Two differentially active alternative promoters control the expression of the zebrafish orphan nuclear receptor gene *Rev-erb α*

Tomoko Kakizawa, Shin-ichi Nishio, Gerard Triqueneaux, Stephanie Bertrand, Juliette Rambaud and Vincent Laudet

Laboratoire de Biologie Moléculaire de la Cellule, CNRS UMR 5161, INRA LA 1237, IFR 128 BioSciences Lyon-Gerland, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon cedex, France

(Requests for offprints should be addressed to V Laudet; Email: vincent.laudet@ens-lyon.fr)

T Kakizawa and S-i Nishio contributed equally to this work

Abstract

The orphan nuclear receptor Rev-erb α (NR1D1) plays an important role in the regulation of the circadian pacemaker and its expression has been shown to be regulated with a robust circadian rhythm in zebrafish and mammals. In addition, in zebrafish its expression has been shown to be developmentally regulated. In order to analyze the mechanisms of the *zfRev-erb α* gene regulation, we have isolated its 5'-upstream region. We found that two promoters control the *zfRev-erb α* expression. The first one (ZfP1) is characterized by a very high degree of sequence identity with the mammalian P1 promoter and contains, as the mammalian P1, a functional Rev-erb α -binding site (RevDR2). Inhibition of *zfRev-erb α* activity in zebrafish embryos using antisense-morpholino knockdown results in an increase of *zfRev-erb α* gene expression suggesting that *zfRev-erb α* is repressing its own transcription *in vivo*. In addition, we show that ROR orphan receptors also regulate *in vitro* and *in vivo* *zfRev-erb α* gene expression through the same RevDR2 element. In contrast, the second promoter ZfP2 is strikingly different from the mammalian P2: its sequence is not conserved between zebrafish and mammals and is not regulated by the same transcription factors. Together, these data suggest that ZfP1 is orthologous to the mammalian P1 promoter, whereas zebrafish ZfP2 has no mammalian ortholog and does not function like ZfP1 to control Rev-erb α expression.

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Introduction

Rev-erb α belongs to the nuclear receptor (NR) superfamily, which includes receptors for steroids, thyroid hormones, retinoic acid, and vitamin D, as well as orphan receptors. In mammals, Rev-erb α is encoded on the opposite strand of the thyroid hormone receptor α gene (Lazar *et al.* 1989, Miyajima *et al.* 1989). Rev-erbs form a group of orphan NRs, with three different genes: *Rev-erb α* (NR1D1; NRs Nomenclature Committee, 1999; also called ear-1), *Rev-erb β* (NR1D2 also called RVR, ear-1b, BD73, or HZF-2; Bonnelye *et al.* 1994, Dumas *et al.* 1994, Pena-de-Ortiz & Jamieson 1997), and *Rev-erb γ* that has been lost specifically in mammals (Bertrand *et al.* 2004; SB). To date, no ligand has been identified for the Rev-erbs. Interestingly, their *Drosophila* homolog encoded by the *E75* gene contains a heme molecule that can interact with nitric oxide and carbon monoxide suggesting that *E75* could be a gas sensor (Reinking *et al.* 2005). It is not yet known if this feature is also conserved in mammalian Rev-erbs. The ligand-binding domain of Rev-erbs lacks the C-terminal

AF2-AD domain, which plays a role in activation, an observation in line with the notion that Rev-erbs act as transcriptional repressors. Rev-erb α has been shown to bind DNA as a monomer on a specific sequence called Rev-erb-response element (RevRE), which contains an AGGTCA motif (Harding & Lazar (1993)) and also as a homodimer to RevDR2 elements composed of one classic RevRE followed by an AGGTCA motif separated by two nucleotides, most often CT (Harding & Lazar 1995, Adelmant *et al.* 1996). In phylogenetic trees, Rev-erbs are related to the retinoic acid-related orphan receptor (ROR), consistent with the fact that these receptors share the same response element (Becker-Andre *et al.* 1993, Giguere *et al.* 1994, Laudet 1997). It has been reported that there is a crosstalk between Rev-erbs and RORs and that they regulate gene expression with opposed activities through RevRE elements (Forman *et al.* 1994, Retnakaran *et al.* 1994). Recently, this regulatory antagonism was also observed in the circadian pathway and it has been shown that Bmal1 is up- or down-regulated by ROR and Rev-erb respectively (Preitner *et al.* 2002, Triqueneaux *et al.* 2004, Akashi &

Takumi 2005, Guillaumond *et al.* 2005). Indeed, the promoters of human and rat *Rev-erb α* were isolated and it has been established that they are regulated by *Rev-erb α* and ROR with opposite activity through a RevDR2 element located close to the transcriptional start site (Adelmant *et al.* 1996, Raspe *et al.* 2002).

The biological function played by *Rev-erb α* has remained unclear, until it was observed that *Rev-erb α* expression is strongly circadian in most of the tissues in mammals and zebrafish (Balsalobre *et al.* 1998, Delaunay *et al.* 2000, Torra *et al.* 2000). Indeed, genetic and functional evidence suggests that *Rev-erb α* is a major player in the control of circadian clocks (reviewed in Emery & Reppert 2004). In mammals, *Rev-erb α* directly regulates the major clock gene *Bmal1*, which acts on the positive limb of the pacemaker driving circadian clocks (Preitner *et al.* 2002). In addition, we and others have previously reported that the promoter of human *Rev-erb α* is activated by CLOCK-BMAL1 heterodimer and repressed by PER and CRY proteins (Preitner *et al.* 2002, Triqueneaux *et al.* 2004), suggesting that *Rev-erb α* expression is directly under the control of the circadian clock. All these data suggest that *Rev-erb α* is a primary determinant of the feedback loop that regulates *Bmal1* transcription (Preitner *et al.* 2002, Emery & Reppert 2004, Yin & Lazar 2005).

In addition, it has been reported that the expression of *Rev-erb α* increases during adipogenesis and decreases during myogenesis. Therefore, a role of *Rev-erb α* has been proposed in myogenic differentiation or adipocyte differentiation (Chawla and Lazar 1993, Downes *et al.* 1995, Laitinen *et al.* 2005). In line with these findings, it has been shown that *Rev-erb α* controls the expression of numerous genes important for lipid homeostasis, such as *ApoA1* or *ApoCIII* (Vu-Dac *et al.* 1998, Gervois *et al.* 1999, Coste and Rodriguez 2002, Laitinen *et al.* 2005). Other evidence suggests that *Rev-erb α* is tightly regulated; in humans, it has been shown that the activity of its promoter is down-regulated by *Rev-erb α* itself through the RevDR2 element (Adelmant *et al.* 1996), although the *in vivo* significance of this regulation has never been rigorously tested. It has also been shown that in mammals, *Rev-erb α* is regulated by *PPAR α* through the RevDR2 site (Gervois *et al.* 1999, Canaple *et al.* 2006). During zebrafish embryogenesis, *Rev-erb α* expression has been observed to be both circadian and developmentally regulated. The expression was shown to start at 24-hours post-fertilization (hpf) specifically in the pineal gland, then the day after (48 hpf) in the pineal gland and retina and then, after 1 more day (72 hpf) in the pineal gland, retina, and optic tectum (Delaunay *et al.* 2000).

Given this striking expression pattern, we thought that the zebrafish would be useful to decipher the mechanisms by which this expression is tightly controlled. The zebrafish is an excellent model system for an *in vivo* promoter analysis, because of its transparency

during embryonic development, and since it is possible to interfere with gene expression using morpholino (MO) injections (Nasevicius & Ekker 2000). In this study, we isolate the 5'-region of the zebrafish *Rev-erb α* gene and we found that in zebrafish, as in mammals, *Rev-erb α* expression is controlled by two promoters, ZfP1 and ZfP2. These promoters drive expression of two *Rev-erb α* isoforms with similar repressive properties. We show that ZfP1 is conserved and functionally similar to the mammalian P1 promoter, whereas ZfP2 is divergent both in its genomic organization and function.

Materials and methods

Plasmid constructions

Full-length cDNA of zf*Rev-erb α* (zf*Rev-erb α* 1) and a version encoding a short isoform (zf*Rev-erb α* 2) were isolated by using reverse-transcription coupled to PCR (RT-PCR) from adult zebrafish total RNA. These cDNAs were subsequently subcloned into pCDNA3 and pCSII+ vectors (Invitrogen). The primers used for PCR and cloning are indicated below. The position of each primer is indicated according to the numbering system of the sequence of the promoter region depicted in Fig. 1. zf*Rev-erb α* 1 5' primer: 5'-CATAATGACTT-TACTGGGGCTC-3' (positions +257 to +274). zf*Rev-erb α* 2 5' primer: 5'-TCCATGTACAGTGAGAATTC-3' (positions +3717 to +3736). zf*Rev-erb α* common 3' primer: 5'-TCAGGCATCAATGCGGAAAGACAG-3' (reverse) positions 1979–2002 of the zf*Rev-erb α* cDNA (Genbank accession number: AY391444).

To study the genomic 5'-region, a 14 kb genomic DNA fragment containing the entire zf*Rev-erb α* coding region was isolated from a genomic DNA library in λ FIX II (a generous gift of Christine and Bernard Thisse). A 6.5 kb fragment containing the 5'-regulatory region was subcloned, sequenced (Fig. 1), and further characterized (see Fig. 2A). It contains a 3079 bp region upstream of exon 1, an intron 1 of 3350 bp, and a part of exon 2 (see Fig. 1 and Supplementary Fig. 2B). This sequence has been deposited in Genbank with the following accession number: AY336123. This large fragment, called zf*Rev-erb α* full, was obtained by LA PCR (TaKaRa Shuzo, Kyoto, Japan) using the following pair of primers: Full 5' primer: 5'-CCGCTCGAGCCTGCTGGCTGGATTATGGTACAC-CACTGCGCT-3' (sense; positions –2858 to –2827). Full 3' primer: 5'-TCGGGATCCGATCCAATGTATGATATCACCCACCTGGTGGT-3' (reverse; positions +3650 to +3683). The amplified product was subcloned into XL-TOPO vector (Invitrogen).

The constructs containing the zf*Rev-erb α* first (ZfP1) or second (ZfP2) promoters were obtained by PCR amplification using zf*Rev-erb α* full as a template. The

Figure 1 Sequence of the zfRev-erbα 5' regulatory region. The two promoters ZfP1 and ZfP2 are indicated together with the transcriptional start sites as determined by RNAase protection (stars). The ATG in exons 1 and 2 are boxed. The main response elements discussed in the text are also indicated by different colors. The region which is highly conserved between zebrafish P1 and mammalian P1 is in italic.

Figure 2 Intron–exon structure of the 5′-UTR of the *zfRev-erb α* gene. (A) Comparison of the zebrafish, human *Rev-erb α* promoter structures. The yellow and red circles indicate the different types of RevREs. The green rectangles indicate the putative E-boxes. Black lines under P1 promoters indicate the highly conserved region. (B) Schematic of the *zfRev-erb α* gene and generation of the two N-terminally distinct isoforms, *zfREV-ERB α 1* and *zfREV-ERB α 2*, from two different promoters, ZfP1 and ZfP2. Numbers under the genomic scheme indicate the position of each exon. Transcription start sites are indicated by arrows. Untranslated sequences are shown as dotted squares and translated ones as solid line squares. The amino acid number of each isoform is indicated. The accession number of ENSEMBL is ENSDARG0000033160.

amplified products (3215 and 3389 bp respectively) were subcloned into pCR2.1-TOPO and sequenced to check the absence of artifactual mutations. From these clones, a *XhoI*–*Bam*HI fragment containing the *Rev-erb α* promoter was excised and subcloned into pGL2basic and pd2EGFP.

The primers used were as follows. In each case, the position of the primer in the sequence shown in Fig. 1 as indicated: ZfP1 5′ primer: 5′-GAGAGCTCGCGGCCG-GAGCTC-3′ (on λ FIX II). ZfP1 3′ primer: 5′-TCGTCGCACCAAATACGTGCGC-3′ (positions +121 to +136). ZfP2 5′ primer: 5′-CCGCTCGAGGA-CACGAACAACACAGGTAAGACGCTTT TAGAA-3′ (positions +293 to +325). ZfP2 3′ primer: 5′-TCGGGATCCGATCCAATGTATGATATCACCC-CACCTGG TGGT-3′ (positions +3650 to +3682).

Mutations of RevDR2 were produced by PCR using the following oligonucleotides. pRevDR2WT: 5′ primer: 5′-GAGCTCCTCTTTGACTTCGACTAC-3′ (positions –231 to –214). 3′ primer: 5′-GGATCCCGCACCAAATACG TCGCG-3′ (positions +121 to +136). pRevDR2M5: 5′ primer: 5′-GTTCTGGAGAAAGTCCTAGCCTGGGC-CACGAGTC-3′ (positions –154 to –121). 3′ primer: 5′-GACTCGTGGCCCAGGCTAGGACTTTCTC-CAGAAC-3′ (positions –154 to –121). pRevDR2M3: 5′ primer: 5′-GGAGAAAGTGTGTACACCCCGGTC-GAGTCCGGT CAC-3′ (positions –149 to –114). 3′ primer: 5′-GTGACCCGACTCGACCCGGGTGTGACA-CACCTTCTCC-3′ (positions –149 to –114). pRevDR2-M53: 5′ primer: 5′-GAGAAAGTCCTAGC-CACCCGGTCGAGTCGGG TCACATG-3′ (positions

–148 to –111). 3' primer: 5'-CATGTGACCCGACTC-GACCGGGTGGCTAGGACTTTCTC-3' (positions –142 to –111). The integrity of each construct was verified by sequencing of both strands.

Cell culture, transient transfection, and reporter assays

COS1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.25 μ g/ml streptomycin at 37 °C in 7% CO₂. Transfection was carried out in COS1 cells using ExGen500 (Euromedex, France). Briefly, cells were plated in 24-well plates, 12 h prior to transfection. Luciferase reporter (100 ng) was co-transfected with the indicated expression vectors together with an expression vector encoding the β -galactosidase gene for normalization (pCMV-SPORT- β -gal vector; Life Technologies; Triqueneaux *et al.* 2004). After a 12-h incubation, the medium on the cells was replaced by fresh medium. Cells were harvested after 24–48 h for reporter assays. Luciferase activity was determined and is shown as relative light units normalized to the amount of β -gal activity. Each transfection was conducted in triplicate and data represent the mean $\pm$ s.d. of at least three independent experiments.

Electrophoretic mobility shift assays (EMSA)

Synthetic oligonucleotides representing each strand of sequences were radiolabeled with 32-P- γ -ATP using polynucleotide kinase and purified on a polyacrylamide gel. Proteins were expressed using TNT reticulocyte lysate kit (Promega). Radiolabeled probes (10 fmol, 20 000–30 000 cpm) were then incubated with binding proteins in 15 μ l reaction mixture containing 10 mM KPO₄ buffer (pH 8.0), 1 mM EDTA, 80 mM KCl, 1 μ g poly(dI-dC), 1 mM dithiothreitol, 0.5 mM MgCl₂, 5 mg BSA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 1 mM leupeptin, and 1 mM pepstatin. These reactions were incubated for 30 min at room temperature and analyzed on a 5% polyacrylamide gel in tris-borate-EDTA (TBE) buffer. Electrophoresis was performed at a constant voltage of 200 V at 4 °C in the same buffer. The oligonucleotides for EMSA were as follows: RevRE wildtype: 5'-TCTGGA-GAAAGTGTGTCAGTGGGCCACGAGTCGGGTCACATGGACACAT-3'. RevRE mutant: 5'-TCTGGA-GAAAGTCCTAGCCACCCGGTCGAGTCGGGTCA-CATGGACACAT-3'.

Zebrafish

Zebrafish (*Danio rerio*) were kept at 28 °C in a 14 h light:10 h darkness cycle (LD 14:10) with the light on at

0900 h (ZT0) and the light off at 2300 h (ZT14). Adults were crossed overnight, resulting in spawning, and fertilization around ZT0 the next morning. Embryos were collected after spawning for MO injections and then raised at 28 °C in Petri dishes. To prevent pigmentation, 0.2 mM 1-phenyl-2-thiourea (PTU; Sigma) was added to the water at 12 hpf.

Whole mount *in situ* hybridization

Embryos at 48 hpf stage were fixed in 4% paraformaldehyde in PBS overnight at 4 °C and then stored in methanol. Whole mount *in situ* hybridization was performed as described by *Thisse et al.* (2004).

The *zfRev-erb α* exon 1 and 3'-end probes for *in situ* hybridization were PCR amplified using the following primers and subsequently subcloned into pCSII+ vector. Exon 1 probe: 5' primer: 5'-TGAATGAACCATATTTCACT-3' (positions 61–80 of the *zfRev-erb α* cDNA, see Fig. 1). 3' primer: 5'-TGTGTTGTTTCGTGTCTACTG-3' (positions 288–307). 3'-end common probe: 5' primer: 5'-TCCCAACCGTACCAGCCCTG-3' (positions 346–365). 3' primer: 5'-TTGTTGGGTGAATTGCGTGC-3' (positions 575–594).

All probes for *zfRev-erb β* and γ and the five *zfROR* genes are available to VL under request.

MOs injections

MOs were designed with sequences complementary to the cDNA encoding *zfRev-erb α 1* (MO1) and *zfRev-erb α 2* (MO2) around the transcriptional start codon based on the recommendations of the manufacturer (GeneTools). The MO sequences were as follows: MO1: 5'-TGCAGGGTTTTGACAGAATTTCCAA-3' positions 234–257; see red boxes in Supplementary Fig. 2B. MO2: 5'-TGGACACAGGGCTGGTACGGTTGGG-3' positions 347–365.

For the control, we used the standard control MO from GeneTools: 5'-CCTCTTACCTCAGTTACAATTAT-3'. We injected the zebrafish wild-type embryos as described (Nasevicius & Ekker 2000) at one- or two-cell stage with 0.5–2 pmol MOs diluted in sterile water (see Supplementary Materials and methods section).

In vivo expression assays

For microinjection of promoter constructs into zebrafish embryos, plasmid DNA was purified using a plasmid isolation kit (Qiagen). The DNA was diluted to a final concentration of 50 ng/ μ l in injection solution (0.1 M KCl and 0.05% phenol red). Circular plasmid was injected into one-cell stage embryos with or without pSG5-r(rat) ROR β expression vector using a microinjector (Femotek, Eppendorf). Injected embryos were

raised at 28 °C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 10–5% methylene blue) and were collected at different times after fertilization. The pGS5-rROR β construct is a generous gift of Michael Becker-André.

Fluorescent microscopy

Embryos and larvae were anesthetized by immersing them in 0.017% 3-aminobenzoic acid ethyl ester methanesulfonate salt solution (Sigma) and then mounted in 3% methyl cellulose (Sigma) or 4% LMP agarose (Invitrogen) on glass slides. They were observed under a Zeiss upright fluorescence microscope (Axio-plan2) equipped with a Zeiss filterset 10.

Supplementary materials and methods

RNAase protection assay

The RNase protection probes were amplified by PCR from the λ FIX II genomic clone using the following primers: ZfP1: 5' primer: 5'-CTCTTTGACTTCGACTAC-3' (positions –231 to –214). 3' primer: 5'-CGCACCAAA-TACGTGCGC-3' (positions +121 to +138). ZfP2: 5' primer: 5'-CAGTTCCTAGAAAGTGCTC-3' (positions +3421 to +3440). 3' primer: 5'-GATGATCCGAAG-CAGGTTG-3' (positions +3762 to +3781).

PCR products were cloned into pCR2.1-TOPO vector (Invitrogen). After linearization with BamHI, antisense-labeled RNA probes were transcribed with T7 polymerase (Roche). These 370 nucleotide- and 361 nucleotide-long probes contain sequences complementary to the 5'-flanking regions of the zfRev-erbs plus 42 bp of the pCR2.1-TOPO vector (see Supplementary Fig. 1A). RNase protection analyses were carried out as previously described (Neel *et al.* 1995) using total RNA extracted from adult zebrafish liver at two different circadian time points.

RT-PCR analysis

Total zebrafish RNA was isolated from adult zebrafish liver by using Trizol Reagent (Invitrogen). The cDNA was prepared by reverse transcription of total RNA with random primers (Promega) and M-MLV reverse transcriptase (Invitrogen).

The RT-PCR analysis of the transcripts from the ZfP2 promoter was done with the following primers: Forward primers (upstream of exon 2, Supplementary Fig. 1B): F1: 5'-ACCTCTTCTTTACCACCAGGTGGGTGAT-3' (positions +3638 to +3667). F2: 5'-GTCTTAC-TAGACGAACAGTGTTGTAAATAT-3' (positions +3608 to +3637). F3: 5'-CTGAGAACATTT-TAACTCCCTCTTTTGA-3' (positions +3578 to

+3607). F4: 5'-CGTACCCAAAAGAGGGAGAAA-TAAAAACA-3' (positions +3548 to +3577). F5: 5'-GAAGTATTTATGGGAACATTTTAAGGAGGA-3' (positions +3518 to +3547).

Reverse primer (in exon 3): 5'-ACCTTGCACAGCAA-CACCATTCATTCA-3' (positions +4053 to +4080 of the zfRev-erb α genomic fragment, see Fig. 1). PCR parameters were as follows: initial denaturation at 95 °C for 10 min followed by 32 cycles of 1 min at 95 °C, 30 s at 55 °C, 40 s at 72 °C with a final elongation at 72 °C for 7 min.

Inhibition of translation *in vitro* by MOs

The ability of each MO to specifically inhibit the translation of the relevant isoform was assessed by *in vitro* reticulocyte-lysate translation assay (Supplementary Fig. 2A). We performed *in vitro* translation of Rev-erb α 1 and Rev-erb α 2 using the Promega kit according to the manufacturer's instructions.

Results

Characterization of the zebrafish Rev-erb α promoter

In order to isolate the zebrafish Rev-erb α regulatory region, we screened a zebrafish genomic DNA library with a zfRev-erb α cDNA fragment encoding exons 1 and 2. We isolated and sequenced a genomic clone of 6.5 kb length including 3.1 kb upstream of the first exon (Figs 1 and 2A). The sequence includes a region that is highly conserved between the zebrafish P1 promoter and the mammalian P1 promoter (61% sequence identity from positions –231 to –8; see Figs 1 and 2A; Triqueneaux *et al.* 2004). This region is located just upstream of zebrafish exon 1 suggesting that it may correspond to a promoter orthologous to mammalian P1. We used two probes ZfP1 and ZfP2 to determine the transcriptional start sites by RNAase protection assay. With one probe including exon 1 (ZfP1 probe), we observed two transcriptional start sites (as observed for mammals) at –12 bp and +1 upstream of the first coding exon 1 (Supplementary Fig. 1, panel A left). Since the *Rev-erb α* gene in mammals contains a second promoter downstream of exon 1 (Fig. 2A), we analyzed the 3'-region of the genomic zebrafish clone with the ZfP2 probe and the RNAase protection assay revealed several protected bands (Supplementary Fig. 1, panel A right). At least three transcriptional start sites could be detected upstream of exon 2 (about –225, –115, and –65 bp). Importantly, most of these protected bands were confirmed by an RT-PCR analysis performed with an overlapping set of primers inside intron 1 (Supplementary Fig. 1B). Sequencing and computer analysis of this region revealed that a TATA-like box is present at

–20 bp from exon 2 (+3631 in Fig. 1), which suggests that this region corresponds to a functional promoter. We next tested the transcriptional activity of the two genomic regions (3.2 kb upstream of exon 1 and 3.4 kb upstream of exon 2 respectively) by fusing them to a luciferase reporter gene. These constructs were transiently transfected into COS1 cells. The activities of both constructs were about 20-fold higher than the PGL2 basic control (Fig. 3A). These values are similar to the

level of activity measured for the mammalian P1 and P2 promoters (data not shown; Triqueneaux *et al.* 2004).

Taken together, these results strongly suggest that the 6.5 kb *zRev-erbα* 5'-flanking region contains two genuine promoters that we call ZfP1 and ZfP2. Since exons 1 and 2 each contain an initiation codon in frame to the main ORF of the cDNA, these two promoters can generate two isoforms with distinct N-termini: a long one of 637 amino acids called *zRev-ERBα1*, from ZfP1, and a short one of 599 amino acids called *zRev-ERBα2*, from ZfP2 (Fig. 2B). Of note, ZfP1 can also generate the *zRev-ERBα2* isoform by alternative translation initiation of the mRNA. This situation is reminiscent of the mammalian *Rev-erbα* gene organization except that in mammals the P2 promoter is upstream of a non-coding exon 1 (Fig. 2A).

Characterization of a functional *Rev-erbα* regulatory element (RevDR2) in the ZfP1 promoter

The sequence of the two promoters revealed that a potential RevDR2 element is present at positions –145 to –127 bp of ZfP1 promoter. This element is located in the conserved region of the promoter. In addition, a non-conserved monomeric RevRE element is present upstream in ZfP2. To investigate the roles of these elements, ZfP1 or ZfP2 reporter constructs and expression vectors encoding the long or short isoforms of *zRev-erbα* were co-transfected into COS1 cells. As shown in Fig. 3A, ZfP1 was repressed by both isoforms of *zRev-ERBα* with the same efficiency, whereas ZfP2 activity was not influenced by *zRev-ERBα*. This suggests that *REV-ERBα* regulates its own expression through the RevDR2 element of ZfP1, but does not recognize the monomeric RevRE in ZfP2. This also shows that both zebrafish *REV-ERBα* isoforms are potent transcriptional repressors.

To investigate the role of the RevDR2 site, we constructed a short version of the ZfP1 (–230 to +138 bp; Fig. 3B, upper). To directly test the importance of the RevDR2 sequence, mutations were introduced into the 5' half-site of the RevDR2 (M5), the 3' half-site (M3), or of both the 5' and 3' half-site (M53). The basal promoter activity was not significantly affected by mutations in the RevDR2 element (data not shown). As shown in Fig. 3B, the shorter ZfP1 construct was still significantly repressed by *zRev-ERBα1* and *zRev-ERBα2*. Interestingly, and as expected, this construct was also activated by *rRORβ* expression, as the full-size ZfP1 reporter constructs (Fig. 3B; data not shown). In contrast, when the mutants M5 or M53 were used, ZfP1 activity was not influenced by *zRev-ERBα* (either $\alpha1$ or $\alpha2$) or *rRORβ*. On the other hand, a small repression by *REV-ERBα* and an activation by *rRORβ* were observed when the M3 mutant was used. This result was expected since

Figure 3 Transcriptional activity of the *zRev-erbα* promoters. (A) COS1 cells were transfected with 100 ng of a 3.2 kb ZfP1 (left) or a 3.4 kb ZfP2 (right) promoter luciferase reporter vector and 100 ng *Rev-erbα* isoforms, *Rev-erbα1* or *Rev-erbα2* expression vectors. DNA amount of each transfection was adjusted by additional pCDNA3 control plasmid. (B) Mapping of the RevDR2 element in ZfP1. COS1 cells were transfected with 100 ng indicated expression vector, *zRev-erbα1*/*zRev-erbα2* *RORβ* or empty pCDNA3 vector and ZfP1 luciferase reporter constructs (100 ng) containing the wild type or three different mutated versions of the RevDR2 element (–145 bp to –127 bp), pRevDR2-M5, pRevDR2-M3, or pRevDR2-M53. Relative luciferase activities are shown after normalization with an internal control of β -gal activity. Each transfection was done in triplicate and the data represent the mean $\pm$ S.D. of at least three independent experiments.

this mutant still includes a bona fide monomeric RevRE sequence. To validate this observation, we carried out EMSA experiments using a labeled RevDR2 element and *in vitro* translated zfRev-erbα and rRORβ proteins (Fig. 4). As expected, these data show that zfRev-erbα and rRORβ bind to the wild-type zfRevDR2 site, whereas the DNA binding was almost abolished when mutations were introduced in the 5' and 3' AGGTCA motifs.

Taken together, these results indicate that Zfp1 is regulated by zfRev-erb and rROR through a zfRevDR2 site and that an intact RevDR2 element is required for the ROR-mediated activation and Rev-erb-mediated repression of Rev-erbα.

The zfRev-erbα gene is regulating its own transcription *in vivo*

It has been previously reported that zebrafish Rev-erbα is expressed in pineal gland, retina, and optic tectum during early embryonic development and that its expression follows a circadian rhythm (Delaunay *et al.*

Figure 4 Both zfREV-ERBα and RORβ bind to the RevRE site within the *zfRev-erbα* gene promoter. Electrophoretic mobility shift analyses were performed using *in vitro* translated zfRev-erbα, RORβ, and β-gal protein with radiolabeled wild type or mutated probes containing the RevDR2 site located in -152 to -103 bp of the *zfRev-erbα* promoter. The mutated probe corresponds to RevRE M53 sequence. Positions of zfREV-ERBα and rRORβ binding are indicated by arrows. The competition experiments were performed by adding a tenfold molar excess of the unlabeled wild-type RevDR2 oligonucleotide (lanes 3, 5, and 7).

2000). We have recently shown that the pineal expression of zfRev-erbα depends on CLOCK and BMAL1 that directly interact with an E-box located in the Zfp1 promoter (Triqueneaux *et al.* 2004). To elucidate the effects of zfRev-erbα on its own gene expression *in vivo*, we used MO antisense oligonucleotides (MO) to prevent Rev-erbα mRNA translation (Nasevicius & Ekker 2000). We designed two different MOs, each specifically targeting the translation of a distinct isoform of Rev-erbα (see Supplementary Fig. 2A and B). Thus, MO1 and MO2 were used to inhibit the expression of zfRev-erbα1 and zfRev-erbα2 respectively. We confirmed that the protein synthesis of each of the zfRev-erbα isoforms was effectively inhibited by these MOs in an *in vitro* translation system (Supplementary Fig. 2A). We focused our analysis on the earliest zfRev-erbα expression, which starts in the pineal gland at 24-h development and becomes prominent at 48-hpf. To distinguish the expression of each isoform of Rev-erbα, we compared the signal generated by two probes that have the same size (250 bp): an exon 1 probe, specific of zfRev-erbα1, and a common 3' probe that recognizes both zfRev-erbα1 and zfRev-erbα2 mRNA. Using these two probes, we found that Rev-erbα1 transcripts are barely detectable in the pineal gland, whereas they are found at later stages in retina and optic tectum (Fig. 5A; data not shown). In contrast, the common probe recapitulates the known expression pattern of the gene (Fig. 5F; data not shown). This result suggests that it is mainly Rev-erbα2 that is expressed in the pineal gland.

The injection of the control MO at the one-cell stage did not modify the expression of Rev-erbα mRNA in the pineal gland at 48 hpf (Fig. 5B and G). In contrast, the injection of MO1 that inhibits translation of zfRev-erbα1 mRNA significantly increases expression levels of zfRev-erbα1 mRNA (Fig. 5C and H). No effect on Rev-erbα1 was observed when the MO2 was injected (Fig. 5D and I). The injection of both MOs also leads to an increase of zfRev-erbα1 mRNA expression (Fig. 5E). Essentially identical results were observed when the expression of both isoforms was monitored using a common probe (Fig. 5F-J): MO1 led to a clear increase of zfRev-erbα1, whereas MO2 seems to have no clear effect and MO1 plus MO2 show the same effect than MO1 alone. Nevertheless, these results should be interpreted keeping into account that our detection of the transcripts emanating from Zfp2 is only indirect, with a common probe detecting both isoforms. As expected, the expression of *Otx5* mRNA (used as a control) was not altered by any of the MO injections (Fig. 5K-O; Gamse *et al.* 2002). These data show that the knockdown of zfRev-erbα with MO1 increases the expression of Rev-erbα itself *in vivo* suggesting that in the pineal gland, the zfRev-erbα1 expression is efficiently repressed by zfRev-erbα1, but not by zfRev-erbα2.

Figure 5 Whole mount *in situ* hybridization analysis of *Rev-erbα* expression in the pineal gland. The wild-type embryos (A, F, and K), control (B, G, and L), MO1 (C, H, and M), MO2 (D, I, and N), or MO1 + MO2 (E, J, and O) morpholino-injected embryos were analyzed by whole mount *in situ* hybridization at 48 hpf. (A–E) Expression of *Rev-erbα* was analyzed using a *Rev-erbα1* specific probe designed in exon 1 (see the scheme below the figure; left). (F–J) Expression of *Rev-erbα* analyzed by a common 3'-end probe that recognize both *Rev-erbα1* and *Rev-erbα2* transcripts (see the scheme below the figure; right). (K–O) The expression of *Otx5* at 48 hpf in embryos was analyzed as a control for normal pineal gland development. Embryos were raised in 14 h light:10 h darkness (LD 14:10) conditions. At ZT0 (0900 h when the light is on), embryos were fixed.

Rev-erbα and ROR relationships *in vivo*

Our transient transfection experiments clearly show that in addition to be controlled by *zfREV-ERBα* itself, *zfRev-erbα* expression is activated by *rRORs*. To gain insights into the *in vivo* relevance of this observation, we first decided to compare the expression patterns of *zfRev-erbα* with those of other *Rev-erb* and *ROR* genes present in the zebrafish genome. We have performed a systematic screen for the expression of all NRs genes present in zebrafish (SB and VL in preparation) and we found five *Rev-erb* genes (one *Rev-erbα*, two *Rev-erbβ*, and two *Rev-erbγ*; see Bertrand *et al.* 2004) and five *ROR* genes (two *RORα*, one *RORβ*, and two *RORγ*). The large number of genes is explained by the ray-finned fish specific genome duplication that occurred early on during the evolution of actinopterygians (Jaillon *et al.* 2004) as well as by the selective loss of several NR genes at the base of the mammalian lineage (Bertrand *et al.* 2004). Figure 6 shows the expression patterns of these genes at 48 hpf. One can observe that *Rev-erbα*, *Rev-erbβ-A*, *Rev-erbβ-B*, and *Rev-erbγ-B* are all expressed in the retina, the optic tectum, the hindbrain, and the

pineal (Fig. 6, top panels A–C and E), whereas *Rev-erbγ-A* (Fig. 6, top panel D) is ubiquitously expressed in the head. All *ROR* genes (*RORα-A*, *RORα-B*, *RORβ*, *RORγ-A*, and *RORγ-B*; Fig. 6 bottom F–J) are expressed at 48 hpf in the retina and the optic tectum. In addition, *RORα-B* and *RORβ* transcripts can be detected in the cerebellum, *RORβ* being also expressed in the lateral line neuromasts. *RORγ-A* transcripts are also detected in pineal. We noticed that four out of the five *Rev-erb* genes and all the *ROR* genes are expressed in retina and optic tectum. Moreover, except in the hindbrain, *ROR* genes are expressed in all territories, whereas *Rev-erb* genes show a restricted expression (Table 1). These expression data strongly suggest that *in vivo* *zfRev-erbα* expression can effectively be controlled by *ROR* genes.

To assess if this regulation occurs in an *in vivo* context, we cloned the *ZfP1* and *ZfP2* promoters upstream of a GFP reporter gene and injected them into early embryos in the presence or absence of a rat *RORβ* expression vector. We observed that when injected alone, *ZfP1* induces GFP expression in 2.5% of the 201 injected fishes, mainly in the retina as well as

Figure 6 Expression patterns of Rev-erbs (upper) and RORs (bottom) transcripts in 48 hpf zebrafish embryos. Lateral views of the head, anterior to the left. Arrows indicate expression in the pineal gland. (A) *Rev-erbα* is expressed in pineal gland (arrow). (B and C) Both *Rev-erbβ* genes are expressed in retina (R), optic tectum (OT), hindbrain (Hb), and pineal gland, the expression level seems weaker than that of *Rev-erbα*. (D) *Rev-erbγ-A* transcripts are found ubiquitously in the head. (E) *Rev-erbγ-B* is expressed weakly in retina, optic tectum, and more strongly in the hindbrain. No pineal expression was detected. (F) *RORα-A* has a very specific expression pattern in retina and optic tectum. (G) *RORα-B* is expressed similarly in retina and optic tectum plus in hindbrain and cerebellum. (H) The unique *RORβ* gene is mainly expressed in retina and more weakly in optic tectum, cerebellum, and lateral line neuromasts (LLN). (I) *RORγ-A* is the only ROR expressed in the pineal gland. It is also found in retina and weakly in the optic tectum. (J) *RORγ-B* transcripts are localized in retina and optic tectum. Embryos were raised in 14 h light:10 h darkness (LD 14:10) conditions. At ZT0 (0900 h when the light is on), embryos were fixed.

in olfactory bulbs, brain, optic tectum at 60 hpf (see Fig. 7). This scattered expression pattern is explained by the strong mosaicism after injection of reporter constructs into zebrafish embryos (Gibbs & Schmale 2000). In the presence of *RORβ*, we found a significant increase (5.7% at 36 hpf and up to 23.4% when observed at 60 hpf) of GFP positive cells driven by *Zfp1*. In contrast, we did not note any effects of *RORβ* on *Zfp2* (Fig. 7A). This suggests that *RORβ* acts, *in vivo*, as a potent activator of *Zfp1* but not of *Zfp2*.

Discussion

In this paper, we show that *Rev-erbα* expression in zebrafish is controlled by two promoters that differentially regulate its expression. In addition, these promoters control the formation of two N-terminally different isoforms: *Zfp1* generates a transcript that encodes for *Rev-erbα1* (the longer form) and can, by alternative translation through an internal AUG

sequence, also generate the shorter *Rev-erbα2*. In contrast, *Zfp2* controls the expression of *Rev-erbα2* only. This situation is reminiscent to what has been found in mammals, where two isoforms generated by alternative promoter usage and/or alternative translation have also been described (Triqueneaux *et al.* 2004). Interestingly, our data also reveal that *Zfp1* is evolutionary conserved, whereas *Zfp2* is divergent and regulated in a different way in mammals and fish.

Evolutionary considerations

Several lines of evidence suggest that *Zfp1* is orthologous to its mammalian counterparts: i) there are about 60% sequence identity in the proximal region between the mammalian and fish promoters; ii) the main sites controlling the expression of the gene (the E-box in which the circadian CLOCK-BMAL1 complex binds and the RevDR2) are conserved and organized in a very similar way; and iii) functionally, these

Table 1 Tissue specific expression of zebrafish *Rev-erb* and *ROR* genes at 48 hpf

Tissue	Gene					ROR				
	Rev-erb									
	α	β-A	β-B	γ-A	γ-B	α-A	α-B	β	γ-A	γ-B
Pineal gland	++	+	+	u	—	—	—	—	++	—
Retina	—	+	+	u	+	++	++	++	++	++
Optic tectum	—	+	+	u	+	++	++	++	+	+
Hindbrain	—	+	+	u	++	—	++	—	—	—

++ , Strong expression; + , weak expression; u, ubiquitous expression; — , no expression.

Figure 7 ROR β controls *zfRev-erbα* expression *in vivo*. (A) Quantitative results of *in vivo* microinjection of ZfP1 or ZfP2 (white bars) and co-injection with ROR β expression vector (colored bars) into one-cell stage embryos. A much higher proportion of ZfP1 and ROR β co-injected embryos exhibit EGFP expression at either 36 or 60 hpf. ZfP2 promoter activity is very weak irrespective of the co-injection of a ROR β expression vector. Quantitative results are indicated below the histogram. (B–D) Three representative examples of GFP positive fish showing GFP expression in retina (B and C) as well as in other locations (C and D), such as olfactory bulbs and otic capsule. These three fishes were co-injected with ZfP1-GFP and ROR β expression vectors. (B and C) Anterior is to the left and dorsal is up. The asterisk indicates retinal cells that show strong GFP expression. Arrowheads indicate retina nonspecific GFP expression. (D) Dorsal view. L, lens. Scale bar = 50 μ m.

regulations are conserved *in vivo* as well as *in vitro*. Such a degree of conservation between mammals and fish promoters is not frequent and further highlights the importance of the regulations we characterized in this paper.

In contrast, sequence comparison suggests that the P2 promoter in mammals and fish are very different. First, the structural organization of the zebrafish *Rev-erbα* gene exhibits some differences with its mammalian ortholog. If two alternative promoters generating two

isoforms are present in both species, the position of the second promoter is different. In mammals, P2 is present upstream of an alternative non-coding exon E1B, whereas the ZfP2 is located directly in front of exon 2. Secondly, the regulation of the two promoters is different. In mammals, P2 is regulated by Rev-erb/ROR, whereas none of these regulators act on zebrafish P2. In contrast, we observed that ZfP2 is regulated by Otx factors, whereas mammalian P2 promoters are not (Nishio *et al.* submitted). All these data suggest that the P2 promoters from fish and mammals are not orthologous or have strongly diverged.

Sequence comparison and functional analysis of the Rev-erb α 1 and Rev-erb α 2 proteins clearly suggest that the zebrafish and mammalian isoforms are very similar. The two isoforms are closely related in terms of their DNA binding and repression activity. This suggests that if there are functional differences, they are probably quite subtle. Both Rev-erb α 2 correspond to truncations of an N-terminal part of the A/B region that notably contains some essential phosphorylation sites (Yin *et al.* 2006). In mammals, the Rev-erb α 2 isoform, which is truncated in its NH2 extremity, is not phosphorylated in this region of the protein and hence exhibits a much higher stability (data not shown). It is an interesting evolutionary situation that divergent alternative promoters control the expression of evolutionary conserved isoforms. One explanation for this situation may be linked to the strong pineal expression of ZfP2, since it is well known that the function of the pineal gland has been drastically modified between early vertebrates, such as teleost fishes and mammals (see Nishio *et al.* submitted). It is clear that more work has to be done to really understand the biological role *in vivo* of the Rev-erb α 2 isoforms in both mammals and zebrafish.

The ZfP1 promoter is the target of Rev-erb α autoregulation

In the ZfP1 promoter, we found at position –145 a classical RevDR2 element, which is located in the conserved region of the promoter. It is known that Rev-erb α and ROR receptors recognize similar response elements with a 5' A/T-rich region and a A/GGGTCA motif, while they have opposite effects on gene transcription (Forman *et al.* 1994, Giguere *et al.* 1994, Retnakaran *et al.* 1994). We demonstrated that this element confers the Rev-erb α /ROR responsiveness (repression and activation respectively). We have also shown that Rev-erb α and ROR can directly bind to this RevRE in EMSA. Thus, as previously discussed, the sequence, position, and function of this RevDR2 appear to be highly conserved in vertebrates. Its close proximity to the neighboring E-boxes that mediate

the activation of Rev-erb α by CLOCK-BMAL1 suggests that some sort of crosstalk may occur between the regulation of the *Rev-erb α* gene by Rev-erb/ROR and CLOCK-BMAL1. This hypothesis remains to be addressed experimentally.

We have previously shown in mammals that the Rev-erb α P1 promoter is down-regulated by the Rev-erb α protein through its binding to the RevDR2 element (Adelmant *et al.* 1996). The *in vivo* relevance of this regulation was questioned when Rev-erb α knockout mice were generated that show no obvious changes in *Rev-erb α* gene expression (Chomez *et al.* 2000). Our results demonstrate that the knockdown of zfRev-erb α with MOs results in the accumulation of Rev-erb α transcripts in the pineal. This accumulation suggests that Rev-erb α represses its own transcription *in vivo*. The MO targeting Rev-erb α 1 induced an accumulation of the transcripts from ZfP1, which is consistent with the idea that it is the RevDR2 site in ZfP1 that is instrumental for this regulation. Since there is no specific probe to visualize the transcripts emanating from ZfP2, it is not possible to know if the activity of ZfP2 is also modulated by MO injection. Nevertheless, given the results of our transient transfection assays showing that ZfP2 is not regulated by Rev-erb α , modulation of ZfP2 activity by the MO injections appears highly unlikely.

In sharp contrast, when we injected MO2 targeting Rev-erb α 2 expression, we did not note any effects on the Rev-erb α transcripts. These data suggest that, in terms of Rev-erb α autorepression, during early embryogenesis in the pineal gland, Rev-erb α 1 plays a more important role than Rev-erb α 2. This is quite surprising given the expression patterns that we observed at 48 hpf, when in the pineal zfRev-erb α 1 is much less expressed than zfRev-erb α 2. Obviously, there is still a lot of work to be done to elucidate the respective roles of Rev-erb α 1 and Rev-erb α 2 and the crosstalk regulation of their expression during zebrafish embryogenesis.

RORs regulates Rev-erb α expression

Our results also show that ROR β is able to activate the expression of Rev-erb α in the RevDR2-dependent manner and strongly suggest that this regulation occurs *in vivo*. We did not note an expression of the ZfP1 construct in the pineal gland, but this was not surprising given the highly mosaic activity of exogenous promoters in zebrafish using transient GFP expression assay (Gibbs & Schmale 2000). Interestingly, no effect of ROR β expression vector injection was found on ZfP2 promoter. This suggests that *in vivo* RORs activate only ZfP1. This is in general agreement with the fact that (according to our *in situ* hybridization data with isoform specific probes) ZfP2 appears to be specifically active in the pineal gland, whereas ZfP1 is apparently more

active in the retina (Fig. 5; data not shown). Given the strong expression of *ROR* genes in retina, if *Zfp2* would have been regulated by RORs, then a specific mechanism would be necessary to explain its lack of activity in retina.

The expression patterns of the five *Rev-erb* and the five *ROR* genes clearly show that there is a common principle controlling the expression of these ten genes. Indeed, most of them are expressed in both retina and optic tectum. All the non-ubiquitously expressed *Rev-erb* genes also show expression in the pineal, where *ROR γ -A* is expressed at this stage. These data suggest that *Rev-erb* and *ROR* genes may be implicated in common signaling pathways acting during development of organs receiving and integrating light input in zebrafish.

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