Transcriptional activation of zebrafish *cyp11a1* promoter is dependent on the nuclear receptor Ff1b

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Abstract

The cytochrome P450scc (cholesterol side-chain cleavage enzyme) encoded by *CYP11A1* catalyzes the first step in steroidogenesis by converting cholesterol to pregnenolone, and thus, controls the synthesis rate of steroid hormones. In mammals, steroidogenic factor 1 (SF1) has been implicated in the cAMP-mediated transcriptional activation of *CYP11A1* promoter. In zebrafish, Ff1b has been established as the homolog of SF1. To assess the dependency of *cyp11a1* expression on Ff1b, the putative promoter of zebrafish *cyp11a1*, spanning 1.7 kb, was isolated and bioinformatic analysis revealed two conserved FF1 response elements (FREs) that potentially bind Ff1b. Transfection studies in cell lines of different lineages confirmed that this promoter fragment contained the necessary regulatory elements required for its basal transcription. Truncation and mutagenesis studies performed in Y1 adrenocortical cells revealed that only the proximal FRE was essential for transcriptional activation. Electrophoretic mobility shift assay, however, indicated that Ff1b bound to both FREs, while their *in vivo* occupancy was confirmed using a chromatin immunoprecipitation assay. Lastly, the *cyp11a1* promoter was able to direct EGFP expression specifically to the interrenal gland and genital ridge when transiently expressed in microinjected zebrafish embryos, and the promoter activity is potentiated by *ff1b* overexpression as measured from luciferase reporter activity in zebrafish embryos.

Journal of Molecular Endocrinology (2009) **43**, 121–130

Introduction

The biosynthesis of active steroid hormones from cholesterol in the adrenal cortex and the gonads involves five distinct steroid hydroxylases that belong to the cytochrome P450 gene family and two hydroxysteroid dehydrogenases (Payne & Hales 2004). In teleost fish like zebrafish, cortisol, which acts as both glucocorticoid and mineralocorticoid, is synthesized in the adrenocortical homolog interrenal gland (Gallo & Civinini 2003, Bury & Sturm 2007). Although the active steroids in zebrafish are slightly different from those in mammals, the enzymes involved in steroidogenesis are identical. Among them, the cholesterol side-chain cleavage enzyme (P450scc, also known as CYP11A1) catalyzes the first and rate-limiting step in the steroidogenesis pathway by converting cholesterol to pregnenolone. Thus, the gene product of *CYP11A1* occupies a key regulatory point in the biosynthesis of steroids (reviewed in Chung *et al.* (1997) and Guo *et al.* (2003, 2007)).

Steroid secretion and production are stimulated by adrenocorticotropin (ACTH) in the adrenals and by gonadotropins in the gonads. Acting through cAMP, they stimulate acute steroidogenesis by increasing the availability of cholesterol for CYP11A1 in the inner

mitochondrial membrane (Jefcoate *et al.* 1987). cAMP also activates the transcription of steroid hydroxylases, leading to a sustained maintenance of steroidogenesis on a longer term basis (John *et al.* 1986). The transcriptional regulation of mammalian *CYP11A1*, including human (Morohashi *et al.* 1987), mouse (Rice *et al.* 1990), rat (Oonk *et al.* 1990), sheep (Pestell *et al.* 1993), and bovine (Ahlgren *et al.* 1999), has been intensively studied. Key *cis*-acting elements and their corresponding *trans*-acting factors, like steroidogenic factor 1 (SF1), AP-2, Sp1, TreP-132, and c-jun/AP-1, have been implicated in their transcriptional activation, particularly in relation to cAMP stimulation (Guo *et al.* 2007). The synthesis of steroid hormones is restricted to steroidogenic tissues and this is due to the developmentally important SF1, which is the central regulator responsible for tissue-specific expression of steroid hydroxylases, including *CYP11A1* (Bakke *et al.* 2001, Kelly *et al.* 2004, Li *et al.* 2004). This also demands extensive coordination among the hypothalamus, pituitary, and steroidogenic tissues, and SF1 again acts to fully integrate the transcriptional responses needed for steroidogenesis (Hammer & Ingraham 1999, Parker *et al.* 2002, Val *et al.* 2003, de Souza *et al.* 2006). Despite being well characterized in terms of gene and protein structure, expression profiles, post-translational

modifications, interacting proteins, and its indispensable role in the regulation of steroidogenesis and the development of steroidogenic organs (Bakke *et al.* 2001, Val *et al.* 2003), very little is known about the molecular mechanism by which SF1 regulates its downstream target genes.

Ff1b (Nr5a1a), the ortholog of SF1 in zebrafish, is similarly the earliest known molecular marker for interrenal development (Chai & Chan 2000). Ff1b also recognizes the same DNA response element as SF1 and LRH1 (Liu *et al.* 2003), both of which belong to the FF1 (NR5) subfamily of nuclear receptors. These DNA response elements will be termed as FF1 response elements (FREs) hereinafter. Knocking down *ff1b* by antisense morpholinos completely disrupted the formation of interrenal gland, as characterized by the down-regulation of *cyp11a1* transcripts and β -HSD enzymatic activity (Chai *et al.* 2003, Hsu *et al.* 2003), and led to impaired osmoregulatory functions (Chai *et al.* 2003). These are reminiscent of findings from SF1^{-/-} mice (Luo *et al.* 1994, Sadovsky *et al.* 1995). The similarity between the two organisms suggests a conserved regulatory program for adrenocortical development over 400 million years of evolution.

In view of the apparent conservation of the biosynthesis pathway for steroids between mammals and teleost (McGonnell & Fowkes 2006, Bury & Sturm 2007), it is reasonable to postulate that like mammalian SF1, Ff1b is primarily responsible for regulating the steroid hydroxylases in zebrafish. Here, we report the cloning and characterization of the 5' promoter region of zebrafish *cyp11a1*. The relative importance of the distal FRE (FREd) and proximal FRE (FREp) was addressed using deletion and site-directed mutagenesis analyses. The occupancy of the FREs by Ff1b was determined using electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP). Lastly, the ability of the *cyp11a1* promoter to drive tissue-specific expression was examined in zebrafish embryos by transient transgenesis.

Materials and methods

Cloning and sequence analysis

The putative promoter region for the zebrafish *cyp11a1* was isolated using a modified genome walking method (Siebert *et al.* 1995). Two microgram genomic DNA was separately digested with restriction enzymes that leave 3' overhangs, namely, BstXI, NsiI, PstI, SacI, and SphI. After phenol/chloroform extraction, each pool of digested DNA genomic fragments was subjected to homopolymeric dC-tailing in a 40 μ l reaction containing 0.2 mM dCTP, 10 mM Tris-Cl (pH 8.4), 25 mM KCl, 1.25 mM MgCl₂, and 38.4 U terminal

transferase for 1 h at 37 °C. The polymeric (dC) tail served as a priming site for the anchored adapter primer, ANC1 (5'-GAGGTCGACGGGIIIGGGIIGG-GIIGH-3'). Hemi-nested PCR was performed for each pool of genomic DNA fragments using the Expand Long Template PCR System (Roche) with ANC1 and gene-specific primers *cyp11a1r2*; 5'-CCATAATTCCG TGGACATTCCTG-3', followed by *cyp11a1r3*; 5'-TGAAGGCAAGGACACTGAGCAG-3'. From 25 μ l primary amplified products, 1 μ l was used as template for secondary PCR. Amplified fragments were cloned using pGEM-T vector and sequenced. A 1.7 kb DNA fragment was recovered from the amplification of the SphI genomic DNA fragments. Potential transcription factor binding sites (TFBS) were determined using MatInspector (Cartharius *et al.* 2005).

Reporter plasmids construction

The promoter fragment of zebrafish *cyp11a1* was cloned into pGL3-Basic luciferase reporter plasmid (Promega) and pEGFP-1 reporter plasmid (Clontech). It was also used as a template to generate the 1.5, 1, and 0.5 kb fragments using primers containing flanking KpnI and BglII restriction sites, and subsequently cloned into pGL3-Basic vector. The proximal and FREd were mutagenized separately, or simultaneously using the Quickchange site directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with the following primers, 5'-CCTTCCTTTAGCATGTTGACCTCGGT-GAAAGTAAATGCGGATC-3' for FREd and 5'-CCTTTAGTAGTTTCATTCACCGTGACCTGTGTGG-TGTTTTGGC-3' for FREp.

Cell culture

The mouse L β T2 gonadotrope and human HepG2 hepatoma cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1 mM sodium pyruvate. The mouse MA-10 Leydig tumor cell line was maintained in Waymouth's medium supplemented with 15% horse serum (HS) and 25 mM HEPES (pH 7.4), and 293T monkey kidney cells were maintained in RPMI medium supplemented with 10% FBS. The mouse Y1 adrenocortical and Chinese hamster ovary CHO-K1 cell lines were maintained in Ham's F12K medium supplemented with 10% HS and 2.5% FBS, and 10% FBS respectively. All cells were maintained at 37 °C in 5% CO₂ with penicillin (100 U/ml) and streptomycin (100 μ g/ml) added into the culture media. Cells were grown to 80–90% confluency prior to seeding for transfections or routine passages. All basal media were purchased from Invitrogen.

Transient transfection and dual-luciferase reporter assay

On the day before transfection, cells were plated in 24-well plates with a density of $0.8\text{--}1.5 \times 10^5$ cells per well. Transient transfections were carried out using FUGENE 6 transfection reagent (Roche) except for MA-10 cells, which were transfected using Lipofectamine 2000 (Invitrogen). Cells were transfected with $0.4\text{ }\mu\text{g}$ each luciferase reporter plasmid and 2 ng pRL-SV40 as a transfection efficiency control. After 24 h, cells were harvested and lysed in $1 \times$ passive lysis buffer provided in the dual Luciferase reporter assay system (Promega). Luciferase activity in $20\text{ }\mu\text{l}$ homogenate was assayed using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wilbad, Germany). The firefly luciferase activity conferred by the respective *cyp11a1-luc* constructs was normalized by the *Renilla* luciferase activity conferred by pRL-SV40. Data are presented as the mean $\pm$ S.E.M. of results from four independent transfections. The transfections were repeated at least once to ensure the reproducibility of the data.

Electrophoretic mobility shift assay

Ff1b proteins were produced by *in vitro* translation from pcDNA3.1ff1b (Liu *et al.* 2003) using the TNT T7 quick coupled transcription/translation system (Promega). To verify the identity of Ff1b protein, the translated proteins were biotinylated by adding Transcend tRNAs (Promega) to the coupled transcription–translation reaction and the presence of a 50 kDa band, representing the Ff1b protein, was confirmed by chemiluminescence. EMSA was performed using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). Oligonucleotides containing the FRED (5'-GTTGACCTGGGAAAAGTAAATG-3'), FREp (5'-GTAGTTCATTGTCCTT-GACCTGTG-3'), the corresponding mutants, FREDmut (5'-GTTGACCTCGGTGAAAGTA-AATG-3'), and FREpMut (5'-GTAGTTCATTACCGTGACCTGTG-3') were synthesized and labeled using the Biotin 3' end DNA labeling kit (Pierce). For each EMSA reaction, $8\text{ }\mu\text{l}$ *in vitro* translated mixture was mixed with 20 fmol biotin-labeled DNA probes and $1\text{ }\mu\text{g}$ Poly dI $\times$ dC in $1 \times$ binding buffer (10 mM Tris, 100 mM KCl, 1 mM dithiothreitol; pH 7.5) in a total reaction volume of $20\text{ }\mu\text{l}$ and incubated at 25°C for 20 min. Unbound DNA probes were resolved from protein–DNA complexes by electrophoresis on a 5% polyacrylamide gel. After electrophoresis, DNA oligonucleotides were transferred onto a ζ -probe membrane (Bio-Rad) by electroblotting and u.v. crosslinked. Biotin-labeled DNAs were visualized with streptavidin-bound HRP and Luminol/Enhancer

chemiluminescent substrate (Pierce) and chemiluminescence detected by exposure to X-ray film (Fujifilm, Tokyo, Japan).

Chromatin immunoprecipitation

ChIP assays were performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA). Ovarian cell suspension was prepared from freshly dissected ovary tissue of adult zebrafish in $1 \times$ PBS. Cells were fixed with 1% formaldehyde for 15 min, washed with chilled $1 \times$ PBS, and resuspended in $200\text{ }\mu\text{l}$ SDS lysis buffer. Following lysis for 10 min on ice, lysate was sonicated 14 times for 10 s separated by a 4-min interval, at 20% of maximum amplitude using the Vibra Cell sonicator VC 505 (Sonics and Materials Inc., Newton, CT, USA) equipped with a 3 mm tip. After sonication, one-tenth of supernatant was removed as input and the remaining was diluted 10-fold prior to immunoclearing by $75\text{ }\mu\text{l}$ salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4°C . Immunoprecipitation was performed overnight at 4°C with $5\text{ }\mu\text{g}$ anti-Ff1b antisera #1660 (Biogenes, Berlin, Germany). The region that the Ff1b antibody recognizes (CAYLHQEQSGRGKLE) is unique to Ff1b and the Ff1b antibody does not cross-react with other Ff1 isoforms in Western blotting (data not shown). The pre-immune serum (PIS) from the same rabbit was used as negative control. Immunoprecipitated agarose was sequentially washed for 4 min each in 1 ml low-salt, high-salt, and lithium chloride immune complex wash buffers, and finally washed twice with Tris/EDTA buffer. Histone complexes were finally eluted from the antisera by freshly prepared elution buffer (1% SDS, 0.1 M NaHCO_3). Cross-linking of histone-DNA (including the input samples) was reversed by 5 M NaCl at 65°C for 4 h. DNA fragments were extracted by phenol/chloroform extraction and ethanol precipitation with the addition of glycogen ($20\text{ }\mu\text{g}/\mu\text{l}$).

An aliquot of the extracted DNA ($1\text{ }\mu\text{l}$) was used for PCR analysis. A pre-denaturation of 5 min at 95°C was followed by 30 cycles (30 s at 95°C , 30 s at 56°C , and 1 min at 72°C), and a final elongation of 10 min at 72°C . To detect enrichment in the proximity of FRED, primers spanning -1669 and -1501 were used; FREDChIPF, 5'-CGGTGTGAAGCAATATGCAT-3', and FREDChIPR, 5'-CCAAGGTCAACATGCTAAAGG-3'. For FREp, primers spanning -249 and -1 were used; FREpChIPF, 5'-CGGATGTGTTTGATTCAAGG-3', and FREpChIPR, 5'-GGCACTCTCTCTCTACTTC-3'. As negative control, primers spanning from -471 to -255 region of the zebrafish *keratin8* promoter, where no consensus FRE is present, were included in the PCR analysis; *keratin8F*, 5'-GCGCCCCACCCAGATAAAA-3',

and keratin8R, 5'-ATGTTGTCACACCCAGCCTG-3'. PCR reactions were resolved on 1.5% agarose gel and visualized by SYBR Safe staining.

Microinjection of EGFP and luciferase reporter plasmids into zebrafish embryos

The *cyp11a1* promoter-EGFP plasmid (100 ng/μl) was diluted in 1× Danieau's buffer and 0.1% phenol red. Microinjection was carried out under a dissection microscope using a Nanoliter 2000 microinjector (WPI). One- to two-cell stage zebrafish embryos were microinjected with 2.3 nl plasmid solution. Embryos were monitored for GFP expression under u.v. microscope (Zeiss Axiovert 25) from 28 hours post fertilization (hpf) onwards. EGFP fluorescence was subsequently captured using confocal microscope (Olympus IX70) equipped with FV300 imaging software.

To quantify the *cyp11a1* promoter activity, zebrafish embryos were injected with 4.6 nl DNA solution containing 50 ng/μl of the respective *CYP11A1* promoter-luciferase constructs, 5 ng/μl internal control, pRL-CMV, and 100 ng/μl pcDNA3.1ff1b (Liu *et al.* 2003). Embryos were checked 4 h later and only those that were healthy and showed strong phenol red staining were retained for luciferase assay. Embryos were harvested and dechorionated at 14 hpf. Embryo extract was prepared from 25 embryos by pipetting up and down in 20 μl of 1× passive lysis buffer and luciferase activity was determined using 20 μl homogenate.

Results

Conserved *cis*-acting elements are identified in zebrafish *cyp11a1* promoter

A 1.7 kb 5'-flanking region of the zebrafish *cyp11a1* was recovered by genome walking and verified by DNA sequencing. The putative promoter sequence matched 158 905–160 637 of chromosome 25 (contig AL929050.6; Zebrafish Ensembl genome database, Zv7). Two TATA boxes were located in the proximity of the FREp but the transcription start site has yet to be determined experimentally. When the promoter sequence was analyzed for potential TFBSs using MatInspector, binding sites for Ff1b, AP-1, Sp1, and factors that bind cAMP response elements (CREs) were evident, and were conserved when compared with pufferfish *Tetraodon nigroviridis*, mouse, and human (Fig. 1). These factors have been previously implicated in the transcriptional regulation of *CYP11A1* promoter (Guo *et al.* 2007). Besides, several bindings sites for known co-regulators of SF1 including Sox proteins,

Figure 1 Conservation of *cis*-elements predicted in the 5' flanking promoter region of zebrafish *CYP11A1* in comparison with the equivalent 2 kb region in the gene promoter of tetraodon, human, and mouse. Binding sites of the transcription factors indicated were predicted using the web-based MatInspector program. Only binding sites for transcription factors that have previously been implicated in the transcriptional regulation of *CYP11A1* promoter were shown. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0029>.

GATA factors, and WT1 have also been identified in these promoters (data not shown). At least two CREs were mapped for all species. Importantly, the two identified FREs were highly conserved across zebrafish, human, and mouse.

The 1.7 kb *cyp11a1* promoter is transcriptionally active in different cell lines

We evaluated the ability of the putative *cyp11a1* promoter to activate luciferase activity in cell lines derived from steroidogenic and non-steroidogenic lineages. In Y1 adrenocortical cells, luciferase activity was 12.2-fold above the basal level conferred by pGL3-Basic (Fig. 2). In CHO-K1 ovarian, MA-10 testis, and LβT2 cells, luciferase activity was increased by 4.5-, 6.7- and 3.5-fold respectively. Intriguingly, luciferase activity was increased by 18-fold in the non-steroidogenic 293T embryonic kidney cells, but was, however, inactive in HepG2 liver carcinoma cells. These results indicate that the regulatory *cis*-elements within this *cyp11a1* promoter are sufficient to activate gene expression in steroidogenic cell lines. Subsequent transfection studies were performed in Y1 cells, as the adrenal cortex represents one of the major tissues where *cyp11a1* is endogenously expressed and, thus, should provide the best cellular niche for the investigation of the zebrafish *cyp11a1* promoter activity.

Figure 2 Promoter activity of the zebrafish *cyp11a1* promoter in cell lines from different lineages. The 1.7 kb zebrafish *CYP11A1* promoter was cloned upstream of the luciferase gene in pGL3-Basic vector, and transfected into Y1 (adrenocortical), MA-10 (Leydig), CHO-K1 (ovarian), LβT2 (gonadotrope), HepG2 (hepatoma), and 293T (kidney) cell lines. Renilla luciferase expression vector, pRL-SV40, was co-transfected for normalization of transfection efficiency. Data are expressed as normalized luciferase activity in relative light unit (RLU) measured from cells transfected with *cyp11a1-luc* or promoterless pGL3Basic vector (adjusted to 1.0) from one representative experiment, and represent the mean of luciferase activity $\pm$ S.E.M. ($n=4$).

FREp is the major mediator of *cyp11a1* promoter activity

Various 5' deleted fragments of the 1.7 kb promoter were generated by PCR and cloned into pGL3-Basic for the assessment of their activities. Luciferase assay revealed that the truncation of this putative *cyp11a1* promoter down to 1 kb did not affect luciferase activity significantly (Fig. 3). Even when FRED was truncated, as in both the 1.5 and 1 kb promoter fragments, luciferase activity was comparable with that of 1.7 kb fragment. Only when the promoter was truncated down to 500 bp, the luciferase activity was reduced to 44.4%. Nevertheless, the 500 bp promoter was still functional, probably due to the intact FREp.

To further explore their functions, we mutagenized the FRED and FREp individually or in combination, such that three nucleotides within the core or flanking recognition site were mutagenized discontinuously in reminiscent of the mutagenesis performed in human *CYP11A1* promoter (Hu *et al.* 2001, Hsu *et al.* 2004; Fig. 4). Transient transfections into Y1 cells showed that mutation in the FREp reduced luciferase activity by 89.6%, while the mutation in FRED only marginally reduced the luciferase activity by 11.6%. When both FREp and FRED were mutated it rendered the *cyp11a1* promoter completely inactive. The results demonstrate that the FREp and FRED do not have similar effects in gene activation. In agreement with the findings from human *CYP11A1* promoter, the FREp, which is situated in the basal promoter, was found to be the major

regulator for the promoter activity *in vitro*, whereas the function of the FRED in basal gene expression is less obvious (Hu *et al.* 2001, Hsu *et al.* 2004).

Ff1b binds to both FRED and FREp *in vitro*

To determine if Ff1b binds to the two FREs selectively, oligonucleotides containing the FRED and the FREp were synthesized, biotin-labeled, and EMSA was carried out. Ff1b proteins were transcribed and translated *in vitro* from the pcDNA3.1Ff1b overexpression plasmid. The identity of Ff1b, which corresponds to a ~ 50 kDa band in a SDS-PAGE, was verified by biotin-labeling and immunoblotting (data not shown). As shown in Fig. 4, no band shift was observed when the biotin-labeled oligonucleotides were incubated with reaction mixtures using the pcDNA3.1 as template (lanes 1 and 6). Mobility complexes were only observed when the reaction mixtures containing Ff1b proteins were used (lanes 2 and 7). Ff1b proteins, however, did not bind the mutated version of the biotin-labeled FRED and FREp (lanes 3 and 8). The Ff1b-FRE complexes were effectively competed by a 200-fold molar excess of the corresponding unlabeled FREs (lanes 4 and 9). The Ff1b-FRE complexes were, however, unaffected by a

Figure 3 The proximal FRE is crucial for the activation of the zebrafish *cyp11a1* promoter. (A) Transcriptional activity of various truncated *cyp11a1* promoter fragments was measured by luciferase assay in Y1 cells. The relative positions and nucleotide sequences of distal Ff1b response element (FRED) and proximal Ff1b response element (FREp) are indicated. (B) The two FREs were site-directed mutagenized either individually or simultaneously, with mutated residues indicated in lower case. Transcriptional activities of the mutagenized promoters were assessed in Y1 cells. Renilla luciferase expression vector, pRL-SV40, was co-transfected for normalization of transfection efficiency. Data are expressed as normalized luciferase activity in relative light unit from one representative experiment, and represent the mean of luciferase activity $\pm$ S.E.M. ($n=4$).

Figure 4 Ff1b binds to both the distal and proximal FRE (FRED and FREp) in electrophoretic mobility shift assay. Biotin-labeled wild-type or mutated oligonucleotides (30 bp) of FRED or FREp were incubated with rabbit reticulocyte in which pcDNA3.1 vector (lanes 1 and 6) or *ff1b* overexpression plasmid, pcDNA3.1ff1b, (lanes 2–5 and 7–10) was used as template for *in vitro* transcription and translation. For competition assay, ~200-fold molar excess of the respective unlabeled FRE or mutated FRE was used.

200-fold molar excess of the unlabeled mutated FREs (lanes 5 and 10). These observations demonstrated the specificity of Ff1b binding to both FREs.

Following the discovery that Ff1b binds to both the FRED and FREp, we used EMSA competitive binding assays to determine the relative binding affinity of Ff1b to both the FREs. The binding of Ff1b to biotin-labeled

Figure 5 Competitive binding of Ff1b to the FRED and FREp in electrophoretic mobility shift assay. (A) Biotin-labeled FRED or (B) FREp oligonucleotides (30 bp) were incubated with rabbit reticulocyte in which *ff1b* overexpression construct, pcDNA3.1ff1b, was used as template for *in vitro* transcription and translation. For competition, 10-, 50-, 100-, and 200-fold molar excess of the respective unlabeled FRE were used.

FRED (Fig. 5A) and to biotin-labeled FREp (Fig. 5B) was progressively reduced by increasing concentrations of unlabeled FREp and FRED at 10-, 50-, 100-, and 200-fold molar excesses. The difference in binding affinity of Ff1b to FRED and FREp, was however, only marginal (Fig. 5).

Ff1b binds to both FRED and FREp *in vivo*

ChIP assay was carried out to determine if Ff1b occupied the two FREs *in vivo*. Chromatin was immunoprecipitated by Ff1b antiserum from ovarian tissues, where Ff1b is abundantly expressed. In agreement with the EMSA results, Ff1b bound to both FRED and FREp *in vivo* as shown by the enrichment of the two promoter regions in PCR analyses following ChIP (Fig. 6). This enrichment did not take place when the PIS was used instead of Ff1b antiserum. Likewise, the enrichment was not seen on *keratin8* promoter where no consensus FRE was present. This is the first *in vivo* demonstration of the association of Ff1b to one of its target gene promoter.

The *cyp11a1* promoter targets EGFP specifically to the interrenal and genital ridge

To investigate if the 1.7 kb fragment was sufficient to direct the tissue-specific expression of *CYP11A1* in zebrafish, it was cloned into pEGFP-1 reporter vector. When the *cyp11a1*-EGFP plasmid was microinjected into zebrafish embryos at 1–2 cell stages, the 1.7 kb promoter was able to target EGFP expression specifically to the interrenal gland and genital ridge (Fig. 7A). Nearly 10% of the microinjected embryos showed EGFP fluorescence at these tissues from ~32 hpf onwards.

To quantify the promoter activity and to assess its responsiveness to *ff1b* overexpression in zebrafish, the *cyp11a1-luc* reporter plasmid was microinjected into

Figure 6 Ff1b binds to both distal and proximal FREs *in vivo* as revealed by ChIP. Chromatin was prepared from freshly dissected zebrafish ovary and immunoprecipitated with antiserum against Ff1b or pre-immune serum (PIS) from the same rabbit. Genomic DNA extracted was analyzed by PCR using primers covering the nucleotide positions; –1669 and –1501 for FRED, –249 and –1 for FREp, and from –471 to –255 for *keratin8* promoter, which does not contain any consensus FRE.

Figure 7 The 1.7 kb zebrafish *cyp11a1* promoter directs tissue-specific expression of EGFP in zebrafish embryos and over-expression of *Ff1b* enhances its promoter activity. (A) The zebrafish *cyp11a1*-pEGFP construct was microinjected into zebrafish embryos at 1- to 2-cell stages. EGFP fluorescence was captured by confocal microscope equipped with a 488 nm laser. (B) Embryos at the 1- to 2-cell stages were injected with the different combinations of reporter and *ff1b*-expression plasmids. pRL-CMV was included as a normalization control for firefly luciferase activity. Embryos ($n=25$) were harvested at 14 hpf for determination of luciferase activity. Data are expressed as normalized luciferase activity measured from one representative microinjection experiment $\pm$ S.E.M. ($n=4$). Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0029>.

zebrafish embryos and transactivation assay was carried out as previously described (Liu *et al.* 2003). As shown in Fig. 7B, the 1.7 kb zebrafish *cyp11a1* promoter was activated by 4.4-fold as compared with the promoterless pGL3-Basic vector. When the *ff1b* overexpression plasmid, *pcDNA3.1ff1b*, was co-injected, the luciferase activity driven by the *cyp11a1-luc* was further enhanced by 3.4-fold. When both the FREs were mutated, the promoter construct (FREpdMut) was completely inactive. Taken together these findings provide the first *in vivo* evidence that *Ff1b* potentiates the activation of the zebrafish *cyp11a1* promoter and that a 1.7 kb promoter fragment is sufficient to activate tissue-specific expression in interrenal gland and genital ridge.

Discussion

This study reports the cloning and functional characterization of a 1.7 kb promoter fragment of zebrafish *cyp11a1*. Bioinformatics analysis revealed conserved modules of *cis*-elements within the *cyp11a1* promoter in comparison with other species including tetraodon, human, and mouse. By transient transfection studies, we showed that the 1.7 kb promoter was capable of activating luciferase activity in a variety of steroidogenic cell lines and that the transcriptional activation was largely dependent on an intact FREp. Our EMSA and ChIP results confirmed the binding of *Ff1b* to both FREd and FREp. Most importantly, the 1.7 kb promoter could direct tissue-specific expression of EGFP in zebrafish embryos in a developmentally regulated fashion and the overexpression of *Ff1b* further potentiated the activity of the *cyp11a1* promoter *in vivo*.

From the study of the human *CYP11A1* promoter, many transcription factors, including AP-1, SF1, Sp1, TReP-132, AP-2, NF-1, and Ets, have been identified to be important for its transcriptional activation (Guo *et al.* 2007). In the zebrafish *cyp11a1* promoter, only binding sites for SF1, Sp1, and AP-1 have been mapped with confidence. Nevertheless, the presence of the two highly conserved FREs is consistent with the role of SF1 acting as a central regulator of the transcriptional activation of steroid hydroxylases. The identification of CRE and binding sites for transcription factors that have been implicated in SF1 transcriptional activity or in the activation of *CYP11A1* promoter, like Sox proteins (De Santa *et al.* 1998), GATA factors (Tremblay & Viger 1999), WT-1 (Shen *et al.* 1994), and AP-1 (Li *et al.* 1999, Huang *et al.* 2001), within the 1.7 kb promoter indicates a conserved mode of transcriptional regulation across different species. The importance of these transcription factors on the activation of the zebrafish *cyp11a1* promoter, however, needs to be further evaluated.

Despite the conservation of *cis*-acting elements within the 1.7 kb promoter region, the zebrafish and human *CYP11A1* promoters are not functionally equivalent. In Y1 cells, the 1.7 kb human promoter was transcriptionally more active ($\sim 70\%$ higher; data not shown) than the zebrafish promoter, but it was completely inactive when microinjected into zebrafish embryos as measured by luciferase and EGFP reporter activity (data not shown). This is intriguing considering that the same 1.7 kb human promoter could direct LacZ expression specifically to the adrenals and gonads in transgenic mice (Hu *et al.* 1999, 2001). In zebrafish, *cyp11a1* expression has also been detected in the brain and yolk syncytial layer (Hsu *et al.* 2002, 2006). Nevertheless, the 1.7 kb *cyp11a1* promoter does not direct EGFP expression to these tissues in our transient

transgenesis studies in zebrafish embryos. These expression domains are likely to be independent of Ff1b regulation and the *cis*-elements responsible are likely to lie outside the 1.7 kb promoter. Indeed, the expression of *Cyp11a1* for rat in the central and peripheral nervous system has been shown to be independent of SF1, as *Sf1* is not expressed in these tissues (Zhang *et al.* 1995, Hammer *et al.* 2004).

Not unexpectedly, the 1.7 kb zebrafish *cyp11a1* promoter is transcriptionally active in steroidogenic cell lines like Y1, MA-10, and CHO-K1, and in LβT2, which originated from pituitary gonadotropes. This is attributed to the presence of SF1, which replaces Ff1b in the activation of the zebrafish *cyp11a1* promoter. However, the high levels of *cyp11a1* promoter activity in 293T cells, which is of kidney origin, is surprising and indicates the presence of another *trans*-acting factor that is capable of assuming the functional role of SF1. This is not new for the transcriptional regulation of *CYP11A1* expression. In placenta tissue, which does not express SF1, AP-2 assumes the role of activating the transcription of *CYP11A1* (Ben Zimra *et al.* 2002). Intriguingly, the endogenous presence of LRH1 in HepG2 cells (Baiz *et al.* 2009), which is a paralogue of SF1 and binds the same FRE (Fayard *et al.* 2004), did not activate the zebrafish *cyp11a1* promoter. This observation, once again, has highlighted the potential roles of other transcription factors that interact with SF1 or Ff1b in regulating the tissue-specific activity of the *cyp11a1* promoter.

Why is it necessary to have two FREs in the promoter of *cyp11a1*? Previous studies on the human promoter have pointed to an explanation that while FREp is responsible for the basal activity, FREd is responsible for hormonal regulation and tissue selectivity (Guo *et al.* 2007). This difference in functionality of the two FREs is due to the different *cis*-acting elements associated with individual FREs. The same feature is seen in the zebrafish *cyp11a1* promoter, where FREd is surrounded by a CRE and binding sites for AP-1 and GATA factors, while FREp is surrounded by TATA boxes and binding sites for transcription factors such as Sox and Wt-1. Transfection studies involving DNA constructs carrying different combinations of the mutated FREs have ascertained the pivotal role of the FREp in the basal activity of *cyp11a1* promoter. Thus, at the proximal promoter, Ff1b bound to FREp regulates the basal promoter activity by interacting with transcription factors in the Pol II initiation complex such as TFIIB, CBP/p300, and AP-1 (Monte *et al.* 1998, Li *et al.* 1999). Although the decrease in promoter activity is marginal following the truncation and mutation of FREd, the results are indicative of its possible role in basal transcription of the *cyp11a1* promoter.

Despite the stronger influence of the FREp mutation on basal promoter activity, EMSA and ChIP assays showed that Ff1b binds to both the distal and FREp *in vitro* and *in vivo*. Differential binding of SF1 to its two binding sites has been demonstrated in the human and rat *CYP11A1* promoters (Clemens *et al.* 1994, Hu *et al.* 2001). This difference in the functionality of the two FREs has added one more layer of complexity in Ff1b transcriptional activity. It will be interesting to dissect the molecular events controlling Ff1b binding to the two FREs in terms of hormonal regulation and to check whether this is related to the dosage-dependent effect. Although our current data suggests Ff1b as the predominant regulator of *cyp11a1* promoter, they do not preclude the possible involvement of other Ff1 isoforms that can potentially bind the FREs, particularly if they are co-expressed in the same tissues.

The ability of the zebrafish *cyp11a1* promoter to specifically target EGFP expression to the interrenal gland and genital ridge is reminiscent of the human *CYP11A1*, where a 1.7 kb promoter is sufficient to direct LacZ expression to adrenals and gonads in transgenic mice (Hu *et al.* 2001). The low percentage of embryos (~10%) expressing EGFP at the two tissues, however, might be indicative that the 1.7 kb promoter is relatively weak, and additional *cis*-elements outside of this fragment might be important to fully recapitulate the endogenous expression level. The characterization of additional 5' flanking sequence would certainly be important. More importantly, by combining microinjection and transient transactivation assays in zebrafish embryos, we provided the first *in vivo* evidence on the responsiveness of the 1.7 kb *cyp11a1* promoter to Ff1b, indicating that this is a viable approach to study the activation of other promoters that are dependent on Ff1b.

In conclusion, a 1.7 kb promoter region of zebrafish *cyp11a1* has been characterized in terms of its basal transcriptional activity and its regulation by Ff1b. The direct influence of Ff1b on the activity of zebrafish *cyp11a1* promoter highlighted how the transcriptional regulation could be conserved even after 400 million years of evolution (Kumar & Hedges 1998). Our findings also ascertained the orthology of Ff1b to its mammalian counterpart SF1, although it remains to be seen if *ff1d*, which is also an ortholog of SF1 (von Hofsten *et al.* 2005, Kuo *et al.* 2005), also retains the ability to regulate the expression of *cyp11a1* and other Ff1b responsive genes.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National University of Singapore and a BMRC grant (BMRC07/1/21/19/527) from A*STAR. S I was supported by a NUS Postgraduate Research Scholarship.

Acknowledgements

We thank Prof. Chung Bon-chu (Institute of Molecular Biology, Academia Sinica, Taiwan) for her generosity in sharing her unpublished data and the human *CYP11A1* promoter constructs with us. We also like to thank Professors Martin Lee (Department of Physiology, NUS) and Pamela Mellon (UCSD) for their kindness in sharing the MA-10 and LβT2 cell lines with us respectively.

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Received in final form 26 April 2009

Accepted 26 May 2009

Made available online as an Accepted Preprint 28 May 2009