2004). However, the KO mice were lighter in weight than wild type mice and cells derived from these KO mice displayed genomic instability that was enhanced by heat stress, underwent senescence sooner, and displayed a higher frequency of chromosomal aberrations and a significant increase in aberrant spermatocytes following heat stress. Considering these observations, it is possible that an accumulation of these genetic defects in later generations could lead to significant developmental problems.

Due to the high degree of conservation and likely redundant function, *Hspa1a* and *Hspa1b* are often referred to collectively as stress induced HSP70. Although the studies presented here focus on mouse *Hspa1b*, due to the high degree of similarity it is likely that the promoter of *Hspa1a* is also poised for transcriptional activation during the earliest stages of embryogenesis through mechanisms identified for Hspa1b.

There exist a number of epigenetic mechanisms by which paternal DNA patterns of expression are passed to daughter cells including DNA methylation (reviewed in (Suzuki & Bird 2008)) and occupancy and/or post-translational modifications of histones and other factors (reviewed in (Corpet & Almouzni 2008)). This list could be expanded to include non-coding RNA molecules (reviewed in (Mattick & Makunin 2006)). In the unique case of *Hspa1b and most likely Hspa1a*, our research suggests that the binding of HSF2, and possibly HSF1, to the HSE promoter element maintains the promoter in an “accessible” chromatin confirmation throughout mitosis (Xing *et al.* 2005) and spermiogenesis (Wilkerson *et al.* 2008).

Mature spermatozoa have traditionally be thought of as merely inert cells with the sole function of delivering paternal DNA to the ovum. However, an ever-increasing number of studies suggest a functional organization exists in sperm chromatin. In spermatozoa, protamines serve as the major DNA binding proteins around which chromatin is organized and compacted (reviewed in (Balhorn 2007)). Research has shown that insufficient levels of protamine leads to male infertility with sperm displaying both structural and functional defects (Cho *et al.* 2001). It seems that other DNA-interacting proteins can contribute to sperm chromatin organization. In humans for example, approximately 15% of sperm chromatin is bound by histones with the remaining 85% bound by protamines (Gatewood *et al.* 1990). The nucleohistone fraction displays hypersensitivity to nuclease cleavage suggesting less DNA compaction (Wykes & Krawetz 2003). It is thought that certain genes that may be important during early embryogenesis could be bound in this nucleohistone portion of chromatin or be directed for expression through other epigenetic mechanisms. In support of this hypothesis, a study found that the postnatally expressed *Hbb-b1* gene was found in the protamine bound DNA fraction while the embryonically expressed *IGF-2* gene was found in the histone bound DNA fraction (Wykes & Krawetz 2003). In addition to chromatin binding factors, it is possible that nuclear matrix attachment regions (MARs) may play a role in organizing sperm chromatin (Martins *et al.* 2004). An intact sperm nuclear matrix appears to be critical to the formation of the male pronucleus (Ward *et al.* 1999).

Although the results presented here are focused on mechanisms that could lead to expression of stress-induced HSP70 expression during the early stages of embryogenesis, there appears to be a role for inducible HSP70 in mature spermatozoa. Previous studies have shown that HSPA1A localizes to the sperm tail and the equatorial region of the sperm head in human spermatozoa (Mitchell *et al.* 2007) and in porcine spermatozoa localization was found in the equatorial region (Spinaci *et al.* 2005). The equatorial region of the sperm head suggests a role

in sperm-oocyte fusion. When porcine spermatozoa are incubated with anti-HSP70 antibodies, there is a marked reduction in fertilization which is likely due to a disruption in sperm-ooycte fusion (Spinaci *et al.* 2005). It is also thought that HSPA1A has a role in human fertility based on a study showing that the presence of antibodies against HSPA1A in seminal plasma inhibits fertilization (Bohring & Krause 2003). Another study implicated reduced expression of HSPA1A in certain subtypes of male infertility (Cedenho *et al.* 2006).

Based on the important cytoprotective role of HSPA1B, bookmarking the *Hspa1b* promoter would provide a mechanism by which *Hspa1b* could be expressed during the earliest stages of embryogenesis in the event of cellular stress. Bookmarking the *Hspa1b* gene in spermatozoa makes particular sense given the need for molecular chaperones to handle the large number of proteins that are translated during embryogenesis and the various stresses that can occur, including osmotic and pH changes. The ability to rapidly express the HSPA1B protein would clearly promote embryonic viability.

MATERIALS AND METHODS

Animals

All CD-1 mice used in this study were adult males (at least 8–10 weeks of age) obtained from Harlan (Indianapolis, IN). Animals were maintained in the Division of Laboratory Animal Resources (DLAR) and studies performed according to approved Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Kentucky (Lexington, KY).

Preparation of Sperm Nuclei

Sperm nuclei were prepared as previously described (Wilkerson *et al.* 2008). Briefly, mature sperm was obtained from the caudal epididymides of adult male CD-1 mice by repeatedly puncturing with a 20G needle. Sperm were then gently flushed from the caudal epididymides using phosphate buffered saline (PBS). Sperm were pelleted, quickly frozen in liquid nitrogen, and then resuspended in cold PBS to lyse any red blood cells present. Centrifugation of the Percoll (Sigma Chemical Co., USA) gradient was performed according to established protocols (Hishinuma & Sekine 2004) with the following modifications. Sperm, washed three times in cold PBS then resuspended in 1mL of PBS, were loaded on a discontinuous Percoll gradient created by layering 1.5mL of 35%, 45%, 75% Percoll (v/v) in a 15mL conical tube. Centrifugation was performed at 700xg for 30 minutes. Sperm nuclei were prepared using cetyltrimethylammonium bromide (CTAB) according to published protocols (Balhorn *et al.* 1977) with minor modifications (Maione *et al.* 1997). Immediately prior to the CTAB incubation, the sperm suspension was sonicated for 3–5 sec at 20% power to dissolve clumps and assist in tail removal. Following CTAB incubation, the sperm suspension was passed through siliconized glass wool to remove any remaining tail debris.

Western Blots

Protein extracts were prepared from sperm nuclei using buffer C (20mM Hepes pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT) followed by brief sonication on ice. Protein extracts were separated on a 8% SDS polyacrylamide gel and western blots were performed using mouse monoclonal antibodies against the largest subunit (Rpb1) of RNA Polymerase II (Covance) according to our established protocols (Hilgarth *et al.* 2003; Lubert & Sarge 2003; Xing *et al.* 2004). The secondary antibody, α-IgG-HRP, was obtained from an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Arlington Heights, IL). Samples for Western blots were prepared from two different groups of animals, with at least two animals used for each preparation.

Chromatin Immunoprecipitations

All chromatin immunoprecipitations (ChIP) were performed as previously published (Wilkerson *et al.* 2008; Worrad *et al.* 1994) with the following modifications. ChIP assays were performed at least twice with each batch of DNA. Pre-cleared chromatin was incubated with 5μg of mouse monoclonal RNA Polymerase II or control mouse IgG (Sigma), and rotated at 4°C for 16 hours. DNA was purified using a QIAquick PCR Purification Kit (Qiagen Inc.) and eluted in 50μl of 10mM Tris (pH 8.5). Immunoprecipitated DNA and input samples obtained prior to immunoprecipitation were analyzed by quantitative real time PCR with a Stratagene Mx 4000 system using Brilliant SYBR Green QPCR master mix (Stratagene) and the following primers (shown 5′ to 3′) from Integrated DNA Technologies (Coralville, IA):

Hspa1b: (+) CCGCAACAGTGTCAATAGC, (−) CCTTGAGTAATCGGAGTTGTGG

Hbb-b1: (+) TTGCTCCTCACATTTGCTTCTG, (−) ACTTCATCGGAGTTCACCTTTC

Hist1h4b: (+) ACGAAGCCCGCCATC, (−) TTGGCGTGCTCCGTGTAGGT

Hist1h1t: (+) GCAGTGAGCAGATATGCAAGA, (−) CCAACAGTGATGGGGTAGTG

Tnp2: (+) GATGTCACCGTGAGAAGGGTACAA, (−) ACAAAGCCCTGCCTCTCATCTTCA

The sequence used for the upstream *Hspa1b* primer differs at 4 of 19 nucleotides from the sequence of *Hspa1a*, including the 3′ terminal nucleotide of the primer. This sequence difference, under the stringency conditions employed during PCR amplification, would be expected to discriminate *Hspa1b* from *Hspa1a*. Samples were checked for specific amplification using dissociation curves analysis software. PCR products were also assayed on polyacrylamide gels with ethidium bromide staining to ensure they were of the expected size (data not shown). The Ct values were normalized to input DNA (DNA before immunoprecipitation step) and IgG controls using the formula 2^[(Ct IgG-Ct Input)−(Ct Ab-Ct Input)] (where Ab=RNA Polymerase II, or IgG). Data is represented as fold differences relative to IgG. The data shown represent quantitative PCR results from three independent sperm purifications and ChIP assays. Error bars represent standard error of the means. Statistical significance was determined using a two-tailed, unpaired t-test.

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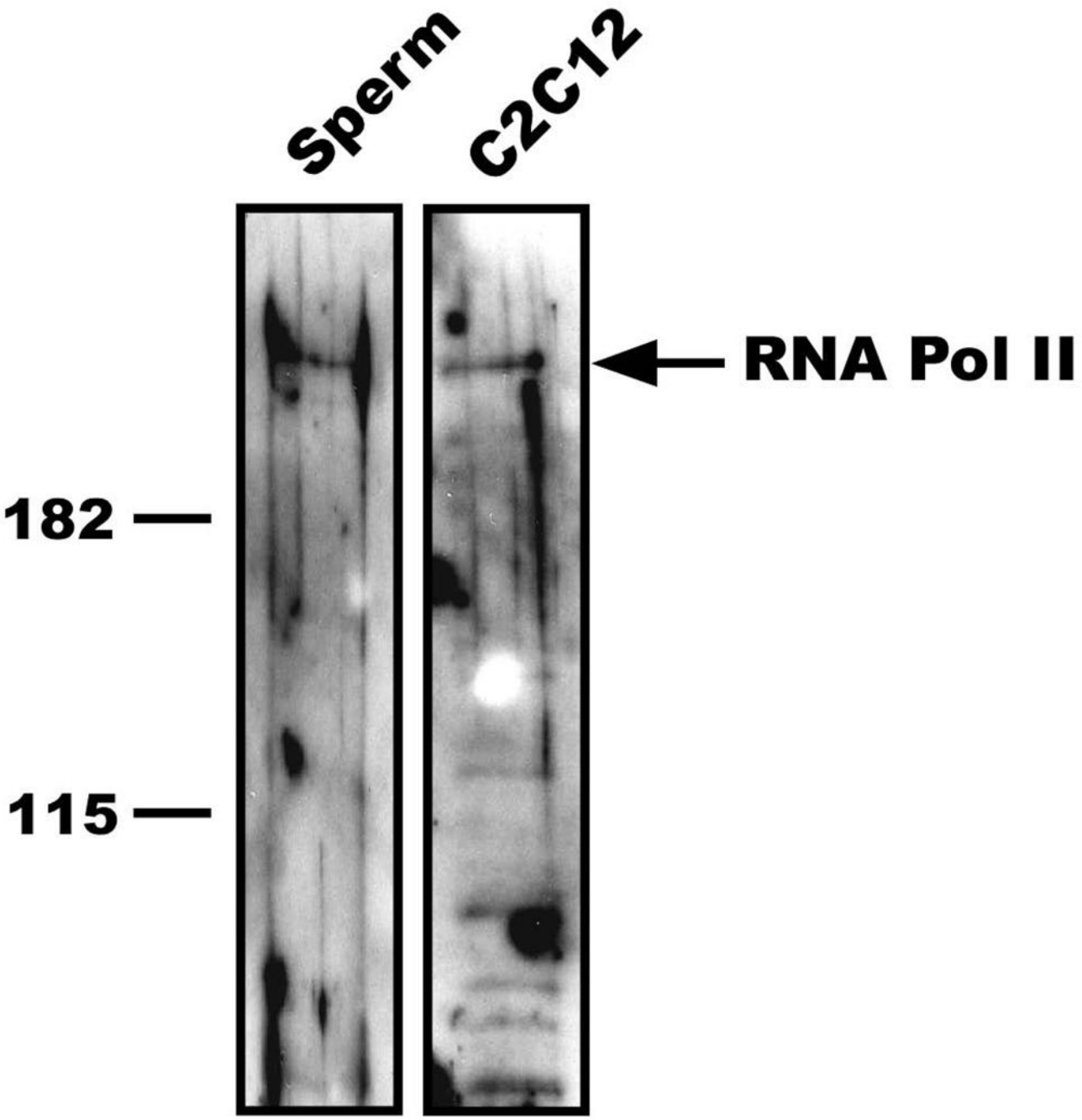

Figure 1. RNA Polymerase II is present in mouse sperm nuclei

Protein extracts prepared from mouse sperm nuclei or a mouse C2C12 cell line (as a control) were separated on a 8% SDS polyacrylamide gel and assayed by western blot using mouse monoclonal antibodies against the largest subunit (Rpb1) of RNA Polymerase II (Covance). Prestained protein markers were used to indicate molecular weight (kDa). Images shown are from the same gel but separated by 2 empty lanes to eliminate cross contamination. Images are representative of results obtained from extracts prepared from two different groups of mice (two mice per group).

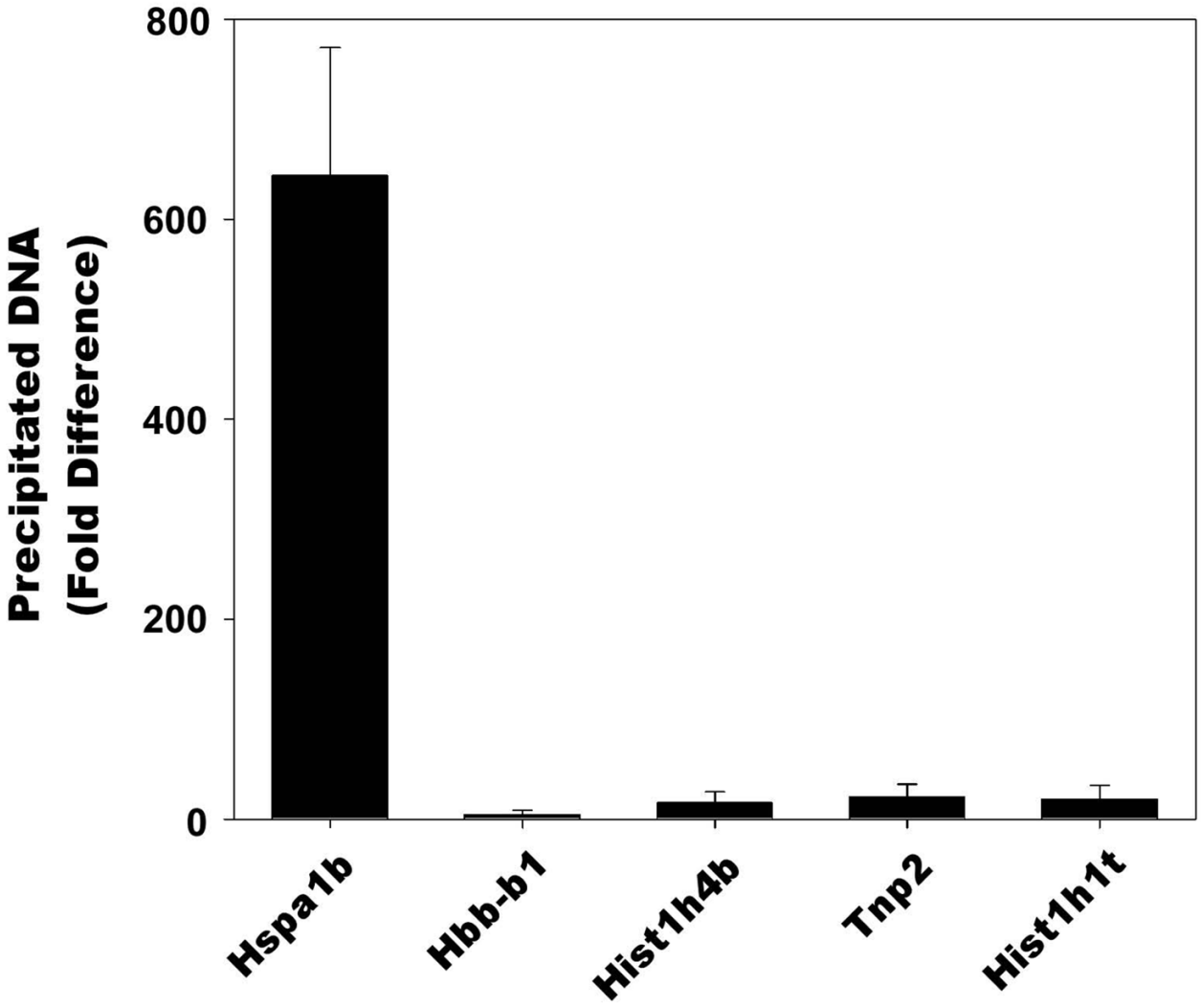

Figure 2. Chromatin IP analysis of RNA Polymerase II binding in mature spermatozoa

Purified mouse cauda epididymal spermatozoa were crosslinked, lysed, sonicated, and subjected to immunoprecipitation using mouse monoclonal antibodies against the largest subunit of RNA Polymerase II or mouse IgG as a negative control antibody. Data is represented as fold difference relative to IgG. The precipitated DNA fragments were subjected to quantitative PCR using primers that amplified the proximal promoters of the Hspa1b, Hbb-b1, Hist1h4b, Tnp2, and Hist1h1t genes.

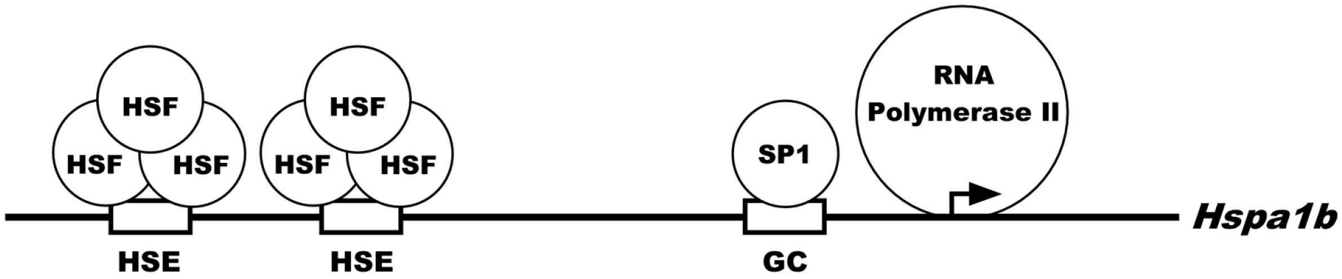

Figure 3. In vivo Promoter Occupancy of the Hspa1b gene in Mature Spermatozoa

In mature spermatozoa, the promoter of the mouse *Hspa1b* gene is bound by a number of transcription factors known to be important for expression of this gene. There are two heat shock elements (HSEs) located at −140 and −110 that are known binding sites for HSF1 and HSF2 and a GC box located at −50 that is bound by SP1. RNA Polymerase II is also bound to the proximal promoter region of *Hspa1b* and further demonstrates the transcriptional readiness of this gene.