Female-enriched and thermosensitive expression of steroidogenic factor-1 during gonadal differentiation in *Pleurodeles waltl*

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Abstract

In the urodele amphibian *Pleurodeles waltl*, sex differentiation is genetically controlled, that is, ZZ male vs ZW female, but may be influenced by temperature, which induces a female-to-male sex reversal. We investigated whether steroidogenic factor 1 (SF-1) could be involved in *Pleurodeles* sex differentiation or in temperature-dependent sex reversal by cloning a *Pleurodeles* SF-1 cDNA and examining its developmental expression. The 468-amino-acid deduced protein is highly conserved in comparison with other species. In ZZ and ZW control larvae, SF-1 mRNA is detected at the first stage of the thermosensitive period (TSP) in the gonad-mesonephros-interrenal complex (GMI). By the end of TSP at stage 55, SF-1 is expressed in the gonad (Gd) and in the mesonephros-interrenal (MI) both in ZZ and ZW larvae. During this stage, a transient, ZW-specific increase of SF-1 transcription occurs not only in Gd but also in MI, this increase starting earlier in Gd than in MI. Therefore, in *P. waltl*, an SF-1 upregulation occurs after the onset of the ovarian-specific increase of aromatase mRNA expression. At the end of metamorphosis, the SF-1 transcription level in Gd and MI is nearly the same in both ZZ and ZW larvae. Besides, after long-term heat treatment leading to sex reversal, SF-1 mRNA upregulation is not observed in ZW larvae, in either Gd or MI. However, SF-1 expression is not decreased after a 48-h heat shock applied at the end of the TSP, suggesting that temperature has no inhibitory effect by itself in long-term heat treatment. Estradiol benzoate treatments show that, at the end of the TSP, SF-1 gene transcription could be controlled by the estrogen level. This is in accordance with the female-enriched SF-1 expression and the decreased SF-1 expression following long-term, sex-reversing heat treatment, which is known to decrease aromatase expression and activity. Thus, it is unlikely that SF-1 is directly involved in *Pleurodeles* temperature-dependent sex reversal.

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Introduction

The steroidogenic factor-1 gene (SF-1) encodes a nuclear orphan receptor belonging to the nuclear receptor superfamily (Lala *et al.* 1992, Whitfield *et al.* 1999). In various species, SF-1 is known to be expressed in several tissues involved in steroidogenesis and/or reproduction, such as the adrenal glands in mammals and corresponding organs in other species, such as the interrenal tissue in amphibians (Mayer *et al.* 2002) as well as brain and gonad (Val *et al.* 2003). SF-1 plays a key role in the development of both the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes (Ikeda *et al.* 1993), since mice with a SF-1 gene homozygous disruption show complete adrenal and gonadal agenesis and ventromedial hypothalamus dysgenesis (Luo *et al.* 1994, Ikeda *et al.* 1995, Shinoda *et al.* 1995). A failure of gonad and adrenal development has also been detected in a human case of male-to-female sex reversal exhibiting a heterozygote mutation in the SF-1 gene (Achermann *et al.* 1999). Furthermore, the SF-1^(-/-) XY

mice have a female phenotype (Luo *et al.* 1994). These results agree with an early expression of SF-1 in the genital ridges of both sexes throughout the undifferentiated stage (Ikeda *et al.* 1994), leading to adrenal formation and gonadal differentiation (Schnabel *et al.* 2003). At a later stage of development, as histologic sex differentiation of the gonad begins, several vertebrate species exhibit a sexually dimorphic gonadal expression of SF-1 in their developing gonads. A higher level of SF-1 mRNA is found in the male differentiating gonad in mouse (Ikeda *et al.* 1994), rat (Hatano *et al.* 1994), man (Hanley *et al.* 2000), pig (Pilon *et al.* 1998) and turtle (Wibbels *et al.* 1998), whereas an opposite situation is observed in chicken (Smith *et al.* 1999), alligator (Western *et al.* 2000) and American bullfrog (Mayer *et al.* 2002). In mammals, SF-1 controls the expression of many enzymes required for steroid hormone production, including aromatase (Parker & Schimmer 2002). For instance, in rat and man, SF-1 binds to the aromatase promoter and activates its transcription (Lynch *et al.* 1993, Michael *et al.* 1995). SF-1-binding sites have also

been identified in the promoter region of aromatase gene in chicken (Kudo *et al.* 1996), *Xenopus* (Akatsuka *et al.* 2005), and many teleost fishes (Tanaka *et al.* 1995, Tong & Chung 2003, Yoshiura *et al.* 2003, Gardner *et al.* 2005). Alternatively, estrogen could also control SF-1 transcription, as suggested in the red-eared slider turtle model (Fleming & Crews 2001), in which estradiol treatment was shown to produce SF-1 upregulation, although it was not proved that the effect was direct or indirect.

In the urodele amphibian *Pleurodeles waltli*, steroid hormones play a role in sex differentiation since the application of estradiol to the rearing water during a hormone-sensitive period induces ZZ genetic male larvae to differentiate into functional neofemales (Gallien 1951). Moreover, a similar treatment performed with an aromatase inhibitor can induce a complete female-to-male sex reversal (Chardard & Dournon 1999), demonstrating that the aromatase enzymatic complex plays a very important part in the process of female differentiation (Kuntz *et al.* 2003a). Indeed, aromatase activity measurements in gonad revealed up to 40-fold higher activity in ZW larvae than in their ZZ counterparts at the onset of ovarian differentiation (Chardard *et al.* 1995). However, the gonadal level of endogenous aromatase transcripts in ZW larvae was only 1.5-fold higher than in ZZ, suggesting the involvement of an unknown post-transcriptional regulation (Kuntz *et al.* 2003b).

Temperature can also interfere with the ZZ/ZW genetic sex determination and sex ratio, since ZW larvae reared at 32 °C during the thermosensitive period (TSP) differentiate into functional neomales (Dournon & Houillon 1984). The masculinizing temperature was shown to inhibit aromatase activity (Chardard *et al.* 1995) and could act as a direct or indirect repressor of estrogen synthesis, since estradiol benzoate can counteract the masculinizing effect of a high rearing temperature (Zaborski 1986).

Since the aromatase gene is differentially expressed during sexual differentiation and considering that this expression can be indirectly affected by temperature, we focused our attention on SF-1, a known potential regulator of aromatase. We first isolated SF-1 cDNA containing a putative open reading frame. Expression analysis during gonadal development of ZZ and ZW larvae revealed the presence of SF-1 mRNA early during the TSP in the gonad–mesonephros–interrenal complex (GMI), either when aromatase mRNA is present in low amounts in the undifferentiated gonad (Gd) or at the time of aromatase expression increase in ZW larvae. A transient, female-specific upregulation of SF-1 expression was observed at the end of TSP, not only in Gd but also in the mesonephros–interrenal (MI), suggesting a role of SF-1 in the differentiating ovary and in mesonephric or interrenal steroid synthesis. Amounts

of SF-1 mRNAs were significantly decreased in ZW larvae subjected to long-term sex-reversing heat treatment, in either Gd or MI, but not in the brain. However, these changes in SF-1 gene expression, rather than being directly regulated by temperature during the TSP, seem to be related to changes in aromatase mRNA expression and activity leading to low estrogen levels. Indeed, estradiol benzoate treatments of ZZ larvae clearly induce upregulation of SF-1 mRNA expression. Therefore, SF-1 does not appear to be directly involved in temperature-dependent sex-reversal in *Pleurodeles*.

Materials and methods

Animals

Adult *P. waltli* were reared in fresh water at a controlled temperature of 20 ± 2 °C in our laboratory (standard ZW²⁰ and ZZ²⁰). The ZW or ZZ sexual genotype was determined by electrophoretic patterning of the two W- and/or Z-linked peptidase-I isoforms, as described previously (Chardard *et al.* 1995). Developmental stages were determined by macroscopic observation according to Gallien and Durocher (1957). Stage 55 lasts about 2 months and was chronologically subdivided into stage 55, stage 55+15 days (55^{15d}) and stage 55+30 days (55^{30d}). The TSP from stage 42 to 54 is defined as the period while ZW larvae must be reared at 32 °C in order to obtain 100% sex reversal (Dournon & Houillon 1984). Heat treatment of ZW larvae was run at 32 °C (ZW³²) during the TSP while other ZW or ZZ larvae were maintained at 20 ± 2 °C as a control (ZW²⁰, ZZ²⁰). Heat shocks of 32 °C were applied to ZW larvae (ZW^{hs}) for 48 h at stages 55 and 55^{30d}. Hormonal treatment was run by rearing ZZ larvae at stages 55 and 55^{30d} for 48 h in fresh water containing estradiol benzoate (100 µg/l). Before dissection, animals were anesthetized in a solution of benzocaine (0.03%), and the brain, Gd, MI or GMI was dissected for further analysis. Due to their small size, Gd could not be isolated from the MI before stage 55^{15d}.

SF-1 cDNA isolation

Total RNA (1 µg) from adult testis was reverse transcribed with oligo(dT₁₅) primers and Superscript II mouse Moloney leukemia virus (MMLV) reverse transcriptase (Invitrogen). An aliquot of the reaction was amplified by PCR with the degenerated primers Sf1, Sf2 and Sf4 (Table 1). The amplification was performed in the presence of 0.1 unit Taq DNA polymerase (Invitrogen) in PCR buffer containing 25 mM of each dNTP and 3.5 mM MgCl₂. A 60 °C to 40 °C touchdown PCR program was run at 94 °C for 3 min followed by 20 cycles at 94 °C for 1 min, annealing for 1 min and 72 °C for 1 min. Amplification was then

Table 1 List of PCR primers used

Gene(*)	Name	Primer sequence
SF-1	Sf1	5'-TGAAAGGGITTYTTTAAARMGIAC-3'
SF-1	Sf2	5'-AARTTYGGICCIATGTAYAA-3'
SF-1	Sf4	5'-TCISWCCARCARTTYTG-3'
SF-1	Sf1-5R	5'-CTGGTCGGCCATCTTGACATGAGGCC-3'
SF-1	Sf1-3R	5'-CTGGAAGCGGTGCGGGCAGACAGG-3'
SF-1	Sf1-1	5'-GGGTACACCTACCCACACTT-3'
SF-1	Sf1-5	5'-TCTTGGCCTGTGACCAGGAG-3'
Aromatase	Arom-1	5'-ATTGCAGCACCTGACACGAT-3'
Aromatase	Arom-6	5'-TTGTTCTGTACATTCTCTAA-3'
GAPDH	fwd	5'-ACTACAAAGGACTAGGTCAGG-3'
GAPDH	rev	5'-CCGGTATTGCACTCAACGACC-3'

(*) Aromatase and GAPDH primers were used as a control in RT-PCR analysis of SF-1 expression.

pursued with 30 cycles at 94 °C for 1 min, 40 °C for 1 min and 72 °C for 1 min. Two independent PCR products of 657 and 800 bp were obtained by using two different sets of primers, Sf2/Sf4 and Sf1/Sf4 respectively (Table 1). After separation on agarose gel electrophoresis, the expected fragments were purified, inserted into the plasmid pCR2 (Invitrogen) and transfected in *E. coli* INV α strain (Invitrogen), and both were sequenced (Genome Express, Meylan, France).

The 5' and 3' rapid amplification of cDNA ends (RACE) system (SMART RACE cDNA amplification kit; Clontech) was used to amplify the 5'-end and 3'-end of SF-1 cDNA synthesized from 1 μ g total RNA isolated from adult testis according to the manufacturer's instructions. PCR was performed with 1 unit Taq DNA polymerase (Advantage 2 Polymerase Mix; Clontech) and specific primer for each amplification in the 5'-end and 3'-end regions (Sf1-5R and Sf1-3R respectively) (Table 1). The reaction mixture was heated at 94 °C for 3 min and amplified for the first five cycles as follows: 94 °C for 30 s and 72 °C for 3 min. The next five cycles were 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min. The remaining 30 cycles of PCR were carried out at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min, with a final extension at 72 °C for 10 min. Two fragments of 0.9 and 1.2 kb, isolated from the 5' and 3' regions respectively, were purified (QiaEX II Kit; Qiagen), inserted into the plasmid pGEM-T Easy (Promega), transfected into *E. coli* TG1 strain and sequenced (Genome Express).

Reverse transcription-polymerase chain reaction (RT-PCR) analyses

The detailed protocol for RT has been previously described (Kuntz *et al.* 2003b). Total RNA was extracted from dissected tissues with TRIzol reagent (Invitrogen) and quantified (BioPhotometer; Eppendorf, Le Pecq,

France). Total RNA (1 μ g) was reverse transcribed with random hexanucleotide primers and 100 units MMLV reverse transcriptase in a total volume of 25 μ l. A 2 μ l aliquot of resultant cDNA was PCR amplified with 0.1 unit of Taq DNA Polymerase (Invitrogen) in PCR buffer containing 25 mM of each dNTP, 2.5 mM MgCl₂ and 0.4 pM of each primer in a total volume of 25 μ l. RT-PCR analyses were performed on 'n' samples (n is given in figures and tables), each sample comprising a pool of four or five individuals. Specific primers for *P. walli* were Sf1-1 and Sf1-5, leading to a PCR product of 462 bp in length. The specific primers for the aromatase cDNA were Arom-1 and Arom-6, and the resulting PCR product was 510 bp in length. All the PCR products encompassed one exon/intron boundary based on other vertebrate gene alignments (not shown). To ensure that the RNA was not degraded and to prevent poor transcription quality, GAPDH cDNA was used as a control (see primer sequence in Table 1). The PCR conditions were 30-s denaturation at 94 °C, and 30-s annealing and 2-min elongation at 72 °C, and led to a 290 bp fragment. Annealing temperatures were 52 °C for GAPDH and aromatase primers, and 60 °C for SF-1 primers. We performed 35 cycles for qualitative analyses and 26 or 28 cycles for semiquantitative analyses in Gd and brain respectively. PCR products were then separated in a 1% agarose gel and transferred to nylon membrane (Hybond-N, Amersham) before hybridization with DIG-labeled probes and detection by chemoluminescence under previously described conditions (Kuntz *et al.* 2003b). Semiquantitative RT-PCR analysis was performed by running 28 or 30 PCR cycles for mRNA from Gd or MI respectively. PCR products were separated in agarose gel and compared with GAPDH level. In all cases, amplification was verified to take place in the linear phase of the PCR, and the intensity of the hybridization signal or agarose gel band was normalized to GAPDH by using the GelDoc 2000 (Bio-Rad) and a software package (Quantity One v.4.3.1; Bio-Rad).

Statistical analysis

The relative intensities of SF-1 and aromatase mRNAs were compared after normalization to GAPDH mRNA levels. The results are expressed as mean $\pm$ S.E.M. of several measurements performed on different pools for each group, as indicated in the text. The variation of relative SF-1 mRNA expression between sexes (ZZ²⁰ and ZW²⁰ groups) was analyzed by comparison of means with Student's *t*-test. The statistical significance of SF-1 and aromatase mRNA expression regarding to temperature (ZW³² or ZW^{hs} and ZW²⁰ groups) was estimated by comparing means of the relative SF-1 or aromatase mRNA expression from each group by analysis of variance (one-way ANOVA) followed by the Bonferroni multiple comparison procedure with SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

Results

SF-1 is conserved in *P. waltl*

Pleurodeles SF-1 cDNA sequence (accession no. AY540336, not shown) was obtained by RT-PCR from total RNA extracted from adult testis. Two independent clones of 657 and 800 bp were obtained by degenerated PCR, using different set of primers (Table 1). Then, 5' and 3' RACE was performed in order to isolate the full-length SF-1 mRNA containing a putative 1407 bp open reading frame. The deduced amino-acid sequence was compared with SF-1 sequences from several species: *Rana rugosa*, *Trachemys scripta*, chicken, mouse and man (Fig. 1). The putative encoded polypeptide is composed of 468 amino acids and is related to that of *R. rugosa* although slightly longer than that of *T. scripta* (466 residues), mouse (462 residues) and man (461 residues). Overall sequence identity with *T. scripta* was 81%, with *R. rugosa* 79%, and with mouse and man 63% (Fig. 1). The conserved regions were identified by comparison with the corresponding regions in other species: the first and second zinc fingers corresponding to the DNA binding domain (residues 13–78), the Ad4 BP/SF-1 box (residues 79–106), region II (residues 274–315), region III (residues 364–387) and the AF-2 domain (residues 458–463). The identity rate of each functional domain in SF-1 was also examined. In contrast to the second zinc finger, the first one is highly conserved between species, except for *P. waltl*, which exhibits a single 15-valine-to-alanine amino-acid substitution. Nevertheless, the Pbox ESCKG localized in the first zinc finger is conserved in our model. SF-1 box, region II and region III present also a high degree of identity among most species (75–100%) while the AF-2 motif, essential for transcriptional activation, is entirely conserved. A proline stretch of 7–8 consecutive amino acids is present in the

mammalian SF-1 but absent in chicken, turtle, frog and *Pleurodeles*.

SF-1 is expressed during early gonadal development

The SF-1 mRNA expression pattern in the urogenital system of *Pleurodeles* larvae during the TSP was examined. Qualitative RT-PCR analysis in GMI from ZZ²⁰, ZW²⁰ and ZW³² larvae selected at different stages of their development showed that SF-1 is expressed early in the three types of larvae: SF-1 mRNAs are detected since stage 42, and their expression is maintained thereafter in a sex-independent manner through the whole TSP that ends at stage 54 (Fig. 2). Thus, SF-1 expression occurs before gonadal differentiation, which can be assessed at stage 53 by histologic examination. Besides, temperature appeared to have no silencing effect on SF-1 during this period of development.

Sex- and tissue-specificity of SF-1 expression during gonadal differentiation

SF-1 mRNA expressions were then compared by semiquantitative RT-PCR analyses performed from total RNA isolated either from GMI at stages 54 and 55, or from isolated Gd and MI taken at various subsets of stage 55 during which metamorphosis proceeds, and at stage 56 at which animals are metamorphosed (Table 2). The results were compared with those obtained in brain, another important, steroid-producing organ.

At stage 54, no significant difference could be detected between GMI from ZW²⁰ and ZZ²⁰ larvae. Then, SF-1 expression in GMI was higher in ZW²⁰ individuals with a 2.14-fold higher mRNA level at the beginning of stage 55. After this stage, the Gd was big enough to allow their dissection and their study independently of MI. We observed the expression of SF-1 to be higher in the ovary at stages 55^{15d} (1.55-fold) and 55^{30d} (1.19-fold). This female-enriched expression was transient, since, at stage 56, there was no significant difference between ZW²⁰ and ZZ²⁰ animals. Surprisingly, a high SF-1 expression was observed in female MI at stage 55^{30d} (2.15-fold), whereas no significant difference was found at stages 55^{15d} and 56. In the brain of ZW²⁰ larvae, SF-1 mRNA level observed inside and outside TSP, that is, at stages 54 and 55^{15d} respectively, was not significantly different from those in ZZ²⁰ ones.

Tissue-specific inhibition of SF-1 expression after long-term heat treatment

In order to test whether SF-1 mRNA expression is regulated by temperature, the SF-1 mRNA level in ZW³² larvae was compared with those measured in ZW²⁰ ones in Gd, MI, GMI and brain as before.

<i>P. waltl</i>	MEYTYDEDDLDEL	CPACGDKVSGYHYGLLTCE	ESCKG	FFKRTVQNNKRYTCTENQT	CKIDKT	60					
<i>T. scripta</i>	MDYSYDEDPNQL	CPVCGDKVSGYHYGLLTCE	ESCKG	FFKRTVQNNKHYTCTESQN	SKIDKT	60					
<i>R. rugosa</i>	MDYSYDEDDLDEL	CPVCGDKVSGYHYGLLTCE	ESCKG	FFKRTVQNNKRYTCTENQS	CKIDKT	60					
chicken	MDYSYDEDDLDEL	CPVCGDKVSGYHYGLLTCE	ESCKG	FFKRTVQNNKHYTCTESQN	CKIDKT	60					
mouse	MDYSYDEDDLDEL	CPVCGDKVSGYHYGLLTCE	ESCKG	FFKRTVQNNKHYTCTESQS	CKIDKT	60					
human	MDYSYDEDDLDEL	CPVCGDKVSGYHYGLLTCE	ESCKG	FFKRTVQNNKHYTCTESQS	CKIDKT	60					
Zinc finger I				Zinc finger II							
<i>P. waltl</i>	QRKRCPCYCRFQKCLTVGM	RLEAVRADRMGRAGSNKF	GPMYKRDRAK	QKKKALIRANGFKL	120						
<i>T. scripta</i>	QRKRCPCYCRFQKCLTVGM	RLEAVRADRMGRGRNKF	GPMYKRDRAK	QKKKALIRANGFKL	120						
<i>R. rugosa</i>	QRKRCPCYCRFQKCLNVGM	RLEAVRADRMGRGRNKF	GPMYKRDRAK	QKKKALIRANGIKL	120						
chicken	QRKCCPCYCRFQKCLTVGM	RLEAVRADRMGRGRNKF	GPMYKRDRAK	QKKKALIRANGFKL	120						
mouse	QRKRCPCYCRFQKCLTVGM	RLEAVRADRMGRGRNKF	GPMYKRDRAK	QKKKAQIRANGFKL	120						
human	QRNRCPCYCRFQKCLTVGM	RLEAVRADRMGRGRNKF	GPMYKRDRAK	QKKKAQIRANGFKL	120						
Zinc finger II		Ad4BP/SF-1 box									
<i>P. waltl</i>	ETVPQIVSPTQTEYTI PSNIHSIHSVSKSLPLNTVAMTQVNYDRSPYGT PSLGMTMP-NH					179					
<i>T. scripta</i>	ETVPQIVSPVQTDYNLSSTIIGHISVSKSLPPNPATMT PVDYDRSPYGT PSLGMTVP-SH					179					
<i>R. rugosa</i>	ETVPQIVSQVQTDYSVANNIHTIHPVSKNLPNTAPMT PVEYDRGSYGGPPPIAMTLP-NH					179					
chicken	ETVPQIVSPVQNDYGLSSTIHSIHAMAKTLPNPAAALTPADYERGPYGT PSLAMTVP-GH					179					
mouse	ETGPPPMGVPPPPPPPD---YMLPPSLHAPEKALVSGPPSGPLGDFGAPSLPMAVPGPH					177					
human	ETGPPPMGVPPPPPPAPD---YVLPSSLHGPEPKGLAAGPPAGPLGDFGAPALPMAVPGAH					177					
<i>P. waltl</i>	GALQGYTYPHFPNRTIKSEFPDHYSSPHES-VAPAFVYSDAYQNSFPDPIDPETILNLLQL					238					
<i>T. scripta</i>	GALSSYHYPSFPNRTIKSEYPDHYTNSHES--VASMYMPDAYPNSAPPDIDPEVILKLLQL					237					
<i>R. rugosa</i>	APLSGYHYSSFSQRTIKSEYPDHYSNVHDPSTAGGYVYPEAYTSTSQPDIDPEVILKLLQL					239					
chicken	TPLAGYHYPSFPNRAIKSEYPDHYSAAHEA--VPTYAYPETYPSSSPDPIDPEVILKLLQL					237					
mouse	GPLAGYLYPAFSNRTIKSEYPEPYASPPQ---PGPPYSYPEPFSGGPNVPELILQLLQL					234					
human	GPLAGYLYPAFPGRAIKSEYPEPYASPPQ---PGLPYGYPEPFSGGPNVPELILQLLQL					233					
<i>P. waltl</i>	EPDEPQIKARYLACLQEQGKSRHEKPPSTFGLMC			KMADQTLFSIVEWARSCIYFKELEV			298				
<i>T. scripta</i>	EPDEPQVKVRILACLQEQGKGRHEKLS-TFGLMC			KMADQTLFSIVEWARSCIFFKELEV			296				
<i>R. rugosa</i>	EPDEPQIKARIISCLQEQNKSRHEKLS-MFGLMC			KMADQTLFSIVEWARSCIYFKELEV			298				
chicken	EPDEAQVKARILACLQEQGKGRHEKLS-TFGLMC			KMADQTLFSIVEWARSCIFFKELEV			296				
mouse	EPEEDQVRARIVGCLQEQEPAKSRSDQPAF--FSLLC			RMADQTFISIVDWARRCMVFKELEV			292				
human	EPDEDQVRARILGCLQEPTKSRPDQPA--FGLLC			RMADQTFISIVDWARRCMVFKELEV			291				
Region II											
<i>P. waltl</i>	GDQMILLQNCWSELLVF		DHIYRQIQHGKENSILLVTGQEI		VDSTIAAQAGPALNNLVLKS		358				
<i>T. scripta</i>	GDQMILLQNCWSELLVF		DHIYRQVQHGKEHSMLLV		TGQEVEMATIAAQAGSNLNNLVLRA		356				
<i>R. rugosa</i>	SDQMILLQNCWSELLVF		DHIYRQMHSKENSILLVTGQEI		LSAIAAQAGSTLNNLVLRA		358				
chicken	GDQMILLQNCWSELLVF		DHVYRQLQHGKEHSVLLVTGQ		EVDLSAVAAQAGSILHSLVLRA		356				
mouse	ADQMTLLQNCWSELLVL		DHIYRQVQYQKEDSILLVTGQ		EVELSTVAVQAGSLLHSLVLRA		352				
human	ADQMTLLQNCWSELLVF		DHIYRQVQHGKEGSILLVTGQ		EVELTTVATQAGSLLHSLVLRA		351				
<i>P. waltl</i>	QELVI	QLHSLQVDRQEFVCLKFLILFSVD	EKCLNNTLARN AQEKINAALHEYT				MCHYPH	418			
<i>T. scripta</i>	QELVL	HLHSLQVDRQEFVCLKFLILFSLD	VKYLENHSLAKDAQEKANAALLEYT				ICHYPH	416			
<i>R. rugosa</i>	QELVI	LLHSLQVDRQEFVCLKFLILFSLD	EKFLENHSLAKSAQEKVDSALMEYT				MCHYPH	418			
chicken	QELVL	HLHSPQVDRQEFVCLKFLILFSLD	VKYLENHALAKDAQEKANAALLEYT				VCHYPH	416			
mouse	QELVL	QLHALQLDRQEFVCLKFLILFSLD	VKFLNNHSLVKDAQEKANAALLDYTL				CHYPH	412			
human	QELVL	QLLALQLDRQEFVCLKFIILFSLD	LKFLNNHILVKDAQEKANAALLDYTL				CHYPH	411			
Region III											
<i>P. waltl</i>	CMDKFRLLLLRLTDIRSI			SMQAE		DYLYHKHMSGEVPCNN	LLIEML	HAKRS	468	100% identity	
<i>T. scripta</i>	AADKFRQLLLRLAEIRSL			SMQAE		EYLYHKHLSGEVPCNN	LLIEML	HAKRT	466	81% identity	
<i>R. rugosa</i>	CTDKYRLLLLRLAEIRSI			SMQAE		EYLYHKHLSGEVPCNN	LLIEML	HAKRA	468	79% identity	
chicken	CTDKFRQLLLRLTEV			RALSMQAE		EYLYHKHLSGEVPCNN	LLIEML	HAKRT	466	78% identity	
mouse	CGDKFQQLLLCLVE			RALSMQAE		EYLYHKHLSG	NEMPRNN	LLIEML	QAKQT	462	63% identity
human	CGDKFQQLLLCLVE			RALSMQAE		EYLYHKHLSG	NEMPRNN	LLIEML	QAKQT	461	63% identity

AF-2

Figure 1 Alignment of the amino-acid sequences of SF-1 cDNAs from several vertebrate species. Amino-acid identity with *Pleurodeles* sequence is shown in gray. The conserved regions, zinc finger I, zinc finger II, Ad4BP (Ad4 binding protein)/SF-1 box, region II, region III and AF-2, are framed. The mammalian proline stretch is in boldface.

Figure 2 Analysis of SF-1 mRNA expression in *Pleurodeles* gonad-mesonephros-interrenal complex (GMI). ZZ and ZW larvae raised at 20 °C (ZZ²⁰ and ZW²⁰) and ZW larvae exposed at a temperature of 32 °C (ZW³²) were sampled at various stages of TSP (42–54) and at stage 55. Total RNA was extracted from a pool of GMI dissected from five larvae, reverse transcribed and amplified by PCR for 35 cycles. GAPDH mRNA level is used as a control.

At stage 54, no difference in SF-1 mRNA level was detected in GMI between ZW³² and ZW²⁰ larvae, whereas at stage 55^{15d}, a reduced expression (1·6-fold) was observed (Table 3). In Gd of larvae at the same stage (55^{15d}), a higher difference was observed between heat-treated larvae and controls, heat treatment leading to a 3·7-fold lower expression of SF-1. The inhibitory effect of heat treatment on gonadal SF-1 expression was confirmed at stage 55^{30d} (2·2-fold lower SF-1 mRNA level). Interestingly, at this stage, a similar situation was observed in MI: there was a 3·4-fold lower expression of SF-1 in this organ in ZW³² larvae. Therefore, heat treatment performed from stage 42 to 54 affects SF-1 expression not only in Gd but also in MI. Besides, this

effect appears late, after the end of the TSP. In the brain, SF-1 transcription is not temperature-sensitive either during the TSP (stage 54) or after the end of TSP (stage 55^{15d}).

Gonadal SF-1 expression is not inhibited by 48-h heat shock

The inhibition of SF-1 expression described above is observed after sex-reversing heat treatment is completed, suggesting that temperature does not act directly on SF-1 expression. In order to get more information about the relationship between temperature and SF-1 expression, a 48-h 32 °C heat shock was applied to ZW larvae

Table 2 ZW²⁰/ZZ²⁰ SF-1 mRNA expression ratio in gonad and/or mesonephros-interrenal and brain

Stage of larval development ⁽²⁾	SF-1 expression in ZW ²⁰ /SF-1 expression in ZZ ²⁰ ⁽¹⁾			
	GMI ⁽³⁾	Gonad	Mesonephros-interrenal	Brain
Stage 54	0.78 ⁽⁴⁾ <i>P</i> =0.434 ⁽⁶⁾ <i>n</i> (ZW ²⁰)=5; <i>n</i> (ZZ ²⁰)=6	ND ⁽⁵⁾	ND	0.95 <i>P</i> =0.671 <i>n</i> (ZW ²⁰)=5; <i>n</i> (ZZ ²⁰)=6
Stage 55	2.14 <i>P</i> =0.001 <i>n</i> (ZW ²⁰)=6; <i>n</i> (ZZ ²⁰)=5	ND	ND	ND
Stage 55 ^{15d}	ND	1.55 <i>P</i> =0.021 <i>n</i> (ZW ²⁰)=4; <i>n</i> (ZZ ²⁰)=5	1.29 <i>P</i> =0.123 <i>n</i> (ZW ²⁰)=4; <i>n</i> (ZZ ²⁰)=5	0.91 <i>P</i> =0.515 <i>n</i> (ZW ²⁰)=4; <i>n</i> (ZZ ²⁰)=4
Stage 55 ^{30d}	ND	1.19 <i>P</i> =0.022 <i>n</i> (ZW ²⁰)=4; <i>n</i> (ZZ ²⁰)=4	2.15 <i>P</i> =0.003 <i>n</i> (ZW ²⁰)=4; <i>n</i> (ZZ ²⁰)=4	ND
Stage 56	ND	0.98 <i>P</i> =0.956 <i>n</i> (ZW ²⁰)=6; <i>n</i> (ZZ ²⁰)=6	1.03 <i>P</i> =0.890 <i>n</i> (ZW ²⁰)=4; <i>n</i> (ZZ ²⁰)=4	ND

(1) SF-1 mRNA/GAPDH mRNA expression ratio measured in ZW²⁰ larvae and divided by those measured in ZZ²⁰ larvae. (2) More details about developmental stages are in Materials and methods. (3) Gonad and mesonephros-interrenal attached (see Materials and methods). (4) Significant values (*P*≤0.05) are in bold face. (5) Not determined. (6) The mean of relative SF-1 mRNA level from each group, *n* (ZW²⁰) and *n* (ZZ²⁰), was compared (Student's *t*-test).

(ZW^{hs}); then, aromatase and SF-1 mRNA levels were measured and compared with those in ZW²⁰ larvae in GMI at stage 55 and in Gd or MI at stage 55^{30d} (Fig. 3). As previously described (Kuntz *et al.* 2003b), aromatase was absent from MI (not shown), and its expression in Gd was insensitive to heat shock when applied after the TSP (Fig. 3A). Conversely, SF-1 mRNA level (Fig. 3B) is slightly reduced under heat shock in GMI at early stage 55 (1.2-fold; *P*=0.355) and in MI at stage 55^{30d} (1.34-fold; *P*=0.127), but not in the isolated Gd at stage 55^{30d}, wherein it appears to be significantly upregulated (1.18-fold; *P*=0.026). This result suggests that *Pleurodeles*

SF-1 is not directly downregulated by temperature. Rather, raising temperature can increase SF-1 mRNA level, at least at stage 55^{30d}, by an unknown tissue-dependent mechanism.

Estrogen sensitivity of SF-1 expression after the end of the TSP

The female-enriched expression of SF-1 occurs after the female-specific increase in aromatase expression. Besides, a decreased SF-1 expression is observed after long-term heat treatment, which is known to

Table 3 ZW³²/ZW²⁰ SF-1 mRNA expression ratio in gonad and/or mesonephros-interrenal and brain

Stage of larval development ⁽²⁾	SF-1 expression in ZW ³² /SF-1 expression in ZW ²⁰ ⁽¹⁾			
	GMI ⁽³⁾	Gonad	Mesonephros-interrenal	Brain
Stage 54	1.33 ⁽⁴⁾ <i>P</i> =0.954 ⁽⁶⁾ <i>n</i> (ZW ³²)=4; <i>n</i> (ZW ²⁰)=5	ND ⁽⁵⁾	ND	0.95 <i>P</i> >0.999 <i>n</i> (ZW ³²)=4; <i>n</i> (ZW ²⁰)=4
Stage 55 ^{15d}	0.61 <i>P</i> =0.008 <i>n</i> (ZW ³²)=4; <i>n</i> (ZW ²⁰)=5	0.27 <i>P</i> =0.001 <i>n</i> (ZW ³²)=3; <i>n</i> (ZW ²⁰)=4	ND	1.08 <i>P</i> >0.999 <i>n</i> (ZW ³²)=4; <i>n</i> (ZW ²⁰)=4
Stage 55 ^{30d}	ND	0.45 <i>P</i> >0.001 <i>n</i> (ZW ³²)=4; <i>n</i> (ZW ²⁰)=4	0.29 <i>P</i> >0.001 <i>n</i> (ZW ³²)=4; <i>n</i> (ZW ²⁰)=4	ND

(1) SF-1 mRNA/GAPDH mRNA expression ratio measured in ZW³² larvae and divided by those measured in ZW²⁰ larvae. (2) More details about developmental stages are in Materials and methods. (3) Gonad and mesonephros-interrenal attached (see Materials and methods). (4) Significant values are in bold face. (5) Not determined. (6) The mean of relative SF-1 mRNA level from each group, *n* (ZW³²) and *n* (ZW²⁰), was compared (ANOVA followed by Bonferroni test).

Figure 3 Heat-shock analysis of SF-1 and aromatase mRNA level in gonad (Gd), mesonephros-interrenal (MI) and gonad-mesonephros-interrenal complex (GMI). Expression during early sexual differentiation (stages 55 and 55^{30d}) was measured in ZW²⁰ and compared with those in ZW larvae submitted to a 48-h 32 °C heat shock (ZW^{hs}). Total RNA from a pool of four or five larvae was reversed transcribed and amplified by PCR for 28 cycles. PCR products were agarose gel fractionated, and relative amounts of aromatase (A) and SF-1 (B) mRNAs were normalized to GAPDH mRNA level. Values are means of four or six replicates: the number of pools is indicated by 'n'. Vertical bars indicate the mean standard error. To calculate the *P* value, the mean of relative SF-1 mRNA level from each group, *n* (ZW^{hs}) and *n* (ZW²⁰), was compared (ANOVA followed by the Bonferroni test). **P* < 0.05 vs ZW²⁰.

downregulate aromatase expression in ZW larvae. These results strongly suggest a relationship between estrogens and SF-1 expression in *Pleurodeles*. To test this hypothesis, we performed 48-h estrogen treatments of ZZ larvae (ZZ^{E2}). The mRNA expression of both aromatase and SF-1 was compared with those in ZW²⁰ and ZZ²⁰ larvae in GMI at stage 55 and in Gd or MI at stage 55^{30d} (Fig. 4). We confirmed our previous studies showing the absence of aromatase expression in MI (Kuntz *et al.* 2003b). In Gd, we observed aromatase mRNA expression to be increased in ZZ^{E2} versus ZZ²⁰ larvae (2.1-fold; *P* = 0.003) when estradiol benzoate was applied at stage 55 (Fig. 4A). Such a sensitivity was not observed when the hormonal treatment was performed at stage 55^{30d}. As observed for aromatase, SF-1 mRNA expression is upregulated in GMI (2.4-fold; *P* = 0.002) when estradiol benzoate was applied at stage 55 (Fig. 4B). When the treatment was performed at stage 55^{30d}, such an increase was not observed in Gd,

while a significant difference was still observed in MI between ZZ²⁰ and ZZ^{E2} larvae (1.8-fold; *P* = 0.008). Moreover, in all cases shown in Fig. 4, the SF-1 mRNA level in ZZ^{E2} larvae was not significantly different from that in ZW²⁰ ones. Taken together, these results suggest that mesonephric and/or interrenal SF-1 expression can be increased by circulating estrogens after gonadal differentiation, that is, after the ovarian raise in the level of aromatase expression and activity.

Discussion

Little is known about SF-1 in amphibians, and its involvement in the regulation of steroid hormone synthesis has still to be demonstrated in *P. waltil*. Whether SF-1 is involved in the transcriptional regulation of aromatase in *P. waltil* is one of the questions we address here. This work was also performed in order to

Figure 4 Effect of estradiol benzoate on aromatase and SF-1 mRNA level in gonad (Gd), mesonephros-interrenal (MI) and gonad-mesonephros-interrenal complex (GMI). Total RNA was extracted from ZZ larvae reared in 100 µg/l estradiol benzoate for 48 h (ZZ^{E2}) at stages 55 and 55^{30d}. (A) As a control, aromatase mRNA expression, absent in MI, is shown in GMI and Gd. (B) RT-PCR analysis of SF-1 expression was examined in the conditions described in Fig. 3. Amounts of SF-1 and aromatase mRNAs were normalized to GAPDH mRNA level, and values were analyzed as described in Fig. 3 by comparing the mean of relative mRNA level of the two groups: $n(ZZ^{20})$ and $n(ZZ^{E2})$.

determine whether SF-1 could itself constitute an upstream target of the masculinizing temperature which leads to downregulation of aromatase expression (Kuntz *et al.* 2003b).

We first cloned from adult testis *Pleurodeles* SF-1 cDNA containing an open reading frame that could encode a protein of 468 amino acids. Sequence identity of the deduced protein compared with polypeptides found in other vertebrates showed a high homology, ranging from 81% in *T. scripta* to 63% in man and mouse. As expected, the regions that correspond to known functional domains in the nuclear receptor family

showed higher amino-acid identity than for the protein as a whole. These results suggest that biologic SF-1 function can be conserved in *Pleurodeles* species.

SF-1 was described as a transcription factor expressed in several tissues involved in reproduction and/or steroidogenesis and is currently detected in brain, testis, ovary, and interrenal or adrenal tissue in mouse, chicken, *T. scripta* and frog (Ingraham *et al.* 1994, Kawano *et al.* 1998, Fleming *et al.* 1999, Smith *et al.* 1999, Mayer *et al.* 2002). As in other species, *Pleurodeles* SF-1 transcripts were detected in all adult steroidogenic tissues (Kuntz *et al.* 2004a) as well as in spleen, as

observed in *R. rugosa* (Kawano *et al.* 1998), and, surprisingly, in lung, where aromatase activity is also present (Kuntz *et al.* 2004a). These results suggest a role for SF-1 in the steroid hormone synthesis pathway, as in chicken (Kudo *et al.* 1996), and *Xenopus* (Akatsuka *et al.* 2005) and teleost (Gardner *et al.* 2005) species, where SF-1 consensus binding sites are conserved in aromatase 5'-flanking region. However, *Pleurodeles* SF-1 *cis*-acting targets in the aromatase gene promoter remain to be discovered.

Then we examined *P. waltil* SF-1 expression during sexual differentiation. Qualitative expression analysis revealed the presence of an SF-1 transcriptional activity as early as stage 42, in ZZ²⁰ and ZW²⁰ or ZW³², and this sex- and temperature-independent signal was observed at all stages of the TSP. This result is consistent with those obtained in mouse, where SF-1 transcripts are present in the undifferentiated urogenital ridge in both sexes before the onset of *Sry* expression (Ikeda *et al.* 1993). Such a gonadal expression pattern, prior to sexual differentiation, is also observed in chicken (Smith *et al.* 1999), turtle (*T. scripta*) (Fleming *et al.* 1999), alligator (Western *et al.* 2000) and frog (*R. catesbeiana*) (Mayer *et al.* 2002). This early presence of SF-1 mRNA in *Pleurodeles* GMI is correlated with its role in the first differentiation steps of the two primary steroidogenic organs: the Gd and interrenal tissue, as demonstrated in other species (Luo *et al.* 1994). Furthermore, gonadal SF-1 expression occurs before expression of its steroidogenic target genes (Parker & Schimmer 2002). However, the question of whether SF-1 is expressed during the TSP in Gd, MI or both in *P. waltil* awaits an answer from *in situ* hybridization studies. Such histologic investigations would also determine whether SF-1 expression and activity are localized in aromatase-producing cells.

After the onset of gonadal sex differentiation, the SF-1 expression pattern becomes sexually dimorphic among species. In mouse embryos, SF-1 expression persists during testicular differentiation, but it declines during ovarian differentiation (Ikeda *et al.* 1994). A similar pattern was found in other mammals (Hatano *et al.* 1994, Pilon *et al.* 1998, Hanley *et al.* 2000) and in turtle (*T. scripta*) (Fleming *et al.* 1999), but not in chicken (Smith *et al.* 1999), alligator (Western *et al.* 2000), frog (*R. catesbeiana*) (Mayer *et al.* 2002) or *P. waltil*. In the last mentioned, our semiquantitative analysis showed a transient increase of the SF-1 transcript level in the ovary after the beginning of its differentiation. This sexually dimorphic expression was detected at stages 55^{15d} and 55^{30d}, which develop after the first evidence of histologic sex differentiation that takes place at stage 53. The female-enriched expression was higher at stage 55^{15d} than at stage 55^{30d}, whereas no significant difference was observed at stage 56. The ovarian specific increase in SF-1 expression could appear as early as stage 55, since a 2-14-fold higher expression was

detected in GMI at this stage. However, since at this stage as well as earlier ones, we could not separate Gd from MI, and since both tissues express SF-1, this hypothesis cannot be validated. Surprisingly, in MI, a similar transient increase in SF-1 expression in ZW²⁰ larvae was observed, but it appeared at stage 55^{30d}, that is, later than in Gd. The delay observed for the maximum SF-1 expression in MI suggests that the SF-1 gene may be more directly activated in Gd, where aromatase is expressed, than in MI, where aromatase is not transcribed. We note that expression of SF-1 in mesonephros or interrenal tissue is very poorly described in the literature; to our knowledge, there are no data about it during the period of sex differentiation. It appears that SF-1 gene transcription can be positively regulated by estrogen in *Pleurodeles*. Indeed, we observed that upregulation of SF-1 mRNA expression can be triggered by exogenous stimulation with estradiol benzoate. In Gd, the local production of these hormones can stimulate SF-1 expression earlier than in MI, which does not produce estrogen and is sensitive to the sole circulating hormones. In *T. scripta*, estrogen was also shown to modulate the regulation of SF-1 transcription, since treatment with estradiol applied at a male-producing temperature resulted in upregulation of gonadal SF-1 and female hatching (Fleming & Crews 2001). However, whether estrogens have a direct or indirect effect on SF-1 expression is not known. Therefore, in *P. waltil*, SF-1 expression could be correlated with the high level of steroidogenic activity necessary to support female sexual development, as in chicken embryos, where the developing ovary displays a higher steroidogenic activity than the testis (Woods & Erton 1978, Guichard *et al.* 1979), while the opposite is observed in mammals (Jost 1970). In *Pleurodeles*, the aromatase and SF-1 expression patterns appear to be closely related, both increasing, during ovarian differentiation, respectively at stages 54 (Kuntz *et al.* 2003b) and 55 (this work). However, SF-1 expression decrease at stage 56 cannot be explained by a reduction in aromatase activity, which is still high at this moment (Chardard & Dournon 1999). Instead, this decrease may be related to the end of the hormone-sensitive period, at which aromatase expression is no longer activated by estradiol.

Whether the SF-1 gene, as a possible regulator of aromatase, is a target of temperature in *Pleurodeles* temperature-dependent sex reversal is another question we address. Our results obtained under heat treatment differ from those obtained in *T. scripta*, in which SF-1 mRNAs increase at male-producing temperature but decline at female-producing temperature (Fleming *et al.* 1999), but are closer to those obtained in *R. rugosa*, in which SF-1 mRNA level remains unchanged in the female-to-male sex reversal, while that of P450 aromatase declines (Kato *et al.* 2004). Indeed, in our

model, we detected a significant decrease in SF-1 expression in Gd of ZW³² larvae at stages 55^{15d} and 55^{30d} with regard to that observed in ZW²⁰ ones. Such a significant decrease is also observed in MI at stage 55^{30d}. According to the results of estrogen treatments, this inhibition of the female-specific peak of SF-1 mRNA in both Gd and MI could be due to the previously described defect in aromatase expression and estrogen synthesis induced under the effect of heat treatment leading to sex reversal (Chardard *et al.* 1995). These results support the hypothesis of a regulation of SF-1 gene expression by estrogen level rather than by a direct effect of temperature. This is strengthened by the fact that, when larvae are submitted to a 48-h heat shock, the SF-1 mRNA response is quite different from that observed after long-term heat treatment, since a weak but not significant inhibition of SF-1 transcription can be observed in MI, while mRNA level is significantly increased in Gd. Since this test was run after TSP, this does not mean that SF-1 could be a target of the masculinizing temperature in the course of sex reversal.

Recently, the brain has been suggested to be involved in sex determination in a turtle exhibiting temperature sex determination (TSD), such as *Malaclemys terrapin*, in which temperature-dependent SF-1 mRNA level rises earlier in female than in male brain before gonadal differentiation (Jeyasuria & Place 1997). In *T. scripta*, the brain also seems to be the site of aromatase response to temperature, since differential aromatase activity was detected in brain during the TSP, whereas no differential activity was observed in Gd (Willingham *et al.* 2000, Crews *et al.* 2001). In *Pleurodeles*, our semiquantitative RT-PCR analysis did not show any differential expression of SF-1 in brain from ZZ²⁰, ZW²⁰ and ZW³² larvae taken at the beginning of sex-specific gonadal differentiation. This result is similar to that observed for aromatase expression (Kuntz *et al.* 2004b), suggesting that brain does not significantly interfere in the sexual determination or differentiation of the Gd in *P. waltil* and would not be considered to play a role in temperature-dependent sex reversal.

This work sheds a new light on the agents involved in *Pleurodeles* gonadal differentiation, although aromatase and estrogen seem to remain, at that time, the most important pieces of the puzzle. However, additional results will be necessary to understand the overall regulation of estrogen synthesis and the actual involvement of SF-1 in the complex path of ovarian differentiation and/or determination.

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