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Regulation of testis-specific carnitine transporter (octn3) gene by proximal *cis*-acting elements Sp1 in mice

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Abstract

The mouse octn transporter family consists of three genes, octn1, octn2 and octn3. The gene products octn2 and octn3, which transport carnitine with high affinity, are both expressed in testis, where carnitine is required to maintain sperm cell motility. Here, we focused on the regulatory mechanism of the expression of octn3 in an attempt to determine whether the differential tissue expression profiles of the octn2 and octn3 genes reflect distinct physiological roles of octn2 and octn3. The promoter activity of the octn3 gene was examined by luciferase assay and gel mobility shift assay using the mouse Sertoli cell line TM4 as host cells. Deletion-mutant assay demonstrated that a gene segment of the 5′-untranslated region located at about −500 bp relative to the transcription start site is required for constitutive octn3 transcription. Deletion of the Sp1-binding site within the region resulted in loss of transcriptional activity. In addition, overexpression of Sp1 in TM4 cells led to a further increase of transcription of octn3. These results demonstrated that Sp1-binding sites are necessary and sufficient for constitutive octn3 gene transcription. Furthermore, the expressions of both of octn2 and octn3 genes in TM4 cells were up-regulated by palmitic acid, whereas carnitine increased only the expression of octn2 without any change in octn3 expression. Accordingly, the expressions of octn2 and 3 are regulated by distinct mechanisms, suggesting distinct roles of octn2 and octn3 in carnitine transport.

Keywords: octn3; Promoter; Carnitine; Testis; Sp1

1. Introduction

Carnitine (β-hydroxy-gamma-trimethylaminobutyric acid) plays a physiologically important role in the β-oxidation of fatty acids by facilitating the transport of long chain fatty acids across the mitochondrial inner membrane, and genetic deficiency of carnitine transport causes critical symptoms such as cardiomyopathy, skeletal muscle myopathy, and hypoglycemia [1–3]. Carnitine is involved in the regulation of spermatozoal motility in epididymal plasma, where it accumulates as both free and acetylated carnitine [4,5]. The concentrations of carnitine in epididymal plasma and spermatozoa range from 1 to 63 mM, which is significantly higher than circulating blood levels (10–50 μM) [2]. Previous studies have shown that the role of carnitine in the epididymis is to foster maturation of spermatozoa [4,6]. However, the mechanism of carnitine accumulation in male reproductive tissues is not clear, and the pattern of carnitine transporter expression in these tissues has not been fully elucidated.

The carnitine transport system consists of several proteins, including three that are encoded by the octn carnitine transporter gene subfamily and $ATB^{0,+}$ [7,8]. The mouse octn1, 2, and 3 genes encode proteins that share >87% amino acid identity [7], and they are oriented tandemly within a 161 kb region on the mouse genome [9]. We have reported the specific expression of octn3 as the carnitine transporter in mouse testis, and of octn2 as the high-affinity carnitine transporter in several tissues in humans and mice [7,9,10]. Although both of these carnitine transporters are expressed in the testis, octn2 exhibits sodium-dependent carnitine transport, while octn3 is a sodium-independent carnitine transporter. octn1 exhibits low but significant carnitine transport activity [7,11,12]. Later, a novel carnitine transporter

Abbreviations: AP-1, activator protein 1; CPT I, carnitine palmitoyl transferase I; DMEM, Dulbecco's modified Eagle's medium; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PPAR-α, peroxisome proliferator activating receptor α; octn, organic cation/carnitine transporter

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(CT2) was identified in human testis; it showed similar functional characteristics to mouse octn3, but the two showed only 33% amino acid sequence similarity [13]. Although the orthologous carnitine transporter to mouse octn3 in human has not been identified, these transporters are likely to be important in carnitine regulation in testis, as well as other tissues. Furthermore, the predominant expression of octn3 in testis suggests an important role in male reproductive activity [7].

Koizumi et al. found an animal model for primary systemic carnitine deficiency, the juvenile visceral steatosis (jvs) mouse [14], and later it was clearly demonstrated that mutation in the octn2 gene was responsible for the jvs phenotype [15,16]. jvs mice exhibit epididymal abnormalities such as epididymal hypoplasia [15]. Specifically, the proximal regions of the epididymis of these mice, including the corpus, are dilated, though no abnormal spermatogenic cells were observed in the testis. Further, the distal regions of the epididymis of jvs mice are devoid of spermatozoa [15]. The difference of phenotype between epididymis and testis in jvs mice might be due to differential expression of octns.

The above findings indicate that octn2 and octn3 both play important roles in male reproductive tissues, despite the differential tissue expression profiles. Accordingly, in order to obtain insight into the reason for the presence of multiple carnitine transporter octns, in the present study we studied the regulatory mechanisms of expression of the octn3 gene. The promoter activity was examined by luciferase assay and gel mobility shift assay using the mouse Sertoli cell-derived line TM4 as host cells. The results obtained in the present study demonstrate that Sp1-binding sites are necessary and sufficient for constitutive octn3 gene transcription.

2. Experimental procedures

2.1. Materials

[γ-^{32}P] adenosine triphosphate (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

2.2. Cell culture and treatment

Testicular Sertoli cell line of mice (TM4) was purchased from American Type Culture Collection (Manassas, VA) and cultured in 50% Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) and 50% F-12 medium (F12, ICN Biomedicals Inc., Irvine, CA), containing 2.5% fetal calf serum, 5% horse serum, 14 mM $NaHCO_3$, and 15 mM HEPES (DMEM/F12) in a humidified incubator at 37 °C under 5% CO_2. At 48 h before harvesting cells (36 h after seeding of the cells), the culture medium was replaced with fresh DMEM/F-12 and supplemented with either ethanol or palmitic acid as required. HEK293 cells obtained from American Type Culture Collection were routinely grown in DMEM containing 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator at 37 °C under 5% CO_2.

2.3. Cloning of mouse octn3 promoter region

The 5′-region of the octn3 gene was PCR-amplified using mouse genomic DNA (BD Biosciences, Palo Alto, CA) as a template, with upstream primer p−2939 and downstream primer p+119 (see Table 1; both synthesized by Hokkaido System Science (Sapporo, Japan)) and Ex taq DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan), based on the reported octn3 gene sequence (GeneBank accession # AB018436). Since the upstream and downstream primers are designed to include an internal *Mlu*I restriction site and an internal *Xho*I site, respectively, the resulting PCR products were digested with *Mlu*I and *Xho*I and ligated into the luciferase reporter gene vector pGL3-Basic (Promega Co. San Luis, LA).

2.4. Gel mobility shift assay

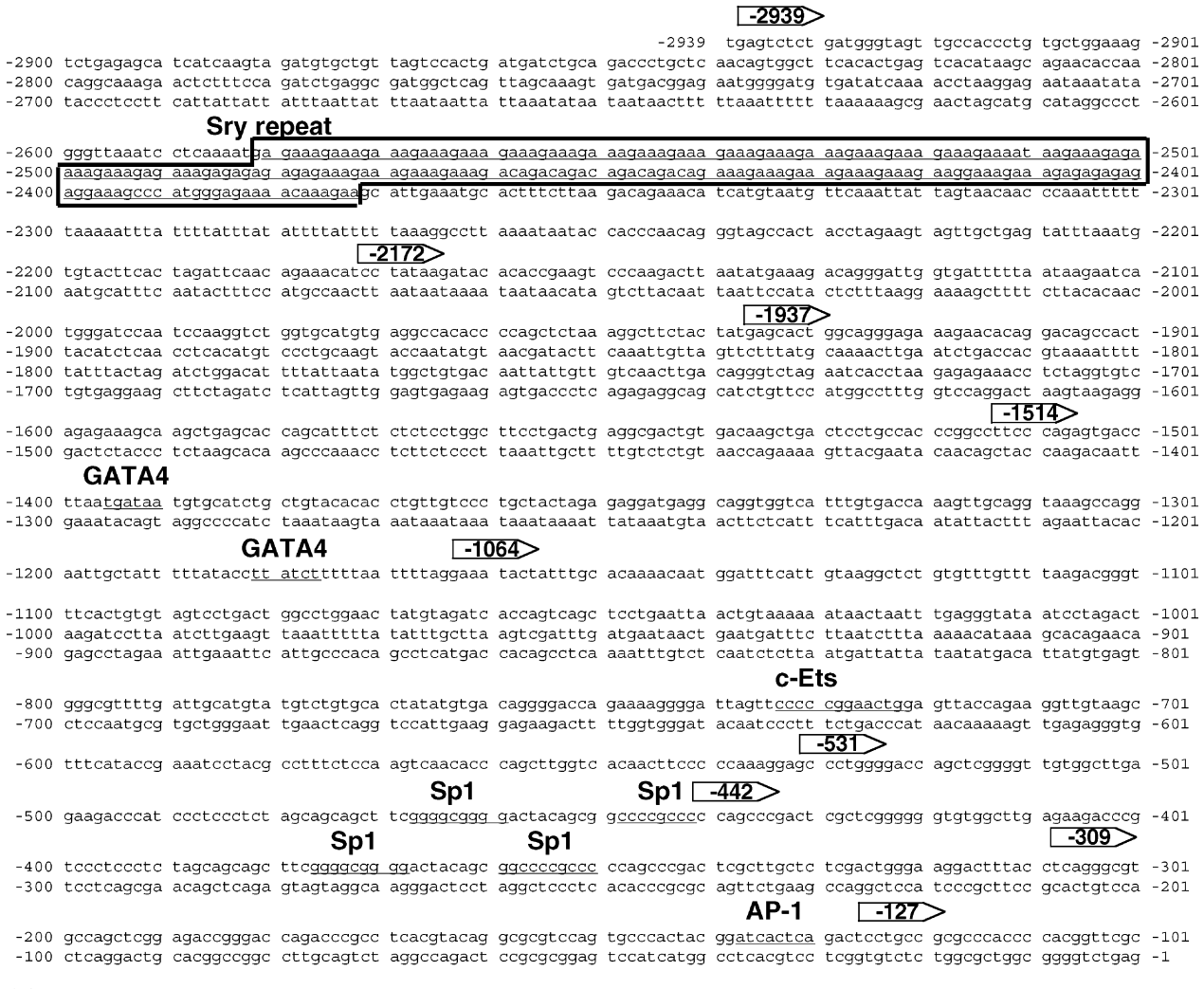
Nuclear extracts were prepared from TM4 cells as described previously [16]. Protein concentration was measured by the method of Bradford [17] using a protein assay

Table 1
Sequences of oligonucleotides used for chimeric plasmid construction, PCR and gel mobility shift assays

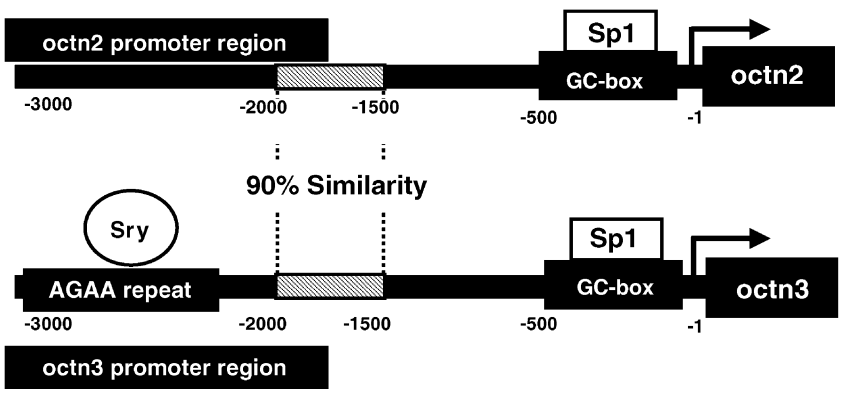
Oligonucleotide	Sequence (5′ to 3′)
octn3	
p−2939	CCACGCGTGAGTCTCTGATGGGTAGTTGC
p−2172	CCACGCGTCCTATAAGATACACACCGAAG
p−1937	GGTACCGAGCACTGCCAGGGAGGAAGAAC
p−1514	CCACGCCCTTCCCAGAGTGACCGAC
p−1064	CCACGCGTGATCACCAGTCAGCTCCTGAA
p−531	CCACGCGTCCTGGGGACCAGCTCGGGGTT
p−442	CCACGCGTCCCAGCCCGACTCGCTCGGGGGGT
p−309	CCACGCGTTCAGGGCGTTCCTCAGCGAACA
p−127	CCACGCGTTCCTGCCGCGCCCACCCCACGG
p+119	TTCTCGAGTGGGCGGCGCACGGTTTGTCC
For PCR	
octn2	TTTCGTGGGTGTGCTGAT
	GTGATGACCCTGATATTCCGT
octn3	ACTGGTGCCTTCAGACCTAC
	TTCAGTTCAATCAGCTTCTGGAC
Sp1 consensus sequence	
wt Sp1 (−467)	GCAGCTTCGGGGCGGGGACTACAGC
wt Sp1 (−447)	TACAGCGGCCCCGCCCCCAGCCCGA
mut Sp1 (−467)	GCAGCTTCGG***TT***CGGGGACTACAGC
mut Sp1 (−447)	TACAGCGGCCCCG***AA***CCCAGCCCGA
per Sp1	ATTCGATCGGGGCGGGGCGAGC
AP-1 consensus sequence	
wt AP-1 (−138)	CTACGGATCACTCAGACTCC
per AP-1	CGCTTGATGAGTCAGCCGGAA

Italic type indicates differences in the sequence of the mutated binding site (mut Sp1) compared with the perfect consensus sequence (per Sp1) and the wild-type Sp1-binding site (wt Sp1).

kit (Bio-Rad Hercules, CA). Oligonucleotides of the sense and antisense strands containing putative Sp1 or AP-1 binding sites were synthesized. The sequences of the oligonucleotides are shown in Table 1 and mapped on the octn3 promoter sequence in Fig. 1. Gel mobility shift assays were carried out as described previously [18]. The Sp1-specific antibody was obtained commercially from Santa Cruz Biotechnology (Santa Cruz, CA).



(A)



(B)

Fig. 1. Nucleotide sequence and structure of the 5′-flanking region of the octn3 and octn2 genes. (A) Nucleotide sequence of the 5′-flanking region of the mouse octn3 gene from −2939 to −1 nt. Nucleotide numbers are relative to the transcription initiation site. Consensus binding sites for putative regulatory elements are underlined, and the respective transcription factors are given above the sequence. Flags denote the size of the deletional constructs used for functional promoter studies. (B) Diagram of the promoters of octn2 and octn3 and positions of putative transcription factor-binding sites.

2.5. Transfections and luciferase assay

Reporter gene constructs were transfected using LipofectAmine 2000 (Gibco BRL Life Technologies), according to the protocol suggested by the manufacturer. Briefly, cells were plated in 24-well plates at approximately 0.5×10^5 cells per well for 24 h before transfection. Before addition of DNA/liposome complexes, cells were rinsed with serum-free medium. For each transfection, 0.8 μg of reporter construct was cotransfected with 0.08 μg of pRL-TK vector (Promega) in 0.5 ml of serum-free medium by incubation at 37 °C for 6 h. After 6 h, the culture medium was changed to medium containing 2.5% fetal calf serum and 5% horse serum, and the cells were incubated for 48 h at 37 °C. Then the cells were rinsed twice with phosphate-buffered saline (PBS) and harvested using passive lysis buffer (Promega). For luciferase assays, cell extracts were mixed with luciferase assay reagent (Promega) for detection in a luminometer (Berthold GmbH and Co. KG, Germany). Luciferase activity is shown as the ratio of firefly/Renilla luciferase activities and data are presented as the mean ± S.E.M. of 3–10 independent transfection experiments. Sp1 expression vectors were gifts from Dr. Alexandrea Stewart [19].

2.6. Site-directed mutagenesis

Site-directed mutagenesis of the Sp1-binding sites (GC-box) was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the oligonucleotides shown in Table 1. Briefly an octn3-derived −531/+119 construct containing mutation was generated by PCR using two complementary oligonucleotides mutated in the Sp1-binding site (sequence mut Sp1 in Table 1) and Pfu DNA polymerase (Stratagene). The product was digested with *Dpn*I to remove the parental DNA template and to identify the DNA containing the mutation. The obtained mutated plasmids were termed mut 447, mut 467 and mut 447/467.

2.7. Reverse transcription–polymerase chain reaction

Total RNA was prepared from TM4 cells using ISOGEN (Wako Pure Chemical Industries, Tokyo, Japan). The total RNA content was determined by measuring the absorbance at 260 nm. mRNA level was analyzed using semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR). Single-strand cDNAs were constructed using an oligo(dT) primer (Invitrogen Corp., Carlsbad, CA). These cDNAs provided templates for PCRs using specific primers (Table 1) at a denaturation temperature of 94 °C for 30 s, an annealing temperature of 58–62 °C for 30–60 s, and an elongation temperature of 72 °C for 30 s in the presence of deoxynucleotides (dNTPs) and Ex Taq polymerase (Takara Shuzo Co. Ltd., Tokyo). Annealing time and temperature were changed as required, depending on the genes. The PCR cycle numbers were titrated for each primer pair to confirm that amplification was performed within a linear range. PCR products were analyzed by 2% agarose gel (w/v) electrophoresis and the gels were stained with ethidium bromide for visualization. mRNA levels were quantified by using light capture (Atto Co., Tokyo). PCR amplification data were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The sets of primers specific for the nucleotide sequences of the transporters are shown in Table 1. The quantitation of each gene was repeated at least three times using RNA sources isolated from independently cultured cells, and the results were statistically analyzed by the use of Student's *t*-test.

3. Results

3.1. Analysis of the 5′-flanking region of the mouse octn3 gene

The transcription initiation site of octn3 was reported by Tamai et al. [7]. The 5′-flanking region of the octn3 gene was PCR-amplified from mouse genomic DNA (GeneBank accession # AB182365). Fig. 1 shows the sequence of 5′-flanking 2939 nt relative to the transcription initiation site, indicated as −1. Potential transcription factor recognition sites were identified by using the program TRANSFAC 4.0 (Biobase GmbH, Germany) and included DNA elements such as GC-box at −467 to −462, −447 to −442, −376 to −371 and −356 to −351, sry repeat at −2582 to −2301, GATA-4 at −1396 to −1391, −1182 to −1177, c-Ets at −734 to −722, and AP-1 at −138 to −130. Comparison of the nucleic acid sequence of the octn3 promoter region with the putative mouse octn2 promoter region, of which the transcription start site was identified using 5′-RACE [20], revealed that octn3 and octn2 exhibited a high degree of similarity, with 90% similarity at −2000 to −1500 (Fig. 1B).

3.2. Constitutive activity of the octn3 enhancer–promoter region in TM4 cells

To functionally characterize the octn3 promoter activity, a series of promoter deletions was constructed in reporter gene vector pGL3-Basic that expresses firefly luciferase. Transfection of the p−2939 Luc plasmid into TM4 cells, which contained the promoter region from −2939 to −1 nt, stimulated luciferase activity about five- to six-fold compared with the pGL3-Basic (no promoter insert) (Fig. 2A). Promoter activities of all deleted constructs between p−2939 Luc and p−127 Luc were compared with that of pGL3-Basic (Fig. 2A). The construct p−531 Luc stimulated luciferase activity eight-fold compared with pGL3 basic. p−531 Luc, which includes four Sp1-binding sites

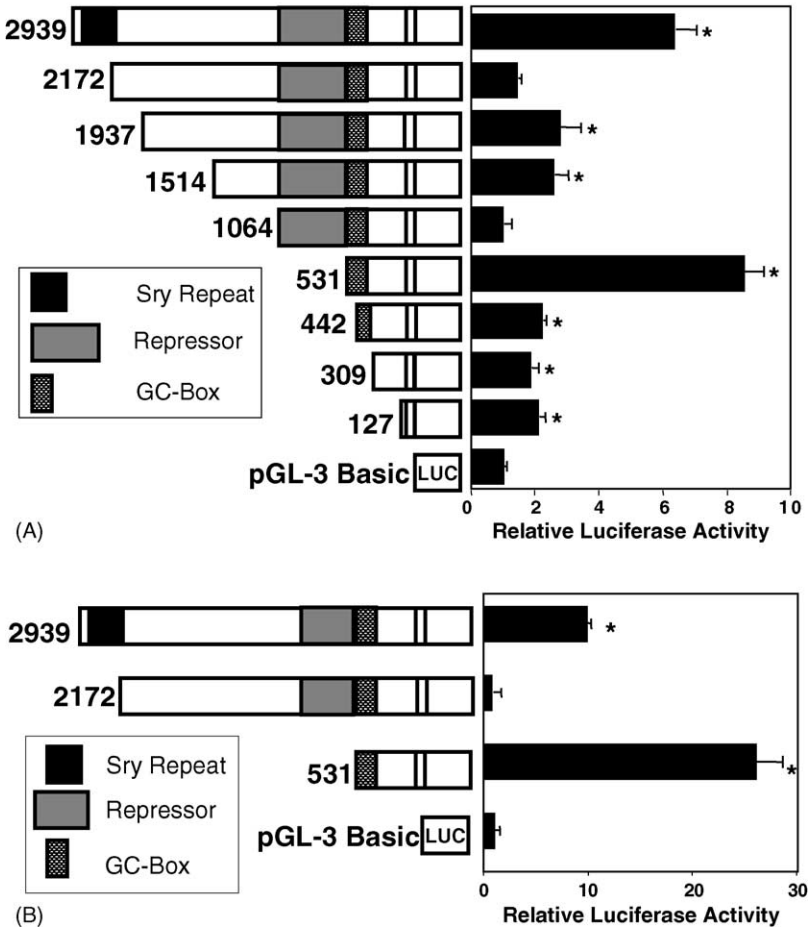


Fig. 2. Analysis of octn3 promoter function in cell lines. The mice testicular-derived cell line TM4 (A) and the kidney-derived cell line HEK293 (B) were transiently transfected with nine or four chimeric promoter constructs ranging from nt −2939 to −127 relative to the transcription start site and extending to +119 nt. Promoter fragments were inserted into the pGL3-Basic luciferase vector. Transfection efficiency was normalized by cotransfection of pRL-TK, and promoter activity was measured as relative light units of firefly luciferase per unit of Renilla luciferase. Promoter activity is shown as the factor of induction of luciferase over background activity measured in cells transfected with pGL3-Basic alone (Basic). Results are expressed as the mean ± S.E.M. of 5–9 independent transfection experiments. Black box, gray box, or patch box show putative sry binding sites, negative regulatory region and Sp1-binding sites, respectively. An asterisk indicates a significant difference ($p < 0.05$) compared with the activity of pGL3-Basic reporter construct.

(Fig. 1A), was sufficient to confer residual promoter activity in TM4 cells. Other constructs, p−1937, p−1514, p−442, p−309, and p−127 Luc, stimulated luciferase activity two-fold compared with the pGL3-Basic, while p−2172 and p−1064 Luc did not. The p−1064 Luc has an active silencer element, c-Ets [21] at −734 to −723. Since the sequence from −2582 to −2301 contains a putative sry binding site, which is an important testis-specific transcriptional factor [22], this region may be associated with testis expression of octn3. Accordingly, we examined whether this region is associated with tissue-specific expression of octn3 or not. octn3 is weakly expressed in kidney [7], so we used HEK293 cells (human kidney-derived cell line) as the host cells. We observed octn3 promoter activity (p−2939) in testicular-derived TM4 cell line, as well as the kidney-derived HEK293 cell line (Fig. 2B). The putative sry binding sites did not provide a tissue-specific regulation (Fig. 2). Furthermore, transfection of p−531 Luc into HEK293 cells stimulated luciferase activity about 26-fold compared with pGL3-Basic (Fig. 2B). Therefore, p−531 Luc was sufficient to confer residual promoter activity in both TM4 and HEK293 cells, and Sp1-binding sites should be included to control the basal promoter activity of octn3.

3.3. Identification of putative Sp1-binding sites in octn3 promoter region

To identify the composition of nuclear proteins that interact with the *cis*-acting motif, we used gel mobility shift assay. Gel mobility shift assays were conducted using TM4 cell-derived nuclear extracts and a putative Sp1-binding motif oligonucleotide within mouse octn3 promoter. The octn3 promoter region from −467 to −1 nt

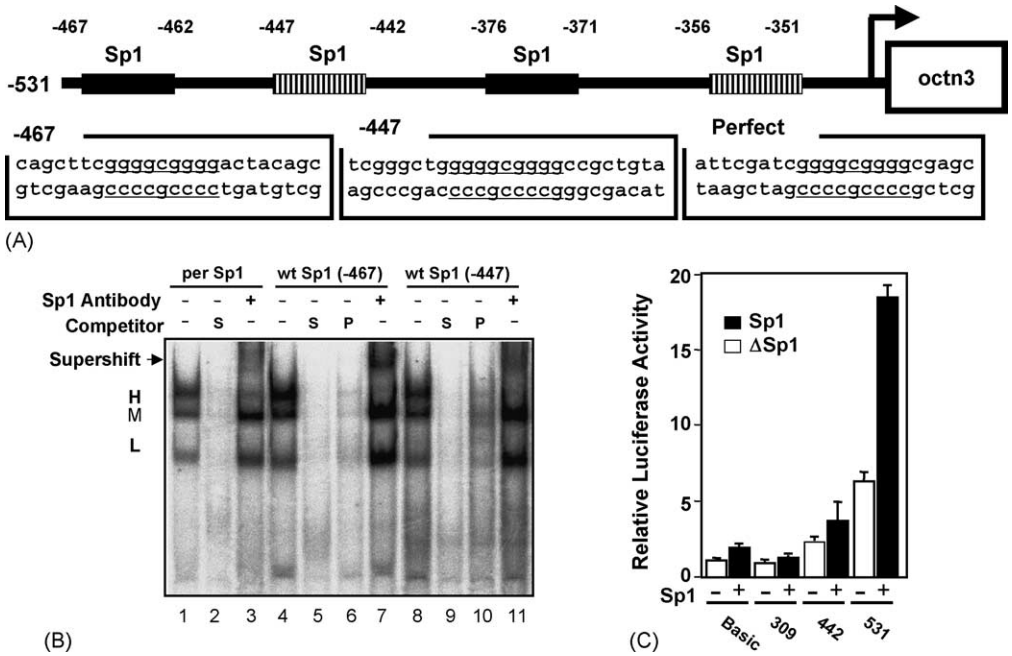


Fig. 3. Gel mobility shift competition assay and supershift analysis of Sp1 bound protein complex. (A) Schematic sequence of the −531 region is shown and includes the underlined Sp1 at −467, −447, −376 and −356. Nucleotide sequences of perfect oligonucleotide that was confirmed to bind Sp1 and the Sp1 sequence (−467 and −447) in the octn3 promoter sites are shown. (B) Gel mobility shift assays were carried out with nuclear extract from TM4 cells. Lanes 1, 4, and 8, probe alone; lanes 2, 5 and 9, probe with 5 pmol of unlabeled oligonucleotide (s: self competition); lanes 6 and 10, probe with 5 pmol of unlabeled oligonucleotide (p: perfect competition); lanes 3, 7 and 11, probe with Sp1 specific antibody (Sp1 antibody). The three shifted protein complexes are labeled from largest to smallest as H, M, L. (C) TM4 cells were co-transfected with the −531 Luc, −442 Luc, or −309 Luc reporter gene constructs plus Sp1 (Sp1, closed bars) or empty vector as a control (open bars).

included four putative Sp1-binding sites (Fig. 3A). As shown in Fig. 3B, three protein complex bands, L, M, and H, were seen (Fig. 3B, lanes 1, 4, and 8). The protein–DNA complexes shown in Fig. 3B were further analyzed using antibody directed to Sp1 nuclear protein (Fig. 3B, lanes 3, 7, and 11) and Sp1-specific oligonucleotide competitors (Fig. 3B, lanes 2, 5, 6, 9, and 10). The three shifted complexes binding to the octn3 oligonucleotide were all subject to competition by perfect Sp1 oligonucleotide (Fig. 3B, lanes 2, 6, and 10), which has exactly the same sequence as the Sp1-binding site, and by an excess of self oligonucleotide with the same sequence as the probe oligonucleotide (Fig. 3B, lanes 5 and 9). Shifted complex band H was supershifted by Sp1 specific antibody (Fig. 3B, lanes 3, 7, and 11). The results shown in Fig. 3B demonstrate that the slower-migrating protein complex (H) contains Sp1, while the two faster-migrating (M and L) complexes may contain Sp3 (Fig. 3B, lanes 3, 7, and 11) [23].

3.4. Stimulation of the octn3 promoter in TM4 cells by induced expression of Sp1

To investigate whether exogenously expressed Sp1 affects octn3 promoter function in TM4 cells, an expression plasmid coding for Sp1 was introduced into TM4 cells together with several octn3 promoter constructs. As shown in Fig. 3C, cotransfection of the Sp1 plasmid enhanced octn3 promoter-driven luciferase activity three-fold (p−531) compared with cotransfection of empty vector lacking Sp1. However, Sp1 did not activate p−442 and p−309 Luc (Fig. 3C). The p−531, p−442, and p−309 Luc included four, two, and no Sp1-binding sites, respectively (Fig. 3A). This result indicated that two Sp1-binding sites (−467 to −462 and −447 to −442) are important for octn3 basal promoter activity.

3.5. Effect of mutation of Sp1-binding sites on octn3 expression

To assess the importance of Sp1-binding to octn3 promoter regions for basal promoter function, mutations of the Sp1-binding sites were introduced into p−531 Luc by site-directed mutagenesis. The resulting reporter gene plasmids mut 467 and mut 447 Luc contained mutations in the −465 to −464 (GG to TT) and −444 to −443 (CC to AA) regions, respectively (Table 1). Introduction of these mutations abrogated Sp1 binding in mobility shift assays (Fig. 4A). As illustrated in Fig. 4B, mutation of the Sp1-binding site resulted in reduction of basal promoter activity of p−531 Luc in TM4 cells to less than half. Furthermore, the octn3 promoter activity of the double mutant mut 447/467 Luc could no longer be stimulated by overexpression of Sp1 (Fig. 4C). These results indicated

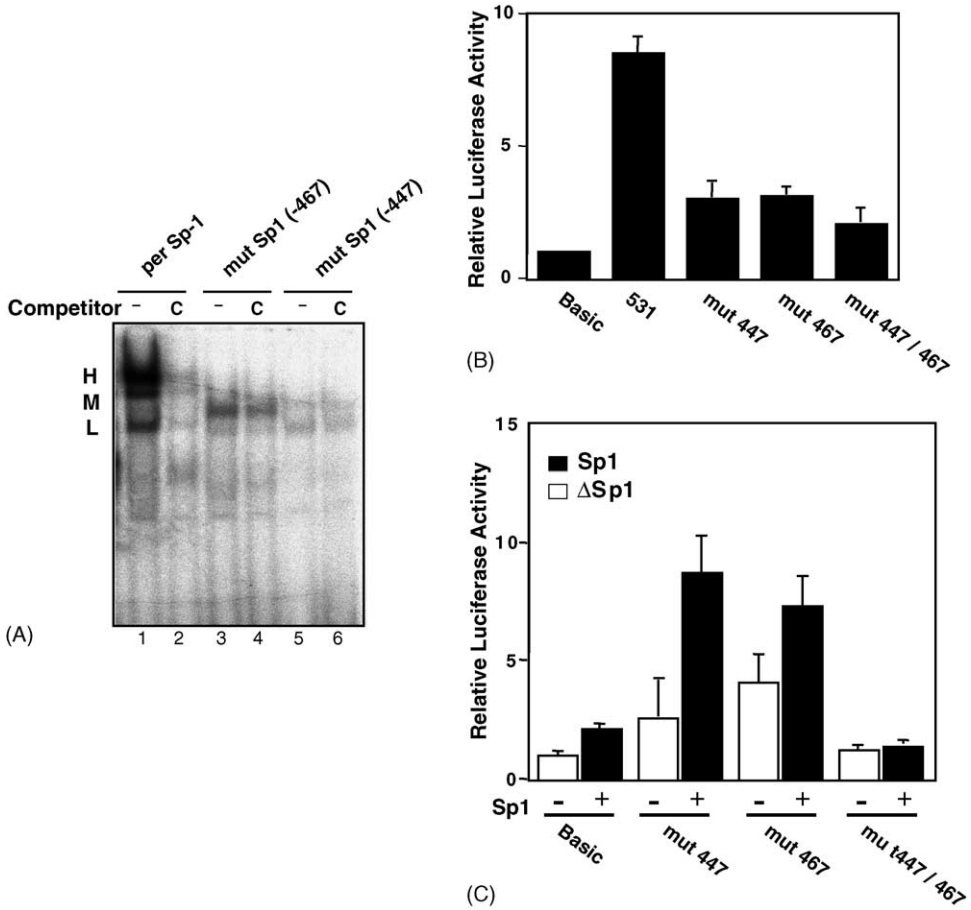


Fig. 4. Loss of octn3 promoter activity by mutagenesis of the Sp1-binding site. (A) An oligonucleotide with a mutation in the proximal Sp1 elements mut Sp1 (−467) and mut Sp1 (−447) does not bind to Sp1. (B) TM4 cells were transfected with p−531 Luc or mutants (mut 447, mut 467 or dual mutant mut 447/467) plasmids containing the −531/+119 region of the octn3 gene. (C) TM4 cells were cotransfected with the mut 447, mut 467 or mut 447/467 Luc reporter gene constructs plus either Sp1 expression plasmid (Sp1, closed bars) or empty vector as a control (open bars). Luciferase activity is shown as the ratio firefly/Renilla luciferase and data represent the mean ± S.E.M. of 3–5 independent transfection experiments.

that Sp1 is sufficient for basal promoter activity of the octn3 gene.

3.6. Effects of carnitine and palmitic acid on transcriptional activation of octn2 and octn3

Beyond its well-known metabolic effect, carnitine has more complex roles in regulating gene expression and the activity of caspase [24]. In addition, long-chain fatty acids rapidly modulate the transcription of several genes involved in their own metabolism [25]. Therefore, we examined the effects of carnitine and palmitic acid on transcriptional activation of octn2 and octn3. Expression of both octn3 mRNA and octn2 mRNA was activated 1.6-fold relative to the ethanol control by cultivation with palmitic acid at 100 μM for 12 h ($n = 3$, normalized to G3PDH, $p < 0.05$, Fig. 5A). In addition, carnitine activated the expression of octn2 mRNA 1.4-fold relative to the ethanol control, but had no effect on expression of octn3 mRNA ($n = 3$, normalized to G3PDH, $p < 0.05$, Fig. 5A).

3.7. Identification of putative AP-1 binding sites in octn3 promoter

Palmitic acid regulates expression of several genes through a peroxisome proliferator activated receptor α (PPARα) or AP-1 pathway [26]. octn3 promoter included a putative AP-1 binding site at −138 to −130, though we could not find PPARα binding sites in the octn3 promoter region up to −2939. Therefore, we examined whether AP-1 could bind this region of the octn3 promoter using gel mobility shift assays with TM4 cells nuclear extract and AP-1 oligonucleotide. As shown in Fig. 5B, a single protein complex was observed (Fig. 5B, lane 3). The shift of the binding complex with octn3 oligonucleotide was subject to competition by perfect AP-1 oligonucleotide (Fig. 5B, lane 5). In addition, P-531 Luc, which contains an AP-1 binding site (Fig. 1A), was activated by palmitic acid, while carnitine had no effect on octn3 promoter activity or mRNA (Fig. 5C). Accordingly, AP-1 bound at the −138 to −130 region in octn3 promoter and stimulated octn3 promoter activity.

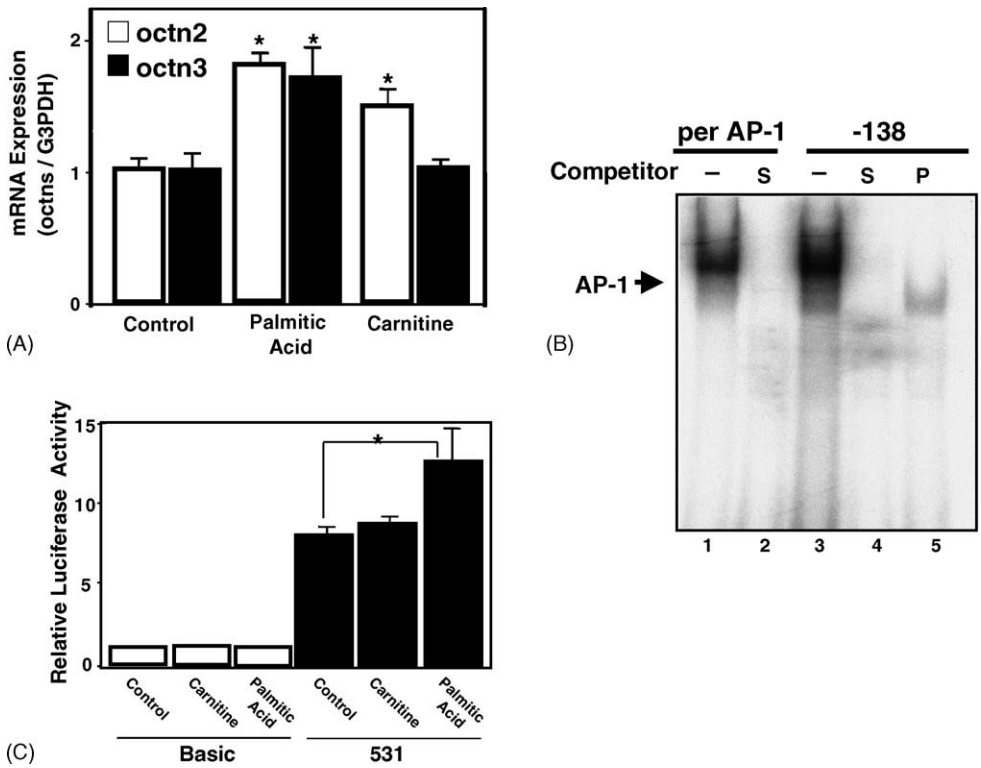


Fig. 5. Effect of carnitine and palmitic acid on promoter activity of octn3 in TM4 cells. (A) RT–PCR of RNA from TM4 cells in medium treated with ethanol (control), palmitic acid (100 μM), or carnitine (100 μM) for 12 h. (B) Gel mobility shift assays were carried out with nuclear extracts from TM4 cells and oligonucleotide sequences that include the binding site of AP-1. Lanes 1 and 3, probe alone; lanes 2 and 4, probe with 5 pmol of unlabeled oligonucleotide (s: self competition); lanes 5, probe with 5 pmol of unlabeled oligonucleotide (p: perfect competitor). (C) TM4 cells were transfected with control vector (pGL3 Basic) or p−531 Luc containing the −531/+119 region of the octn3 gene. TM4 cells were cultured in either the absence (control) or presence of carnitine or palmitic acid (both 100 μM) for 12 h. Luciferase activity is shown as the ratio of firefly/Renilla luciferase activities and data represent the mean ± S.E.M. of 3–5 independent transfection experiments.

4. Discussion

The present study deals with the characterization of the promoter region of mouse octn3, which is predominantly expressed in testis, and its implications for the role of octn3 in comparison with that of the sodium-dependent carnitine transporter octn2.

The promoter region of octn3 contains several consensus recognition sites for both ubiquitously expressed transcription factors, such as Sp1 and AP-1, and testis-specific transcriptional factors, including sry [22]. The octn3 promoter region had four consensus sequences for Sp1 close to the transcription initiation site (Fig. 1A), and an examination of basal promoter activity in a mouse testis-derived cell line (TM4 cells) supported the involvement of Sp1 in the expression of octn3. Binding of Sp1 to this region of the octn3 promoter was confirmed by both gel mobility shift assays and supershift analysis using an anti-Sp1 antibody (Fig. 3). Coexpression of exogenous Sp1 stimulated octn3 promoter activity up to three-fold in TM4 cells (Fig. 4). Furthermore, targeted mutation of the Sp1-binding site (GGGCGG), the so-called GC-box, abolished not only the inducibility of octn3 promoter activity by Sp1, but also the basal promoter activity in TM4 cells (Fig. 4). These results demonstrated that Sp1 controls basal promoter activity for octn3 expression. In particular, two binding sites for SP1 (−467 to −462 and −447 to −442) were important for octn3 basal promoter activity (Fig. 3). Consensus sequences for Sp1 have been identified in the promoter regions of a variety of genes, though their role in gene regulation is not yet well understood [27]. Some researchers have suggested that Sp1 is involved in cell differentiation, as it is expressed at high levels in hematopoietic stem cells, fetal cells, and spermatids [28,29]. octn3 exists in spermatogenic cells at various differentiation stages from spermatids to spermatozoa (unpublished observation). This finding is consistent with the expression profile of both Sp1 and octn3, and it is thought that octn3 plays an important role in spermatogenesis. Others have speculated that Sp1 is a major transcription factor for housekeeping genes, as the Sp1 consensus sequence is commonly found in the promoter region of those genes [30]. However, the basal transcriptional regulation of octn3 was controlled by Sp1 (Fig. 6), though the expression of octn3 was limited in testis. It is possible that there is a tissue-specific co-factor for transcriptional regulation of octn3.

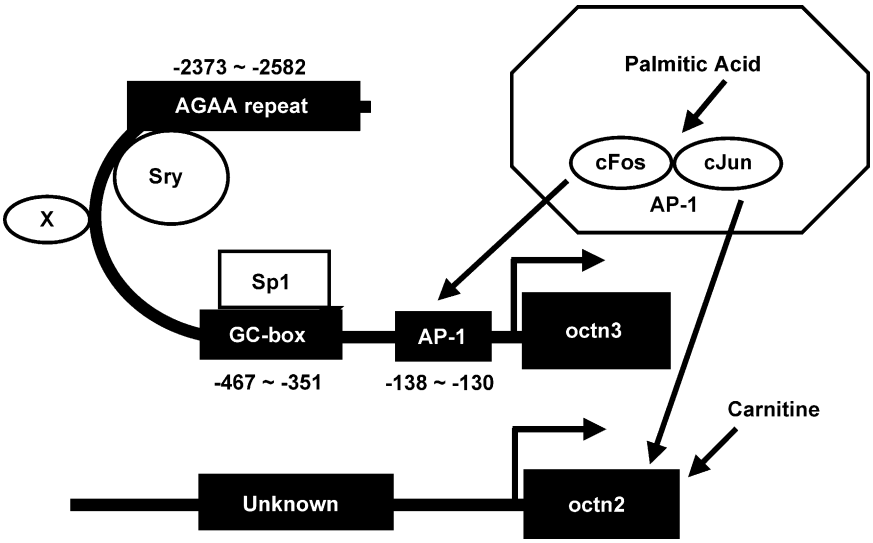


Fig. 6. Model of the octn3 promoter region. Basal promoter activity of octn3 is regulated by Sp1; there are four Sp1-binding sites within −531 nt from the transcription initiation site. The region of −1064 to −531 includes a negative regulatory region, indicated as X. The −2582 to −2373 nt region contained AGAA repeats that were recognized by sry. Palmitic acid up-regulates the activity of the octn3 promoter. Carnitine up-regulates octn2, but not octn3, possibly via transcriptional factor AP-1.

The promoter of octn3 contains putative sry binding sites at −2373 to −2582, whereas the promoter of octn2 does not. To identify which transcriptional factor could be responsible for testis-enriched expression of octn3, luciferase assay was performed using TM4 cells and a kidney-derived cell line, HEK293 (Fig. 2). However, octn3 promoter activity (p−2939 Luc) was observed in testicular-derived cell line, TM4, as well as HEK293 cell line (Fig. 2B). This observation suggests that the sry binding sites of the octn3 promoter are not likely to contribute to the testis specificity. The promoter region of octn3 included GATA-4 binding sites at −1396 to −1391, −1182 to −1177. When those sites were deleted, octn3 promoter activity was decreased (Fig. 2A). In addition, it was reported that GATA-4 regulates testis expression of Dmrt1 [31]. Accordingly, it was thought that GATA-4 is involved in the predominant expression in testis.

In mice, the genital ridges are first evident at embryonic day 9.5. At embryonic day 10.5, a critical switch in gonadal development occurs, in which XY gonads express the testis-determining gene sry [22], initiating the differentiation of the supporting cell precursors as Sertoli rather than granulosa cells [32]. octn3 was detected only in 7-day embryo before birth [7], and therefore it might be involved in testis development. More studies will be required to establish what transcription factor regulates the testis-specificity of the octn3 gene. At present, no octn3 counterpart in human has been found. However, predominant expression in testis, sodium-independent transport of carnitine, and affinity of carnitine and other characteristics of mouse octn3 are very similar to human CT2 [12]. Accordingly, it is expected that there are common mechanisms to maintain carnitine in testis and the clarification of the regulation mechanisms of mouse octn3 will be helpful to understand the carnitine disposition in human testis.

It has been suggested that long-chain fatty acids rapidly modulate the transcription of several genes involved in their own metabolism [25]. Carnitine palmitoyl transferase I (CPT I), which is a rate-limiting enzyme in mitochondrial β-oxidation of fatty acids, is induced by long-chain fatty acid [33,34]. CPT I expression is regulated by PPARα and AP-1 (composed of c-fos and c-jun). In addition, long-chain fatty acids such as palmitic acid and oleic acid cause transcriptional activation of the nur-77 and c-fos genes, which are immediate-early response genes [26]. In this study, it was shown that expression of octn2 and octn3 is up-regulated by palmitic acid (Fig. 5). In addition, carnitine did not affect the expression of octn3 in TM4 cells, whereas octn2 expression was up-regulated by carnitine. These results suggest that the regulatory mechanisms of the two octn genes are distinct (Fig. 6). Vescovo et al. reported that carnitine can prevent apoptosis of skeletal muscle cells and is effective in the treatment of congestive heart failure-associated myopathy [24]. In contrast, palmitic acid induces apoptosis in the β-cell line RINm5F [35]. Accordingly, it was thought that octn3 and octn2 are associated with apoptosis. In spite of the functional overlaps of octn3 and octn2 in carnitine transport, the two transporters may have different roles, considering the different regulation mechanisms of their genes. Accordingly, more research will be required to elucidate the physiological significance of the presence of multiple carnitine transporters, octns.

In conclusion, this study provided the evidence that Sp1-binding sites are necessary for constitutive octn3 gene transcription. The expression of octn3 was enhanced by

factors associated with β-oxidation, such as palmitic acid, but not by carnitine and this regulatory mechanism is presumably mediated by transcription factor AP-1. Furthermore, it appears that the expressions of octn2 and octn3 are regulated by different mechanisms.

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