We previously isolated 80 TISP (transcript induced in spermiogenesis) genes whose transcription is dramatically induced during spermiogenesis. Our analysis here of the expression of these genes in the testis of the cAMP-responsive element modulator (CREM)-null mouse reveals that 54 TISP genes are under the transcriptional regulation of CREM. One CREM-regulated gene is Tisp40, which encodes a basic leucine zipper (bZip)-type transcription factor bearing a transmembrane domain that generates the two proteins Tisp40α and Tisp40β. Both of these proteins function by binding to UPRE (unfolded protein-response element) but do not recognize CRE motifs. We show here that Tisp40α mRNA is generated under the direct transcriptional regulation of CREM. CREM and Tisp40 form a heterodimer, which functions through CRE but not through UPRE. Furthermore, binding ability of CREM to CRE is dramatically up-regulated by forming a heterodimer with Tisp40αΔTM, a truncated form of Tisp40α that lacks the transmembrane domain. We confirmed that Tisp40 and CREM actually bind to the Tisp40 promoter in vivo by chromatin immunoprecipitation assay. Finally, we demonstrate that the Tisp40ΔTM-CREM heterodimer acts as a recruiter of HIRA, a histone chaperone, to CRE. Taken together, we propose that Tisp40 is an important transcriptional regulator during spermiogenesis.

Spermatogenesis is a complex process that produces mature sperm. It involves three phases, namely, proliferation of spermatogonia, meiosis, and spermiogenesis. During spermiogenesis, sperm nuclei become tightly packed with proteins, first by small basic proteins called transition proteins and then by protamines (1). Thus, it was believed that none or only very few genes are expressed after meiosis (2). Challenging this belief, however, is our previous report that shows many spermatid-specific genes (TISP, transcript induced in spermiogenesis) are up-regulated in the round spermatids after meiosis (3).

It is known that the bZip-type transcription factor CREM acts as a master regulator of spermatogenesis (4). CREM generates many alternatively spliced forms. One of these is called ICER (inducible cAMP early repressor), which is an inactive form of CREM that suppresses CRE-mediated gene expression during meiosis. In post-meiotic cells, however, CREM generates active forms called CREMα, -β, and -γ, which up-regulate many CRE-mediated genes (4, 5). CREM-null mice display male infertility; while the spermatogonia differentiate normally into round spermatids, all the elongating spermatids are eliminated by apoptosis during spermiogenesis, which results in the lack of late spermatids (6–8). Therefore, it is thought that CREM is an essential factor for spermiogenesis.

Some spermiogenic genes, such as the genes for transition proteins (Tnp1, Tnp2, protamine (Prm)1, Prm2, and angiotensin-converting enzyme (ACE), have been identified as the direct transcriptional targets of CREM (6, 7). However, CREM probably regulates the expression of many other spermiogenic genes because defects in the Tnp1, Tnp-2, Prm1, Prm2, and ACE genes alone cannot fully explain the male infertility of CREM-null mice (6, 7). For example, mice whose Tnp1 and Tnp2 genes are both knocked out can still produce epididymal sperm, unlike CREM-null mice (6, 7, 9). Thus, identification of other novel target genes of CREM would shed light on the mechanisms regulating spermiogenesis.

CREB (cAMP-response element-binding protein)-binding protein (CBP) acts as a co-activator of many transcription factor through its histone acetyl transferase activity, which is recruited by transcription factors on promoter regions and acetylates histones (10). In case of CREB, recruitment of CBP to the promoter region by CREB is dependent on its phosphorylation (11). Once recruited, CBP acetylates histones to facilitate new transcription. In contrast, CREM is not phosphorylated in male germ cells. Consequently, instead of CBP, ACT (activator of CREM in testis) is believed to function as the trans-activator of CREM in male germ cells (7, 12, 13). Prior to chromatin condensation, ACT is exported by Kif17b, a testis-specific kinesin, independent of its motor domain, and this export is inhibited by leptomycin B (14, 15). Notably, while ACT-null mice display sperm abnormality, they can nevertheless produce offspring (16). This observation reveals that the trans-activation of CREM is retained in ACT-null mice, which indicates that another, as yet unknown, factor can also act as an activator of CREM, probably by complementing ACT function.

Although CREM is an essential factor for spermiogenesis, precisely how CREM regulates the transcription of haploid-specific genes remains unknown. Here we report that a novel regulator, Tisp40, acts as a...
recruiter of chromatin modifier to CRE by forming heterodimer with CREM during mouse spermiogenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—To prepare pCMV-3FLAG-Tisp40α/βΔTM, pCMV-6Myr-CREMα (wt and S117A), pCDNA3-CREMα (wt and S117A), and several reporter plasmids, we generated cDNA fragments containing the appropriate open reading frames and the promoter regions by PCR. The lack of artificial point mutations from PCR was confirmed by DNA sequencing of the generated plasmid DNA. p5XATF6 (UPRE)-GL3 is a kind gift from Prof. K. Mori (Kyoto University).

**ChIP Assay**—Preparation of RNA from the testes of CREM(+/-) mice and Northern blot analysis were performed as described previously (3). TISP cDNA inserts cut out of the plasmids by EcoRI/NotI restriction enzyme were radiolabeled by [32P]dCTP with the random primer DNA labeling kit (TaKaRa, Japan) and used as hybridization probes. The Northern blots were probed with radiolabeled gyceraldehyde-3-phosphate dehydrogenase cDNA as a loading control. GST-Tisp40αΔTM was expressed in Escherichia coli and purified on glutathione-Sepharose 4B. HA-CREM was expressed with the TnT expression system (Promega). We mixed GST-Tisp40αΔTM with HA-CREM on glutathione-Sepharose 4B and conducted Western blot analysis after extensive washing. HA-CREM expressed in TnT lysates was used as a positive control whereas GST alone served as a negative control. Western blot analysis was performed as described previously (17).

**Cell Culture, Transient Transfection, Luciferase Assay, Co-immunoprecipitation Assay, and EMSA**—The culture of HeLa S3 cells, their transient transfection, the luciferase assay and EMSA (electrophoresis mobility shift blot analysis after extensive washing) have been described (17). For the co-immunoprecipitation assay, transfected HeLa S3 cells were extracted in binding buffer (25 mM Hepes-KOH, pH 7.8, 150 mM KCl, 5 mM MgCl2, 2.5 mM CaCl2, 1 mM DTT, 0.05% Nonidet P-40) supplemented with protease inhibitor mixture (Sigma) and 1,000 units micrococcal nuclease for 1 h at 4 °C. The lysates were centrifuged by 17,400 g for 15 min at 4 °C. The supernatant was then incubated with protein G-Sepharose with rotation for 1 h at 4 °C and precipitated by centrifugation. The supernatant was subsequently incubated with anti-Myc antibody (PL14;MBL), anti-CREM antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or control IgG with rotation overnight at 4 °C. Protein G-Sepharose was then added to this mixture, incubated with rotation for 1 h at 4 °C, and precipitated by centrifugation. After extensive washing, the precipitated proteins were subjected to Western blot analysis using anti-Tisp40 antibody or anti-CREM antibody as the probes. To detect the endogenous interaction between CREM and Tisp40, we used two tests from adult mice and 1 μg of anti-CREM antibody, anti-Tisp40 antibody, or rabbit IgG.

**In Vivo Electroporation Assay**—Reporter DNA fragments containing the Tisp40 upstream region, the luciferase gene, and the SV40 poly(A) signal were amplified from the recombinant luciferase constructs by PCR with the following oligonucleotide pair: RV Primer3, 5'GACTGCTATGGCCGCCGG-3' and 1,000 units micrococcal nuclease for 1 h at 4 °C. The lysates were centrifuged by 17,400 g for 15 min at 4 °C. The supernatant was then incubated with protein G-Sepharose with rotation for 1 h at 4 °C and precipitated by centrifugation. After extensive washing, the precipitated proteins were subjected to Western blot analysis using anti-Tisp40 antibody or anti-CREM antibody as the probes. To detect the endogenous interaction between CREM and Tisp40, we used two tests from adult mice and 1 μg of anti-CREM antibody, anti-Tisp40 antibody, or rabbit IgG.

**ChIP Assay**—Germ cells from two testes were fixed in 500 μl of 1% formaldehyde/phosphate-buffered saline(−) for 5 min at 4 °C. The fixed germ cells were sonicated (Output 7, ON 30 s OFF 60 s, 5 cycles at 4 °C, using handy sonic PR-20, TOMY, Tokyo) after phosphate-buffered saline(−) was washed two times. 200 μl of sonicated chromatin-protein complexes were diluted in 1.8 ml of ChiP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 16.7 mM Tris-HCl, pH 8.0). Diluted chromatin-protein complexes were immune-precipitated by antibody against CREM, Tisp40, or HIRA (mixed WC15 and WC19), respectively, and antibody against rabbit or mouse IgG (negative control) at 4 °C overnight after blocking with 800 μg of single strand DNA. The immune complexes were precipitated with protein G-Sepharose followed by washing with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), LiCl buffer (0.25 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0), and TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The immune-purified complexes of chromatin proteins were eluted in 50 ml of elution buffer (1% SDS, 0.1 M NaHCO3, and 10 mM DTT) twice, and 4 μl of 5 mM NaCl were added to eluted mixtures and incubated for 4 h at 65 °C. Then, 0.2 μl of 0.5 mM EDTA, 4 μl of 1 M Tris-HCl, pH 6.8, and 0.2 μl of 20 mg/ml protease K were added and incubated for 1 h at 45 °C. The chromatin DNA were purified by PheOH/CHCl3 extractions and EtOH precipitations. Purified chromatin DNA was used as PCR templates. PCR was conducted for 35 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min (3 min in the final cycle).

**Northern Blot Analysis, In Vitro Pulldown Assay, and Western Blot Analysis**—Preparation of RNA from the testes of CREM(−/−) mice and Northern blot analysis were performed as described previously (3). TISP cDNA inserts cut out of the plasmids by EcoRI/NotI restriction enzyme were radiolabeled by [32P]dCTP with the random primer DNA labeling kit (TaKaRa, Japan) and used as hybridization probes. The Northern blots were probed with radiolabeled gyceraldehyde-3-phosphate dehydrogenase cDNA as a loading control. GST-Tisp40αΔTM was expressed in Escherichia coli and purified on glutathione-Sepharose 4B. HA-CREM was expressed with the TnT expression system (Promega). We mixed GST-Tisp40αΔTM with HA-CREM on glutathione-Sepharose 4B and conducted Western blot analysis after extensive washing. HA-CREM expressed in TnT lysates was used as a positive control whereas GST alone served as a negative control. Western blot analysis was performed as described previously (17).

**RESULTS**

**The Expression of 54 TISP Genes Is Regulated by a CREM-dependent Pathway**—To determine how many spermatid-specific genes are directly up-regulated by CREM, we examined the effect of the CREM-null mutation on the expression of 80 TISP genes that we previously comprehensively identified as mouse spermatid-specific genes (3). Northern blot analysis revealed that the expression of 54 TISP genes is dramatically reduced in the CREM(−/−) mice (Fig. 1), which suggests that these genes are under the transcriptional control of CREM. Comparison of the intensities of the bands allowed us to classify these 54 TISP genes into three classes (A−C, see Fig. 1). The 38 class A TISP genes showed no expression in the CREM(−/−) testis. Consistent with previous reports (6, 7), protamine1 (TISP18), protamine2 (TISP1), and transition protein2 (TISP28) belong to this class. TISP40 is also a member of this group. The 11 class B clones, which showed intense expression in the CREM(+/+) testis, still exhibited weak expression in the
CREM(+/−) testis. The 5 class C clones showed at least two bands; one was expressed at almost equal levels in the CREM(−/−) and CREM(+/+) testes, whereas the other band was predominantly found only in the CREM(+/+) testis. The expression of the remaining 26 TISP genes did not appear to differ between the CREM(−/−) and CREM(+/+) testes (data not shown), despite the expression restricted to the spermatids (3). This suggests a novel transcriptional pathway exists during spermiogenesis that is independent of CREM.

Tisp40 mRNA Is Directly Transcribed by CREM through the CRE2 Site—We previously showed that TISP40 generates two proteins named Tisp40α and Tisp40β, and that transcription of the mRNAs for these proteins occurs after step 5, which is later than the transcription of the CREM gene (17). Because both of these mRNAs, which will be referred to hereafter as Tisp40α/β mRNAs, were not observed in CREM-null mice, we speculated that the transcription of the Tisp40α/β mRNAs is under the direct control of CREM. To test this assumption, we performed promoter assays. Compared with Tisp40α mRNA, Tisp40β mRNA contains an extra 5'-region that adds 55 amino acids that may act as an acidic trans-activational domain (17). We found two CRE motifs between the transcriptional start sites of Tisp40α and Tisp40β. We named the upstream CRE site, CRE1, and the downstream CRE site, CRE2 (Fig. 2A).

**FIGURE 2.** Electrophoresis mobility shift assay to assess the importance of CRE sites in the TISP40 gene promoter. A, schematic presentation of the promoter region of TISP40 gene, which contains two CRE-like motifs (arrows) denoted as CRE1 and CRE2. Arrowheads indicate the transcriptional start sites of Tisp40α and Tisp40β. HA-CREM expressed in vitro was used to detect its binding to the 32P-labeled oligonucleotide probes representing wild-type (wt) and mutated (mt) CRE1 and CRE2 sites (lanes 2, 4, 6, and 8). In vitro-expressed HA tag alone incubated with the 32P-labeled probe served as negative controls (lanes 1, 3, 5, and 7). C, supershift assay with the anti-HA antibody (3F10) was used to show that the shifted band actually contains HA-CREM. The binding of the antibody generates a band that migrates more slowly (arrowhead) relative to the band when the antibody is absent (arrows). D and E, competition assays were used to confirm the binding of CREM to CRE1 and CRE2. Excess amounts (4×, 10×, or 20×) of unlabeled wt or mt CRE1 or CRE2 oligonucleotide probes were added to the reaction mixture containing HA-CREM and the 32P-labeled CRE1 and CRE2 oligonucleotide probes. The wt CRE2 competitor efficiently reduced the ability of CREM to bind to CRE1 and to CRE2 sites, unlike wt or mt CRE1 or mt CRE2.
To determine whether CREM actually binds to these CRE sites, we performed EMSA and found that in vitro expressed HA-CREM\(\alpha\) fusion protein bound to both the CRE1 and CRE2 sites (Fig. 2B, lanes 1, 2, 5, and 6), but not to mutated CRE1 and mutated CRE2 sites (lanes 3, 4, 7, and 8). To confirm that the HA-CREM\(\alpha\)-shifted CRE band actually contains HA-CREM\(\alpha\), we performed a supershift assay with anti-HA antibody (3F10), where the antibody was allowed to associate with HA-CREM\(\alpha\) before the radiolabeled CRE probe was added to the reaction mixture. This confirmed that the HA-CREM\(\alpha\)-shifted band indeed contained HA-CREM\(\alpha\) (Fig. 2C, lane 6). However, we could not detect HA-CREM\(\alpha\) bound to the CRE1 site by the supershift assay, probably because the interaction of HA-CREM\(\alpha\) with the CRE1 site is very weak (lane 3). Successful competition using 4-, 10-, or 20-fold molar excesses of CRE1 or CRE2 oligonucleotides also confirmed CREM\(\alpha\) associates specifically with wild-type CRE1 and CRE2 (Fig. 2D, lanes 1–5 and 11–15). In contrast, competition assays using mutated CRE1 and CRE2 site oligonucleotides did not inhibit the binding between HA-CREM\(\alpha\) and CRE very efficiently (Fig. 2D, lanes 6–10 and 16–20). Competition assays to compare the ability of CREM\(\alpha\) to bind to CRE1 (wt) and CRE2 (wt) showed that CRE2 (wt) efficiently reduced the binding activity of CREM\(\alpha\) to both CRE1 and CRE2, whereas CRE1 (wt) only reduced the binding of CREM\(\alpha\) to CRE1 (Fig. 2E). These results indicate that CREM\(\alpha\) prefers to bind to the CRE2 site rather than to the CRE1 site (Fig. 2, B–E).

We next determined the putative cis-element required for the expression of Tisp40α/β mRNA in vivo. To do so, we performed the in vivo electroporation assay that permits transient expression of reporter plasmids in the testes of mice; this assay was previously used to identify the cis-element in haploid spermatid-specific genes (18). We thus constructed reporter plasmids that harbored a deletion or a point mutation in the Tisp40α/β promoter (Fig. 3A), transferred them into the seminiferous tubules of the murine testes by electroporation and measured the luciferase activity. The results were normalized with full-length Tisp40 promoter-driven Renilla luciferase activity. As shown in Fig. 3A, the relative reporter expression of the 5′ deletion mutants −2071 to −151, −971 to −151, −311 to −151, −220 to −151, and −101 to −151 (lines 1, 2, 4, 5, and 6, respectively) was not significantly reduced when compared with the activity of the full-length Tisp40 promoter. In contrast, the relative reporter expression of the −2071 to −971 3′ deletion mutant (line 3) and the −50 to +151 5′ deletion mutant (line 7) was reduced to about 70% of the expression driven by the full-length Tisp40 promoter. These data indicate the region −101 to −50 of the Tisp40α promoter region is important for the expression of Tisp40α. This region contains the CRE2 site but not the CRE1 site. Consequently, we then prepared Tisp40α reporter plasmids bearing mutations in the CRE1 and/or CRE2 sites and transferred them into the testes by electroporation. We found that the reporter expression of theΔCRE1 promoter was not reduced (line 8), but the reporter expression of theΔCRE2 single mutant (line 9) and theΔCRE1/2 double mutant (line 10) were reduced to similar levels as the−2071 to −971 3′ deletion mutant (line 2) or −50 to +151 5′ deletion mutant (line 7). These data are consistent with the results obtained by EMSA (Fig. 2) and indicate that the CRE2 site is essential for Tisp40α mRNA expression.

Finally, to confirm that CREM\(\alpha\) actually up-regulates Tisp40α mRNA, we performed luciferase assays by cotransfecting HeLa cells with a reporter plasmid containing the Tisp40α/β promoter upstream of firefly luciferase cDNA with or without the 6Myc-CREM\(\alpha\)-expressing vector. The results were normalized by thymidine kinase promoter-driven Renilla luciferase...
expressed by using the *in vitro* transcription-translation system (TNT).

We next examined the association between the Tisp40a/β and CREMγ proteins in cultured cells by performing co-immunoprecipitation assays after transfecting HeLa cells with pCMV-6Myc-Tisp40a/βΔTM along with pCDNA3-CREMγ or empty vector. We first confirmed the transfection was successful by detecting the bands for 6Myc-Tisp40a/βΔTM and CREMγ in the whole cell extract (WCE) with anti-Myc monoclonal antibody (PL14) and anti-CREM polyclonal antibody, respectively (Fig. 4B, upper two panels). That anti-Myc antibody successfully immunoprecipitated 6Myc-Tisp40a/βΔTM (lanes 3–6, second panel from the bottom) was also confirmed by detecting the relevant bands in the Western blot. When the WCE was immunoprecipitated by anti-Myc antibody and probed with anti-CREM antibody, a band for CREMγ (indicated by an arrow) was detected only when Tisp40aΔTM (lane 5, bottom panel) or Tisp40bΔTM (lane 6, bottom panel) was produced together with CREMγ. This indicates that CREM associates with the Tisp40 proteins in HeLa cells. We could not perform reciprocal co-immunoprecipitation experiments, because the anti-CREM antibody nonspecifically recognized 6Myc-Tisp40a/βΔTM proteins when they were immunoprecipitated with anti-CREM antibody (arrowheads in bottom panel and data not shown).

Finally, we confirmed the physiological association of Tisp40 with CREM in the mouse testis by performing a co-immunoprecipitation assay using whole testis cell extract. As shown in Fig. 4C, CREM proteins were precipitated with anti-CREM antibody but not with control IgG (Fig. 4C, upper panel, lanes 1–3). When precipitated with anti-CREM antibody, Tisp40 protein was co-immunoprecipitated (Fig. 4C, lower panel, lanes 1–3). Here, the reciprocal co-immunoprecipitation with anti-Tisp40 antibody was confirmed (lane 4). These data suggest that Tisp40ΔTM and CREMγ form a heterodimer in the mouse testis.

**CREMγ Inhibits the Trans-activation of Tisp40δ through UPRE**—It has been shown that Tisp40δ binds to UPRE but not to CRE (17). We thus investigated whether the Tisp40δ-CREM heterodimer functions through UPRE or CRE. When we cotransfected HeLa cells with the pGL3–5XUPRE reporter plasmid (kind gift from Prof. K. Mori) together with the CREMγ-expressing vector and/or the Tisp40δΔTM-expressing vector and measured the luciferase activity, we found that Tisp40δΔTM activated transcription through UPRE but its activity was suppressed by CREMγ (Fig. 5, lanes 1–3). Furthermore, CREMγ (S117A), which is a trans-inactive mutant, also suppressed the trans-activity of Tisp40δΔTM (lane 4). These data suggest that the CREM-Tisp40δΔTM heterodimer does not function through UPRE and that the ability of CREMγ to inhibit the trans-activity of Tisp40δΔTM is not because of its transcriptional activity.

**The Binding Ability of CREMγ with CRE Is Increased by Tisp40δ**—Many CREB/CREM family proteins regulate transcription by binding to the CRE motif. However, Tisp40δ binds to UPRE but not CRE and regulates transcription through UPRE (17). Because the trans-activation of Tisp40δΔTM through UPRE was inhibited by CREMγ as shown above (Fig. 5), we speculated that the Tisp40δΔTM-CREMγ heterodimer may function as a trans-activator through CRE. To test this, we cotransfected HeLa cells with the pGL3–5XCREC construct derived from the somatostatin (SOM) gene promoter together with or without vectors expressing CREMγ, Tisp40δΔTM, or Tisp40δΔTM. Luciferase assays revealed that Tisp40δΔTM greatly enhanced the trans-activation of CREM (Fig. 6A, lanes 1, 5, 6, and 7). Furthermore, Tisp40δΔTM, a
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trans-inactive subtype, also enhanced the trans-activation of CREM in a dose-dependent manner (lanes 1–4).

The CRE promoter in the pGL3–5XCRE construct consists of artificial five tandem promoters. To exclude the possibility of artificial trans-activation because of this artificial construct, we examined if Tisp40 enhanced the trans-activation of CRE through the Tisp40α/βΔTM promoter region that contains the natural CRE sites. As we expected, the trans-activation activity of CREM co-transfected with Tisp40α/βΔTM was much greater when the −2071→ +151 Tisp40α/β or −971→ +151 Tisp40α promoter were co-expressed than when the −2071→ −971 Tisp40β promoter was co-expressed (Fig. 6B).

The activation of CREM and CREB requires the recruitment of CBP (CREB-binding protein), which in turn requires that CREM and CREB are phosphorylated at Ser133 or Ser117, respectively. However, it is believed that CREM is not phosphorylated at Ser117 in the testis. This would suggest that the recruitment of Tisp40α/β to CREM does not require the phosphorylation of the Ser117 residue of CREM. To test this notion, we investigated whether CREM (S117A) can also be activated by Tisp40α/βΔTM. Luciferase assays revealed that Tisp40α/βΔTM activated CREM (S117A), albeit with slightly lower efficiency (5–20% lower than that of wild-type CREMω) (Fig. 6C).

The above observations indicate that the Tisp40-mediated enhancement of CREMω trans-activity required the part of the Tisp40α promoter region that contains the CRE2 site. As shown in Fig. 6, A and B, the inactive subtype Tisp40α also enhanced the trans-activation of CREM. We speculated that this activation might be caused by the enhanced interaction of CREM with the CRE site. To test this, we performed EMSA with purified GST-Tisp40αΔTM and in vitro expressed HA-CREMω. HA-CREMω bound to the CRE site of SOM and the CRE2 site of Tisp40 (Fig. 6D, lanes 2, 6 and Fig. 2), whereas GST-Tisp40αΔTM could bind to neither CRE site (lanes 3 and 4). However, the interaction of HA-CREMω with both CRE sites was greatly enhanced (lane 4) and the band was slightly shifted (lane 6) when GST-Tisp40αΔTM was present. These data clearly demonstrate that the heterodimer consisting of CREMω and Tisp40ΔTM functions through CRE but not through UPRE (Figs. 5 and 6, A–D).

FIGURE 5. Luciferase assays to show CREMω (wt) and CREMω (S117A) both inhibit the UPRE-mediated trans-activity of Tisp40α/βΔTM. HeLa cells were cotransfected with 0.2 μg of pGL3–5XUPRE and 0.1 μg of each 3FLAG vector alone (negative control), Tisp40α/βΔTM, CREMω (wt) or CREMω (S117A), respectively. After 48 h of transfection, the cells were harvested and the cell lysates were subjected to luciferase assays. Average values of three independent experiments are shown. The results were normalized against Renilla luciferase activities obtained from pTK-hRL, which served as an internal control. All data were expressed as standard deviations of the mean for three independent experiments.

FIGURE 6. Promoter assays to show the effect of Tisp40α/βΔTM on the trans-activation of CREM through CRE. A, luciferase assays to show that CREMω activates transcription through CRE and that Tisp40α/βΔTM enhances this activity in a dose-dependent manner. HeLa cells were cotransfected with the reporter plasmid pGL3–5XCRE with or without vectors expressing CREMω and/or Tisp40α/βΔTM. After 48 h of transfection, the cells were harvested and the cell lysates were subjected to luciferase assays. B, luciferase assays to show that Tisp40α/βΔTM enhances the trans-activation of CREM only when CREM associates with the −971→ +151 promoter region of Tisp40α/β. This region is used to transcribe Tisp40α mRNA. HeLa cells were transfected with the indicated plasmids, and the cell lysates were subjected to luciferase assays. C, luciferase assays to show that the trans-activation through CRE of both wild-type CREM (wt) and mutated CREM (S117A) is enhanced by cotransfection with Tisp40α/βΔTM or Tisp40αΔTM. The results were normalized against Renilla luciferase activities obtained from pTK-hRL, which served as an internal control. All data were expressed as standard deviations of the mean for three independent experiments. D, EMSA to show that the binding selectivity and activity of the CRE site from the SOM gene (lanes 1–4) and the CRE2 site of Tisp40α (lanes 5–8) were enhanced when HA-CREMω and GST-Tisp40αΔTM were cotransfected (lanes 4 and 8) as compared with HA-CREMω alone (lanes 2 and 6) or GST-Tisp40αΔTM alone (lanes 3 and 7). E, CHIP assay to show that interaction CREM and Tisp40 with Tisp40 promoter in vivo. Lane 1, INPUT; lane 2, CHIP by rabbit IgG; lane 3, CHIP by anti-Tisp40 antibody; lane 4, CHIP by anti-CREM antibody.
Both Tisp40 and CREMt Bind to the CRE Motif in Vivo—Next, we performed chromatin immunoprecipitation assays to confirm that Tisp40 and CREM actually bind to the TISP40 promoter in vivo. For this purpose, we used the Tisp40 promoter region (−220 to +151), which contains both CRE1 and CRE2. We found that the TISP40 promoter region was actually co-immunoprecipitated with anti-CREM and anti-Tisp40 antibodies (Fig. 6E). The result suggests that Tisp40/TM-CREM cooperates with CREM to bind to the CRE at the midstage of mouse spermiogenesis.

Tisp40ΔTM-CREM Heterodimer Functions as a Recruiter of Histone Chaperone HIRA to CRE Motif—Recently, Adham et al. reported that the levels of certain CREM target genes, such as Prms and Tnps, are not reduced in Creb3l4/TISP40−/− deficient mice (32). We independently generated TISP40−/− mice and obtained similar results (data not shown). However, we additionally found the reduced levels of histone removal from testis chromatin in TISP40−/− deficient mice. Thus, we think that Tisp40 acts as a recruiter of a chromatin modifier that facilitates histone removal, such as histone (de)acetylase, methylase, or histone chaperone. Indeed, we purified complexes associated with the trans-regulational region of Tisp40 or CREM by performing pulldown assays (Fig. 7, A and B). Then, Tisp40 or CREM-associated proteins were analyzed by nano LC-ESI MS/MS analysis after separated by SDS-PAGE, and several proteins were successfully identified (Fig. 7B and supplemental Table 3). Among them, we focused on HIRA because it is a replication independent H3.3-specific histone chaperone (33). We found that HIRA was not only a partner of the CREM complex but also a component of the present in the Tisp40

I. Nagamori, K. Yomogida, I. Masahito, M. Okabe, N. Yabuta, and H. Nojima, unpublished data.
complex (Fig. 7C). Interestingly, the intensity of the HIRA band in Tisp40β (lane 2) complex is significantly weaker than those of Tisp40α and CREM complexes (lanes 1 and 3). Next, we tried to demonstrate an endogenous interaction between Tisp40 or CREM and HIRA by performing co-immunoprecipitation assays. Although we could not detect any interactions between HIRA and CREM or Tisp40, we found that HIRA interacted less efficiently with CRE in TISP40(−/−) testis (Fig. 7D).

Finally, luciferase assay revealed that Tisp40α ATM-CREMα-dependent transcriptional activity through CRE is activated by HIRA, but not by HIRA alone (Fig. 7E, lanes 1–3 and 5). In this assay, to emphasize the effects of Tisp40 and HIRA, expression of CREMα was reduced below the detection level of transcriptional activity induced by CREMα alone through CRE. Consistent with the results obtained by pulldown assays (Fig. 7, C and E), Tisp40β ATM-CREMβ-dependent transcriptional activity was not enhanced by addition of HIRA (lanes 4 and 6). As described above, HIRA is the histone H3.3-specific chaperone and H3.3 was deposited in the transcriptional active region (34). While their report support HIRA stimulation of transcription (Fig. 7E), it was reported that HIRA could repress the transcription of histones (35). We think HIRA actually represses the transcription of histones, and CRE-mediated transcription stimulated by HIRA seems to be caused by overexpression. Because, HIRA was not recruited to CRE (Fig. 7D) and histone removal was delayed in TISP40(−/−) testis, we speculated that real role of HIRA on CRE is histone removal during mid spermiogenesis. From these data, we conclude that Tisp40 and CREM play a pivotal role in the recruitment of HIRA to the CRE in testis.

**DISCUSSION**

In the present study, we found many (54) of the 80 known spermatid-specific TISP genes are novel transcriptional targets of CREM (Fig. 1, supplemental Tables 1 and 2). These genes include putative signal transducers such as TISP46 (Cdc14 phosphatase) and TISP47 (testis-specific Ser/Thr kinase), and putative scaffold proteins such as TISP50 (Shippo1), TISP19 (actin-like-7B), TISP51 (a kinase anchoring protein 110kDa), TISP52 (AKAP3/4), and TISP56 (actin-like-7a). TISP50 (Shippo1) is localized in both the elongated spermatid and the sperm tail and seems to act as a supporter of sperm conformation (19), while AKAP110 regulates PKA activity and sperm motility (20, 21) and AKAP3/4 is essential for sperm tail conformation (22, 23). Other novel TISP targets of CREM are actin family genes such as TISP19 (actin-like-7β), and TISP61 (actin-like-7α), which are known to be important in maintaining the cytoskeleton. Moreover, TISP69 (sperizin) is a RING finger protein that may function as an E3 ubiquitin ligase to promote the proteasome-mediated degradation of spermatid proteins (24, 25). Indeed, many proteins are poly- or mono-ubiquitinated during spermiogenesis (26). Our discovery that these TISP genes are in the CREM transcriptional pathway explains why male CREM-null mice are infertile: the absence of CREM would lead to poor expression of the cytoskeleton components, supporters of sperm conformation, signal transducers and regulators of proteasome-mediated proteolysis, which would cause abnormal sperm maturation. We believe that further characterization of the roles these TISP genes play in the CREM pathway may unveil the detailed molecular mechanism of spermiogenesis. It is known that many bZIP proteins form not only homodimers but also heterodimers or tetramers. For instance, c-Fos and c-Jun form a heterodimer (AP-1), whereas ATF6α or ATF6β forms a heterotetramer with NF-Y A, B, and C (27, 28). Similarly, it is thought that CREM forms a heterodimer with CREB and ATF1. However, unlike CREM, neither CREB nor ATF1 are expressed in spermatids. Thus, it was thought that CREM functions only as a homodimer in spermiogenesis. In the present study, however, we showed that CREM complexes with Tisp40, a new membrane-bound bZIP-type transcription factor that is expressed in postmeiotic male germ cells.

Regulated intramembrane proteolysis (Rip) is a widely conserved process found in species ranging from bacteria to humans (29). SREBPs and ATF6 are well known transcription factors that are activated via the Rip pathway. We previously reported that Tisp40 (17), like other transcription factors such as OASIS (30) and CREB1 (31), normally localizes in the endoplasmic reticulum and is activated via the Rip pathway. When cells are exposed to various types of stress, such as sterol depletin or the endoplasmic reticulum stress, these proteins are translocated into the Golgi apparatus. Subsequently, they are released from the membrane by two step proteolytic cleavage involving first site 1 protease (S1P) and then site 2 protease (S2P). The cleaved portion then enters the nucleus, where it acts as an activated transcription factor that regulates the transcription of its targets gene(s). We previously showed that Tisp40 is subjected to two step cleavage by S1P and S2P, after which it enters the nucleus (17). We show here that the nuclear forms of the Tisp40 proteins (Tisp40α/β ATM) associate with CREMα not only in vitro but also in vivo (Fig. 4). Moreover, the trans-activity of Tisp40β through UPRE is inhibited by CREMα (Fig. 5) whereas in contrast, Tisp40α/β ATM enhanced the trans-activation of CREM through CRE in cultured cells independent of phosphorylation of CREMα at Ser117 (Fig. 6, A–C). This enhancement is caused by an increase in the binding ability of CREM to CRE by Tisp40 (Fig. 6D).

Considering many TISP genes are not expressed in the testis of CREM-null mice (Fig. 1), whereas in ACT-null mice, the expression of these spermatid-specific genes is almost normal (16), we first hypothesized that the CREM-Tisp40 heterodimer functions as a highly active transcription factor in haploid spermatids. However, Adham et al. (32) and our group independently found that some of CREM-regulated genes are normally expressed in TISP40/CREB3α-deficient mice. Moreover, we found that histones were abnormally retained in later stage germ cells of TISP40-deficient testis. Thus, we think that Tisp40 acts as a recruiter of histone modifier to CRE site in cooperation with CREM. We here identified HIRA as one of the association partners of Tisp40 and CREM by pulldown and nano LC-ESI MS/MS (Fig. 7). Because HIRA is a histone H3.3 chaperone, we speculated that histone H3 was included in the Tisp40 and CREM complexes (33). Actually, histone H3 was included in the Tisp40 complex. Unexpectedly, however, histone H3 was not included in CREM complex (Fig. 7C).

It remains unclear where in chromatin regions H3.3 is deposited during spermatogenesis in mammalian cells. However, in Drosophila, H3.3 is properly deposited in chromatin during first meiotic prophase cells and elongating spermatids cells, and HSP70 genes rapidly lose histone H3 and acquire variant H3.3 histones (36, 37). From these data, H3.3 is thought to be linked to not only transcriptional active regions but also histone replacement, and then deposited and removed by HIRA (38).

Although it remains unclear whether all histones, which are abnormally retained in TISP40(−/−) testis, are directly regulated by HIRA surrounding the CRE, these data imply that both Tisp40 complex and CREM complex include HIRA but Tisp40 complex alone can function as a histone regulator. Considering that binding of Tisp40 with CRE is required for forming a heterodimer with CREM (Fig. 6, A–C) that recruitment of HIRA to CRE is Tisp40-dependent (Fig. 7D), that histone H3 (presumably H3.3) is included only in Tisp40 complex, and that histone removal is delayed in TISP40(−/−) mice, we here propose that Tisp40 forms a heterodimer with CREM to bind to CRE site for recruitment of HIRA to the CRE in cooperation with CREM.
ment of HIRA surrounding the CRE site to deposit and/or remove H3.3, and thus facilitate histone exchange during midspermiogenesis.

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