Transient expression analysis of the mouse ornithine decarboxylase antizyme haploid-specific promoter using in vivo electroporation

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Abstract The testicular isoform of the ornithine decarboxylase antizyme (OAZt) gene is expressed exclusively in the haploid spermatids of mice. The 357-bp region, which includes a TATA-less promoter and an untranslated region, is sufficient for OAZt gene expression in the spermatids of transgenic mice. In this study, in vivo transient transfection to living mouse testes was used to define the transcriptional regulatory elements of the OAZt gene promoter. We found that the 10-bp element that contains an initiator (Inr) plays a central role as the core promoter, in combination with a downstream element, while two cyclic adenosine monophosphate-responsive element (CRE)-like sites in the upstream region also contribute to promoter activity. The electrophoretic mobility shift assay showed binding of the testis-specific factors to these elements. Our results show that the in vivo DNA transfer technique enables detailed analysis of haploid germ cell-specific gene regulation in mice.
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Key words: Haploid gene expression; Mouse; Testis; In vivo electroporation; Promoter

1. Introduction

Mammalian spermatogenesis involves a complex developmental program in which the stem cells of spermatogenesis, namely spermatogonia, transform into spermatozoa. This process is dependent upon precise, developmental stage- and germ cell-type-specific gene expression [1]. The chromatin of testicular germ cells is composed of somatic cell-type histones until the spermatocyte stage. During the meiotic prophase, several testicular histone variants are synthesized. The differentiation of spermatocytes proceeds to spermiogenesis, during which the histones are displaced from chromatin, first by transition proteins and later by protamines, thereby producing the nucleoprotamine of the spermatozoon [2]. The DNA–protamine interaction causes chromatin condensation, which results in the conversion of the transcriptionally active nucleus into the quiescent nucleus of the spermatozoon [3]. This

unique genome DNA/protein status may affect the temporally regulated expression of a group of genes in the haploid spermatids. Indeed, unique structural features have been described for the haploid-specific promoters [4]. Therefore, it is particularly important to study the molecular mechanisms that underlie the regulation of gene expression during the haploid phase of spermatogenesis.

Since permanent spermatogenic cell lines that could be used for transient expression experiments are only beginning to be established [5,6], most germ cell promoter studies to date have been carried out in transgenic mice [7–9]. Previously, we analyzed the upstream regulatory region of the testicular isoform of the ornithine decarboxylase antizyme (OAZt) gene, which is expressed exclusively in haploid germ cells in the testis. The mouse OAZt (mOAZt) essential regulatory region, which lies between positions −133 and +242 and includes two cyclic adenosine monophosphate-responsive elements (CREs) and an initiator (Inr), but no TATA box, was subjected to transgenic expression analysis [10].

In order to define the *cis*-element within this 375-bp region, we performed an in vivo transient expression assay, since the transgenic approach is cumbersome. Previously, the activities of a haploid-specific promoter and an enhancer were detected only in spermatids using the in vivo transient expression method, which consisted of DNA transfer into seminiferous tubules followed by in vivo electroporation [11,12]. However, the definition of the essential transcriptional regulatory elements of a haploid-specific promoter, using a series of deletion constructs, has not been performed.

Our study, using an in vivo transient transfection assay, is the first attempt to identify the individual regulatory elements in a haploid-specific promoter that contribute to gene expression in the germ cell. A series of constructs, in which deletions of the mOAZt promoter region were linked to the luciferase reporter gene, were used in the assays. These transfection assays showed that the 10-bp Inr-containing region and a 43-bp downstream region play crucial roles in transcription, and that two upstream CREs also contribute to total promoter activity.

2. Materials and methods

2.1. Plasmid constructs for promoter analysis
The reporter construct that contains the 5′-flanking and 5′-untranslated regions of the mOAZt gene cloned in the pGL3-basic (Promega) vector has been described previously [10]. Deletion constructs were produced using polymerase chain reaction (PCR)-amplified DNA

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fragments. The pGL3-basic vector was also used as the negative control. The *Renilla* luciferase (RL) gene under the control of the −133/+242 region of the mOAZt gene was generated as the standard construct for in vivo transfection.

2.2. In vivo electroporation assay

Reporter DNA fragments containing the mOAZt 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′-CTAGCAAAATAGGCTGT-CCC-3′ and RV Primer4, 5′-GACGATAGTCATGCCCCGCG-3′. The PCR product was purified with the SUPREC™ PCR column (TaKaRa). The control fragment was also amplified from the RL plasmid, using the oligonucleotide pair: RL5, 5′-TTTTGCTCA-CATGGCTCGAC-3′ and RL3, 5′-CGCACATTTCCCCGAAAA-GT-3′. For each transfection, 10 μl of DNA solution containing 0.5 μg/μl of each PCR reporter product, 0.5 μg/μl of control −133/+242-RL fragment, 100 μM of general caspase inhibitor (Z-VAD-FMK, R&D Systems) and 0.1 μg/μl of trypan blue (Nacalai) were injected into the seminiferous tubules of 5-week-old ICR mice (Shizuoka Laboratory Animal Center). The injection via the efferent ducts was performed according to the method of Ogawa et al. [13]. Electron pulses were delivered by an electric pulse generator (Electroporator CUY-21; Tokiwa-Science). The testes were held between a pair of tweezers-type electrodes (Tokiwa-Science), and square-form electric pulses were applied eight times in four different directions at 40 V for 50 ms, at intervals of 950 ms.

2.3. Luciferase assay

20 h after the operation, the transfected testes were collected for the luciferase assay. The seminiferous tubules were obtained from the testes and cut into small fragments. The paste was suspended in 1 ml of phosphate-buffered saline (PBS), mixed by pipetting, and maintained at room temperature for 2 min. 500 μl of supernatant was harvested, and centrifuged at 2000 rpm for 1 min at room temperature. The pellets were suspended in 250 μl of accessory lysis buffer, and the luciferase assays were performed sequentially using the Pica-Gene Dual SeaPansy™ luminescence kit (Nippon Gene), according to the manufacturer's directions. The luciferase activities were standardized with the levels of RL activity in each experiment.

2.4. Electrophoretic mobility shift assay (EMSA)

The mouse testicular germ cell fraction was collected essentially as described previously [14]. Nuclear protein extracts for EMSA were prepared from testis germ cell fractions and liver cells, as described previously [15]. The fluorescent Cy5.5 end-labeled probe G5 was synthesized. Other probes (G7 and G8) were added two additional guanines at the 5′-terminals, which were labeled with FluoroLink™ Cy5.5-dCTP (Amersham), using the Klenow fragment (TaKaRa).

DNA–protein binding reactions were carried out at 25°C for 30 min in 10–15 μl of binding buffer (20 mM HEPES–KOH (pH 7.8), 31 mM KCl, 80 mM ethylenediamine tetraacetic acid (EDTA), 8% glycerol, 5 mM dithiothreitol (DTT), and 0.5% Tween 80), to which was added 400 fmol of the labeled probes, 3 μg of testis or liver nuclear extract, and 0.5 μg of poly-dI:dC (Amersham). For probe G8, the binding reaction was performed at 16°C. For the competition experiments,

unlabeled oligonucleotides were added to the reaction mixture prior to addition of the probe. For the gel supershift analysis, 3 μg of testis nuclear protein extract and 2 μg of anti-CRE modulator (CREM) antibody (CREM-1 X-12; Santa Cruz Biotechnology) were preincubated at 4°C for 60 min. The reaction mixtures were electrophoresed on a 5% polyacrylamide gel (38:2) with 0.5× TAE buffer at 30 mA for 30 min at 4°C. For probe G8, 2.5% glycerol was added to the acrylamide gel and running buffer. The gels were analyzed using the Odyssey Infrared Imaging System (LI-COR).

3. Results and discussion

3.1. In vivo transfection analysis of haploid spermatid gene regulation

The 375-bp promoter region of the mOAZt gene has two CRE-like motifs, TGACATAA and TGACCTCA, and one Inr-like sequence, TCATAAT, but lacks a canonical TATA box (Fig. 1). To identify the regulatory elements in this promoter region, we employed an in vivo transfection assay, which is a combination of DNA transfer into the seminiferous tubules of the testis and subsequent electroporation. To date, haploid spermatid-specific regulation of two genes has been confirmed by this technique, using either LacZ or EGFP as the reporter [11,12]. However, transient expression analysis for the identification of regulatory motifs has not been described previously. Thus, our study is the first attempt to achieving this goal. The DNA fragment that includes the sequence upstream of the mOAZt gene was fused with the luciferase gene, and introduced into male germ cells. The −133/+242 region of the mOAZt gene showed 10-fold higher luciferase activity than the negative control (lacking the promoter) (Fig. 2A), which demonstrates that differences in promoter activities can be evaluated by this method.

3.2. CRE motifs contribute to mOAZt promoter activity

In order to define the regulatory region in more detail, we made a series of 5′-deletion constructs, and tested their promoter activities. The transfections were repeated 6–12 times, to yield reproducible results. As shown in Fig. 2A, a deletion that extended up to position −33 and that removed 100 bp upstream resulted in no significant reduction in promoter activity. A further deletion of the region from −33 to −5 resulted in a two-fold decrease in promoter activity. This indicates that this 29-bp fragment, which contains two CRE-like motifs, plays a role in the optimization of mOAZt promoter activity. However, this fragment does not appear to be critical for testis-specific expression of the mOAZt gene. Several tar-


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-133  CCACACCCGACTCAGTATATAACTTTTAAAGCATTTTAATTATTGCTATAGTTGT  -79

                                                        CRE
      AAAGTATATACTAGTTGAATATGAACCTTGGTTAGTGGGAACTGCAGTGACATAAGCAAG  -19

          CRE              Inr
      TGACCTCATGTCTATGTCATAATGCCTGCCAGGTCACAATGGACAGGGTGGCTTGAACCT  +42
                    +1

      CTGCGACTTATCCGCTGAACCTCACTTTGCCGCAGGAGGCACTGAACAGAGAAACTGCCT  +102

      TGTAACAGGTCCCGCCCCTCTCTCTACTCCCTTTCTTATATCAAGAGGGGAAAAACACGG  +162

      AACTATCTCTATCCATTCTGGTCACCATTCGCCTATTACCTCTACTGTTACAAATACCGG  +222

      ATCACCCTCCGGGAGAA  +242
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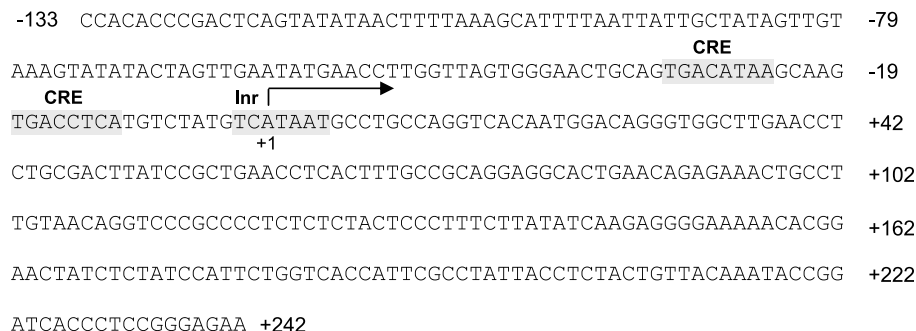
Fig. 1. Promoter region of the mOAZt gene. The fragment that contains the 133-bp upstream and 242-bp downstream regions is sufficient for the regulation of the haploid-specific expression of this gene. The transcription start site is indicated by a broken arrow. The gray boxes represent the locations of the CREs and the Inr.
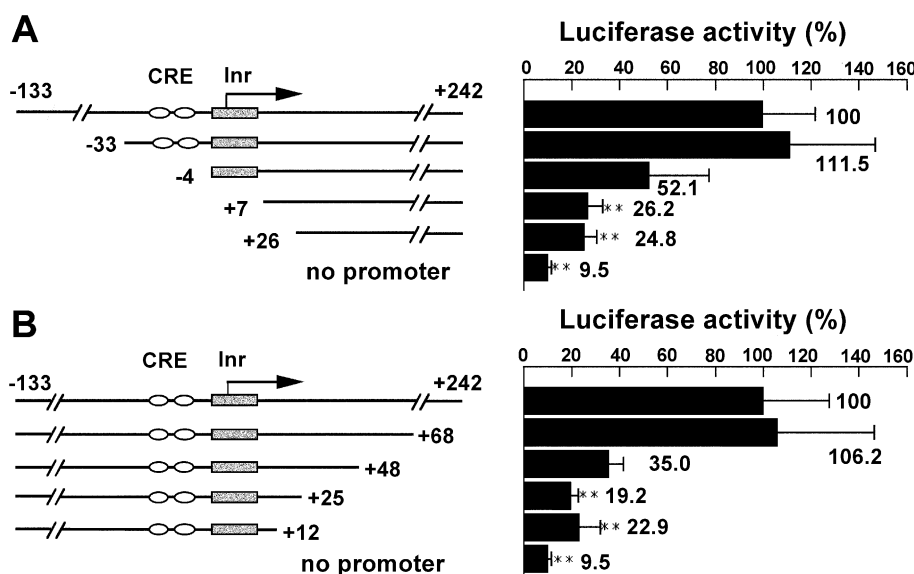
Fig. 2. Deletion analysis of mOAZt promoter activity using in vivo transient transfection. The mOAZt promoter was deleted sequentially either upstream (A) or downstream (B) of the transcriptional start site, and linked to the luciferase reporter gene. The deletion constructs were transfected into the seminiferous tubules of mouse testes. The positions of the 5′- and 3′-end points of the constructs are indicated as distances from the transcription start site. The pGL3-basic plasmid was transfected as the promoter-less control. The luciferase activities, which are normalized to that of RL driven by the −133/+242 promoter construct, are presented in the bar graph as mean values, with error bars showing the standard deviations (S.D.). The transfections were repeated at least three times. The asterisks (**) in A and B indicate that the values are significantly ($P < 0.01$) lower than those for the −4/+242 or −133/+48 constructs, respectively.

get genes for CREMτ-mediated activation have been identified in haploid germ cells [16]. Moreover, targeted disruption of the CREM gene results in a complete blockade of germ cell differentiation at the first steps of spermiogenesis [17,18]. Thus, CREMτ may contribute to the regulation of mOAZt through CRE-like motifs.

To assess this possibility, we used EMSA to examine the binding between the CREM that was present in the testis and the 29-bp region. Mouse testicular germ cell nuclear extracts were incubated with the G5 (−33/−5) CRE-containing oligonucleotide, and several testis-specific DNA–protein complexes were observed (Fig. 3B, compare lanes 1 and 6). The binding of labeled G5 to the shifted bands, with the exception of DNA–protein complex number 2, was competitively inhibited by the addition of an excess of unlabeled G5, but not by an excess of the non-specific oligonucleotide. To evaluate whether the DNA–protein complexes contained isoforms of CREM, we performed supershift analysis with the anti-CREM antibody (Fig. 3B, lanes 7 and 8). The anti-CREM antibody disrupted the gel shift pattern, which indicates that some CREM isoforms are able to bind to these CRE motifs. The CREM gene generates both repressors (CREMα, β, γ) and activators (CREMτs) via alternative cell-specific splicing. Although CREMτ transcripts are extremely abundant, particularly in the haploid spermatids of the testis, other CREM variants are present at low levels. These data indicated that certain CREMτs upregulate the mOAZt gene via CRE motifs in the gene upstream region.

3.3. The Inr-containing motif functions as a core promoter together with a downstream element

The 5′-deletion that extended up to position −4 resulted in 50% of full activity in the transient transfection assay, which suggests that the core promoter of the mOAZt gene is located in a more distal region. An additional deletion up to position +7, which removed a 10-bp sequence that included the transcriptional start site and the Inr, caused a two-fold reduction, as compared to the level of expression of the −4/+242 region. This activity appears to be the basal level, since an additional deletion to position +26 caused no significant further reduction in promoter activity (Fig. 2A). The −4/+6 region may be responsible for the initiation of transcription, thereby acting as the core promoter. The functions of several Inr-containing promoters that lack a TATA box are supported by important downstream sequences, which include the downstream promoter element (DPE) and downstream core element (DCE) [19]. To examine whether the intergenic region of the mOAZt gene also contributes to the promoter activity of this gene, we created several 3′-deletion constructs, and examined the promoter activities by in vivo transfection. The transfections were repeated three to nine times, to yield reproducible results. As shown in Fig. 2B, the construct that was deleted to +68, i.e. a 174-bp deletion of the downstream region, showed no significant reduction in promoter activity. A further 20-bp deletion, which removed the region from +68 to +49, reduced by three-fold the reporter gene activity. An additional deletion to position +25 reduced the promoter activity to the basal level. A deletion to position +12 caused no further reduction in promoter activity. These results reveal that the +26/+68 region downstream of the transcription start site contributes significantly to mOAZt promoter activity. We named the +26/+68 region the 'OAZt downstream element' (ODE). Both the −4/+6 and +26/+68 regions are indispensable for mOAZt transcription, because the loss of either reduces the promoter activity to the basal level. The ODE sequences are highly conserved among the mouse, human, and rat OAZt genes (Fig. 4). Furthermore, almost identical sequences (GCTTGAACCTC and GCTGAACCTC) were observed in the
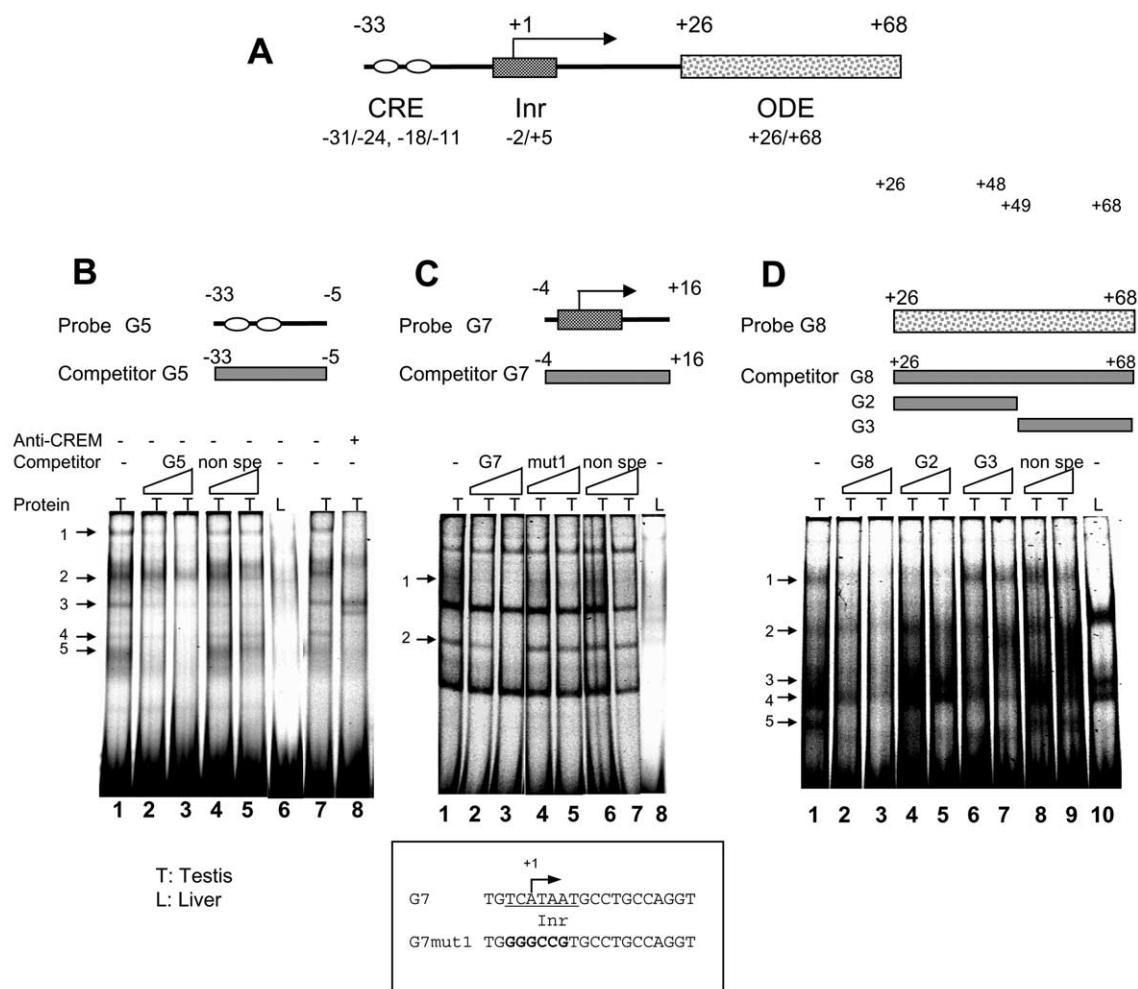
Fig. 3. Testis-specific factors bind the three different functional sequences in the mOAZt promoter. A: Schematic structure of the mOAZt promoter. B–D: Three different fragments of the mOAZt promoter, including the CRE (G5), Inr (G7), and ODE (G8), were end-labeled and incubated with mouse testicular germ cell (T) and liver (L) nuclear extracts. Wild-type (G5, G7, G8, G2, and G3), non-specific (non spe), and mutant (mut1) competitors were used at 10- or 100-fold molar excess. The supershift assay was performed with the anti-CREM antibody (+).

+33/+43 and +56/+65 regions of the mouse ODE, as well as in other species. This level of conservation indicates that ODE plays an important role in haploid-specific expression in mammals.

The widely recognized DPE is a conserved motif that is located between residues +28 and +34, and includes the common GATCG sequence motif, which is found in various species from *Drosophila* to humans [20,21]. The DCE of the human β-globin gene, which is located between resides +10 and +45, is an additional example of a downstream element [22,23]. The DPE and DCE function in concert with Inr, to increase transcription factor IID (TFIID)–promoter complex formation and/or to stabilize the complex through direct interactions with TATA box-binding protein (TBP) associated factors (TAFs). Thus, the downstream elements and Inr function together as a single core promoter unit. This is especially true for the downstream element and Inr-containing motif, which indicates that ODE also facilitates the binding of TFIID to a TATA-less promoter, although ODE does not share homology with the other downstream elements, and exists at a different position in the promoter. The unidentified unique sequence of ODE indicates that certain binding factors are specifically expressed in testicular germ cells.

To examine this possibility, we performed EMSA (Fig. 3C and D). Incubation of the nuclear extract with probe G7 (−4/+16), which contains Inr together with its flanking region, produced several testis-specific DNA–protein complexes (compare lanes 1 and 8 in Fig. 3C). Two of the complexes (arrowed) were sequence-specific and competed with unlabeled G7, but not with the non-specific oligonucleotide (Fig. 3C, lanes 2, 3, 6 and 7). On the other hand, G7mut1, which has a 6-bp mutation at position −2/+4, did not compete with these DNA–protein complexes, even when added at 100-fold molar excess (Fig. 3C, lane 5), which suggests that the testis-specific binding of the DNA–protein complex requires the sequence that lies in the −2/+4 region. Using probe G8 (+26/+68), which contains ODE, five sequence-specific DNA–protein complexes were observed (arrowed in Fig. 3D). Complexes 1 and 5 were testis-specific (compare lanes 1 and 10 in Fig. 3D). Complex 4 showed the strongest band, the intensity of which was reduced significantly by the addition of unlabeled G3, but not G2. This result was consistent with the data from the 3′-deletions in the transient transfection assay. On the other hand, complex 1, which is the testis-specific band, competed with the G2 competitor. These data suggest that the +26/+68 region contains the target

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human:    C----CAG-T-----A--CAG-----AA-GTCTTCAAGTTCTATTTTTTAA-TCATT
mouse:    CA---C----ACCCGACTCAGTATATAAC-TTTTAAAG--C-A--TTTTAATT-ATT      -90
rat  :    CAGGGCTGTTTGTCAAC-CAGTATCT-ACGTTTTAAAG--C-A--TTTTAACT-ATT
          *     *         *  ***      *  * ** ***   * *  ****** * ***


human:-C-ATA-CAGCCATAACA-TGCATACTAGTCGAATATGAGCCTTGGATAAGGGATTAGGG
mouse:GCTATAGTTG---TAA-AGTATATACTAGTTGAATATGAACCTTGG-T------------      -47
rat  :GCTATAATTG---TAA-AGTATATACTAGTTGAATATGAACCTTGGCT---GG--CA--G
       * ***     *   *** * *  ******** ******* ****** *


human:TAGATAAGCAGCAGAGTG-GAGTCCT-GAGGTGACATAAGGGC---TGACCCCTTATCCA
mouse:-----------TA--GTG-G-GAACTGCA-GTGACATAA--GCAAGTGACCTCATGTCTA      -5
                                    CRE                CRE
rat  :--G---A-CA-GAG-GGGCG-GGACTACA-GTGACATAA--CCAAGTGACATCATGTCTA
              *   *  *  *  **  *  ********    *    **** *  * **  *


human:TGTCATAGTGCC--CACTGGGTCACAATGCCACCA-GGTTGCCTAAACCTCTGCCACCCA
                                         +26          +48
mouse:TGTCATAATGCCTGC-C-AGGTCACAATG-GA-CAGGGTGGCTTGAACCTCTGCGACTTA      + 52
       Inr                                                      ODE
rat  :TGTCATAGTGCCTGC-C-AGGTCACAATG-GA-CAGGGTGGCCTGAACCTCTGCAACTCA
       ****** ****  * *  *********  * ** *** ** * ********* **   *


human:-CCTGTGAACTTCACTTTGCCACAGGGAGGCACTGAACTGAAAAACTGCCTTGTAAGAGG
                   +68
mouse:TCCGCTGAACCTCACTTTGCCGCA-GGAGGCACTGAACAGAGAAACTGCCTTGTAACAGG      + 111
rat  :TCCTCTGAACCTCACTTTGCCGCAGGGGAGGCACTGAACAGAGAAACTGCCTTGTACCAGG
       **  ***** ********* ** ************* **  ************   ***


human:TGTCGCCCCTCTGTCTACTCCCTTTCTTATATCAAGAGGGGAAAAACACGTAACTACCTC
mouse:TCCCGCCCCTCTCTCTACTCCCTTTCTTATATCAAGAGGGGAAAAACACGGAACTATCTC      + 171
rat  :TCCCGCCCCTCTCTCTACTCCCTTTCTTATATTAAGAGGGGAAAAACACGGAACTGCCTC
        *  ******** ******************* ***************** ****   ***


human:TACCCGA-TCTGGTCACCATACGCCTATTACCTTTACTGTTACAAGTACCGGATCACTCT
mouse:TATCC-ATTCTGGTCACCATTCGCCTATTACCTCTACTGTTACAAATACCGGATCACCCT      + 230
rat  :TACCC-ATTCTGGTCACCATACGCCTATTACCTCTACTGTTACAAATACCGGATCACCCT
       ** ** * ************ ************ ********** *********** **


human:CCGGGAGAAGATG
mouse:CCGGGAGAAGATG +243
rat  :CCGGGAGAAGATG
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Fig. 4. Comparison of genome sequences among the human, mouse and rat promoter regions of the OAZt gene. The transcription start site of the mOAZt gene is indicated by a broken arrow. The translation initiation codon is indicated in bold. Asterisks show identical nucleotides among the three species. The CREs, Inr, and ODE in the mOAZt gene are shaded. The repeat sequences in the downstream region (described in the text) are indicated by open boxes. The sequence data for the mouse, human, and rat OAZt genes have been deposited in DDBJ/Gen-Bank under the accession numbers: AB083045, AL589765.19, and AC133978.2, respectively.

sequences for several transcriptional factors. From these results, we speculate that interactions between Inr and its flanking region and ODE promote the haploid-specific transcription of the mOAZt gene.

In this report, we describe the identification and characterization of a functional downstream element in the mouse haploid-specific gene, i.e. the OAZt promoter. Many of the genes that are transcribed specifically in spermatogenic cells have been reported as having TATA-independent promoters [4], and some of these promoters use Inr. However, the significance of the downstream elements has not been reported for testis-specific genes, with the exception of two *Drosophila* genes, the downstream elements of which modulate promoter activity in combination with Inr [24,25]. Thus, this is the first report of the existence of a downstream element in a mammalian testis-specific promoter.

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