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Differential *DMRT1* Expression in the Gonads of *Podarcis sicula* (Reptilia: Lacertidae)

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DM domain · *DMRT1* expression · Reptiles · Spermatogenic cycle · Testis

Abstract
DMRT genes encode a large family of transcription factors which share an unusual cysteine-rich DNA-binding motif, the DM domain. DM family members have been studied in the context of sexual development; in particular, the *DMRT1* gene appeared to be the one most directly involved in sex determination, but its activity is largely unexplored and possible downstream targets of this factor have yet to be identified. *DMRT1* of the lacertid lizard *Podarcis sicula (PsDMRT1)* was isolated as a model to study differential gene expression during the seasonal reproductive cycle of an ectothermal species. The adult testis of *P. sicula* exhibits full activity in spring, complete regression in summer and a slow autumnal recrudescence without spermiation. We cloned a 591-bp partial ORF of the *PsDMRT1* fragment, whose putative amino acid sequence contains the conserved DM domain. Northern blot analysis of mRNA from different tissues of *P. sicula* individuals captured in spring demonstrated *DMRT1* transcripts only in testis. Semi-quantitative RT-PCR and in situ hybridization experiments showed peak *PsDMRT1* expression in spring, lower expression in autumn and no expression during the period of gonad regression. A possible correlation between androgen level variations and *PsDMRT1* transcripts is hypothesized and discussed. Finally, data showed that *PsDMRT1* is expressed only in spermatogenic cells, before the second meiotic division, suggesting that its role is confined to the proliferation and maintenance of spermatogonia and spermatocytes.

DMRT1 (doublesex and mab-3 related transcription factor-1) is an evolutionarily conserved transcription factor belonging to a family of important sexual regulators that share a unique cysteine-rich DNA-binding motif, known as the DM domain [Erdmann and Burtis, 1993; Zhu et al., 2000; Brunner et al., 2001]. Homologues of the *DMRT1* gene have been isolated in invertebrates and vertebrates and their conserved function in the control of sex determination has been demonstrated [Raymond et al., 1999; Zarkower, 2001; Volff et al., 2003; Kobayashi et al., 2004; Wang et al., 2006]. In vertebrates, the *DMRT1* gene is expressed throughout testicular differentiation both in pre-meiotic germ cells and in differentiating Sertoli cells. *Dmrt1*-null mutant mice have severely dysgenic testes in which Sertoli cells and germ cells fail to differentiate properly after birth [Kim et al., 2007; Lei et al., 2007]. Early embryonic expression of the gene therefore seems to be an integral part of normal gonadal development in vertebrates.

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The function of *DMRT1* has been studied in several species where sex differentiation is influenced by environmental factors, such as temperature changes. Its expression is upregulated at male-producing temperatures in turtles, alligators and salamanders [Kettlewell et al., 2000; Torres Maldonado et al., 2002; Rhen et al., 2007; Ferguson-Smith, 2007; Hoshi et al., 2008]. *DMRT1* downregulation, together with upregulation of the P450 aromatase gene *(CYP19A1)* in species with temperature-dependent sex determination such as salamander, produces sex reversal from genetic males to phenotypic females [Murdock and Wibbels, 2006; Sakata et al., 2006]. In contrast, scant data are available on the expression pattern of *DMRT1* in the adult gonad and its possible correlation to spermatogenesis. *Dmrt1* expression reappears after birth in mouse testis germ cells, but not in ovary, and is found both in stem and in proliferating spermatogonia [Kim et al., 2007; Lei et al., 2007].

In some teleostean species *dmrt1* expression is confined to the testis. Variation in expression levels according to spermatogenic stage suggested a specific role for it in adult testicular function [Marchand et al., 2000; He et al., 2003; Fernandino et al., 2006].

The spermatogenic cycle of reptiles, particularly Lacertilia, living in temperate regions is very sensitive to external temperature changes [Angelini and Ghiara, 1984]. However, there are no data on the involvement of *DMRT1* in the spermatogenic process of ectothermal vertebrates with seasonal reproductive patterns.

In this work we isolated the *DMRT1* gene of the lacertid lizard *Podarcis sicula (PsDMRT1)* and analyzed its expression in the different phases of the male gonad cycle, which is characterized by full activity in spring, complete regression in summer and slow autumnal recrudescence without spermiation [Angelini et al., 1979].

Materials and Methods

Animals and Samples

The tissues used in this study were from adult *P. sicula* individuals captured in the neighborhood of Naples (Italy).

Isolation and Sequencing of P. sicula DMRT1 *cDNA*

The *P. sicula DMRT1* sequence corresponding to the 591 bp of complete ORF was isolated from total RNA extracted from sexually mature testes with Tri-reagent (Sigma-Aldrich, St. Louis, Mo., USA) by RT-PCR using 1 μg total RNA, oligo-dT primers and Superscript III (Invitrogen Life Technologies, Carlsbad, Calif., USA). Amplification (30 cycles) was performed using AmpliTaq Gold (Applied Biosystems, Foster City, Calif., USA) and *Calotes versicolor* primers (forward: 5′-ACA AGC GGT TCT GCA TGT GG-3′; reverse: 5′-CAT CCT GTA CTG GGA GCC CA-3′) in a Perkin-Elmer Gene Amp PCR System 2400 (PE, Waltham, Mass., USA) with the following conditions: denaturing at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. cDNA was cloned into a pCR II vector (Invitrogen Life Technologies) and sequenced in both directions by PRIMM (Naples, Italy) (GenBank accession number GQ372846). All sequence information was processed using the BlastN and BlastP programs; multiple alignments were generated with ClustalW. EMBL sequence accession numbers for *DMRT1* are: *C. versicolor,* AF464141*; Chinemys reevesii*, AB365876; *Gallus gallus*, AF211349; *Homo sapiens*, AF130728; *Mus musculus*, AF202778.

Northern Blot

For transcript analysis, total RNA extracted from several tissues (e.g. ovary, heart, liver, and testis) of *P. sicula* individuals captured in spring was isolated as described above. In brief, 20 μg of total RNA per sample was separated on 1% formaldehyde agarose gel and blotted onto a nylon membrane (GE Healthcare, Amersham, UK). The *P. sicula DMRT1* cDNA fragment was labeled using a ^{32}P-labeled dCTP (NEN Co, Boston, Mass., USA) and the random primer DNA labeling kit (Roche, Mannheim, Germany). RNA blots were hybridized in a mixture of 0.5 M $NaHPO_4$, 7% SDS, 1% BSA, and 1 mM EDTA at 60°C. Filters were washed at 60°C in 2× SSC, 2% SDS and then in 0.2× SSC, 0.2% SDS before exposure to an X-ray film (Kodak, USA). An 18S-fragment was used to reprobe the blots to confirm equal loading of RNA.

Semi-Quantitative RT-PCR

Total RNA was extracted from testis of specimens captured in May, August and October using Tri-reagent. RT-PCR was performed using 1 μg of total RNA as described above. The reaction was performed with *P. sicula DMRT1*-specific primers: forward 5′-GCGTGACTGCCAGTGTAAAA-3′ and reverse 5′-CCTTCTGTTGGTGCAGTTGA-3′ under the following conditions: denaturing at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 s.

In situ Hybridization in Sections

P. sicula DMRT1 cDNA was used as a template to synthesize digoxigenin-labeled antisense and sense probes, with the digoxigenin-labeled UTP and Sp6 or T7 RNA polymerase, according to the manufacturer's recommendations (Roche). *P. sicula* testis tissue was fixed in Bouin's fixative, dehydrated in ethanol and processed for embedding in paraffin according to standard techniques. Then, 7-μm-thick paraffin sections were placed on Superfrost Plus slides (Carlo Erba, Milan, Italy), deparaffinized in xylene and rehydrated in graded ethanols. Sections were hybridized overnight at 60°C in 40% formamide, 1× Denhardt's solution, 5× SSC, 200 μg/ml tRNA (Sigma) and 100 ng of sense or antisense digoxigenin-labeled RNA on each slide. Slides were washed in 0.5× SSC, 20% formamide for 1 h at 60°C, and exposed to RNase A at 37°C for 30 min. After a wash in 0.5× SSC, 20% formamide, for 30 min at 60°C, they were incubated overnight in anti-digoxigenin fluorescein-conjugated antibody diluted 1:2,000 in 2% blocking reagent solution (both from Roche). After several washes in 1× PBS, 0.1% Tween 20, sections were mounted and observed under an Eclipse 1000 UV photomicroscope (Nikon, Tokyo, Japan).

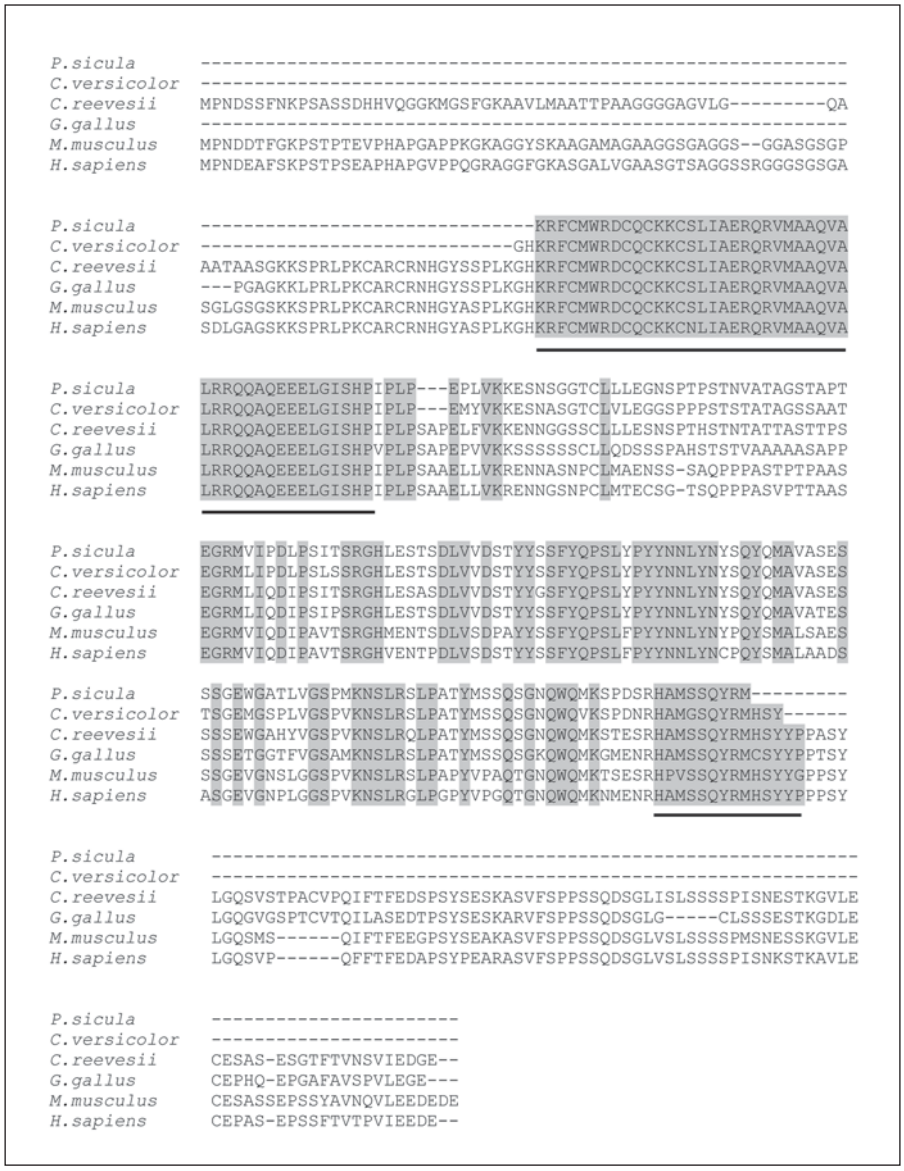

Fig. 1. Multiple alignments of *P. sicula* DMRT1 and DMRT1 of other vertebrates. Identical amino acid regions are shown in gray boxes. DM domains in DMRT1 and the male-specific motif in DMRT1 of vertebrates are underlined. GenBank accession numbers of the *DMRT1* sequences used are reported in Materials and Methods.

Results

Isolation and Sequence Analysis of P. sicula DMRT1

A partial sequence of *P. sicula DMRT1* cDNA was amplified by RT-PCR from total RNA of high gonad activity adult testis using *C. versicolor* primers [Sreenivasulu et al., 2002]. The primers were designed on the DM domain region within the male-specific motif. The fragment isolated from *P. sicula* was 591 bp long and encoded 197 amino acids, including the DM domain towards the protein C-terminus, with a partial male-specific motif. The amino acid sequence derived from *P. sicula DMRT1* demonstrated high homology to the *DMRT1* sequences of *Pelodiscus sinensis* (85% identity), *C. versicolor* (88% identity), *C. reevesii* (86% identity), *Oncorhyncus mykiss* (52% identity), *Danio rerio* (53% identity), *Xenopus laevis* (66% identity), *G. gallus* (78% identity), *M. musculus* (71% identity), and *H. sapiens* (70% identity).

The most conserved sequence was located in the DM domain (fig. 1): the alignment of the DM domain from the same species showed 90–100% identity. The amino acid region outside the DM domain before the male-specific motif showed high homology to *P. sinensis* (82% identity), *C. versicolor* (86% identity), and *C. reevesii* (82% identity), and lower homology to other vertebrate species, such as *D. rerio* (44% identity), *X. laevis* (62% identity), *G.*

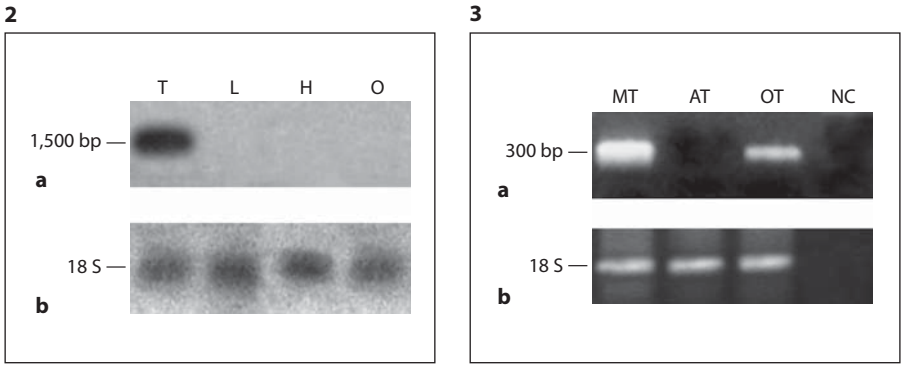

Fig. 2. Northern blot analysis of total RNA isolated from *P. sicula* tissues. **a** *P. sicula DMRT1* cDNA was used to probe male adult tissues collected in May: testis (T), liver (L), heart (H), ovary (O). **b** Reprobing with 18S was done as a loading control.

Fig. 3. a RT-PCR analysis of *DMRT1* in *P. sicula* testes collected in May (MT), August (AT) and October (OT). NC: Negative control, no retro-transcriptase was added. **b** RT-PCR of the same total RNA with 18S primers.

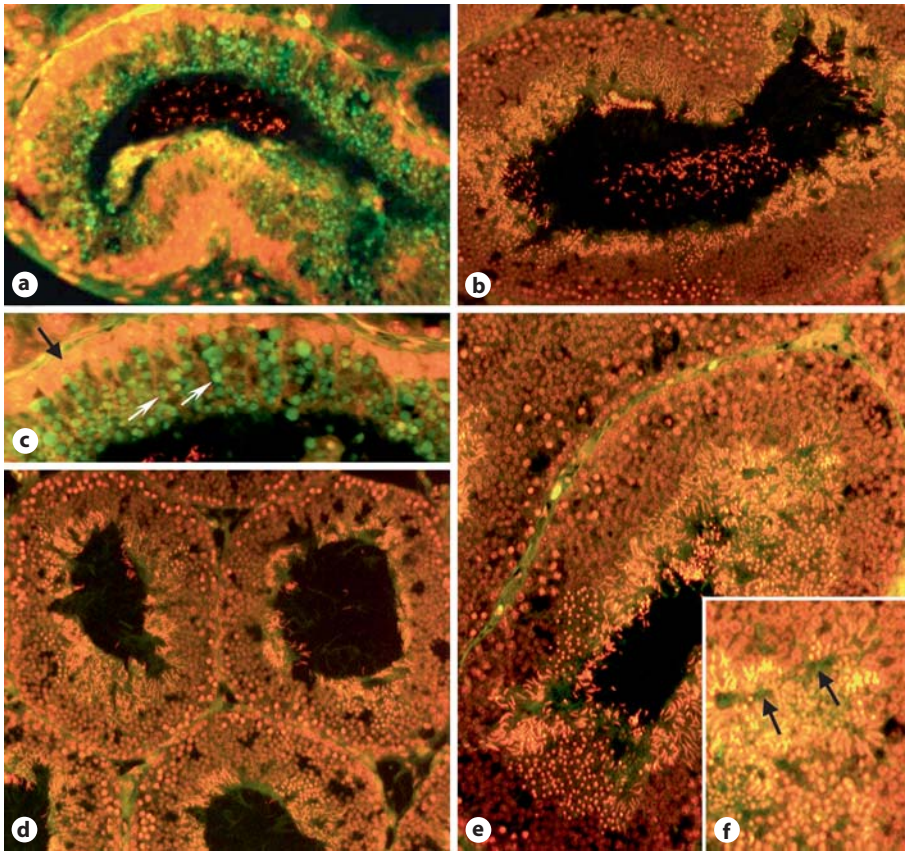

Fig. 4. In situ hybridization on sections from paraffin-embedded testis collected in various months with *P. sicula DMRT1*. **a**, **c** May: strong signal on primary and secondary spermatocytes (white arrows). **b** May: section hybridized with a sense riboprobe as a negative control. **d** August: absent hybridization signal. **e**, **f** October: positive signal (black arrows) only for a few spermatocytic cells.

gallus (73% identity), *M. musculus* (66% identity), and *H. sapiens* (64% identity). Alignment of the *P. sicula* DMRT1 protein sequence with *C. versicolor, C. reevesii, G. gallus*, *H. sapiens* and *M. musculus* DMRT1 confirmed these data.

DMRT1 Expression in Tissues

Northern blot analysis of total RNA from *P. sicula* liver, heart, testis (in different phases of the annual spermatogenic cycle), and ovary (spring) disclosed a major 1,500-bp band (fig. 2) only in testes collected in May, the period when spermatogenesis is most active. Fainter bands were also seen in October, when gonad activity resumes. *DMRT1* mRNA was not expressed in August, a time of complete gonad regression (fig. 3).

In situ Hybridization in Sections

Sections of adult testis in different stages of the annual spermatogenic cycle were hybridized with sense and antisense *DMRT1* probes (fig. 4). As in the Northern

blots, mRNA was highly expressed in May. In in situ localization experiments a strong fluorescent signal was seen in primary as well as secondary spermatocytes, whereas no signal was detected in spermatozoa or somatic Sertoli cells. Sections from the regression period showed no mRNA expression, while a faint signal was detected in spermatogonia in October, when gonad activity resumes.

Discussion

The *DMRT* genes encode a large family of transcription factors that share an unusual cysteine-rich DNA-binding motif, the DM domain. DM family members have been studied primarily in the context of sexual development. *DMRT1* appeared to be the gene most closely involved in sex determination; however, the way in which its activity is mediated is largely unexplored, and possible downstream targets have yet to be identified [Hong et al., 2007].

In this work, cloning of a partial *DMRT1* ORF fragment of 591 bp from the lizard *P. sicula* disclosed a high similarity of its putative amino acid sequence to other sequences isolated in reptiles, and high homology to the DM domain of *DMRT1* of other vertebrates, confirming the evolutionary conservation of the gene throughout the animal kingdom.

We also investigated the possible involvement of *DMRT1* in *P. sicula* spermatogenesis to establish whether it is differentially expressed in the 3 phases of its seasonal spermatogenic cycle.

A first intriguing finding from Northern blot as well as in situ hybridization studies was that *DMRT1* is highly expressed in adult *P. sicula* testis during the reproductive season, though not in other tissues, ovary included. In this phase, spanning from the end of February to mid or late June, intense spermatogenesis is demonstrated by well-developed tubules and large lumina containing all stages of spermatogenic cells [Angelini and Ghiara, 1984; Angelini and Botte, 1992]. Semi-quantitative RT-PCR assays, performed to identify variations in *P. sicula DMRT1* mRNA in the 3 phases of the spermatogenic cycle, showed *DMRT1* upregulation in this period, while in situ hybridization experiments showed high gene expression in the first stages of germ cells, but not in spermatozoa or supporting Sertoli cells, as seen during gonad differentiation. These data agree with those reported for the protogynous hermaphroditic groupers *(Epinephelus coioides)*, where *DMRT1* is also confined to specific spermatogenesis stages and cells in reverting males [Xia et al., 2007], suggesting that in ectothermal vertebrates *DMRT1* might play a functional role during spermatogenic cell differentiation from spermatogonia to spermatocytes.

During gonad regression, from late July to early August, when degenerative phenomena occur in all seminiferous tubules and the gonadosomatic index is lowest, *P. sicula DMRT1* expression was undetectable.

In the testes collected in autumn, showing a revival of spermatogenesis and resumption of spermiation, *P. sicula DMRT1* transcripts were again expressed, albeit at a much lower level, and *DMRT1* mRNA was detected in a small number of type B spermatogonia and in spermatocytes.

The proliferative stage of *P. sicula* male gonads in spring is supported by an increase both in plasma and in testicular levels of androgens. Testosterone peaks in the mating season (spring), then plummets in June and remains low throughout hibernation, when another androgen, dehydroepiandrosterone, reaches peak levels. During the refractory period low levels of androgens are coupled to critical estrogen concentrations [Andò et al., 1990; Angelini and Botte, 1992].

Manipulation of sex determination and/or sex reversal with exogenous steroid hormones in fish, amphibians and reptiles with temperature-dependent sex determination clearly demonstrated that exogenous steroids correlate with *DMRT1* expression [Shibata et al., 2002; Aoyama et al., 2003; He et al., 2003; Xia et al., 2006].

The present study points to a possible correlation between androgen level variations and *DMRT1* transcripts. It may thus be hypothesized that endogenous steroid hormones influence or modulate *DMRT1* transcription during the reproductive cycle of *P. sicula*. Our data also highlight that *P. sicula DMRT1* is expressed only in spermatogenic cells, before the second meiotic division, suggesting that its role is confined to the proliferation and maintenance of spermatogonia and spermatocytes. Although clarification of the mechanisms involved in these processes requires further investigation, these data provide new insights into the role of *DMRT1* in adult gonads.

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