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

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Abstract

The orphan nuclear receptor Rev-erbα (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-erbα gene regulation, we have isolated its 5'-upstream region. We found that two promoters control the zfRev-erbα 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-erbα-binding site (RevDR2). Inhibition of zfRev-erbα activity in zebrafish embryos using antisense-morpholino knockdown results in an increase of zfRev-erbα gene expression suggesting that zfRev-erbα is repressing its own transcription in vivo. In addition, we show that ROR orphan receptors also regulate in vitro and in vivo zfRev-erbα 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-erbα expression.

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Introduction

Rev-erbα 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-erbα 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-erbα (NR1D1; NRs Nomenclature Committee, 1999; also called ear-1), Rev-erbβ (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-erβ 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-erbα has been shown to bind DNA as a monomer on a specific sequence called Rev-erbα-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-erbz were isolated and it has been established that they are regulated by Rev-erbz 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-erbz has remained unclear, until it was observed that Rev-erbz 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-erbz is a major player in the control of circadian clocks (reviewed in Emery & Reppert 2004). In mammals, Rev-erbz 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-erbz is activated by CLOCK-BMAL1 heterodimer and repressed by PER and CRY proteins (Preitner et al. 2002, Triqueneaux et al. 2004), suggesting that Rev-erbz expression is directly under the control of the circadian clock. All these data suggest that Rev-erbz 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-erbz increases during adipogenesis and decreases during myogenesis. Therefore, a role of Rev-erbz 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-erbz controls the expression of numerous genes important for lipid homeostasis, such as ApoA1 or ApoCIII (Vu–Dac et al. 2000, Coste and Rodriguez 2002, Laitinen et al. 2005). Other evidence suggests that Rev-erbz is tightly regulated; in humans, it has been shown that the activity of its promoter is down-regulated by Rev-erbz 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-erbz is regulated by PPARz through the RevDR2 site (Gervois et al. 1999, Coste and Rodriguez 2002, Laitinen et al. 2005).

During zebrafish embryogenesis, Rev-erbz 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 (72hpf) 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-erbz gene and we found that in zebrafish, as in mammals, Rev-erbz expression is controlled by two promoters, ZIP1 and ZIP2. These promoters drive expression of two Rev-erbz isoforms with similar repressive properties. We show that ZIP1 is conserved and functionally similar to the mammalian P1 promoter, whereas ZIP2 is divergent both in its genomic organization and function.

Materials and methods

Plasmid constructions

Full-length cDNA of zfRev-erbz (zfRev-erbz1) and a version encoding a short isoform (zfRev-erbz2) 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. zfRev-erbz1 5’ primer: 5’-CATATGACTT-TACTGGGGCTC-3’ (positions +257 to +274). zfRev-erbz2 5’ primer: 5’-TCCATGTACACTGAATTCTC-3’ (positions +3717 to +3736). zfRev-erbz common 3’ primer: 5’-TCAGGCTATCATGGGAAAACAGG-3’ (reverse) positions 1979–2002 of the zfRev-erbz cDNA (Genbank accession number: AY391444).

To study the genomic 5’-region, a 14 kb genomic DNA fragment containing the entire zfRev-erbz coding region was isolated from a genomic DNA library in λFIX II (a generous gift of Christine and Bernard Thisset). 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 zfRev-erbz full, was obtained by LA PCR (TaKaRa Shuzo, Kyoto, Japan) using the following pair of primers: Full 5’ primer: 5’-CCGGCTCAGGTCTGGGATTATTGGTACAC-3’ (sense; positions −2858 to −2827). Full 3’ primer: 5’-TCGGGTATCCATCCATTTGTA-TTCACCCCACACTGGTGGT-3’ (reverse; positions +3650 to +3683). The amplified product was subcloned into XL-TOPO vector (Invitrogen).

The constructs containing the zfRev-erbz first (ZIP1) or second (ZIP2) promoters were obtained by PCR amplification using zfRev-erbz full as a template. The
Figure 1 Sequence of the zfRev-erb\textsubscript{a} 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.
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–BamHI fragment containing the Rev-erbα 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′-GAGAGCTCGCGGCCGC-GAGCTC-3′ (on λFIX II). ZfP1 3′ primer: 5′-TCGTCGCACCAAATACGTGCGC-3′ (positions C121 to C136). ZfP2 5′ primer: 5′-CCGCTCGAGGA-CACGAACAACACAGGTAA GACGCTTT TAGAA-3′ (positions C293 to C325). ZfP2 3′ primer: 5′-TCGGGATCCGATCCAATGTATGATATCACCC-CACCTGG TGGT-3′ (positions C3650 to C3682).

Mutations of RevDR2 were produced by PCR using the following oligonucleotides. pRevDR2WT: 5′ primer: 5′-GAGCTCCTCTTTGACTTCGACTAC-3′ (positions K231 to K214). 3′ primer: 5′-GGATCCCGCACCAAATACG TGCGC-3′ (positions C121 to C136). pRevDR2M5: 5′ primer: 5′-GTTCTGGAGAAAGTCCTAGCCTGGGC-CA CGAGTC-3′ (positions K154 to K121). 3′ primer: 5′-GACTCGTGGCCCAGGCTAGGACTTTCTC-CAGAAC-3′ (positions K154 to K121). pRevDR2M3: 5′ primer: 5′-GGAGAAAGTGTGTCACA CC CGGGTCGAGTCGGGTCACATG-3′ (positions K149 to K114). 3′ primer: 5′-GTGACCCGACTCGACCGGGTGTGACA-CACTTTCTCC-3′ (positions K149 to K114).

Figure 2 Intron–exon structure of the 5′-UTR of the zfRev-erbα gene. (A) Comparison of the zebrafish, human Rev-erbα 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-erbα gene and generation of the two N-terminally distinct isoforms, zfREV-ERBα1 and zfREV-ERBα2, 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 are shown as solid line squares. The amino acid number of each isoform is indicated. The accession number of ENSEMBL is ENSDARG00000033160.
−148 to −111). 3’ primer: 5’-CATGTGACCCGACT-GACCGGTGGCTAGGACTTTCTC-3’ (positions −T42 to −11). 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 µg/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-b-gal vector; Life Technologies; Triqueneaux et al. 2004). After a 12h 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 ± 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-γATP 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 µl reaction mixture containing 10 mM KPO4 buffer (pH 8.0), 1 mM EDTA, 80 mM KCl, 1 µg 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-GAAATGTGTGACTGGGAGCCGGATCAGGGTTGATGACACAT-3’. RevRE mutant: 5’-TCTGGA-GAAATGTGTGACTGGGAGCCGGATCAGGGTTGATGACACAT-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 Thisset al. (2004).

The zfRev-erbβ 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’-TCTGGA-GAAATGTGTGACTGGGAGCCGGATCAGGGTTGATGACACAT-3’ (positions 61–80 of the zfRev-erbz cDNA, see Fig. 1). 3’ end primer: 5’-TCTGGA-GAAATGTGTGACTGGGAGCCGGATCAGGGTTGATGACACAT-3’ (positions 288–307). 3’-end common probe: 5’-TCTGGA-GAAATGTGTGACTGGGAGCCGGATCAGGGTTGATGACACAT-3’ (positions 346–365).

All probes for zfRev-erbβ 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-erbz1 (MO1) and zfRev-erbz2 (MO2) around the transcriptional start codon based on the recommendations of the manufacturer (GeneTools). The MO sequences were as follows: MO1: 5’-TCTGGA-GAAATGTGTGACTGGGAGCCGGATCAGGGTTGATGACACAT-3’ (positions 234–257); see red boxes in Supplementary Fig. 2B. MO2: 5’-TCTGGA-GAAATGTGTGACTGGGAGCCGGATCAGGGTTGATGACACAT-3’ (positions 347–365).

For the control, we used the standard control MO from GeneTools: 5’-CCTTACCTCAGTTACATTTAT-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/µl 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) RORβ expression vector using a microinjector (Femojet, 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-rRORβ 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 (Axioplan2) equipped with a Zeiss filterset 10.

Supplementary materials and methods

RNAase protection assay

The RNase protection probes were amplified by PCR from the λFIX II genomic clone using the following primers: ZIP1: 5’ primer: 5’-CTCTTTGACTTGGACTACC3’ (positions −231 to −214). 3’ primer: 5’-GGACACAAATTACGTGCGG-3’ (positions +121 to +138). ZIP2: 5’ primer: 5’-CAGTTCGAGACGGCTC3’ (positions +3421 to +3440). 3’ primer: 5’-GATGATCCGAAGCGGTTG-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). RNA 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 ZIP2 promoter was done with the following primers: Forward primers (upstream of exon 2, Supplementary Fig. 1B): F1: 5’-ACCTGTTTTTCACTACCGGTTGGT-GAT-3’ (positions +3638 to +3667). F2: 5’-GTCTTAGTAGCAGAAGTGTGTAATAT-3’ (positions +3608 to +3637). F3: 5’-CTGAGAACACATT-TAAAACACTCTCTTTTGA-3’ (positions +3578 to +3607). F4: 5’-GTACCCCAAGGAGGAGAAA-TAAAACA-3’ (positions +3548 to +3577). F5: 5’-GAAGATTTTATGGGACACCTTTTAAGGAGA-3’ (positions +3518 to +3547).

Reverse primer (in exon 3): 5’-ACCTGTGACCGAACA-CCATTCCATTCA-3’ (positions +4053 to +4080 of the zfRev-erzb 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-erzb1 and Rev-erzb2 using the Promega kit according to the manufacturer’s instructions.

Results

Characterization of the zebrafish Rev-erbα promoter

In order to isolate the zebrafish Rev-erbα regulatory region, we screened a zebrafish genomic DNA library with a zfRev-erbs 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 ZIP1 and ZIP2 to determine the transcriptional start sites by RNAase protection assay. With one probe including exon 1 (ZIP1 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. 1A, panel A left). Since the Rev-erbα 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 ZIP2 probe and the RNase 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
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 zfRev-erbz 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 zfREV-ERBz1, from ZfP1, and a short one of 599 amino acids called zfREV-ERBz2, from ZfP2 (Fig. 2B). Of note, ZfP1 can also generate the zfREV-ERBz2 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 zfREV-erbz were co-transfected into COS1 cells. As shown in Fig. 3A, ZfP1 was repressed by both isoforms of zfREV-erbz with the same efficiency, whereas ZfP2 activity was not influenced by zfREV-ERBz. This suggests that REV-ERBz 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-ERBz 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 zfREV-ERBz1 and zfREV-ERBz2. Interestingly, and as expected, this construct was also activated by rRORΔ 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 zfREV-ERBz (either z1 or z2) or rRORΔ. On the other hand, a small repression by REV-ERBz and an activation by rRORΔ were observed when the M3 mutant was used. This result was expected since

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**Figure 3** Transcriptional activity of the zfRev-erbz promoters. (A) COS1 cells were transfected with 100 ng of a 3-2 kb ZIP1 (left) or a 3-4 kb ZIP2 (right) promoter luciferase reporter vector and 100 ng Rev-erbz isoforms, Rev-erbz1 or Rev-erbz2 expression vectors. DNA amount of each transfection was adjusted by additional pcDNA3 control plasmid. (B) Mapping of the RevDR2 element in ZIP1. COS1 cells were transfected with 100 ng indicated expression vector, zfRev-erbz1-zfRev-erbz2 RORβ or empty pcDNA3 vector and ZIP1 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 ± 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. 2007). 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-erb1 and zfRev-erb2 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-erbα1 itself in vivo suggesting that in the pineal gland, the zfRev-erbα1 expression is efficiently repressed by zfRev-erbα2, but not by zfRev-erbα2.
Our transient transfection experiments clearly show that in addition to being 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 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

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**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.
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 RORα, 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

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

| 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 pinel expression was detected. (F) RORα-A has a very specific expression pattern in retina and optic tectum. (G) RORβ-B is expressed similarly in retina and optic tectum plus in hindbrain and cerebellum. (H) The unique RORγ 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.

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

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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

Figure 7 RORβ controls zfRev-erbα expression in vivo. (A) Quantitative results of in vivo microinjection of ZfP1 or ZfP2 (white bars) and co-injection with RORβ expression vector (colored bars) into one-cell stage embryos. A much higher proportion of ZfP1 and RORβ 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 RORβ 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 RORβ 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 μm.
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 ZIP2 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 ZIP2 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\textsubscript{1} and Rev-erb\textsubscript{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\textsubscript{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\textsubscript{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 ZIP2, 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\textsubscript{2} isoforms in both mammals and zebrafish.

The ZIP1 promoter is the target of Rev-erb\textsubscript{2} 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\textsubscript{2} and ROR receptors recognize similar response elements with a 5' A/T-rich region and a AGGGTCA 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\textsubscript{2}/ROR responsiveness (repression and activation respectively). We have also shown that Rev-erb\textsubscript{2} 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\textsubscript{2} by CLOCK-BMAL1 suggests that some sort of crosstalk may occur between the regulation of the Rev-erb\textsubscript{2} 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\textsubscript{2} P1 promoter is down-regulated by the Rev-erb\textsubscript{2} protein through its binding to the RevDR2 element (Adelmant et al. 1996). The in vivo relevance of this regulation was questioned when Rev-erb\textsubscript{2} knockout mice were generated that show no obvious changes in Rev-erb\textsubscript{2} gene expression (Chomez et al. 2000). Our results demonstrate that the knockdown of zfRev-erb\textsubscript{2} with MOs results in the accumulation of Rev-erb\textsubscript{2} transcripts in the pineal. This accumulation suggests that Rev-erb\textsubscript{2} represses its own transcription in vivo. The MO targeting Rev-erb\textsubscript{1} induced an accumulation of the transcripts from ZIP1, which is consistent with the idea that it is the RevDR2 site in ZIP1 that is instrumental for this regulation. Since there is no specific probe to visualize the transcripts emanating from ZIP2, it is not possible to know if the activity of ZIP2 is also modulated by MO injection. Nevertheless, given the results of our transient transfection assays showing that ZIP2 is not regulated by Rev-erb\textsubscript{2}, modulation of ZIP2 activity by the MO injections appears highly unlikely.

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

RORs regulates Rev-erb\textsubscript{2} expression

Our results also show that ROR\textbeta is able to activate the expression of Rev-erb\textsubscript{2} in the RevDR2-dependent manner and strongly suggest that this regulation occurs in vivo. We did not note an expression of the ZIP1 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\textbeta expression vector injection was found on ZIP2 promoter. This suggests that in vivo RORs activate only ZIP1. This is in general agreement with the fact that (according to our in situ hybridization data with isoform specific probes) ZIP2 appears to be specifically active in the pineal gland, whereas ZIP1 is apparently more

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active in the retina (Fig. 5; data not shown). Given the strong expression of ROR genes in retina, if ZIP2 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 RORγ-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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