A Genomic Region Transcribed into a Long Noncoding RNA Interacts with the Prss42/Tessp-2 Promoter in Spermatocytes during Mouse Spermatogenesis and its Flanking Sequences Can Function as Enhancers

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Abbreviations: 3C, chromosome conformation capture; BAC, bacterial artificial chromosome; cDNA, complementary DNA; CIAA, chloroform isoamylalcohol; DIG, digoxigenin; EC, embryonal
carcinoma; HS, hypersensitive site; IncRNA, long noncoding RNA; PBS, phosphate buffered saline; PCR, polymerase chain reaction; Prss, protease serine; RACE, rapid amplification of complimentary DNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; Tessp, testis-specific serine protease; TSA, tyramide signal amplification; TSS, transcriptional start site; TTS, transcriptional termination site

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SUMMARY

Spermatogenesis is precisely regulated by many meiotic stage-specific genes, but their regulatory mechanisms are not fully understood. The Prss/Tessp gene cluster is located on the mouse chromosome 9F2-F3, and the three genes, Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4 on the cluster, are specifically activated in pachytene spermatocytes during meiosis. To elucidate a mechanism of their activation, we searched for DNase I hypersensitive sites (HSs) and long noncoding RNAs (lncRNAs) at the Prss/Tessp locus. We found eight DNase I HSs, three of which were testicular germ cell-specific at or close to the Prss42/Tessp-2 promoter, and a testis-specific lncRNA, lncRNA-HSVIII, which was transcribed from 3’ to the Prss42/Tessp-2 gene. By in situ hybridization, lncRNA-HSVIII was localized in nuclei of most pachytene spermatocytes and in cytosols of pachytene spermatocytes at stage X and spermatids. Chromosome conformation capture assay showed that the chromatin at lncRNA-HSVIII specifically interacted with the Prss42/Tessp-2 promoter in primary and secondary spermatocytes. By reporter gene assay, a 5.8-kb genome sequence, encompassing the entire lncRNA-HSVIII sequence and its flanking regions, significantly increased Prss42/Tessp-2 promoter activity, but transfection of this construct did not change the lncRNA-HSVIII expression, which indicated that the increased promoter activity was likely to be dependent on enhancer activity. Indeed, we found that both upstream and downstream regions of the lncRNA-HSVIII sequence significantly increased Prss42/Tessp-2 promoter activity. Our data indicate the direct interaction of a genomic region at lncRNA-HSVIII with the Prss42/Tessp-2 promoter in spermatocytes and suggest that adjacent sequences to the lncRNA function as enhancers for the Prss42/Tessp-2 gene.
Spermatogenesis is a process to generate spermatozoa, but its mechanistic details, e.g., how genes are specifically activated at particular stages during meiosis, especially in primary spermatocytes, remains to be elucidated (Grimes, 2004; Shima et al., 2004; Wang et al., 2005; Johnston et al., 2008; Goldberg et al., 2010; Ou et al., 2010). We have studied three paralogous mouse genes, Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4, which constitute a gene cluster on the mouse chromosome 9F2-F3 and are activated in primary spermatocytes at the late pachytene stage (Yoneda et al., 2013). The three genes encode serine proteases that are considered to play crucial roles in the progression of meiosis at different stages, together with another homologous gene, Prss41/Tessp-1 (Yoneda and Kimura, 2013; Yoneda et al., 2013). Functional significance and spermatocyte-specific expression of the Prss/Tessp genes indicate that this gene cluster can serve as an excellent model for studying the mechanisms of gene activation during meiosis.

Classically, germ cell-specific genes were considered to be controlled solely by their proximal promoters, because many studies reported that minimum promoters were sufficient to activate such genes by generating transgenic mice (Reddi et al., 2007; Kehoe et al., 2008). In addition, general transcription factors that are exclusively expressed in testicular germ cells are known to play important roles in gene activation during meiosis (DeJong, 2006; Goodrich and Tjian, 2010). However, it is becoming clear that distal enhancer elements are required for the germ cell-specific gene activation. For example, the enhancer located between –4.8 kb and –1.3 kb of the transcriptional start site (TSS) was necessary for full activation of the mouse Ccna1 gene (Lele and Wolgemuth, 2004), and we recently found an enhancer for the spermatocyte-specific mouse Tcam1 gene (Kurihara et al., 2014). Therefore, the mechanisms of gene activation during male meiosis should be much more complicated.

In general, the regulation of chromatin structure is key to gene activation, and DNase I hypersensitivity reflects the chromatin state. DNase I hypersensitive site (HS) is a genome region with loose chromatin which is often marked with epigenetic modifications leading to the formation of
active chromatin (Jenuwein and Allis, 2001; Alabert and Groth, 2012; Mercer et al., 2013) and associated with promoters and enhancers (Cockerill, 2011; Iwafuchi-Doi and Zaret, 2014). Enhancers increase the transcription rate of their target genes by physically interacting with promoters, as revealed by the innovative chromosome conformation capture (3C) assay (Dekker et al., 2002; Tolhuis et al., 2002). Recently, some enhancers were found to be controlled by long noncoding RNAs (lncRNAs) (Zaratiegui et al., 2007; Umlauf et al., 2008; Ponting et al., 2009; Wilusz et al., 2009; Wang and Chang, 2011), which were extensively transcribed from mammalian genome in many tissues including the mouse testis (Bao et al., 2013; Sun et al., 2013; Liang et al., 2014; Necsulea et al., 2014). In those cases, lncRNAs were necessary for the dynamic chromatin rearrangement allowing enhancers to contact promoter regions (Sanyal et al., 2012; Lai et al., 2013; Li et al., 2013). However, whereas regulatory mechanisms of various tissue-specific genes have been identified (Arvey et al., 2012; Natarajan et al., 2012; Todeschini et al., 2014), it is unclear how DNase I HSs and lncRNAs are related to the specific gene activation in the testis during meiosis.

In this study, we attempted to reveal the mechanism of activation of the Prss/Tessp gene cluster. We found eight DNase I HSs at the locus and a testis-specific lncRNA, lncRNA-HSVIII, which was transcribed from 3’ to the Prss42/Tessp-2 gene. Interestingly, in spermatocytes, the chromatin at lncRNA-HSVIII interacted with the Prss42/Tessp-2 promoter, in which germ cell-specific DNase I HSs were present. However, the lncRNA-HSVIII transcription was unlikely to be related to the Prss42/Tessp-2 gene regulation, and instead, adjacent sequences to the lncRNA possessed enhancer activity for the Prss42/Tessp-2 promoter. The study provides a model for a novel mechanism of gene activation during male meiosis.

RESULTS

DNase I HS Mapping at the Mouse Prss/Tessp Locus
To identify cis-regulatory sequences for Prss/Tessp genes, we performed DNase I HS mapping on the Prss/Tessp genes and their adjacent regions in testicular germ cells and liver cells. The germ cell fraction was prepared as previously described (Yoneda et al., 2013), and we confirmed that more than 70% of cells in the fraction were germ cells based on the marker gene expression in this study. The liver was used as a tissue which did not express any of the cluster genes. The nuclei from germ cells and liver cells were treated with DNase I, and DNAs were purified, digested with restriction enzymes, and subjected to Southern blot analysis.

We first examined two restriction fragments, digested with HindIII and BamHI, which covered a 38-kb region encompassing the Prss42/Tessp-2 gene (Fig. 1A). By HindIII digestion, we detected a mother band at the position of 20 kb as expected and three additional bands: two at 11.9 kb and 1.2 kb in both native germ cells and liver cells, and one at 9.9 kb only in the liver (Fig. 1B). These DNase I HSs corresponded to 13.3 kb, 11.3 kb, and 2.7 kb upstream of the Prss42/Tessp-2 gene, and we named them HSI, HSII, and HSIII (Fig. 1A and B). The band corresponding to HSI was observed before DNase I treatment in the liver, possibly due to endogenous DNase activity. By BamHI digestion, we observed a mother band at 22 kb as expected and four additional bands: two at 11.1 kb and 4.9 kb in both tissues, one at 6.7 kb only in the liver, and the other one at 3.9 kb only in native germ cells, although the signal intensity was different between the two cell types (Fig. 1C). These DNase I HSs corresponded to the promoter, gene body, and 3’ flanking region of the Prss42/Tessp-2 gene.

The presence of DNase I HSs at the Prss42/Tessp-2 promoter was consistent with the active transcription of this gene in native germ cells, but we also detected a band corresponding to this promoter in liver cells by BamHI digestion (Fig. 1C, arrowhead). Thus, we performed fine mapping at this region by digestion with EcoRI. The Southern blot analysis resulted in the detection of a mother band at 3.6 kb as expected in both cells. In germ cells, three additional bands were observed at 2.4 kb, 1.5 kb, and 1.2 kb, whereas no clear band was detected in the liver (Fig. 1D). The lack of DNase I HS in the liver was not due to insufficient DNase I treatment, because the gel image after ethidium bromide staining indicated a similar extent of digestion to germ cell nuclei (Fig. S1). The DNase I HSs
in the EcoRI fragment were considered to be germ cell-specific. As a result of mapping these DNase I HSs at the locus, the 2.4-kb band corresponded to the signal at 3.9 kb by BamHI digestion, and the other two bands were presumed to be detected as one BamHI fragment at 4.9 kb. We named these DNase I HSs HSIV, HSV, and HSVI (Fig. 1C and D), and then, the DNase I HSs located downstream to them were named HSVII and HSVIII (Fig. 1C).

Consequently, we found eight DNase I HSs at the Prss42/Tessp-2 locus: two of them (HSII and HSVII) were liver-specific, and three at or close to the Prss42/Tessp-2 promoter (HSIV, HSV, and HSVI) were present only in testicular germ cells (Fig. 1A).

**Characterization of lncRNA-HSVIII Transcribed from 3’ to the Prss42/Tessp-2 gene**

We next assessed noncoding transcription at the Prss/Tessp locus because lncRNAs are important for regulation of various genes (Rinn and Chang, 2012) and there are many lncRNAs expressed in the testis (Bao et al., 2013; Sun et al., 2013; Liang et al., 2014). Based on transcriptomic data (accession numbers: SRX135150, SRX135160, and SRX135162), we found that several intergenic regions were specifically transcribed in the testis at the Prss/Tessp locus. As a result of reverse transcription-polymerase chain reaction (RT-PCR) analyses, we identified a novel lncRNA transcribed from the downstream region of the Prss42/Tessp-2 gene and named it lncRNA-HSVIII (Fig. 2A). This lncRNA was specifically detected by RT-PCR in samples of total RNA from whole testis (Fig. 2B). We then examined which types of cells expressed lncRNA-HSVIII by fractionating testicular cells. We prepared germ, Sertoli, and Sertoli/Leydig cell fractions, and the expression of marker genes indicated that the fractionation was successful (Fig. S2). By RT-PCR, we detected the transcript in germ and Sertoli/Leydig cell fractions but not in Sertoli cell fraction (Fig. 2C). The Sertoli cell fraction did not contain any other cell types (Fig. S2) and showed no signal for lncRNA-HSVIII (Fig. 2C), so the signal in the Sertoli/Leydig cell fraction was likely to originate from Leydig cells. Therefore, lncRNA-HSVIII was presumed to be expressed in germ cells and Leydig cells, and the stronger signal in the germ cell fraction might represent its main role in meiosis. We also examined subcellular localization of this
In long non-coding RNA (lncRNA) by RT-PCR with nuclear and cytosolic RNAs of germ cells, and found that it was localized predominantly in the nucleus but was also present in the cytosol (Fig. 2D).

These results suggested that lncRNA-HSVIII was involved in the germ cell-specific regulation of Prss/Tessp genes. Thus, we decided to determine the full-length sequence of lncRNA-HSVIII by rapid amplification of cDNA ends (RACE) analyses. Using a 5’RACE procedure, in 20 subclones, we detected one TSS of lncRNA-HSVIII. On the other hands, 3’RACE resulted in the identification of three transcriptional termination sites (TTSs) by sequencing 10 subclones. Interestingly, there were the repeat of GAAAA sequence in the 3’ region, and the subclones had different numbers of this sequence. Seven subclones had a final adenine of the 7th GAAAA sequence as TTS, and the 3rd and 10th GAAAA was TTS in one and two subclones, respectively. Thus, we determined the 7th GAAAA as the 3’ end of lncRNA-HSVIII. Consequently, the full-length of lncRNA-HSVIII was determined to be 2665 nucleotides long (Fig. 2A) and it had a poly(A) tail. The nucleotide sequence was deposited to DDBJ/EMBL/GenBank databases under the accession number LC060751.

To further analyze the expression pattern of lncRNA-HSVIII, we performed in situ hybridization with the highly sensitive tyramide signal amplification (TSA) system using adult testes. We detected many signals as red dots with the antisense probe of lncRNA-HSVIII, whereas few dots were observed with the sense probe (Fig. 3A and B). Consistent with the RT-PCR result of fractionated testicular cells (Fig. 2C), positive signals were present not only in germ cells but also in nuclei and cytosols of Leydig cells (Fig. 3C, arrows). In seminiferous tubules, positive red signals were observed at all seminiferous epithelial stages. At stage V, a few signals were observed in nuclei of pachytene spermatocytes and in cytosols of spermatids (Fig. 3D). While the signals in spermatids remained, those in nuclei of pachytene spermatocytes increased at stage VIII (Fig. 3E). At stage X, more red dots were observed in nuclei of some pachytene spermatocytes and in cytosols of the others (Fig. 3F). These results indicated that the lncRNA-HSVIII transcription was activated in early pachytene spermatocytes and enhanced at late pachytene stages, during which the transcripts were localized to nuclei, and at the end of the pachytene spermatocyte stage, lncRNA-HSVIII changed its localization to cytosols and the cytosolic
RNAs were retained in spermatids.

**Spermatocyte-Specific Interaction of the Chromatin at lncRNA-HSVIII with the Prss42/Tessp-2 Promoter**

In pachytene spermatocytes, it was possible that *lncRNA-HSVIII* transcription contributed to activation of the *Prss/Tessp* cluster genes. In general, cis-acting lncRNAs require the interaction of genomic regions transcribed into them with their target promoters or mediate the interaction of enhancers with promoters for gene activation (Zaratiegui et al., 2007; Umlauf et al., 2008; Ponting et al., 2009; Wilusz et al., 2009; Wang and Chang, 2011;). Thus, we investigated whether the chromatin at *lncRNA-HSVIII* interacted with any of *Prss/Tessp* promoters by 3C assay. Since many lncRNAs interacted with their target promoters via their 3’ regions (Lai et al., 2013; Xiang et al., 2014), we designed an anchor primer at 3’ of the *lncRNA-HSVIII* region and examined the interaction with HSI, HSIV, and promoters of the three *Prss/Tessp* genes (Fig. 4A). The interaction frequency was normalized to that of the control in which a bacterial artificial chromosome (BAC) clone encompassing the *Prss/Tessp* cluster was digested and ligated. The value was further normalized to the frequency at the *Ercc3* locus which is thought to show positive interaction at similar levels in many tissues (Palstra et al., 2003). Relative interaction frequency at the *Ercc3* locus was set to 1.0.

We first performed 3C assay using testicular germ cells and liver cells. While we did not detect significant levels of interaction with any regions in liver cells, HSIV and the *Prss42/Tessp-2* promoter were found to interact with the *lncRNA-HSVIII* region in germ cells (Fig. 4B). The interaction frequency with HSIV in germ cells was significantly higher than that in the liver and was comparable to the level at the *Ercc3* locus. The frequency with the *Prss42/Tessp-2* promoter was also significantly higher in germ cells than in the liver, and the level was about one third of that at the *Ercc3* locus.

When we performed this experiment without DNA ligase, no significant signals were detected by PCR (data not shown).

We next fractionated germ cells by a cell sorter into four meiotic stages; spermatogonia, primary
spermatocytes, secondary spermatocytes, and spermatids/spermatozoa, as previously described (Yoneda et al., 2013). By performing 3C assay with the fractionated cells, we detected significant levels of interaction in spermatocytes but not in spermatogonia and spermatids/spermatozoa (Fig. 4C). In primary spermatocytes, HSIV and the Prss42/Tessp-2 promoter interacted with the lncRNA-HSVIII region at significantly higher levels than in spermatogonia, and the interaction frequency with HSIV increased in secondary spermatocytes (Fig. 4C). The interaction disappeared in the fraction of spermatids and spermatozoa (Fig. 4C). Taken together with our previous findings that Prss42/Tessp-2 was activated in primary spermatocytes and its transcription was maintained in secondary spermatocytes but declined in spermatids/spermatozoa (Yoneda et al., 2013), these results suggested that the interaction of the chromatin at lncRNA-HSVIII with upstream regions of the Prss42/Tessp-2 promoter was necessary for the spermatocyte-specific Prss42/tessp-2 activation. We assumed that either lncRNA-HSVIII transcription or enhancer elements within or close to it were involved in the Prss42/Tessp-2 gene activation.

Enhancer Activity in the Region Encompassing lncRNA-HSVIII

To determine whether lncRNA-HSVIII transcription was related to Prss42/Tessp-2 activation or the genomic sequence encompassing it functioned as an enhancer for Prss42/Tessp-2, we prepared a reporter construct containing a 5.8-kb EcoRI fragment fused with the luciferase gene driven by the Prss42/Tessp-2 promoter (Fig. 5). This 5.8-kb fragment encompassed the entire sequence of lncRNA-HSVIII as well as its 1.9-kb promoter and 1.2-kb 3’ sequence (Fig. 5A). For comparison, we also prepared a construct without the 5.8-kb fragment and that with a 6.7-kb λ.HindIII fragment (Fig. 5B and C). In luciferase reporter assays, we used two established mouse cell lines, an embryonal carcinoma (EC) cell line, P19TG1, and a hepatic tumor line, Hepa1-6, because both endogenously expressed Prss42/Tessp-2 mRNA (data not shown).

We introduced the constructs into P19TG1 and Hepa1-6 cells and measured luciferase activity two days later. The activity was significantly higher in the construct with the 5.8-kb fragment than
those without it and with the λDNA in both cells (Fig. 5B and C). To examine whether this enhancement of Prss42/Tessp-2 promoter activity was associated with IncRNA-HSVIII transcription, we measured the IncRNA-HSVIII level in P19TG1 and Hepa1-6 cells transfected with the construct with or without the 5.8-kb fragment. By quantitative RT-PCR, levels of the IncRNA-HSVIII transcript were not significantly different between the two constructs in P19TG1 cells (Fig. 6). In Hepa1-6 cells, we could not detect any levels of the IncRNA-HSVIII transcript with either construct (data not shown). Thus, IncRNA-HSVIII was not transcribed from the transgene, which strongly suggested that the 5.8-kb sequence increased Prss42/Tessp-2 promoter activity by functioning as an enhancer element in P19TG1 and Hepa1-6 cells.

We finally assessed which region in the 5.8-kb sequence possessed enhancer activity by separating it into three regions: 2.3-kb upstream region, 2.7-kb IncRNA-HSVIII sequence, and 1.5-kb downstream region (Fig. 5A). As a result of transfection and luciferase activity assay, the IncRNA-HSVIII sequence itself showed no enhancer activity in either P19TG1 cells or Hepa1-6 cells (Fig. 7A). In contrast, both upstream and downstream sequences of IncRNA-HSVIII significantly increased Prss42/Tessp-2 promoter activity in both cells (Fig. 7B). This indicated that the flanking sequences to IncRNA-HSVIII possessed enhancer activity for the Prss42/Tessp-2 gene, which suggests that genomic regions adjacent to IncRNA-HSVIII function as enhancers for the Prss42/Tessp-2 gene in spermatocytes.

**DISCUSSION**

Many studies reported that testicular germ cell-specific genes were controlled exclusively by their proximal promoters (Reddi et al., 2007). However, even if a promoter was sufficient to germ cell-specific gene activation, the transgene was often silenced or the expression level varied between transgenic lines (Robinson et al., 1989; Zambrowicz et al., 1993; S. Li et al., 1998; Reddi et al., 1999;
Han et al., 2004). This indicates that other elements such as insulators and distal enhancers are required for germ cell-specific gene regulation. Actually, the presence of enhancers functioning during male meiosis was reported for some genes (Lele and Wolgemuth, 2004; Kurihara et al., 2014). Our current data provide another evidence of the potential presence of enhancers for a spermatocyte-specific gene and highlight the complicated mechanism for gene activation during meiosis.

In this study, we first searched for DNase I HSs to identify cis-regulatory sequences that were involved in the activation of Prss/Tessp genes. Although we found eight DNase I HSs, three germ cell-specific ones were all positioned in the Prss42/Tessp-2 promoter or close to it (Fig. 1). This might suggest that the Prss42/Tessp-2 promoter possessed major activity for the spermatocyte-specific gene activation, as exemplified by the Pgk2 and Prm1 promoter containing DNase I HSs and being mostly sufficient for their meiotic stage-specific activation (Peschon et al., 1987, 1989; Robinson et al., 1989; Kumari et al., 1996; Kramer et al., 1998; Martins and Krawetz, 2007). However, ubiquitously distributed DNase I HSs could possibly function for tissue-specific gene expression, and enhancers were not necessarily associated with DNase I hypersensitivity. Therefore, while the Prss42/Tessp-2 promoter was obviously important for its spermatocyte-specific expression, we thought that there were distal cis-regulatory elements of this gene. In fact, we found potential enhancer sequences at 3’ to the Prss42/Tessp-2 gene and focused on their characterization.

The presence of potential enhancers for Prss42/Tessp-2 but not for the other two cluster genes is remarkable given that Prss42/Tessp-2 mRNA is expressed at the highest level in three cluster genes (Yoneda et al., 2013). Although it is unclear whether Prss44/Tessp-4 and Prss43/Tessp-3 genes are mostly regulated by their promoters, the chromatin interaction of potential enhancer elements with the Prss42/Tessp-2 promoter but not with the other Prss/Tessp promoters suggests different regulatory mechanisms for the cluster genes. While the Prss42/Tessp-2 gene may be regulated by a complicated mechanism involving several regulatory elements, proximal promoters may be sufficient for activating the other two genes.
Long range chromatin interaction is very important in the regulation of many genes in various tissues (Sipos and Gyurkovics, 2005; Bartkuhn and Renkawitz, 2008; Ghirlando et al., 2012), but there have been no such reports in testicular germ cells. Here we provide such an example, but more importantly, we revealed dynamic changes of the chromatin conformation during meiosis. Our data clearly indicated the stage-specific interaction of the IncRNA-HSVIII region with the Prss42/Tessp-2 promoter as follows: in spermatogonia, the chromatin at IncRNA-HSVIII did not interact with the Prss42/Tessp-2 promoter, then these two sites physically interacted in primary and secondary spermatocytes, but their interaction disappeared in spermatids (Fig. 4). The stages at which this long range chromatin interaction was observed coincided with those when Prss42/Tess-2 mRNA was expressed (Yoneda et al., 2013). This strongly suggests that the dynamic chromatin rearrangement is critical to Prss42/Tessp-2 activation.

For Prss42/Tessp-2 activation, we tested two possibilities: enhancer activity of genomic sequences encompassing IncRNA-HSVIII and the activation by IncRNA-HSVIII transcription. Although they were not mutually exclusive, we attempted to distinguish between these possibilities by generating a reporter construct, in which the luciferase gene driven by the Prss42/Tessp-2 promoter was connected to a 5.8-kb fragment encompassing the entire IncRNA-HSVIII and its flanking sequences. As a result, this sequence showed enhancer activity in two cell lines expressing the Prss42/Tessp-2 gene, while IncRNA-HSVIII was not transcribed from the transgene (Figs. 5 and 6). This indicated that the Prss42/Tessp-2 promoter seemed to be regulated by an enhancer, but we further performed two additional experiments to exclude the possibility of IncRNA-mediated gene regulation. First, we overexpressed IncRNA-HSVIII in three cell lines, Hepa1-6, P19TG1, and GC-2spd(ts) cells, but mRNA levels of Prss/Tess cluster genes were not changed in any cells (data not shown). GC-2spd(ts) cells were established from mouse primary spermatocytes (Hofmann et al., 1994). Second, by reporter gene assay, overexpression of IncRNA-HSVIII did not increase Prss42/Tessp-2 promoter activity in Hepa1-6 cells (data not shown). Taken together with the observation that IncRNA-HSVIII was not necessarily localized in nuclei of the late stage of pachytene spermatocytes that were actively
transcribing Prss/Tessp genes (Fig. 3), we conclude that IncRNA-HSVIII does not contribute to transcriptional activation of the cluster.

Even if IncRNA-HSVIII is not related to the regulation of Prss/Tessp cluster genes, it probably contributes to the regulation of meiosis, and interestingly, the function of this IncRNA should be different between meiotic stages. In most pachytene spermatocytes, IncRNA-HSVIII was localized to nuclei, and its expression level dramatically increased at late pachytene stages (Fig. 3). Nuclear IncRNAs generally have one of the two functions: construction of nuclear structures such as paraspeckles and transcriptional activation or silencing (Chen and Carmichael, 2010). At present, we do not know how IncRNA-HSVIII functions in pachytene spermatocytes, but because we did not identify any specific nuclear structure associated with this IncRNA, it might control the transcription of spermatocyte-specific genes. In spermatids and some pachytene spermatocytes at stage X, IncRNA-HSVIII was localized in cytosols (Fig. 3F). Cytosolic IncRNAs reported so far are involved in the regulation of RNA stability and translation (Wu and Brewer, 2012; Atianand and Fitzgerald, 2014). Because many transcripts are under translational arrest in early stages of primary spermatocytes and their translation takes place at later stages (Morales et al., 1991; Langford et al., 1993; Schäfer et al., 1995; Yan et al., 2010), the cytosolic IncRNA-HSVIII may play a role in the translational control at this stage.

Instead of the contribution of IncRNA-HSVIII, we found that genomic sequences adjacent to it could function as enhancers for the Prss42/Tessp-2 gene in EC cells (P19TG1) and hepatic tumor cells (Hepa1-6). Although these cells endogenously expressed Prss42/Tessp-2 mRNA, it is a critical question whether the adjacent sequences to IncRNA-HSVIII really function as enhancers in native spermatocytes. In this context, it is notable that many testis-specific genes were activated in various tumor cells (Simpson et al., 2005), which suggests that activation mechanisms of testis specific-genes are partly common between in the testis and in the cell lines we used. Considering that similar enhancer activity was observed in two unrelated cell lines that expressed Prss42/Tessp-2 mRNA (Figs. 5 and 7), our data supports that the flanking sequences to IncRNA-HSVIII really function as enhancers
for the *Prss42/Tessp-2* gene in the testis.

However, it is also true that cell lines do not completely resemble native tissues. Hepa1-6 cells were derived from mouse hepatoma (Darlington et al., 1980; Darlington, 1987) and have been widely used to study biological events in the liver as well as for other purposes. P19TG1 is an HPRT-deficient subline of P19 EC cell line (Mise et al., 1996), which was established from a teratocarcinoma by transplantation of an E7.5 mouse embryo into the testis (McBurney and Rogers, 1982). P19 cell line is commonly used to study mechanisms of neurogenesis and myogenesis (Bain et al., 1994; van der Heyden and Defize, 2003), but it possibly have similar characteristics to spermatogenetic cells because a factor for germ cell differentiation, *Stra8*, was reported to be induced by retinoic acid (Oulad-Abdelghani et al., 1996). In our assay, some transcription factors that were commonly expressed in the testis and in the two cell lines we used might bind to the flanking sequences to *lncRNA-HSVIII* and enhance *Prss42/Tessp-2* promoter activity.

It is interesting that enhancer activity in the 5.8-kb fragment was divided into two regions: 5’ and 3’ flanking sequences of *lncRNA-HSVIII* (Fig. 7). This suggests that the upstream and downstream regions of *lncRNA-HSVIII* cooperatively function to enhance *Prss42/Tessp-2* promoter activity, which is reminiscent of super-enhancers (Pott and Lieb, 2014). Super-enhancers were first reported in mouse embryonic stem cells and contained several enhancers, each of which was located within 12.5 kb, to activate genes for cell type specification (Whyte et al., 2013). In a super-enhancer, each enhancer interacts with the target promoter, resulting in the formation of complex chromatin structure. If both 5’ and 3’ sequences to *lncRNA-HSVIII* enhance *Prss42/Tessp-2* promoter activity in spermatocytes, they may independently interact with the promoter. Alternatively, the 3’ sequence to *lncRNA-HSVIII* may mainly function as an enhancer, and the 5’ sequence may play an auxiliary role in the *Prss42/Tessp-2* gene regulation. It is unclear whether the flanking sequences to *lncRNA-HSVIII* are parts of a super-enhancer, but we assume that at least the 3’ sequence to *lncRNA-HSVIII* is required for activation of the *Prss42/Tessp-2* gene in spermatocytes during germ cell differentiation.

Finally, we discuss the regulatory mechanism of the gene cluster. The gene cluster usually
consists of several paralogues that were generated by duplication of an ancestral gene in the process of evolution. Indeed, the Prss/Tessp gene cluster contains three paralogous genes and they were estimated to be generated by gene duplication (Yoneda et al., 2013). As discussed above, our current data demonstrated that the IncRNA-HSVIII region interacted with the Prss42/Tessp-2 promoter but not with promoters of the other cluster genes, which indicates that Prss/Tessp cluster genes are controlled by different elements. This is interesting because most gene clusters are controlled together by a single cis-regulatory element such as locus control region (Fraser and Grosveld, 1998; Festenstein and Kioussis, 2000; Sproul et al., 2005; Dean, 2006). Even if cluster genes are expressed at different developmental stages or in different tissues, locus control region regulates the entire cluster (Grosveld et al., 1987; Jones et al., 1995; Su et al., 2000). The regulation of the Prss/Tessp gene cluster is in contrast to the mechanism for other gene clusters, because the Prss42/Tessp-2 gene is possibly regulated by its downstream sequence that possesses enhancer activity, but the sequence does not engage in the regulation of Prss44/Tessp-4 and Prss43/Tessp-3 genes. Therefore, our current data also provide a new insight into the regulation of gene clusters.

MATERIALS AND METHODS

Animals

C57BL6/Crj mice (CLEA Japan Inc., Tokyo, Japan) were maintained on 14 hr light/10 hr dark cycles at 25°C, and fed with enough food and water. The experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

DNase I HS Mapping

Native germ cells were isolated from two adult mouse testes and the purity was checked by the marker gene expression as previously described (Yoneda et al., 2013; Kurihara et al., 2014). The cells
were lysed with 4 ml NP-40 lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40) containing 1×proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Liver nuclei were collected by cutting and homogenizing 0.1 g liver piece in 4 ml NP-40 lysis buffer containing 1×proteinase inhibitor cocktail. The nuclei were collected by centrifugation, washed with 1 ml DNase I digestion buffer (0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 1 mM CaCl₂, 0.1 mM phenylmethylsulfonyl fluoride), and suspended again in 625 µl DNase I digestion buffer. The suspension was divided into five aliquots, and 125 µl DNase I digestion buffer was added to one aliquot and mixed with 250 µl 2×Stop solution (20 mM Tris-HCl (pH 7.5), 100 mM EDTA, 600 mM NaCl, 1% SDS, 200 µg/ml proteinase K). The remaining four aliquots were mixed with 125 µl DNase I digestion buffer containing 40 U/ml DNase I (Takara, Ohtsu, Japan) and treated for 15, 45, 90, and 180 sec at 37°C. The reaction was stopped by adding 250 µl 2×Stop solution to each sample, and all the five samples were incubated at 55°C for overnight to purify the genome DNA. The aliquot without DNase I digestion was used as a sample at a time point of 0 sec. The purified DNA was measured, and approximately 5–10 µg DNA was digested with restriction enzymes, HindIII, BamHI, or EcoRI at 37°C for 20 hr and purified by phenol/chloroform isoamylalcohol (CIAA) extraction and ethanol precipitation. These samples were separated by electrophoresis on 0.7% agarose gels and transferred to Hybond-N+ membranes (GE Healthcare, Piscataway, USA) for Southern blot hybridization.

Southern Blot Hybridization

A 38-kb region encompassing the Prss42/Tessp-2 gene was detected as two fragments resulting from HindIII and BamHI digestion (Fig. 1A). A 679-bp probe, which could detect both fragments, was prepared by PCR amplification with ExTaq polymerase (Takara) and mouse genome DNA. After the PCR amplification, the products were subcloned into a pBluescript II vector, and their sequences were checked by the DNA sequencing analysis. We selected the clone which contained the completely correct sequence, and by restriction digestion, agarose gel electrophoresis, and DNA purification from
the gel, we obtained the probe. The probe was $^{32}$P-labeled and hybridized with the membranes either at 65°C in 0.5 M sodium phosphate (pH 7.2), 0.1 mM EDTA, 5% SDS, 1% bovine serum albumin, and 100 µg/ml herring sperm DNA or at 42°C in 50% formamide, 5×Denhardt’s solution, 5×SSPE, 1% SDS, and 100 µg/ml herring sperm DNA. The membranes were washed with 1×SSC/0.1% SDS at 65°C or 50°C, and the signals were detected by autoradiography using Kodak Biomax MR films. For fine mapping of the Prss42/Tessp-2 promoter region, the genome DNA treated with DNase I was digested with EcoRI, and a 711-bp probe for this fragment was obtained as above (Fig. 1A). The primers for PCR reactions to prepare probes are listed in Table 1.

**RT-PCR Analysis**

Total RNAs were extracted by ISOGEN II (Nippon gene, Tokyo, Japan) according to the manufacturer’s instruction. After the treatment with TurboDNase I (Ambion, Austin, USA), RNAs were reverse-transcribed into cDNAs using Superscript III (Invitrogen, Carlsbad, USA) according to the instruction. PCR was performed by using ExTaq polymerase (Takara). Primer sequences are shown in Table 1.

**5’RACE and 3’RACE**

RACE analyses were performed as previously described (Kurihara et al., 2014; Matsubara et al., 2014). For 5’RACE, reverse transcription was done with RT-1 primer, and the first PCR was performed with GSP1 and Abridged Anchor Primer. The second nested PCR was performed using GSP2 and Abridged Universal Amplification Primer. For 3’RACE, the first and second PCRs were performed using GSP3 and GSP4, respectively, with 3 sites Adaptor Primer. All the products were subcloned into a pBluescript II vector (Stratagene, La Jolla, USA) by the TA-cloning method, and the sequences of at least ten subclones were determined for each experiment. Primer positions are indicated in Fig. 2A and their sequences are shown in Table 1.
Preparation of Germ, Sertoli, and Sertoli/Leydig Cell Fractions

The germ cell fraction was prepared as above. The Sertoli cell fraction was obtained from 11-day-old testes by primary culture of Sertoli cells as previously described (Yoneda and Kimura, 2013). The Sertoli/Leydig cell fraction was obtained from 6-month-old testes as follows. The testes were decapsulated and treated with 0.1% collagenase at 32°C. The floating cells were collected and applied to discontinuous Percoll gradient (20%, 37%, and 53%). After centrifugation, the cells between 37% and 53% were washed and used as the Sertoli/Leydig cell fraction.

Preparation of Subcellular Fractions

Testicular germ cells were lysed in NP-40 lysis buffer on ice for 10 min, and the lysate was centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was used as the cytoplasmic fraction, and the precipitates were washed with the same buffer 2-3 times. The resulting pellet was used as the nuclear fraction.

In situ Hybridization

For in situ hybridization analysis, adult mouse testes were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 3 hr at 4°C. The tissues were washed three times with PBS and dissected into two pieces with a razor blade under a dissecting microscope. In situ hybridization with the TSA Plus system (PerkinElmer) was performed according to the procedure reported previously (Kotani et al., 2013). Briefly, samples were dehydrated, embedded in paraffin, and cut into 7-μm-thick sections. The sense and antisense RNA probes for *lncRNA-HSVIII* were synthesized with digoxigenin (DIG) RNA labeling kit (Roche Molecular Biochemicals). This time, we used a full-length sequence of *lncRNA-HSVIII* (2665 nucleotides) for labeling. After hybridization and washing, the sections were incubated with anti-DIG-horseradish peroxidase antibody (Roche Molecular Biochemicals) (1:500 dilution) for 30 min. The reaction with tyramide-Cy3 was performed according to the manufacturer’s instructions. After the incubation with 10 μg/ml Hoechst 33258 for 10 min, the samples were observed
under an LSM5LIVE confocal microscope (Carl Zeiss).

**Fractionation of Germ Cells into Different Meiotic Stages**

Testicular germ cells were fractionated into different meiotic stages by a cell sorter as previously described (Yoneda et al., 2013).

**3C Assay**

3C assay was performed according to previously published methods with modifications (Hagège et al., 2007; Ho et al., 2008). Native testicular germ cells were obtained as above, and hepatic cells were collected by homogenization of a 0.1 g liver piece in Opti-MEM (Invitrogen). The cells were crosslinked by 1% formaldehyde in Opti-MEM for 10 min, and the crosslink reaction was stopped by adding glycine solution at a final concentration of 0.125 M and incubating the sample for 5 min. After being washed twice with ice-cold PBS, the cells were suspended in 1 ml ice-cold PBS containing 1× proteinase inhibitor cocktail (Roche Molecular Biochemicals) and centrifuged at 700×g at 4°C for 5 min. By suspending the cells in Cold Lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM MgCl2, 1× proteinase inhibitor, 0.2% NP-40) and incubating on ice for 10 min, the crosslinked nuclei were centrifuged at 420×g at 4°C for 5 min. After being washed with ice-cold PBS, the nuclei were suspended in the mixture of 1.2×H buffer (Takara) and 0.1% SDS and incubated at 37°C for 1 hr while shaking at 200 rpm. Then, Triton-X100 was added to the samples at a final concentration of 2% to neutralize SDS, and the mixture was incubated at 37°C for 1 hr while shaking at 200 rpm. 2000 units of PstI (Takara, high concentration) were added to the nuclei and were incubated at 37°C for overnight while shaking at 200 rpm. At this point, the concentration of H buffer became 1×. After the restriction digestion, SDS was added at a final concentration of 1.67% to the reaction, and the sample was incubated at 65°C for 30 min. Then, the sample was split into two portions (one for ligation and the other for negative control), and each of them was mixed with 1× Takara ligation buffer (66 mM Tris-HCl (pH 7.5), 6.6 mM MgCl2, 10 mM DTT, 0.1 mM ATP) to dilute it to approximately 10 times
and with Triton-X100 at a final concentration of 1% to neutralize SDS. After incubation at 37°C for 30 min while shaking at 120 rpm, 1500 units of T4 DNA Ligase (Takara) was added to one portion, and the samples were incubated at 16°C for 4 hr while shaking at 60 rpm, followed by incubation at room temperature for 1 hr. After the ligation, proteinase K was added at a final concentration of 20 µg/ml and the samples were incubated at 65°C for overnight to reverse-crosslink the chromatin and to digest proteins. The samples were purified by phenol/CIAA extraction and ethanol precipitation, and finally dissolved in TE.

As a positive control, 10 µg of BAC DNA encompassing the Prss/Tessp gene cluster (B6Ng01-306015, RIKEN Bioresource Center, Tsukuba, Japan) were digested with 25 units of PstI at 37°C for 2 hr. The digested DNA was purified by phenol/CIAA extraction and ethanol precipitation, and ligated with 50 units of T4 DNA Ligase in 1×Takara ligation buffer at 16°C for 4 hr. The ligated DNA was purified by phenol/CIAA extraction and ethanol precipitation, and dissolved in TE.

3C PCR primers were designed around PstI restriction sites at or close to HSI, HSIV, Prss42/Tessp-2 promoter, Prss44/Tessp-4 promoter, 3’ end of Prss44/Tessp-4, an intergenic region between Prss44/Tessp-4 and Prss43/Tessp-3, and Prss43/Tessp-3 promoter in an antisense orientation (Fig. 4A). The anchor primer was designed at 3’ of the IncRNA-HSVIII sequence in a sense orientation. Quantitative PCR was conducted with KOD SYBR qPCR Mix (Toyobo, Tokyo, Japan) under the condition of 40 cycles of 98°C for 10 sec, 60°C for 10 sec, and 68°C for 1 min. The data was first normalized to those with the BAC clone to equalize the relative PCR efficiency, and interaction frequencies were further normalized to that at the Ercc3 gene locus, which was expected to show similar interaction frequency irrespective of cell- and tissue-types (Palstra et al., 2003). All the primers used for 3C assay are listed in Table 1.

**Plasmid Constructs**

The Prss42/Tessp-2 promoter sequence was amplified by PCR with mouse genome DNA using KOD polymerase (Toyobo) and a primer pair listed in Table 1. The 1636-bp product was blunted and
phosphorylated, and inserted into a pGL-3 Basic vector (Promega Corporation, Madison, USA) at the blunted \textit{Hind}III site. We used the plasmid clone which contained no mutation in the promoter after checking the sequence. The 5.8-kb fragment encompassing the \textit{lncRNA-HSVIII} sequence was obtained by digestion of a mouse BAC clone, B6Ng01-306015 (RIKEN Bioresource Center), with \textit{Eco}RI, and subcloned into a pBluescript II vector (Stratagene). The fragment was further cut out from the vector with \textit{Eco}RI, blunted and phosphorylated, and inserted into the pGL-3 Basic vector containing the \textit{Prss42/Tessp-2} promoter at the blunted \textit{Bam}HI site. A 6.7-kb \(\lambda\textit{Hind}III\) fragment was obtained from a \(\lambda\textit{Hind}III\) marker and inserted into the same vector at the blunted \textit{Bam}HI site after blunted and phosphorylated.

To obtain the full-length sequence of \textit{lncRNA-HSVIII}, we first amplified 2562-bp 5’-half and 259-bp 3’-half of this IncRNA, separately, by RT-PCR using primer pairs shown in Table 1. The two products were separately subcloned into a pBluescript II KS(+) vector at the \textit{Eco}RV site by the TA-cloning method, and their sequences were confirmed by DNA sequencing. Then, we digested both plasmids with \textit{Bgl}II and \textit{Sal}I, and inserted a 109-bp 3’-half into the plasmid containing a 2556-bp 5’-half. After digestion of the plasmid which contained the full-length of \textit{lncRNA-HSVIII} with \textit{Bam}HI and \textit{Sal}I, we ligated the \textit{lncRNA-HSVIII} fragment into \textit{Bam}HI/\textit{Sal}I site of the pGL-3 Basic vector containing the \textit{Prss42/Tessp-2} promoter. Alternatively, the digested \textit{lncRNA-HSVIII} fragment was blunted and phosphorylated, and inserted into the \textit{Sma}I site of the construct.

To obtain upstream and downstream sequences of \textit{lncRNA-HSVIII}, we digested the pBluescript vector containing the 5.8-kb fragment with \textit{SmaI/Hinc}II and \textit{SacI/Eco}RV, respectively. The fragments were blunted and phosphorylated, and inserted into the blunted \textit{Bam}HI site of the pGL-3 Basic vector containing the \textit{Prss42/Tessp-2} promoter.

\textbf{Cell Culture, Transfection, and Reporter Gene Assay}

Hepa1-6 cells were cultured as previously described (Matsubara et al., 2010). P19TG1 cells were cultured with Eagle’s minimum essential medium containing fetal bovine serum (10%) and
L-glutamine (2 mM) in the 37°C incubator supplied with 5% CO₂.

Because molecular weights were greatly different between constructs, we used the same molar amount of DNA in each assay. For reporter assay, Hepa1-6 cells were cultured in 24-well dishes and transfected with constructs by using the GeneJuice reagent (Novagen, Madison, USA) according to the instruction. P19TG1 cells were cultured in 35-mm dishes and transfected by using the FuGENE-HD reagent (Promega Corporation). The day before transfection, $0.5 \times 10^5$ P19TG1 cells were seeded on a 35-mm dish. On the day of transfection, the cell confluency was about 50-80%, and we formed complexes of various constructs with FuGENE-HD transfection reagent and added them to cells, according to manufacturer’s instructions. The cells were collected 48 hours after transfection, and luciferase activity was measured by using Dual-Luciferase Reporter Assay System (Promega Corporation).

The construct we prepared as above contained the Firefly luciferase gene, and in each experiment, we co-transfected it with a pRL-CMV vector (Promega Corporation) in which the Renilla luciferase gene was driven by the CMV promoter. All the constructs were transfected as circular plasmids. We normalized Firefly luciferase activity to Renilla luciferase activity for adjusting transfection efficiency. The activity was further normalized to that of the cells transfected with a pGL3-Basic vector, and relative activity was calculated as the value from a pGL3-Basic vector was set to 1.0.

Statistical Analysis

Results were expressed as the average ± standard deviation (SD) of at least three independent experiments. Student’s $t$-test and Dunnett’s test were performed using Microsoft Excel statistical analysis functions (Microsoft Corporation, Redmond, USA). Differences were considered statistically significant at $P < 0.05$.

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REFERENCES


FIGURE LEGENDS

**Figure 1.** DNase I HS mapping at the mouse Prss/Tessp locus. A: Schematic drawing of the Prss/Tessp gene cluster showing the position of identified DNase I HSs in germ cells and liver cells by
vertical arrows. A 63-kb region corresponding to 110775-110838 kb of the mouse chromosome 9 is drawn. The width of the arrow reflects the signal intensity of each DNase I HS. Three cluster genes are depicted with white boxes and bent arrows indicate the transcriptional direction. An about 38-kb sequence encompassing the Prss42/Tessp-2 gene was searched as three restriction fragments digested with BamHI, HindIII, or EcoRI. Restriction sites and fragment sizes are shown below the gene structure. B: Southern blot analysis detecting DNase I HSs on the HindIII fragment. Nuclei from native testicular germ cells and liver cells were treated with DNase I for 0, 15, 45, 90, or 180 sec, and genome DNAs were purified. After digestion with HindIII, DNAs were transferred to a nylon membrane and hybridized with a radio-labeled probe (a grey box). The membrane was washed, and signals were detected by autoradiography. The Prss42/Tessp-2 gene and its upstream region are drawn at the top with the HindIII fragment and the fragments detected as a result of DNase I treatment. Sizes of DNase I-digested fragments are shown, and positions of DNase I HSs are indicated with their numbers by vertical arrows. Below the structure, the image of the Southern blot analysis are presented, and the bands corresponding to DNase I HSs are indicated by horizontal arrows. Molecular size markers are shown at right. C: Southern blot analysis detecting DNase I HSs on the BamHI fragment. DNase I treatment and Southern blot analysis were performed as in B, and the structure of the Prss42/Tessp-2 and Prss44/Tessp-4 locus and the Southern blot image are indicated as in B. An arrowhead shows the signal which was not detected by EcoRI digestion in liver cells. D: Fine mapping of the Prss42/Tessp-2 promoter region. DNase I treatment of nuclei, restriction digestion with EcoRI, and Southern blot were performed as in B. A promoter region and the first four exons of the Prss42/Tessp-2 gene are drawn at the top. Positions of DNase I HSs are indicated by vertical arrows, and exons are depicted with white boxes connected by bent lines. Below the gene structure, an EcoRI fragment is shown with the fragments detected after DNase I treatment, and the probe for Southern blot is indicated by a grey box. The Southern blot image is presented as in B.

Figure 2. Characterization and expression of lncRNA-HSVIII. A: Schematic drawing of the
Prss42/Tessp-2 gene and lncRNA-HSVIII. Exons of the Prss42/Tessp-2 gene are depicted with black and white boxes. A black box represent a coding region and a white box represents an untranslated region. lncRNA-HSVIII contains no intron. Bent arrows indicate the transcriptional direction of the Prss42/Tessp-2 gene and lncRNA-HSVIII. A vertical arrow shows the position of DNase I HSVIII. Below the structure, primer positions used in 5’RACE and 3’ RACE are indicated by short horizontal arrows. B: Tissue specificity of lncRNA-HSVIII was examined by RT-PCR. Total RNAs were isolated from eight mouse tissues as indicated, and reverse transcription was performed using the oligo(dT) primer with (RT+) or without reverse transcriptase (RT-). The cycle number of each PCR reaction is indicated in the parenthesis at right. The Gapdh gene was examined as an internal control. lncRNA-HSVIII was specifically expressed in the testis. C: Localization of lncRNA-HSVIII in the mouse testis was examined by RT-PCR using cDNAs obtained from fractionated native testicular cells. Germ cell fraction and Sertoli/Leydig cell fraction were isolated from adult mouse testes, while Sertoli cells were collected by primary culture of the cells from immature testes. The expression of marker genes was investigated by quantitative RT-PCR and the results are presented in Figure S2. RT-PCR was performed as in B, and the cycle number of each PCR is indicated in the parenthesis at right. lncRNA-HSVIII was mainly expressed in germ cells and at a lower level in Leydig cells. D: Subcellular localization of lncRNA-HSVIII. Native testicular germ cells were fractionated into the nucleus and cytoplasm, and RT-PCR was performed as in B. Two sets of primers were used to check the quality of the two fractions. Gapdh (in5-ex6) was amplified to detect immature mRNA in the nucleus, and Gapdh (ex5-ex6) was for mature mRNA in the cytoplasm. These two sets of PCR indicate that our subcellular fractionation was successful. The lncRNA-HSVIII signal was detected in both fractions, but the nuclear signal was stronger.

Figure 3. Localization of lncRNA-HSVIII in the mouse testis. In situ hybridization analysis of lncRNA-HSVIII in the adult mouse testis was performed with the TSA system. The testis was fixed with paraformaldehyde, embedded in paraffin, and cut into 7-µm sections. The sections were
hybridized with DIG-labelled sense or antisense probe for \textit{IncRNA-HSVIII} and subsequently incubated with anti-DIG-HRP antibody. The positive signals were detected as red dots by the reaction with tyramide-Cy3, and nuclei were stained with Hoechst33258 (blue). A: A picture of the testis section hybridized with the antisense probe of \textit{IncRNA-HSVIII}. Positive signals (red dots) were observed in all seminiferous tubules and in interstitial Leydig cells. B: A picture of the testis section hybridized with the sense probe of \textit{IncRNA-HSVIII}. Few red dots may be seen. C: An enlarged picture of Leydig cells on the section hybridized with the antisense probe. Positive red signals were present in both nuclei and cytosols of Leydig cells (red dots pointed by arrows). D-F: Enlarged pictures of seminiferous tubules at different stages. Stages are indicated in the pictures. Positive red signals were observed in nuclei of most pachytene spermatocytes and in cytosols of spermatids and some pachytene cells. The cytosolic signals in pachytene spermatocytes are indicated by arrowheads in F. All scale bars represent 50 µm.

**Figure 4.** Spermatocyte-specific interaction of the chromatin at \textit{IncRNA-HSVIII} with the \textit{Prss42/Tessp-2} promoter. A: Schematic drawing of primer positions and restriction sites for 3C analysis. The \textit{Prss/Tessp} cluster was depicted with eight DNase I HSs and \textit{IncRNA-HSVIII} at the top. Bent arrows indicate the transcriptional direction and vertical arrows represent DNase I HSs. Below the structure, positions of \textit{PstI} restriction sites are indicated by vertical lines. Between the lines, primer positions are shown by horizontal arrows. An anchor primer is drawn by a black arrow, and the others by grey arrows. Distance from the restriction site close to the anchor primer is indicated at the bottom. B: Physical interaction was investigated by 3C analysis in testicular germ cells and liver cells. The cells were crosslinked with formaldehyde, and the nuclei were isolated and digested with \textit{PstI} restriction enzyme. After dilution of the sample, the chromatin was ligated, and genome DNA was purified. Quantitative PCR was performed using the anchor primer at a 3’ region of \textit{IncRNA-HSVIII} with another primer at HSI, HSIV, \textit{Prss/Tessp} promoters, and some other regions as indicated in A. \textit{PstI} digestion and ligation was also performed with a BAC clone encompassing the \textit{Prss/Tessp} cluster, and the data obtained from germ and hepatic cells were normalized to those from the BAC clone. To
compare the interaction frequency from different tissues, the data were further normalized to those at the *Ercc3* locus which was set to 1.0. The black bar represents the position of the anchor primer. The *lncRNA-HSVIII* region interacted with HSIV and the *Prss42/Tessp-2* promoter only in germ cells. The data are presented as mean ± SD from three independent experiments. *P*<0.05 and **P*<0.01 relative to the interaction frequency in liver cells. *P* = 0.0475 at HSVI and 0.0056 at the *Prss42/Tessp-2* promoter. 

**Figure 5.** Enhancer activity of the 5.8-kb sequence encompassing *lncRNA-HSVIII*. A: Schematic drawing of the *Prss42/Tessp-2* gene and *lncRNA-HSVIII*. The *Prss42/Tessp-2* locus is drawn as in Fig. 2A. The position of DNase I HSVIII is shown by a vertical arrow. A 5.8-kb *Eco*RI fragment encompassing *lncRNA-HSVIII* is indicated below the gene structure. *Hinc*II and *Sac*I were used to obtain 5’ and 3’ flanking sequences to *lncRNA-HSVIII*, respectively, and their sizes are shown. B and C: Luciferase assay was conducted with constructs indicated at the left side of the graph. The indicated constructs were prepared and transfected into embryonal carcinoma, P19TG1 cells (B) or hepatic tumor, Hepal-6 cells (C), and luciferase activity was measured 48 hr after transfection. The value from the construct without any promoter was set to 1.0 in each experiment. The 5.8-kb sequence significantly increased *Prss42/Tessp-2* promoter activity in both cells. The data are presented as mean ± SD from at least three independent experiments. *P*<0.05 relative to the construct without the 5.8-kb sequence and to the construct with a 6.7-kb *λ*DNA.
Figure 6. No induction of IncRNA-HSVIII transcription by the reporter construct. The expression of IncRNA-HSVIII was measured by quantitative RT-PCR with cDNAs obtained from P19TG1 cells that were transfected with the indicated constructs. The transfection of the construct with the 5.8-kb fragment did not result in the increase of the IncRNA-HSVIII level.

Figure 7. Enhancer activity of upstream and downstream sequences of IncRNA-HSVIII. A: Enhancer activity of the IncRNA-HSVIII sequence was assessed by reporter gene assay. Constructs indicated at the left side of the graph were transfected into P19TG1 cells (top) or Hepa1-6 cells (bottom), and luciferase activity was measured 48 hr after transfection. Relative activity was calculated and presented as in Fig. 5. The IncRNA-HSVIII sequence did not possess enhancer activity in either cells. The data are presented as mean ± SD from at least three independent experiments. B: Enhancer activity of the upstream and downstream sequence of IncRNA-HSVIII was evaluated. A 2.3-kb upstream and a 1.5-kb downstream sequence of IncRNA-HSVIII was obtained by HincII and SacI restriction digestion of the 5.8-kb fragment, respectively, as indicated in Fig. 5A. All the constructs were separately transfected into P19TG1 cells and Hepa1-6 cells, and luciferase activity was measured and presented as in Fig. 5. Both upstream and downstream sequences significantly increased Prss42/Tessp-2 promoter activity in both cells. The data are presented as mean ± SD from at least three independent experiments. *P<0.05 relative to the construct without the 5.8-kb sequence.
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<td>5'-TGA GAT CGT CAC ACG TCC TT-3'</td>
<td></td>
</tr>
<tr>
<td>Plasmid Constructs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tessp-2 promoter</td>
<td>5'-CCA AGT ACA CTG TAG CTG TC-3'</td>
<td>5'-GTC ATC ACG TAG GCC ACC CT-3'</td>
</tr>
<tr>
<td>5' half of IncRNA-HSVIII</td>
<td>5'-TTC ATC TTC CCT CAC TAG TCA TCA TTT G-3'</td>
<td>5'-ACC AGG CTG CTC TG AAC TTA C-3'</td>
</tr>
<tr>
<td>3' half of IncRNA-HSVIII</td>
<td>5'-AGT ATA GAG GAG CTC TTA TC-3'</td>
<td>5'-TTT TCT TTT CTT TTC TCT TTT TTC TTC TTC TTC TCT TGT TGT-3'</td>
</tr>
</tbody>
</table>
Figure 4

(A) Genomic structure showing regulatory elements and their positions relative to Poxv sites.

(B) Relative interaction frequency for Germ cell and Liver.

(C) Relative interaction frequency for various cell types, including Spermatogonia, Primary Spermatocytes, Secondary Spermatocytes, Spermatids, and Spermatozoa.
Figure 5

(A)

(B)

(C)
Figure 6

[Diagram showing relative expression levels of IncRNA-HSVIII]