BIOLOGY OF REPRODUCTION **79,** 283–288 (2008)
Published online before print 23 April 2008.
DOI 10.1095/biolreprod.107.066241

Interaction of HSF1 and HSF2 with the *Hspa1b* Promoter in Mouse Epididymal Spermatozoa[1]

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ABSTRACT

The *Hspa1b* gene is one of the first genes expressed after fertilization, with expression observed in the male pronucleus as early as the one-cell stage of embryogenesis. This expression can occur in the absence of stress and is initiated during the minor zygotic genome activation. There is a significant reduction in the number of embryos developing to the blastocyte stage when HSPA1B levels are depleted, which supports the importance of this protein for embryonic viability. However, the mechanism responsible for allowing expression of *Hspa1b* during the minor zygotic genome activation (ZGA) is unknown. In this report, we investigated the role of HSF1 and HSF2 in bookmarking *Hspa1b* during late spermatogenesis. Western blot results show that both HSF1 and HSF2 are present in epididymal spermatozoa, and immunofluorescence analysis revealed that some of the HSF1 and HSF2 proteins in these cells overlap the 4′,6′-diamidino-2-phenylindole-stained DNA region. Results from chromatin immunoprecipitation assays showed that HSF1, HSF2, and SP1 are bound to the *Hspa1b* promoter in epididymal spermatozoa. Furthermore, we observed an increase in HSF2 binding to the *Hspa1b* promoter in late spermatids versus early spermatids, suggesting a likely period during spermatogenesis when transcription factor binding could occur. These results support a model in which the binding of HSF1, HSF2, and SP1 to the promoter of *Hspa1b* would allow the rapid formation of a transcription-competent state during the minor ZGA, thereby allowing *Hspa1b* expression.

bookmarking, embryogenesis, epididymis, gene regulation, HSF1, HSF2, Hsp70, Hspa1b, *sperm, spermatogenesis, testis*

INTRODUCTION

In the mammalian embryo, a transition takes place such that maternal control of development is shifted to the zygote in a process termed the zygotic genome activation (ZGA) (reviewed in [1]). 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 [2–7], with more stringent transcriptional regulation [8–11]. During the minor ZGA, transcription in the one-cell embryo appears to be relatively promiscuous and opportunistic [12, 13], with the majority of transcription occurring in the male pronucleus [14, 15].

The *Hspa1b* gene is one of the first genes expressed following fertilization, with expression taking place in the absence of stress as early as the one-cell stage of embryogenesis [16, 17]. The importance of *Hspa1b* during embryogenesis is demonstrated by immunodepletion experiments using HSPA1B antibodies [18]. Those studies demonstrated that reduced levels of HSPA1B lead to a significant reduction in embryos developing to the blastocyte stage. However, despite the importance of HSPA1B for embryonic viability, the mechanism responsible for allowing 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 *Myc* genes, remain uncompacted and accessible during mitosis [19–23]. 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. Recently we have found that in somatic cells the *Hspa1b* gene is bookmarked during mitosis by the binding of heat shock factor 2 (HSF2) to the heat shock element (HSE) of the *Hspa1b* promoter [24]. Bookmarking *Hspa1b* during mitosis allows the rapid expression of this cytoprotective gene in early G1 if the cell encounters stress. Relevant to our study, it has been reported that mice lacking HSF2 display increased embryonic lethality, indicating the importance of this factor for embryogenesis [25].

Heat shock factor 1 (HSF1) is a protein that also binds to the HSE of the *Hspa1b* promoter during cellular stress and induces expression of *Hspa1b* (reviewed in [26]). It has been reported that HSF2 interacts with HSF1 [27–29], suggesting the possibility that these two DNA-binding proteins could both be involved in mediating gene bookmarking and facilitating expression of *Hspa1b.* In addition, expression of *Hspa1b* during the earliest stages of embryogenesis is HSF1-dependent, although stress is not required [17, 30]. HSF1 is important for embryogenesis since mouse embryos in mothers lacking HSF1 are unable to develop beyond the zygotic stage and exhibit increased embryonic lethality [31–33].

Based on these reports, we hypothesized that HSF1 and HSF2 could be involved in expression of *Hspa1b* in the male pronucleus of the one-cell embryo. Here we show that HSF1, HSF2, and SP1 are bound to the *Hspa1b* promoter in mature spermatozoa, which is unusual since transcription has ceased [34–36], chromatin has been reorganized and highly compacted [37], and numerous basal transcription factors, transcriptional regulators, and architectural factors are displaced from chromatin by the point of step 10 spermatids [36]. Considering our previous finding that HSF2 can bookmark the *Hspa1b* gene in somatic cells, the results presented here suggest a mechanism by which *Hspa1b* could be expressed in the male pronucleus of the one-cell embryo.

[1]Supported by a Lalor Foundation postdoctoral grant and a National Institutes of Health (NIH) HD grant (F32HD050043) to D.C.W and NIH grants GM61053 and GM64606 to K.D.S.
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Received: 22 October 2007.
First decision: 25 November 2007.
Accepted: 11 April 2008.

ISSN: 0006-3363. http://www.biolreprod.org

MATERIALS AND METHODS

Animals

All CD-1 mice used in this study were adult males obtained from Harlan (Indianapolis, IN). Animals were maintained in the Division of Laboratory Animal Resources, and studies were performed according to approved Institutional Animal Care and Use Committee guidelines at the University of Kentucky (Lexington, KY) and Louisiana State University (Shreveport, LA).

Generation of Antibodies Against HSF1 and HSF2

Affinity-purified goat polyclonal antibodies to HSF1 and HSF2 were prepared by Bethyl Laboratories (Montgomery, TX). These antibodies were raised against the synthetic peptides TISLLTGSEPPKAKDPTVS and YLCELAPAPLDSDMPLLDS, which correspond to the C-terminal sequences of the mouse HSF1 and HSF2 polypeptides (which are identical to the C-terminal sequences of human HSF1 and HSF2).

Preparation of Sperm Nuclei and Spermatids

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 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.) gradient was performed according to established protocols [38] with the following modifications. Sperm, washed three times in cold PBS then resuspended in 1 ml of PBS, were loaded on a discontinuous Percoll gradient created by layering 1.5 ml of 35%, 45%, and 75% Percoll in a 15-ml conical tube. Centrifugation was performed at 700 × *g* for 30 min. Purity was confirmed via bright field microscopy (Supplemental Fig. 1 available at www.biolreprod.org). Sperm nuclei were prepared using cetyltrimethylammonium bromide (CTAB) according to published protocols [39] with minor modifications [40]. 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. Enriched populations of mouse early and late spermatids were obtained by centrifugal elutriation performed in the laboratory of Dr. Sidney Grimes (Louisiana State University) according to established protocols [41, 42]. Enriched cell types were cross-linked with 2% paraformaldehyde, quenched with 1× glycine, washed three times with cold 1× PBS, then frozen on dry ice and shipped to us for chromatin immunoprecipitation (ChIP) analysis.

Western Blots

Protein extracts were prepared from sperm nuclei using buffer C (20 mM Hepes [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM dithiothreitol) followed by brief sonication on ice. Protein extracts were separated on an 8% SDS polyacrylamide gel, and Western blots were performed using goat polyclonal antibodies against HSF1 or HSF2 (described above) according to our established protocols [43–45]. The secondary antibody, α-IgG-horseradish peroxidase, was obtained from an enhanced chemiluminescence kit (Amersham Life Science, Arlington Heights, IL). Samples for Western blots were prepared from at least two different groups of animals, with at least two animals used for each preparation.

Immunofluorescence

Immunofluorescence was performed with goat HSF1 and HSF2 antibodies as well as rabbit HSF1 and HSF2 antibodies according to established protocols in our laboratory [46]. Briefly, nuclei from mouse epididymal spermatozoa were isolated and purified as previously discussed. Purity was confirmed via bright field microscopy. Purified sperm nuclei resuspended in PBS were allowed to adhere to poly-L-lysine (50 μg/ml)-coated coverslips for 15 min, fixed with 4% paraformaldehyde, and permeabilized using 0.5% Triton-X 100/0.5% saponin. Nuclei were probed using goat polyclonal antibodies against HSF1 or HSF2 and rabbit polyclonal antibodies against HSF1 or HSF2. Blots were then incubated with AlexaFluor 488 (Invitrogen)-linked secondary antibodies to visualize HSF1 or HSF2. DNA was visualized using 4′,6′-diamidino-2-phenylindole (DAPI), and images were taken with a Nikon fluorescent microscope with a 100× oil immersion objective and a Nikon spotcam digital imaging camera. Isolation, purification, and immunofluorescence detection were performed twice with each antibody.

Chromatin Immunoprecipitations

All ChIPs were performed at least three times according to established protocols in our laboratory [24] with the following modifications. Precleared chromatin was incubated with 3 μg of goat polyclonal HSF1, goat polyclonal HSF2, or rabbit SP1 antibodies (Santa Cruz Biotechnology, a generous gift from Dr. Dan Noonan) or species-matched control IgG (Sigma) and rotated at 4°C for 16 h. DNA was purified using a QIAquick PCR Purification Kit (Qiagen Inc.) and eluted in 50 μl of 10 mM 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-bl: (+) TTGCTCCTCACATTTGCTTCTG, (–) ACTTCATCGGAGTTCACCTTTC

Hist1h4b: (+) ACGAAGCCCGCCATC, (–) TTGGCGTGCTCCGTGTAGGT

Hist1hlt: (+) GCAGTGAGCAGATATGCAAGA, (–) CCAACAGTGATGGGGTAGTG

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. 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 = HSF1, HSF2, SP1, or IgG). Data is represented as fold differences relative to IgG, which was set to 1. The data shown represent quantitative PCR results from at least three independent sperm purifications and ChIP assays. Error bars represent SEM. Statistical significance was determined using a two-tailed, unpaired *t*-test.

RESULTS

HSF1 and HSF2 Are Present in Mouse Sperm Nuclei

Based on our hypothesis that HSF2, and possibly HSF1, could function in bookmarking the *Hspa1b* gene in mature sperm, we assayed for the presence of these factors in mature spermatozoa. To test this we isolated caudal epididymal spermatozoa from adult CD-1 mice and purified the sperm by centrifugation using a Percoll gradient. To minimize any extranuclear protein contamination, the purified sperm were treated with the detergent CTAB in conjunction with a 3- to 5-sec sonication on ice to further disrupt the sperm membrane and facilitate tail removal. Cells were visualized by light microscopy to confirm the complete removal of tails and to confirm the purity of the samples (Supplemental Fig. 1). Protein extracts were prepared from sperm nuclei and assayed by Western blots using antibodies against HSF1 and HSF2 (Fig. 1). The results presented in Figure 1 are representative of the banding patterns observed from two independent mouse sperm protein isolations and Western blots and indicates that both HSF1 and HSF2 are present in mature spermatozoa. We observed two bands present in the HSF1 blot (top panel) migrating at approximately 80 kDa that likely represent the α- and β-splice variants of this protein and/or different phosphorylation states [47, 48]. The single band observed for HSF2 (bottom panel) migrated at approximately 70 kDa and is consistent with previous reports [48].

Localization of HSF1 and HSF2 in Sperm Nuclei

Based on our Western blot results we wanted to determine specifically where HSF1 and HSF2 were localized in mature spermatozoa and whether they exhibit any colocalization with DNA. Sperm nuclei were prepared as described for Western blots to minimize any extranuclear contamination. Nuclei were allowed to adhere to poly-L-lysine-coated coverslips for 15 min at room temperature. Immunofluorescence analysis was then performed using goat HSF1 or HSF2 polyclonal antibodies followed by incubation with AlexaFluor 488-linked secondary

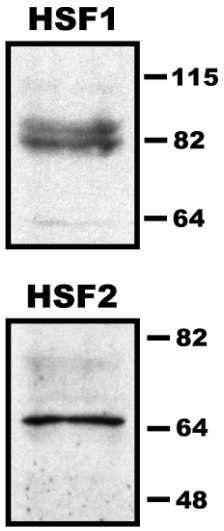

FIG. 1. HSF1 and HSF2 are present in mouse sperm nuclei. Protein extracts prepared from mouse sperm nuclei were separated on an 8% SDS polyacrylamide gel and assayed by Western blot using goat polyclonal antibodies against HSF1 (top panel) or HSF2 (bottom panel). Prestained protein markers were used to indicate molecular mass (kDa). Images are representative of results obtained from extracts prepared from at least two different sets of animals (at least two animals per group).

antibodies (Fig. 2, A and B, middle panels). DAPI staining was used to visualize DNA (left panels). In the merged images (right panels), HSF1 and HSF2 were observed within the DNA-containing region of mature spermatozoa. In addition to staining in the nuclear periphery, we consistently observed punctate staining of HSF1 and HSF2 overlapping the DNA staining. To further confirm the colocalization of HSF1 and HSF2 with DAPI-stained DNA, we repeated the sperm purification and immunofluorescence as described above but used different, previously characterized HSF1 and HSF2 rabbit polyclonal antibodies (Fig. 2, C and D, respectively). The results shown in Figure 2 are representative images from multiple sperm purifications and visualizations. The detection of HSF1 and HSF2 using goat polyclonal antibodies appeared to display slightly brighter punctate labeling compared to the rabbit HSF1 and HSF2 polyclonal antibodies, but the general labeling pattern of HSF1 and HSF2 is very similar.

ChIP Analysis of HSF1 and HSF2 in Mature Sperm

Western blots and immunofluorescence confirmed that HSF2, a factor known to bookmark the *Hspa1b* gene, and HSF1, a factor required for the stress-induced expression of *Hspa1b,* are both present in mature spermatozoa, and each factor colocalizes with DNA. Based on these results, we hypothesized that one or both of these factors could 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, we performed ChIP assays on caudal spermatozoa using polyclonal antibodies against HSF1, HSF2, and goat IgG as a negative control antibody (Fig. 3, A and B). We assayed the binding of HSF1 and HSF2 to the promoter of the *Hspa1b* gene as well as the promoters of the β-globin *Hbb-bl* and histone *Hist1h4b* genes (two negative control genes lacking recognizable HSE promoter elements). DNA fragments precipitated by the indicated antibodies were assayed by quantitative real-time PCR. The results indicate that binding of HSF1 and HSF2 to the *Hspa1b* promoter is statistically higher ($P < 0.01$) than to the *β-globin* and *Hist1h4b* gene promoters, indicating that these two HSFs are present on the *Hspa1b* promoter in the DNA of mature spermatozoa. The goat HSF1 and HSF2 antibodies specifically precipitate *Hspa1b*-containing DNA (black bars) compared with the goat IgG negative control, which displays a lack of specificity for *Hspa1b*-containing DNA (gray bars).

ChIP Analysis of SP1 in Mature Sperm

Based on previous results suggesting that the transcription factor SP1 is important for expression of *Hspa1b* in early-stage embryogenesis, we performed ChIP assays on caudal spermatozoa using rabbit polyclonal antibodies against SP1, and rabbit IgG as a negative control antibody (Fig. 4). We assayed the binding of SP1 to the promoter of the *Hspa1b* gene as well as the promoter of the testis-specific histone *Hist1hlt* gene using quantitative PCR. The *Hist1hlt* gene is expressed exclusively in mid- to late pachytene spermatocytes, with expression regulated in part by the binding of SP1 to the proximal promoter of *Hist1hlt*. We found that SP1 bound to the *Hspa1b* promoter approximately 12-fold more than to the *H1fnt* promoter. The specificity of the SP1 antibodies for precipitating *Hspa1b*-containing DNA (black bars) is demonstrated by

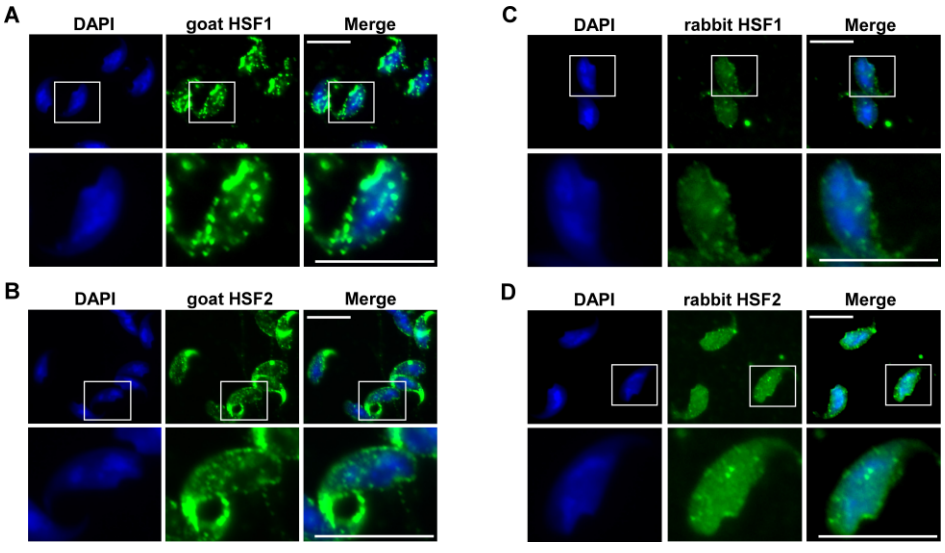

FIG. 2. Immunofluorescent localization of HSF1 and HSF2 in mouse sperm. Purified mouse sperm nuclei were allowed to adhere to poly-L-lysine-coated coverslips, fixed with paraformaldehyde, and permeabilized. Nuclei were probed using goat polyclonal antibodies against HSF1 (**A**, middle lane) or HSF2 (**B**, middle lane) and rabbit polyclonal antibodies against HSF1 (**C**, middle lane) or HSF2 (**D**, middle lane). Primary antibodies were detected with AlexaFluor 488-linked secondary antibodies to visualize HSF1 or HSF2. DNA was visualized using DAPI staining (left lanes), and images were taken with a Nikon fluorescent microscope with a 100× oil immersion objective and a Nikon spotcam digital imaging camera. White boxes highlight specific cells that are enlarged (below indicated image) to show further detail. Images shown are representative of the staining patterns observed from two independent experiments. Bars = 5 μm.

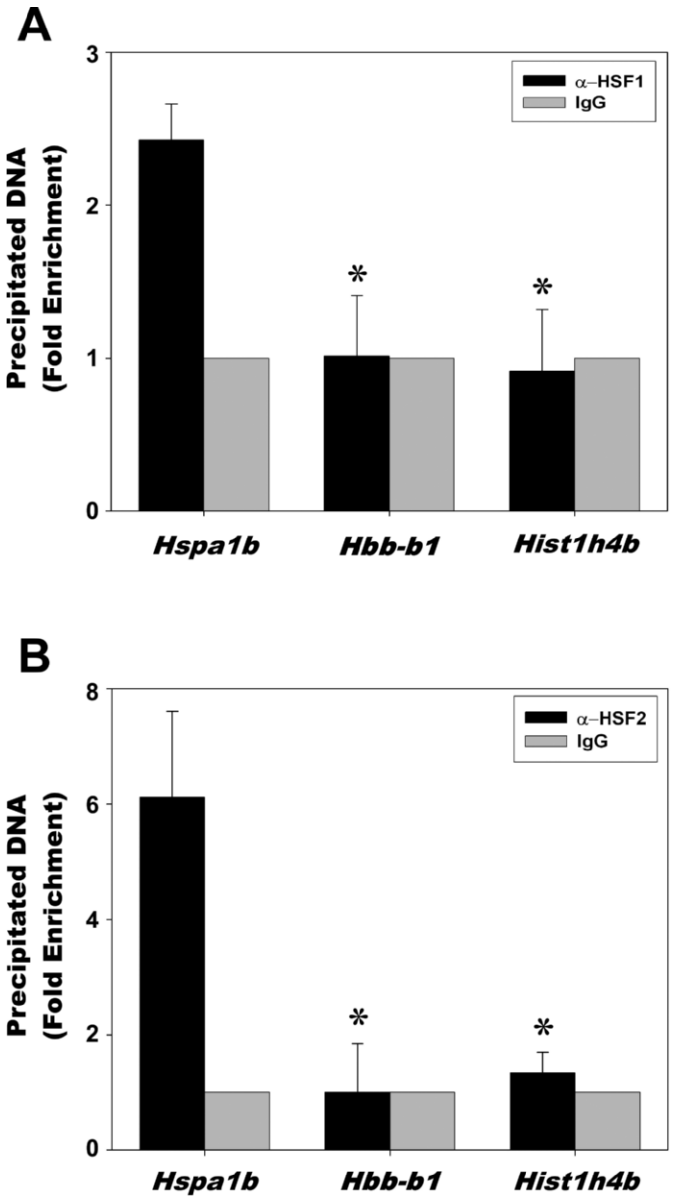

FIG. 3. ChIP analysis of HSF1 and HSF2 binding in mature spermatozoa. Purified mouse caudal epididymal spermatozoa were crosslinked, lysed, sonicated, and subjected to immunoprecipitation using polyclonal antibodies against HSF1 (**A**) or HSF2 (**B**). Goat IgG was used as a negative control antibody and set to =1 (no binding). The precipitated DNA fragments were subjected to quantitative PCR using primers that amplified the proximal promoters of the *Hspa1b,* β-globin *Hbb-b1,* and histone *Hist1h4b* genes. Asterisk (*) indicates a statistically significant difference ($P < 0.01$) between the *Hspa1b* and the controls *Hbb-b1* and *Hist1h4b*.

the lack of *Hspa1b*-containing DNA precipitated by rabbit IgG negative control (gray bars). The binding of SP1 to the *Hist1hlt* promoter was much lower than that observed for IgG; however, this was not due to any detectable problem associated with the amplification, since input DNA was amplified equally well using primers that recognize the *Hspa1b* promoter and *Hist1hlt* promoter (Supplemental Fig. 2).

ChIP Analysis of HSF2 in Spermatids

Once we determined that HSF1, HSF2, and SP1 could reside on the *Hspa1b* promoter in mature sperm, we reasoned that these factors were most likely not binding in sperm due to

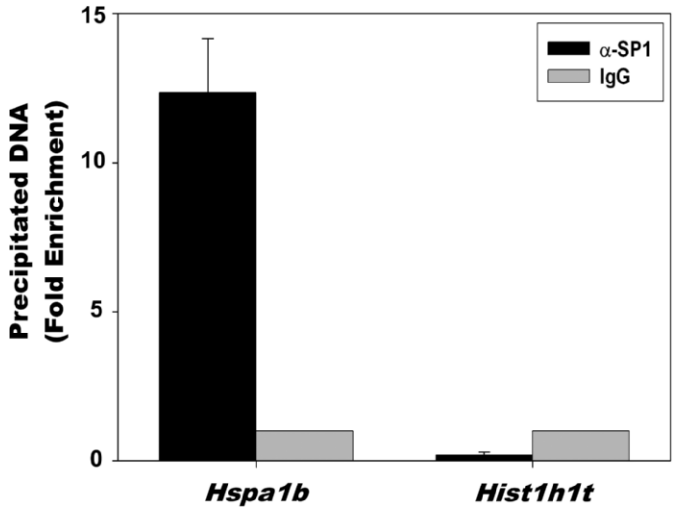

FIG. 4. ChIP analysis of SP1 binding in mature spermatozoa. Purified spermatozoa from mouse caudal epididymides were cross-linked, lysed, sonicated, and subjected to immunoprecipitation using polyclonal antibodies against SP1. Rabbit IgG was used as a negative control antibody and set to =1 (no binding). The precipitated DNA fragments were subjected to quantitative PCR using primers that amplified the proximal promoters of the *Hspa1b* or *Hist1h1t* genes. There was a statistically significant difference ($P < 0.001$) between the results for *Hspa1b* and *Hist1h1t.*

the high level of chromatin compaction. Therefore, we began investigating earlier spermatogenic cells, including early and late spermatids. Since HSF2 is the most likely factor contributing to the "open" chromatin conformation of *Hspa1b* in mature sperm, we performed ChIP assays in early and late spermatids using goat HSF2 antibodies (Fig. 5). From four independent ChIP assays, we consistently found that HSF2 bound to the *Hspa1b* promoter nearly 2.5-fold higher in late

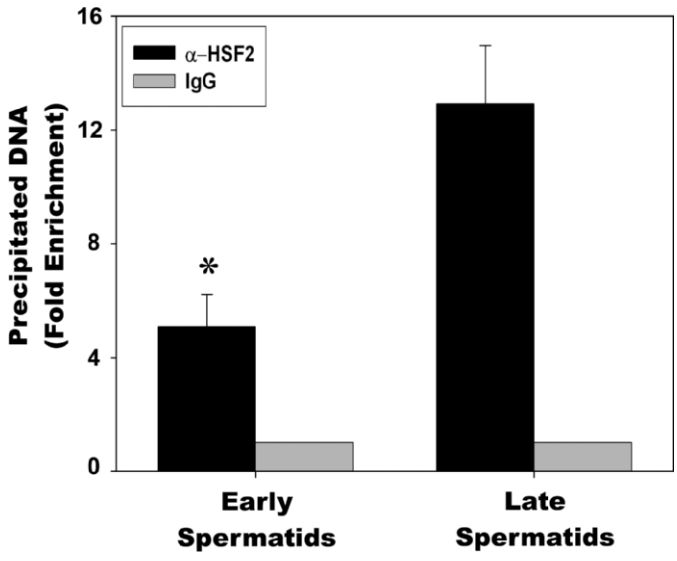

FIG. 5. ChIP analysis of HSF2 binding in spermatids. Early spermatids and late spermatids from mouse testes were isolated by centrifugal elutriation, cross-linked, sonicated, and subjected to immunoprecipitation using goat polyclonal antibodies against HSF2. Goat IgG was used as a negative control antibody and set to =1 (no binding). Precipitated DNA fragments were subjected to quantitative PCR using primers that amplified the proximal promoter of *Hspa1b*. Asterisk (*) indicates a statistically significant difference ($P < 0.01$) between early spermatids and late spermatids.

spermatids than early spermatids. The specificity of the HSF2 antibodies for precipitating *Hspa1b*-containing DNA (black bars) is demonstrated by the lack of *Hspa1b*-containing DNA precipitated by goat IgG-negative control (gray bars).

DISCUSSION

There has been a significant amount of research attempting to elucidate the biological role(s) of HSF1 and HSF2. Some of this research has used mice with mutations in these genes to identify their function. There is good agreement in the literature regarding the importance of HSF1 and HSF2 for male and female fertility [25, 32, 49–51]. However, the role of these factors during embryogenesis is not as clear. One report has implicated *Hsf1* mutations in increased prenatal lethality in mice [33]. Another report also found that mice lacking HSF1 displayed a number of phenotypes including prenatal lethality, the severity and lethality of which was found to be influenced by the genetic background of the *Hsf1* mutant mice [50]. Mutations of *Hsf* have also been studied in Drosophila with embryonic lethality at the 1st or 2nd larval instar [52]. Although there are likely to be non-Hsp gene targets for HSF1 and HSF2 in early embryos, the embryonic defects associated with a lack of these factors could be attributed to a lack of regulation of the *Hspa1b*. The importance of HSF2 during embryogenesis has also been studied in mice. One report found that *Hsf2* mutant mice were viable with normal life spans and behavioral functions, suggesting that *Hsf2* was not essential for embryonic development [53]. However, other reports found that *Hsf2* mutant mice displayed an increase in embryonic lethality [25, 32].

The differences observed for *Hsf1* and *Hsf2* mutant mice could be attributed to the genetic background of the mice, a phenomenon that has been observed in other studies [54, 55]. Another possible explanation is that these factors are able to compensate for each other, as HSF1 and HSF2 are highly homologous (72% identical in DNA-binding domain [56]) and both have been shown to bind to HSE promoter elements. Consistent with this hypothesis, previous work showed that mice lacking both HSF1 and HSF2 have more severe defects than mice lacking HSF1 or HSF2 individually [49]. Also, a recent report has demonstrated an interplay between HSF1 and HSF2 and a potential role for HSF2 in the HSF1-mediated induction of major heat shock genes [29].

Due to our interest in fully understanding the roles of HSF1 and HSF2 during spermatogenesis and early embryogenesis, we began investigating the possibility that HSF1 and HSF2 were functioning to bookmark the cytoprotective *Hspa1b* gene to provide a mechanism by which *Hspa1b* could be expressed in early embryos in the event of cellular stress. In this report we show that both HSF1 and HSF2 are present in mature spermatozoa and are bound to the *Hspa1b* promoter. We have also found that SP1 is bound to the *Hspa1b* promoter. These observations are novel since 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 [37]. The reason for the transcriptional silence in mature sperm could be the removal of many transcription factors from the highly compacted DNA. In support of this hypothesis, a previous study showed that as transcription ceases, a number of basal transcription factors, transcriptional regulators, and architectural factors are displaced from chromatin beginning in step 7 spermatids, with no detectible binding observed by the point of step 10 spermatids [36]. The presence of HSF1 and HSF2 on the *Hspa1b* promoter provides an interesting mechanism by which the *Hspa1b* gene could be rapidly and preferentially expressed in the male pronucleus during early embryogenesis. We have previously shown that HSF2 can bookmark the *Hspa1b* gene in mitotic cells [24]. Furthermore, HSF1 is important for embryogenesis [33, 50] and is required for the expression of *Hspa1b* during early embryogenesis [17]. With the opportunistic and essentially unregulated transcription in the one-cell embryo [12, 13], the presence of HSF1 and HSF2 on the *Hspa1b* promoter would facilitate the rapid assembly of any necessary transcriptional machinery on the *Hspa1b* promoter versus other promoters lacking bound factors at this stage of embryogenesis. Another group using immunolocalization techniques reported that HSF2 was consistently pronuclear in one-cell embryos in the absence of stress [17], which is consistent with our findings. They also found that HSF1 was cytoplasmic in the absence of stress, but it is likely that the HSF1 bound to the *Hspa1b* promoter in the male pronucleus would be below the level of detection by immunolocalization.

Recently our laboratory has shown that HSF2 can bind to the promoters of *Hspb2*, *Hsp90aa1*, and *Fos* in mitotic cells [57]. Other genes, many of which are members of the heat shock superfamily, are also bound by HSF1 and HSF2 [58]. This suggests that HSF1 and HSF2 may have a larger role in the expression of other genes important for early-stage embryonic development.

For many years mature spermatozoa were considered inert cells with the single function of delivering paternal DNA to the ovum. However, as more studies of spermatozoa emerge, it appears that a number of important processes are occurring in spermatozoa. Bookmarking the *Hspa1b* gene in spermatozoa makes particular sense given the crucial 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.

ACKNOWLEDGMENTS

We would like to thank Dr. Sidney Grimes for kindly providing centrifugally elutriated mouse germ cells and Dr. Dan Noonan for providing SP1 antibodies. We would also like to thank other members of our laboratory for insightful discussions during these studies.

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