cAMP-responsive element in TATA-less core promoter is essential for haploid-specific gene expression in mouse testis

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ABSTRACT

Promoters, including neither TATA box nor initiator, have been frequently found in testicular germ cell-specific genes in mice. These investigations imply that unique forms of the polymerase II transcription initiation machinery play a role in selective activation of germ cell-specific gene expression programs during spermatogenesis. However, there is little information about testis-specific core promoters, because useful germ cell culture system is not available. In this study, we characterize the regulatory region of the haploid-specific Oxct2b gene in detail by using in vivo transient transfection assay in combination with a transgenic approach, with electrophoretic mobility shift and chromatin immunoprecipitation assays. Expression studies using mutant constructs demonstrate that a 34 bp region, which extends from -49 to -16, acts as a core promoter in an orientation-dependent manner. This promoter region includes the cAMP-responsive element (CRE)-like sequence TGACGCAG, but contains no other motifs, such as a TATA box or initiator. The CRE-like element is indispensable for the core promoter activity, but not for activator in testicular germ cells, through the binding of a testis-specific CRE modulator transcription factor. These results indicate the presence of alternative transcriptional initiation machinery for cell-type-specific gene expression in testicular germ cells.

INTRODUCTION

Spermatogenesis is a cyclic and continuous process through which spermatogonia undergo proliferation and differentiation, to give rise to mature spermatozoa. Each differentiation step requires the expression of a specific set of precisely regulated genes, some of which are expressed exclusively in testicular germ cells. Many testicular germ cell-specific genes are paralogues of genes that are expressed ubiquitously (1). It has also been reported that more functional intronless paralogues are expressed in pachytene spermatocytes and spermatids than in any other tissues (2–4). Intronless genes are considered to be retroposons derived from paralogues, or ancestral genes, that carry introns. It is generally the case for retroposons that the inserted cDNA which is reverse-transcribed from the mRNA cannot be expressed in any tissue, because the mRNAs lack a promoter in the 5'-flanking sequence of the genomic DNA. This indicates that an upstream sequence, which has no intrinsic promoter activity, has been adapted to function as a promoter. This prediction implies that testicular germ cells provide the appropriate environment for retroposon transcription, which facilitates gene expression from promoter-like sequences. It appears that the rules that govern gene transcription during later stages of spermatogenesis are drastically different from those in other cell types.

The expression of certain haploid-specific genes depends on TATA-containing core promoters (5–9). RNA polymerase II (Pol II), TFIIB and TATA-binding protein (TBP) accumulate in round spermatids, while the amount of mRNA increases in the testsis (10,11). Although the overexpression of the TBP gene has been implicated in the transcription of all the TATA-dependent genes in haploid germ cells, it does not account for the specificity of haploid-specific gene expression. In this context, the cAMP-responsive element (CRE) is present upstream...
of the TATA box in these TATA-dependent haploid-specific promoters. The testis-specific activator isoform of the CRE modulator (CREMt) upregulates promoter activity in sperm- 

atids through CRE binding (12), and spermatogenesis in male CREM-null mice stops at the stage of early spermatids (13,14).

The ACT co-activator binds and modulates CREM

The promoter structure of the gene enables the study of the machinery of initiation complex formation is unique to haploid-specific promoters. The testis-specific activator isoform of the CRE gene, was amplified by PCR from a genomic clone (23), using an upstream BgIII site-containing primer and a downstream HindIII site-containing primer (Table 1). PCR was performed with 200 ng of template DNA in 100 µl of reaction mixture [1 µM of each primer, 800 µM dNTPs, 1× reaction buffer and 1 U of Taq DNA polymerase (TaKaRa Biomedical)]. The PCR conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 1.5 min. After digestion with BglIII and HindIII, the amplified fragment was sub-

cloned into the BglIII and HindIII sites of the pEGFP-1 vector (Clontech), to generate the −566/+35EGFP construct. The −103/+16EGFP construct was cloned in a similar manner into the pEGFP-1 vector, using a primer pair that introduced BglIII and HindIII sites at the 5′ and 3′ ends, respectively (Table 1).

A series of Oxt2b upstream deletion constructs, which included −932/+35Luc, −566/+35Luc, −389/+35Luc, −256/+35Luc, −103/+35Luc, and −50/+35Luc, −389/+5Luc, −389/−16Luc, −389/−50Luc and −566/−50Luc, were cloned into pGL3-Basic (Promega), using primer pairs that introduced BglIII and HindIII sites at the 5′ and 3′ ends, respectively (Table 1).

The Δ−49/−16Luc construct was generated by introducing a synthetic oligonucleotide that contained HindIII restriction sites at either end and extended from −15 to +35 into the HindIII site of −566/−50Luc. The −389/−49/−16Luc, −389/−16/−49Luc and CLmut constructs were generated by introducing a synthetic oligonucleotide that contained HindIII restriction sites at either end and extended from −49 to −16 into the HindIII site of −389/−50Luc. The −49/−16(−389/−49/−16)Luc and −16/−49(−389/−49/−16) Luc constructs, or the −49/−16(−389/−49/−16)Luc and −16/−49(−389/−49/−16) Luc constructs, were generated by introducing a synthetic oligonucleotide that contained HindIII restriction sites at either end and extended from −49 to −16 into the BglIII site of −389/−49/−16 Luc or CLmut, respectively. For the standardization of the in vivo electroporation, the OAZt-pRL construct was generated by cloning the testis-specific ornithine decarbox-
ylase antizyme (OAZt) promoter into the pRL-Null vector (Promega) (18). All the constructs were confirmed by restriction enzyme digestion and DNA sequencing. Plasmid DNA was purified using QIAGEN columns (Qiagen).

### MATERIALS AND METHODS

#### Reporter constructs

For the transgenic analysis, the 600 bp genomic DNA fragment, that extends 566 bp upstream and 35 bp downstream of the transcriptional start site of the Oxct2b gene, was amplified by PCR from a genomic clone (23), using an upstream BgIII site-containing primer and a downstream HindIII site-containing primer (Table 1). PCR was performed with 200 ng of template DNA in 100 µl of reaction mixture [1 µM of each primer, 800 µM dNTPs, 1× reaction buffer and 1 U of Taq DNA polymerase (TaKaRa Biomedical)]. The PCR conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 1.5 min. After digestion with BglIII and HindIII, the amplified fragment was sub-

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ylase antizyme (OAZt) promoter into the pRL-Null vector (Promega) (18). All the constructs were confirmed by restriction enzyme digestion and DNA sequencing. Plasmid DNA was purified using QIAGEN columns (Qiagen).

#### Table 1. The oligonucleotides used for PCR

<table>
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<th>Construct</th>
<th>Forward oligonucleotides</th>
<th>Reverse oligonucleotides</th>
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<tr>
<td>−566/+35EGFP</td>
<td>5′-NNAGACTTCAGAGGAAGCTGTTTCCC-3′</td>
<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
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<tr>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
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<tr>
<td>−389/+35Luc</td>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
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<tr>
<td>−256/+35Luc</td>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
</tr>
<tr>
<td>−103/+35Luc</td>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
</tr>
<tr>
<td>−50/+35Luc</td>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
</tr>
<tr>
<td>−389/+5Luc</td>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
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<tr>
<td>−389/−50Luc</td>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
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<tr>
<td>−389/−16Luc</td>
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<tr>
<td>−389/−50Luc</td>
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<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
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<tr>
<td>−566/−50Luc</td>
<td>5′-NNAGACTTCAGAGGAAGCTGTTTCCC-3′</td>
<td>5′-CTGAAGCTTAGTCGCCCGACAGAGAGAGA-3′</td>
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</table>

Primers for transgenic mouse analysis

| −103TG, 5′-CAGGaATGCCCAAGGGGTGTCCT-3′ | EGFP-N, 5′-CGTCGCGCGGCCATGGCGGTCGCT-3′ |
Generation of transgenic mice

The enhanced green fluorescent protein (EGFP) constructs described above were digested using BglIII and AflIII. The inserted fragments, which included the Ooct2b upstream region, the EGFP gene and the SV40 polyadenylation [poly(A)] signal, were resolved by agarose gel electrophoresis and purified using glass beads (Qiaex; Qiagen). The excised DNA fragments were injected into the male pronuclei of C3HxC57BL/6 F1 eggs. Transgenic founders from the microinjected eggs were identified by PCR using the primer pair – 103TG/EGFP-N (Table 1), and the founders were mated with the C57BL/6 mice. The positive male offspring from this cross were used in subsequent experiments.

Histological analysis

Fluorescent stereoscopic photographs were taken of whole testes from transgenic adult mice. Histological analyses were performed as described previously (24).

In vivo electroporation assay

The reporter DNA fragments that contained the Ooct2b upstream region, firefly luciferase gene and SV40 poly(A) signal were amplified from the recombinant luciferase constructs by PCR using oligonucleotide pair 5′-CTAGCAAAATAGGCTGTCCCC-3′ (RV primer3) and 5′-GACGATAGTCACTCCCCCGG-3′ (RV primer4), which correspond to regions 4760–4779 and 2061–2080 of the pGL3-Basic vector, respectively. DNA fragments for standardization, which contained the OAZt promoter, the Renilla luciferase gene and SV40 poly(A) sequences, were amplified from OAZt-pRL by PCR using the primer pair 5′-TTTGCTCACATGGCTCGAC-3′ (RL5) and 5′-GGTGGACCCAGCCGACAC-3′ (RL6), which correspond to regions 3291–3310 and 1511–1530 of the pRL-Null vector, respectively. The PCR conditions were as described above except for annealing condition, which was performed at 56°C for 1 min. The PCR products were purified using SUPREC™-PCR (TaKaRa Biomedical).

For each transfection, 12 µl of a mixture [that contained 5 µg of each PCR reporter product, 5 µg of the OAZt-pRL PCR product, 100 µM of general caspase inhibitor (Z-VAD-FMK; R&D Systems, Inc.) and 0.1 mg/ml of trypan blue (Nacalai Tesque)] was injected through the efferent duct into the ovulated eggs of C57BL/6 mice. The positive male offspring from this cross were used in subsequent experiments.

Electrophoretic mobility shift assay

The mouse testicular germ cell fraction was collected essentially as described previously (25,26). The germ cells were suspended in 3 ml of buffer A [10 mM HEPES–KOH (pH 7.8), 0.1 mM EDTA, 10 mM KCl, 0.1% NP-40, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/µl aprotinin, 2 µg/µl pepstatin and 2 µg/µl leupeptin] before being incubated for 15 min at 4°C. The solution was mixed by gentle vortexing at 4°C for 5 min and was centrifuged at 2300 g for 5 min at 4°C. Nuclear protein extracts were prepared from testis germ cell fraction and liver cells, essentially as described previously (27). The nuclear pellets were washed in buffer A and resuspended in 1 ml (testis) or 2 ml (liver) of buffer C [50 mM HEPES–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, 2% glycerol, 1 mM DTT, 0.5 mM PMSF, 2 µg/µl aprotinin, 2 µg/µl pepstatin and 2 µg/µl leupeptin]. The suspension was rotated slowly for 30 min at 4°C. The resultant nuclear lysates were centrifuged at 13 000 g for 15 min (testis) or 30 min (liver) at 4°C. The supernatants of the nuclear extracts were aliquoted, frozen in liquid nitrogen and stored at −80°C until use. Protein concentrations were 3–10 mg/ml.

The synthetic oligonucleotides (10 nmol; Table 2) were annealed to produce a double-stranded DNA fragment with extruding GG-dinucleotides at the termini. Each DNA fragment was labeled with 5-amino-propargyl-2′,3′-deoxyctydine 5′-triphosphate coupled to Cy5.5 fluorescent dye (Cy5.5-AP-2, FluoroLink™Cy5.5-dCTP; Amersham Pharmacia Biotech) using the Klenow fragment of DNA Pol I. The binding reactions were performed by incubating 5 µg of nuclear extract and 300 fmol of Cy5.5-labeled probe, with or without competitor, for 30 min at room temperature in 10 µl of binding buffer [20 mM HEPES–KOH (pH 7.8), 31 mM KCl, 80 mM EDTA, 8% glycerol, 1 mM DTT, 0.5% Tween-80, 0.2 µg of poly(d–dC)]. For the supershift assay, 200 ng of antibody was added after the binding reaction and the mixture was incubated for a further 10 min at room temperature. Rabbit polyclonal anti-CREM [CREM-1 (X-12): sc-440], rabbit polyclonal anti-AP-2α [AP-2α (C-18): sc-184] and mouse monoclonal anti-CREM [CREM-1 (240): sc-58] antibodies were purchased from Santa Cruz Biotechnology. The samples were loaded onto 5% polyacrylamide gels made in 0.5× TAE [50 mM Tris–HCl (pH 8.0), 30 mM sodium acetate acid and 10 mM EDTA]. Electrophoresis was performed at 4°C at 250 V for 40 min. Gel imaging was carried out using the Odyssey Infrared Imaging System (LI-COR) at a wavelength of 700 nm.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed with modifications to the protocol described by the manufacturer (Upstate Biotechnology) and Sakakibara et al. (28). The collected germ cell fraction described above was cross-linked in vivo in 1% formaldehyde in phosphate-buffered saline (PBS) at 37°C for 10 min with slow rotation. The cross-linked germ cells were pelleted by centrifugation at 2300 g for 4 min at 4°C and were washed twice in ice-cold PBS containing 0.5 mM PMSF, 2 µg/µl

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**Table 2.** The oligonucleotides used for EMSA

<table>
<thead>
<tr>
<th>Fragment (location)</th>
<th>Sequencea</th>
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<tr>
<td>1(−50/−10)</td>
<td>5′-AGAGTTCCACCTCCCTGCTCTG-TAGGCAGCCGACGCCTG-3′</td>
</tr>
<tr>
<td>2(−50/−31)</td>
<td>5′-AGAGTTCCACCTCCCTGCTCTG-TAGGCAGCCGACGCCTG-3′</td>
</tr>
<tr>
<td>3(−40/−20)</td>
<td>5′-GTTCCTGCTGTCGTAGGCCAGCG-3′</td>
</tr>
<tr>
<td>4(−30/−10)</td>
<td>5′-GGGGTGGAGCCGACGCGGTCG-3′</td>
</tr>
</tbody>
</table>

aThe underlined GG are recessed dinucleotides at 5′ end used for Cy5.5 labeling.
RESULTS

The 0.6 kb 5′-upstream sequence of the Oxct2b gene is responsible for haploid spermatid-specific expression in transgenic mice.

The Oxct2b gene is expressed in late spermatids in the testis (22). To determine the genomic region that is responsible for haploid-specific Oxct2b expression, transgenic mouse lines were generated by using −566/+35EGFP (Figure 1A). Of the six founder lines that were established, almost all (49/52) of the transgenic male pups expressed the EGFP reporter gene. Green fluorescence was observed in the seminiferous tubules of the testes of the transgenic mice, but not in the other tissues examined (Figure 1B and C). No EGFP signals were observed in non-transgenic littermates. To determine which cell type expressed the reporter gene, the testes of −566/+35EGFP transgenic mice were examined histologically. The EGFP signals were faint in the inner layers of the seminiferous tubules at stage IX, but were strong at other stages (Figure 1D). These strong signals were restricted to elongating and elongated spermatids, and were not observed in spermatogonia, spermatocytes, round spermatids or in any other non-spermatogenic cells within the testis (Figure 1D). The EGFP expression pattern was in good agreement with the expression pattern of the endogenous Oxct2b transcripts described previously (22). These results confirm that the 0.6 kb, 5′-upstream sequence is sufficient for haploid-specific expression of the Oxct2b gene.

Identification of a core Oxct2b promoter by in vivo transient transfection

Detailed functional analyses of promoters are usually carried out using cultured cells that transcribe the gene of interest. However, a culture system for spermatogenic cells is not currently available. In this study, to identify the essential elements for Oxct2b transcription in the male germ cells, we used an in vivo transient transfection technique in testicular germ cells, as described previously (24,25). We introduced PCR products that contained Oxct2b promoters fused to a luciferase reporter into testicular germ cells using a combination of DNA injection into the seminiferous tubules and subsequent in vivo electroporation. We made a series of 5′-deletion constructs and tested their promoter activities. As shown in Figure 3A, deletions that extended up to −932 or to −389 resulted in no significant reduction in promoter activity compared to −566/+35Luc, which is the upstream region that was used for the transgenic analysis. Further deletions from −389 to −256, −256 to −103 and −103 to −50 resulted in stepwise reductions of the luciferase activity of −566/+35Luc to 61, 37 and
16%, respectively. These data suggest that the 339 bp region from /C0 389 to /C0 50 is required for high-level promoter activity in testicular germ cells. In addition, /C0 50/+35Luc showed basal promoter activity, because luciferase activity produced by this construct was 2-fold higher than that of the promoter-less pGL3-Basic vector.

The Oxct2b promoter contains the CCAAAGT motif, which is similar to the Inr consensus sequence of PyPyANA/TPyPy (29) that surrounds the transcription initiation site, but lacks a TATA box (Figure 2). The functions of several Inr-containing promoters that lack TATA boxes are supported by important downstream sequences (25). To investigate whether the intergenic region of the Oxct2b gene also contributes to the promoter activity of this gene, we generated several 3′-deletion constructs and examined their promoter activities using in vivo transient transfection. As shown in Figure 3B, the construct that contained a 51 bp deletion to position /C0 16, which included the downstream region and transcription initiation site of the gene, showed no significant reduction in promoter activity. A further 34 bp deletion, which removed the /C0 16 to /C0 49 region, reduced the reporter gene activity to basal levels. These results indicated that the intergenic region of the Oxct2b gene does not contribute to promoter activity, whereas the sequence from /C0 49 to /C0 16 is necessary for Oxct2b promoter activity.

We further verified the functional importance of this fragment, using the internal deletion construct Δ−49/−16Luc. As shown in Figure 4A, the Δ−49/−16Luc construct induced a level of luciferase activity that was not significantly higher than that of the −566/−50Luc construct. This result indicates that the −49/−16 sequence is indispensable for Oxct2b promoter activity. We postulate that the −49/−16 sequence

Figure 2. Sequence alignment of the upstream regions of the Oxct2b (GenBank accession no. AB105455) and Oxct2a (GenBank accession no. AB105454) genes. Identical nucleotides are represented by asterisks; deletions are indicated by dashes. The numbers to the right of each sequence are relative to the transcription start site (23), which is designated as +1. The major putative consensus transcription factor binding sites, as predicted from database analysis using the internet-based TFSEARCH (http://150.82.196.184/research/db/TFSEARCH.html) are boxed (set at a cut-off score of 85). The translational initiation codon ATG at position +60 is highlighted in gray.

Figure 3. Deletion analysis of Oxct2b promoter activity using in vivo transient transfection of testicular germ cells. The Oxct2b promoter was deleted sequentially either upstream (A) or downstream (B) of the transcription start site, and individual deletions were fused to the luciferase reporter gene. The deletion constructs were transfected into the seminiferous tubules of mouse testes. Luciferase activity, which was normalized to that of Renilla luciferase driven by the OAZt promoter (25), is presented as the mean values (±SEM) of three independent experiments that were performed in duplicate. *P < 0.05, **P < 0.01 compared with −566/+35Luc (A) and −389/+35Luc (B) (ANOVA followed by Bonferroni analysis). The luciferase activities of −566/+35Luc (A) and −389/+35Luc (B) were set at 1.0.
includes a core promoter, i.e. a site for the assembly of the pre-initiation complex and for the determination of transcription orientation. We examined whether the direction of the –49/–16 region was critical for Oxct2b promoter activity. Figure 4B shows that the level of luciferase activity of the inverted orientation of the –49/–16 sequence (–389/–49/–16) was reduced to that of the –389/–50Luc construct, which lacks the –49/–16 region. These results demonstrate that the –49/–16 region contains a core promoter to direct transcription of the gene. To examine whether the –49/–16 region contains cis-regulatory elements, such as an enhancer, we positioned the –49/–16 sequence at the 5′ end of –389/–49/–16Luc and performed in vivo transient transfection into testicular germ cells. Figure 4C shows that both orientations of the re-positioned –49/–16 fragment failed to upregulate the luciferase activity of the –389/–49/–16Luc construct. These results suggest that the –49/–16 sequence does not act as a cis-regulatory element. The 20 bp sequence that encompasses the CRE-like motif (Oxct2b-CL) in the promoter region of the Oxct2b gene is conserved in the mouse paralogue Oxct2a and human orthologue OXCT2 (Figure 4D), which implies that this element plays an essential role in Oxct2 gene expression in spermatids.

To confirm that the Oxct2b promoter directs the reporter gene in spermatids, we generated transgenic mice using the –103/–16EGFP construct, which contains the core promoter and an additional 5′-upstream sequence (Figure 5A) that affects promoter activity but not cell type-specificity. Three transgenic lines were established. EGFP expression was detected in the testes of the transgenic, but not in non-transgenic littermates (Figure 5B and C). The EGFP signal in sections of testes from the –103/–16EGFP transgenic mice was detected in the innermost layers of the seminiferous tubules and was restricted to elongating and elongated spermatids (Figure 5D), as seen in the –566/+35EGFP transgenic lines (Figure 1). None of the transgenic lines showed EGFP signals in any organ other than the testis. These results demonstrate that the sequences upstream from –103 and downstream from –16 are not necessary for the accurate transcription of the EGFP reporter gene in the spermatids of transgenic mice.
The Oxct2b-CL motif within the promoter binds the CREM protein in testicular germ cell nuclei

The in vivo transient transfection experiments in combination with the transgene experiments show that the −49/−16 region is necessary for Oxct2b gene expression in haploid spermatids. We identified the CRE-like motif (Oxct2b-CL) TGAGCGCAG in the −29 to −22 region (Figure 2 and 4D), in which 3 nt in the 3′-half of the motif differ from the corresponding sites in the CRE consensus sequence TGACGTCA. To examine whether the transcription factor(s) in the testis nuclear extract could bind the Oxct2b promoter, an electrophoretic mobility shift assay (EMSA) was performed using an oligonucleotide of the −50/−10 region as the probe. Some DNA–protein complex bands were observed using nuclear extracts from the testis (Figure 6A, lane 3). In addition, the most prominent band was not observed in the liver sample (Figure 6A, lane 2). The most prominent gel-shift band was eliminated when the non-labeled −50/−10 oligonucleotide and fragments 3 and 4, all of which contained Oxct2b-CL, were used as competitors (Figure 6A, lanes 5–7). In contrast, fragment 2 (−50 to −31), which lacks Oxct2b-CL, did not compete for this band (Figure 6A, lane 4). Furthermore, a mutant competitor, in which all eight nucleotides of Oxct2b-CL were substituted, could not compete for complex formation (Figure 6B). These results indicate that the testicular nuclear protein binds directly to the Oxct2b-CL motif. The CRE or CRE-like motif is a well-known binding site for the CREB protein and CREM. We tested the specific interactions between CREB or CREM and Oxct2b-CL by supershift analysis with specific antibodies (Figure 6C). The specific band of the complex was removed by the anti-CREM antibody, but not by either the CREB or AP-2α antibody, which indicates that the trans-acting factor involved in this DNA–protein complex is a member of the CREM family. To test whether CREM directly binds to Oxct2b-CL in the core promoter within testicular germ cells, ChIP assays were performed. Testicular germ cell nuclei were cross-linked by formaldehyde and the lysates were immunoprecipitated with CREM or CREB antibodies. PCR amplification was performed using primers designed to amplify a fragment containing the Oxct2b-CL within the Oxct2b promoter. The predicted 215 bp PCR product was amplified from samples derived from CREM immunoprecipitates, but not from samples immunoprecipitated with CREB (Figure 6D). These data demonstrate that CREM interacts with Oxct2b-CL within the testicular germ cell nuclei.

To define in greater detail the binding sequence within the motif, several Oxct2b-CL mutants were used as competitors in EMSA with testis nuclear extracts. In this experiment, a fragment of the −40/−20 region was used as the probe. Substitution mutations in the TGACG sequence abolished the binding capability (m1, m2 and m3 in Figure 6E), whereas mutations in the CAG sequence did not affect binding capability (m4 and m5 in Figure 6E). These results indicate that the 5′-half of the Oxct2b-CL sequence is crucial for protein–binding activity. The binding specificity of Oxct2b-CL was examined further in a competition experiment with the CRE-like motif of the testis-specific angiotensin-converting enzyme (ACEt), the promoter activity of which has been demonstrated to be CRE-like sequence (TGAGGTCA)-dependent in male germ cells (30). The fragment that includes

![Figure 6. The Oxct2b core promoter binds specifically to CREM factor through an Oxct2b-CL motif. (A) The testis nuclear extract binds to the promoter (−50/−10) of the Oxct2b gene. Lane 1, without nuclear extract; lane 2, liver nuclear extract; lanes 3–7, testis nuclear extract without competitor (lane 3), in the presence of a 100-fold molar excess of unlabeled oligonucleotides 1 (lane 7), 2 (lane 4), 3 (lane 5) and 4 (lane 6), which are shown to the left as competitors. The arrow shows testis-specific protein binding. (B) The Oxct2b-CL element in the Oxct2b promoter (−50/−10) binds a testis-specific nuclear protein. A 100-fold molar excess of the unlabeled wild-type (wt) or mutated (mut) promoter fragment was used as the competitor. (C) The CREM factor binds to the Oxct2b promoter (−40/−20) in vitro. EMSAs were performed with testis nuclear extracts in the presence of anti-CREM, anti-CREB or anti-AP-2α antibodies, using a labeled promoter fragment that spanned −40 to −20. (D) The CREM factor binds to the Oxct2b promoter in germ cell nuclei. Formaldehyde-cross-linked chromatin prepared from testicular germ cells was immunoprecipitated with anti-CREM (lane 2) or anti-CREB (lane 3) antibodies. Fragments shown were amplified using PCR performed with specific primers for the Oxct2b promoter. A sample of the total input DNA (lane 1) was included in the PCR analysis. (E) The 5′-half of the Oxct2b-CL motif is responsible for binding the testis-specific protein. The nucleotide sequences of the wild-type probe (−40/−20), mutant competitor (m1–m5) and testis-specific ACEet oligonucleotides are shown in the upper panel. The Oxct2b-CL motif and the positions of the mutations are shown in boldface and lowercase, respectively. The binding reactions were performed in the absence of unlabeled nucleotide (lane 1), and in the presence of a 1-fold (lanes 2, 5, 8, 11, 14, 16 and 18), 10-fold (lanes 3, 6, 9, 12, 15, 17 and 19) or 100-fold (lanes 4, 7, 10 and 13) molar excess of competitor. The arrow indicates the testis-specific DNA–protein complex.
the ACEε CRE-like motif also competed for the testis-specific Oxct2b-CL–protein complex (Figure 6E). These data indicate that CREM binds haploid-specific promoters via the TGAC/GG sequence, regardless of whether the motif is included in a core promoter or regulatory elements.

Oxct2b-CL in the core promoter is responsible for transcription in testicular germ cells

EMSA and ChIP showed that the Oxct2b-CL motif is the binding site in the Oxct2b promoter that is recognized by testis nuclear proteins, including CREM. To confirm that the Oxct2b-CL sequence is necessary for core promoter activity, we generated a mutant construct (CLmut) in which the Oxct2b-CL sequence (TGACGCAG) was changed to CTTTGGGC, which could not bind CREM in the EMSA (Figure 6B). In vivo transient transfection of CLmut into testicular germ cells showed that mutation of Oxct2b-CL diminished promoter activity, with results similar to those obtained with the −389/−50Luc construct, which lacks the core promoter (Figure 7). These results indicate that Oxct2b-CL is essential for promoter activity through binding the CREM transcription factor. Several studies have demonstrated that CRE and CRE-like motifs act as activators or enhancers, but not as core promoter elements in the regulation of CRE and CRE-like motifs act as activators or enhancers, transcription factor. Several studies have demonstrated that CRE and CRE-like motifs act as activators or enhancers, but not as core promoter elements in the regulation of testis-specific gene transcription (31). If Oxct2b-CL is a cis-activator, additional Oxct2b-CL-upregulates the promoter activity of the CLmut construct. To examine this possibility, we repositioned the −49−16 sequence to the 5′ end of CLmut and performed in vivo transient transfection of testicular germ cells. As shown in Figure 7, the levels of luciferase activity of the −49−16CLmut and −16−49CLmut constructs were similar to those seen with CLmut and were significantly lower than those seen with the −389/−49−16Luc construct. These results indicate that Oxct2b-CL does not function as a cis-activator and that no other core promoter elements are present within the −49−16 region.

![Luciferase activity](image)

**Figure 7.** Oxct2b-CL is indispensable for Oxct2b core promoter activity in testicular germ cells. Reporter gene assays with the 5′-upstream constructs, which comprise −389/−49−16, mutated Oxct2b-CL in −389/−49−16 (CLmut), and the correct (−49−16CLmut) and inverted (−16−49CLmut) sequences of −49−16 ligated to the 5′ end of the CLmut and −389/−50, were carried out via in vivo transient transfection of testicular germ cells. The results are presented as described in the legend to Figure 4. **P < 0.01 compared with the −389/−49−16Luc construct (ANOVA followed by Bonferroni analysis). The luciferase activity of −389/−49−16Luc was set at 1.0.

**DISCUSSION**

**In vivo** electroporation is a useful tool for functional analyses of testicular germ cell-specific gene promoters

The highly ordered process of spermatogenesis, in turn, requires a precise and well-coordinated programme to regulate constantly changing patterns of gene expression. The lack of conventional tissue and cell culture systems that recapitulate precisely the coordinated processes of spermatogenesis has hindered the elucidation of the molecular mechanisms that govern gene expression during spermatogenesis. In this study, we have demonstrated the feasibility of using in vivo transient transfection, which is a combination of intratubular DNA injection and subsequent in vivo electroporation, for the functional analysis of testicular germ cell-specific promoters.

In this technique, foreign DNA sequences are introduced into Sertoli and germ cells (32). Thus, the values obtained for the reporter activities in experiments using whole testis represent the accumulated activities of both cell types, although Oxct2b promoter activity could not be detected in the somatic cell line (P. Somboonthum and M. Nozaki, unpublished data), and the haploid-specific promoter is dominantly activated in the spermatids in this system (24). To exclude potential reporter activity in Sertoli cells, we collected the germ cell fraction and performed luciferase assays. Since linearized DNA is more unstable than circular DNA in Sertoli cells (32), we introduced linear PCR products as reporters. Data validation was performed by transgenic mouse analysis. From these data, we conclude that this system functions as a simple assay system for the analysis of the regulatory elements of haploid spermatid-specific genes.

A CRE-like element regulates the Oxct2b core promoter

In this study, we demonstrated that the −49 to −16 region of the Oxct2b gene functions as a core promoter in testicular germ cells, because this 34 bp fragment had 2-fold greater luciferase activity than the promoter-less control, and the inversion of this sequence abolished transcriptional activity in the in vivo transient transfection assays (Figures 3 and 4A and B). The 34 bp region, which contains a CRE-like element (Oxct2b-CL) but lacks any basal promoter elements, such as a TATA box or Inr region, which contains a CRE-like element (Oxct2b-CL) but lacks any basal promoter elements, such as a TATA box or Inr region, which contains a CRE-like element (Oxct2b-CL) but lacks any basal promoter elements, such as a TATA box or Inr region, which contains a CRE-like element (Oxct2b-CL) but lacks any basal promoter elements, such as a TATA box or Inr region, which contains a CRE-like element (Oxct2b-CL) but lacks any basal promoter elements, such as a TATA box or Inr region, which contains a CRE-like element (Oxct2b-CL) but lacks any basal promoter elements, such as a TATA box or Inr

**Figure 4.** Oxct2b-CL in the core promoter cannot function as a cis-activator. In contrast, Oxct2b-CL in the Oxct2b core promoter cannot function as a cis-activator (Figures 4C and 7). Therefore, Oxct2b is a very rare example of how the CRE-like motif is essential for basal promoter activity.
Transcription initiation of protein-encoding genes is a complex process that requires the precise positioning of Pol II on the core promoter DNA. This is accomplished via a series of interactions between Pol II and at least six accessory proteins, namely TFIIA, TFIIH, TFIIA, TFIIIE, TFIIIF and TFIIH, which have been termed GTFs (38,39). Nucleation of the initiation complex occurs by recognition of a specific core element. To date, the TATA box has been considered to be the primary core element responsible for positioning the basal transcription machinery on the promoter. Among the GTFs, TFIIA is a multisubunit protein complex that contains the TBP at its core and TBP-associated factors (TAFs). It is generally accepted that the binding of TBP to the TATA box nucleates the formation of the transcription pre-initiation complex (PIC), either through stepwise assembly of other basal factors or through recruitment of the holoenzyme (40,41). Following the characterization of more promoters of protein-coding genes, it has been noted that many genes lack the TATA box (42). A second core element, Inr, which encompasses the transcription start site, is sufficient for the positioning of the basal transcription machinery in the absence of a TATA box (43). It has also been postulated that the sequence-specific transcription factor Sp1 tethers the PIC to TATA-less promoters by interacting with a component of TFIID (21,44). However, the Oxtc2b promoter lacks the TATA box, Sp1-binding site, and Inr, which suggests that Oxtc2b-CL plays an essential role in PIC formation in testicular germ cells. If this is true, Oxtc2b-CL-binding factor(s) should be involved in the formation of the functional PIC through Oxtc2b-CL, that is, Oxtc2b-CL at −29/−22 may be a platform for the assembly of GTFs and for Pol II to initiate accurate haploid spermatid-specific transcription.

CREM involvement in PIC formation in testicular germ cells

In this study, we found that Oxtc2b-CL (TGACGCAG) is bound specifically to testis-specific CREM via the 5′-half of TGACG. The CREM isoform is the same as the CREM that is bound to the CRE-like motif of the testis-specific ACEt promoter (Figure 6). The ACEt promoter is activated by the CREM testicular isoform through the CRE-like motif (45). CREM is a member of the CREB family and is involved in the regulation of gene expression in response to several signaling pathways. CREM proteins are produced as activators or repressors by the process of alternative splicing. The N-terminal half of the CREM activator contains an activator domain (AD) that is made up of glutamine-rich Q1 and Q2 domains and the P-box, which is phosphorylated by various kinases that regulate the transcriptional potential of these proteins (46). The transis isoform of CREM, which contains the exons that encode AD, is highly expressed after meiosis during spermatogenesis (47,48). However, the transis CREM isoform is not phosphorylated and its transcriptional activity can be stimulated by interaction with the ACT co-activator in spermatids (15). Thus, Oxtc2b-CL should be bound to the non-phosphorylated CREM testicular isoform. The Oxtc2b promoter has no TATA box, which suggests that a certain scaffold of PIC formation is required for Oxtc2b promoter activity. In this regard, full-length CREM and CREMAQ2, which lack the Q2 domain, were able to bind to TBP and TAF130 (TAF4) in GST-pull down assays (49). Therefore, we postulate a possible mechanism whereby the Oxtc2b promoter is activated by a CREM activator, which is mediated by the recruitment of TFIID to Oxtc2b-CL via its interaction with TAF4 and TBP. In this case, PIC formation does not necessarily require a TATA box. Alternatively, the AD of CREM interacts with TFIIAα, as shown by two-hybrid and co-immunoprecipitation analyses. This interaction is restricted to the activator isoform of CREM and does not require phosphorylation. CREM and TFIIA co-localize intracellularly (16). These data indicate that CREM regulates transcription through binding to TFIIA in spermatids. PIC formation in TATA-less Pol II promoters is postulated to require specific interactions between various factors, including TFIIA (50,51). TFIIA has been shown to interact with TBP-related factor TRF2 (TLF/TLP) and TBP (52–56), and TRF2 has been suggested to operate on TATA-less promoters (57–61). Furthermore, in vivo experiments in which the association of TFIIA and TRF2 were studied indicated that the TRF2–TFIIA complex is more stable than the TBP–TFIIA complex, which implies that TRF2 initiates transcription more efficiently through TFIIA binding than does TBP (62). TRF2 is encoded by two mRNAs with common protein-coding sequences, but distinct 5′-untranslated regions. One of these mRNAs is expressed ubiquitously, whereas the other shows a restricted expression pattern and is abundant in the testis (59,61). TRF2-deficient male mice are viable, but sterile due to late arrest of spermatogenesis (63,64). Although the expression of CREM and many other postmeiotic genes is unaltered in TRF2-null mice, several spermiogenesis genes that are transcribed in late-round spermatids appear to be under the control of TRF2 (63). Based on our results, we propose a model in which a testicular CREM-dependent TFIIA–TRF2 complex is formed on Oxtc2b-CL, which serves as an efficient scaffold for the remainder of the PIC in spermatids.

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