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Functional identification of an osmotic response element (ORE) in the promoter region of the killifish deiodinase 2 gene (*FhDio2*)

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Summary

The physiological role played by thyroid hormones (TH) in hydro-osmotic homeostasis in fish remains a controversial issue. Previous studies have shown that in *Fundulus heteroclitus* (killifish) hypo-osmotic stress increases liver iodothyronine deiodinase type 2 (D2) mRNA and D2 activity. In this study we identified two conserved osmotic response element (ORE) motifs in the promoter region of the killifish D2 gene (*FhDio2*) and examined their possible role in the transcriptional regulation of *FhDio2* during hypo-osmotic stress. As assessed by the electrophoretic mobility shift assay, results from *in vivo* and *in vitro* experiments demonstrate that exposure to an abrupt hyposmotic challenge triggers in the liver of killifish a strong nuclear recruitment of a putative osmotic response element binding protein (OREBP). This protein–DNA binding is time-dependent, attains a maximum within 2–8 h after the osmotic stress, and is followed by a significant increase in D2 activity. Furthermore, protein–DNA binding and the subsequent elevation in enzyme activity were blocked by the tyrosine kinase inhibitor genistein. Thus, during hypo-osmotic stress, a putative OREBP kinase-activated pathway stimulates *FhDio2* transcription and enzymatic activity. These data and the fact that D2 is the major enzyme providing local intracellular T_3 suggest that TH plays a direct role in osmoregulation in fish, possibly by participating in hepatic ammonia metabolism. This study provides important insight into the physiological role of TH in hydro-osmotic homeostasis in fish.

Key words: osmotic response element, osmotic stress, deiodinase type 2 gene, *Fundulus heteroclitus*, thyroid hormones.

Introduction

The response to osmotic stress is among the most basic constitutive homeostatic functions in all living systems. It has been suggested that the network of genes and transduction signals involved in its operation, the stress proteome, belongs to a highly conserved core set of proteins (Kultz, 2003). The first osmotic response element (ORE) in a eukaryotic genome was originally described in the rabbit aldolase reductase gene (Ferraris et al., 1996), followed by the characterization of its corresponding transcription factor, the ORE binding protein (OREBP) (Ferraris et al., 1999). Tyrosine phosphorylation is required for OREBP to translocate into the nucleus and activate transcription. Although the pathway responsible for OREBP phosphorylation remains to be fully elucidated, tyrosine kinases p38 and protein kinase A (PKA) have been implicated (Ferraris et al., 2002). In marked contrast to terrestrial vertebrates, information regarding osmotic stress responses in aquatic species is scarce and fragmented. Up to now OREBPs have been amply assessed during hyperosmotic conditions, but these studies have been mainly restricted to *in vitro* experiments and to mammals. Recently the transcriptional regulation in response to changes in environmental salinity has begun to be explored in euryhaline fishes. Two osmotic stress transcription factors have been identified in tilapia gill, the osmotic transcription factor 1 (Ostf1) and the tilapia homolog of transcription factor II B (TFIIB). Both show a specific and transient up-regulation during hyperosmotic stress (Fiol and Kultz, 2005). Ostf1 has strong homology to the mammalian glucocorticoid leucine zipper protein; however, no steroid is required for its induction during hyperosmotic, NaCl-mediated stress in fish (Fiol et al., 2006).

Because of their binding to nuclear receptors, thyroid hormones (TH) are well-known transcription mediators. TH play a central role in regulating diverse homeostatic functions, from the basal metabolic rate and protein synthesis, to development and cell differentiation (Anderson et al., 2000). Nevertheless, the function of TH in hydro-osmotic balance in fish has been a controversial issue (for reviews, see McCormick, 2001; Orozco and Valverde-R., 2005; Klaren et al., 2007). Previous data from our laboratory have shown that in the euryhaline teleost *Fundulus heteroclitus* (Fh), hypo-osmotic stress elicits an increase in liver iodothyronine deiodinase type 2 (D2) activity (Orozco et al., 1998). D2 is known to be the major provider of T_3 at the target cell level; thus, an up-regulation of this enzyme after an osmotic challenge suggested the participation of TH during this homeostatic response. In the present study we used a bioinformatic approach (Heinemeyer et al., 1998) to identify two conserved ORE motifs within the 5′

UTR of the *FhDio2* gene (Orozco et al., 2002a). We then carried out a series of *in vivo* and *in vitro* experiments to examine the possible participation of these ORE motifs in regulating *FhDio2* gene expression during hypo-osmotic stress in *Fundulus heteroclitus*. Our data demonstrate the participation of a putative OREBP-activated pathway in the liver of killifish challenged by abrupt low salinity.

Materials and methods

Bioinformatic search

Using 1.3 kb of the known 5′ UTR of *FhDio2* (GenBank accession no. AY065834), we performed a computational search for transcription factor binding sites (TFBS). To this end we used the public version of TF Search (http://www.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer et al., 1998) with the available vertebrate matrices and a threshold score up to 85.

Animals

Seawater-adapted male *Fundulus heteroclitus* L. (killifish), body mass 4–6 g, were collected from the estuarine creeks of the Matanzas River (St Augustine, FL, USA). After capture, fish were deparasitized and kept in tanks with running seawater (SW) piped directly from the ocean at a temperature of around 28°C. Animals were fed *ad libitum* (Silver Cup, Nelson and Sons, Murray, UT, USA) and maintained on a light:dark cycle of 14 h:10 h. All experimental protocols used in this study were approved by the Institutional Animals Ethics Committee. Experiments were carried out 1 week after acclimatization.

In vivo experiments

For each experiment, fish (N=10–12) were placed into tanks with running SW and constant aeration. At the start of the experiment, the water supply was shut down, and the water volume of each tank was adjusted to 6 l with a 1:1 ratio of salt- and freshwater (50%). Except for the change in water salinity, control and experimental groups were handled in the same manner. Based on previous experiments (Orozco et al., 1998), fish were sacrificed 0.5, 1, 2, 4, 6, 8, 12 and 16 h after the hypo-osmotic challenge. Animals were sacrificed by decapitation, and the liver was collected and divided to measure D2 mRNA concentrations, D2 activity and for nuclear extraction (see below). D2 mRNA was measured in pools.

In vitro experiments

Seawater acclimated fishes (N=2–3) were sacrificed, and their livers were dissected and cut into small pieces of about 2 mm (5 mg wet mass) in the presence of L-15 medium (Invitrogen, Carlsbad, CA, USA). Liver explants were pooled, kept on ice for 30 min, and then randomly assigned to either control or experimental 24-well plates. The L-15 medium in the experimental plates was made hypotonic by simple dilution 1:1, restoring its nutrients with an amino acid stock solution (Sigma-Aldrich, St Louis, MO, USA) and galactose 10 g l^{-1}. The explants were incubated for 6 h in a metabolic bath chamber at 28°C under continuous agitation and saturating humidity with 99% O_2 and 1% CO_2 (Janssens and Grigg, 1994). Explants and the corresponding culture media were separately collected after 2, 4 and 6 h of incubation. Liver fragments were divided into two pools: one to quantitate D2 activity and the other for nuclear extraction. D2 activity was measured in tissue homogenates, and to evaluate tissue viability lactate dehydrogenase (LDH) was measured in the culture media. For the tyrosine kinase inhibitor experiments, explants were pre-treated with genistein 2 h prior before exposure to L-15 hypo-osmotic medium.

Nuclear and cytoplasmic extracts

Control or experimental livers were resuspended in hypotonic buffer (10 mmol l^{-1} Hepes, pH 7.9, 10 mmol l^{-1} KCl, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT) as previously described (López-Bojórquez et al., 2004). The tissue was broken mechanically with a plastic homogenizer at low velocity. To allow cell debris to settle the homogenates were incubated at 4°C for 30 min, and the supernatants were collected. The integrity of the nuclei was evaluated by Trypan-blue stain (1:1).

Nuclei were centrifuged at 800 ***g*** to separate them from the cytoplasmic fraction. The supernatants (cytoplasm) were diluted in an equal volume of dilution buffer (20 mmol l^{-1} Hepes, pH 7.9, 50 mmol l^{-1} KCl, 20% glycerol, 0.2 mmol l^{-1} EDTA, 0.5 mmol l^{-1} PMSF, 1 mmol l^{-1} DTT) for protein quantification. The pellet (nuclei) was recovered, resuspended in hypertonic buffer (50 mmol l^{-1} Tris-HCl, pH 7.9, 400 mmol l^{-1} NaCl, 400 mmol l^{-1} KCl, 10%, glycerol, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT, 0.5 mmol l^{-1} PMSF) and maintained with agitation at 4°C for 30 min. After centrifugation at 16 000 ***g*** for 25 min, the supernatant was resuspended in an equal volume of dilution buffer. Protein was quantified by the Bradford method (BioRad, Hercules, CA, USA). The same protocol was followed to isolate the nuclear and cytoplasmic fraction from the explants in the *in vitro* experiments.

Analytical procedures

LDH assay

The viability of liver explants was assessed by the quantitative determination of LDH in the culture media using the reagents supplied in the SPINREACT kit (SpinReact, Girona, Spain). The activity was normalized to the protein content of the corresponding liver explants for each well. These data were used to normalize D2 activity of the corresponding explant by multiplying D2 specific activity and LDH activity. Normalized D2 results are expressed in arbitrary units.

Deiodination assay

Enzyme activity was measured in duplicate as previously described (Orozco et al., 2000). Briefly, the total volume of the reaction mixture was 100 μl and contained 1 nmol l^{-1} ^{125}I-T_4 [specific activity 1200 μCi μg^{-1} (1 μCi=3.7×10^{10} Bq); NEN-Perkin Elmer, Wellesley, MA, USA], 25 mmol l^{-1} DTT (Calbiochem, Darmstadt, Germany) and 100 μg/tube of liver homogenate protein, and the assays were incubated for 1 h at 37°C. The released acid-soluble ^{125}I was isolated by chromatography on Dowex 50W-X2 columns (BioRad). Enzyme specific activity (fmol ^{125}I mg^{-1} h^{-1}) was calculated as previously described (Pazos-Moura et al., 1991). Protein content was measured by the Bradford method. Since the LDH activity in the culture medium is inversely proportional to cell viability, the final D2 activity is reported as product of both enzymes, in arbitrary units.

Measurement of D2 mRNA

For quantitation, RNA was isolated from livers (TRIzol, Invitrogen), and cDNA was reverse-transcribed (*Superscript*, Invitrogen) from 10 μg RNA using a gene-specific primer (TTCAGAGCTCATCTACTATCGT). The D2 mRNA concentration was measured in duplicate by a competitive PCR as previously described (García-G. et al., 2004). The standard curve ranged from 10^4 to 10^9 molecules μl^{-1}. The oligonucleotides used (sense: CAAACAGGTGAAACTTG-GCT and antisense: TCGTCGATGTAGACCAGC) amplified a product of 270 bp (40 s at 94°, 40 s at 65°, 30 s at 72° for 35 cycles). Identical PCRs from the RNA samples prior to the reverse transcription reaction yielded no detectable products, which indicates that the RNA was not contaminated with genomic DNA. Results are expressed as molecules μg^{-1} total mRNA used in the reverse transcription.

Electrophoretic mobility shift assay (EMSA)

Putative OREBP-binding activity was studied by the use of [^{32}P]-labeled double-stranded oligonucleotides corresponding to ORE1 (5′ TGTAGA **GGGAAAAGCT** GGGACCA 3′) or ORE2 (5′ TTTCAGG **CGGAAAAGTA** ACATTTC 3′) (see below). The probes (200 ng in 1 μl) were labeled with 0.5 μl (5 U) of T4 phosphonucleotide kinase; 2.5 μl of buffer provided in the kit (Roche, Basel, Switzerland) and 3 μl of ^{32}P-γATP (NEN-Perkin Elmer, specific activity 3000 Ci mmol l^{-1}), and water to a final volume of 25 μl. The labeling reaction was mixed with 1 ml of hybridization solution and then passed through a nitrocellulose filter to eliminate excess ^{32}P-γATP. The quality of the labeled products was assessed by electrophoresis in denaturing polyacrylamide gels exposed to photographic film. The binding reaction was performed by incubating 10 μg of either nuclear or cytoplasmic protein with the corresponding probes. The reaction mixture was incubated on ice for 40 min in a buffer containing 20 mmol l^{-1} Hepes, 50 mmol l^{-1} KCl, 20% glycerol, 0.2 mmol l^{-1} EDTA, 0.5 mmol l^{-1} PMSF, 1 mmol l^{-1} DTT, 1 μg μl^{-1} BSA, and polydI/dC (Pharmacia, NY, NY, USA). The reaction mixture was loaded onto a 5 % non-denaturing polyacrylamide gel and resolved at 120 V for 4 h. The gel was dried, and the DNA–protein complexes were visualized by exposure in a Storage Phosphor Screen (Molecular Dynamics, San Francisco CA, USA). The screens were read in a *Phosphorimager* (Storm Molecular Dynamics) and quantified with the ImageQuant software (Molecular Dynamics). The radioactivity of each band was corrected for the background and is presented in arbitrary units.

Statistics

The EMSA figures are a representative experiment from 4–6 repetitions. The graph shows the average of three independent quantitative evaluations of the gel shown.

In the D2 activity experiments, differences among groups were analyzed by one-way ANOVA coupled with a Bonferroni's multiple comparison test (control *vs* treatment). Differences were considered significant when $P<0.05$.

Results

The 5′ UTR of FhDio2 contains two osmotic responsive elements

As shown in Fig. 1A, the computational analysis revealed the presence of several putative TFBS in *FhDio2*. Among them we identified two ORE motifs at positions –322 (ORE1) and –1016 (ORE2). ORE1 was identical to the canonical mammalian ORE consensus motif (Ferraris et al., 1999), and ORE2 only differed in one nucleotide (Fig. 1B).

Hypo-osmotic stress in vivo triggers the nuclear recruitment of a putative OREBP and up-regulates mRNA expression and D2 activity

To examine whether a putative OREBP is associated with *FhDio2* transcription *via* the ORE elements identified in the 5′ promoter region of the gene, we performed EMSA with specific oligonucleotides containing the endogenous ORE1 and ORE2 motifs identified above (Fig. 1). Nuclear extracts from both control and hypo-osmotically stressed fish were obtained at 0.5, 1, 2, 3, 4, 8, 12 and 16 h after the stress. As shown in Fig. 2A, hypo-osmotic stress was accompanied by clear-cut recruitment of cytoplasmic protein into the nuclei. Indeed, this protein–DNA binding occurred in a biphasic mode: an initial protein translocation 2 h after stress, and a second, more intense wave of recruitment 6 h later. Furthermore, the nuclear protein–DNA binding correlated with the parallel disappearance of protein from the cytoplasmic compartment. The protein reappeared in the cytoplasm 12 h after initiation of the experiment. Fig. 3 shows the specificity of the putative OREBP complex. Formation of the complex was specifically blocked by

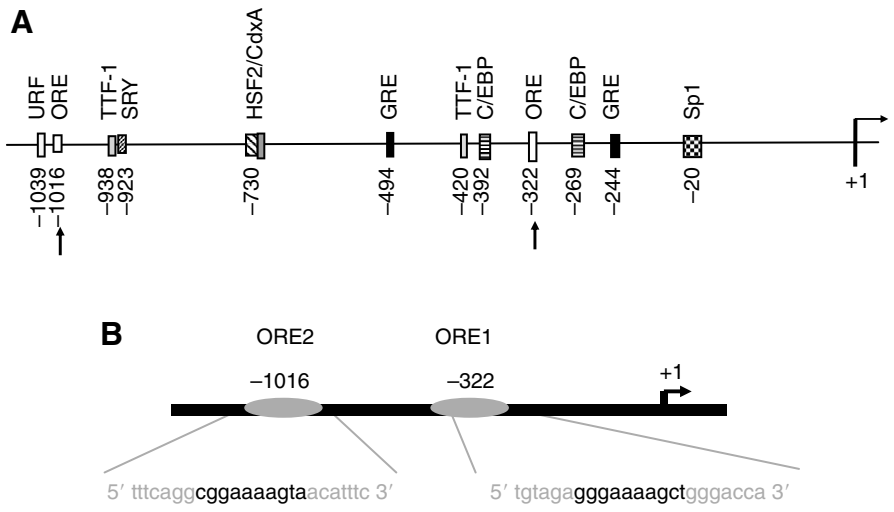

Fig. 1. Putative transcription factor binding sites (TFBS) in the *FhDio2*. (A) Schematic representation of the 1.3 kb promoter region of *FhDio2* showing the localization of putative TFBS. Numbers refer to the position of the starting nucleotide of the element upstream of the transcription start sequence. Black arrows indicate the position of the two putative ORE-binding sites. (B) The sites and the corresponding oligonucleotide sequences of ORE1 and ORE2 are indicated.

competition with a 100-fold excess of the identical but unlabeled oligonucleotide, as well as when nuclear extracts were omitted from the binding reaction or when a random oligonucleotide was used. Closely coupled in time to this initial putative OREBP nuclear recruitment, both the D2 mRNA concentration and D2 activity (Fig. 4) increased significantly. D2 mRNA attained maximum values 8 h after stress, which coincides with the second and more intense wave of the putative OREBP recruitment. The increase in enzyme activity was slower and became significant 12 h after the osmotic challenge.

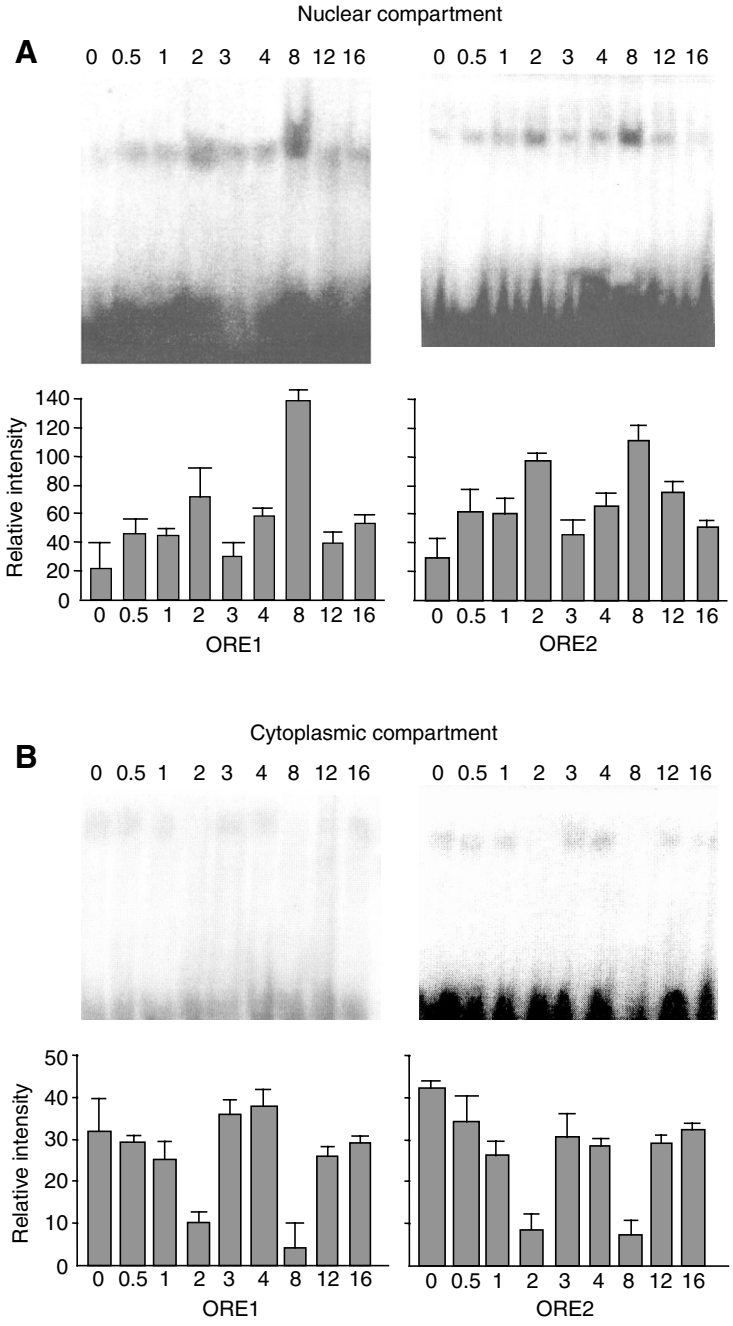

Fig. 2. *In vivo* time course of nuclear recruitment of a putative osmotic response element binding protein (OREBP) after hypo-osmotic stress. (A) Translocation (EMSA, using ORE1 and ORE2 oligonucleotides) of putative OREBPs into the nuclei, and (B) their corresponding disappearance from the cytoplasmic compartment. Protein–DNA binding occurred in a biphasic mode: an initial protein translocation 2 h after hypo-osmotic stress, and a second and more intense wave of recruitment 8 h post-challenge. Panels show representative gels from 4–6 replicates. Below each gel is the corresponding quantification (relative intensity; arbitrary units) of the complexes. Notice the difference in the scales for nuclei (A) and cytoplasmic (B) graphs. Values are means ± s.e.m. of three separate quantifications. *P<0.05.

Liver cells in vitro directly detect extracellular osmotic changes

Consistent with results from the *in vivo* experiments, when liver explants were confronted directly with the hypo-osmotic challenge, there was a significant protein translocation into the nuclei (Fig. 5A). This protein–DNA recruitment is first observed at 2 h and reaches a peak 4 h post-stress. Furthermore, this conspicuous, putative OREBP-translocation response is followed by a significant increase in hepatic D2 activity 2 h later (Fig. 5B).

Tyrosine kinase inhibition blocks putative OREBP binding and D2 activation

To test for the possible participation of tyrosine kinases in the putative OREBP activation, hepatic explants were incubated with 10 μmol l^{-1} genistein 2 h prior to the hypo-osmotic challenge. Subsequently, the explants were challenged with hyposmotic medium for

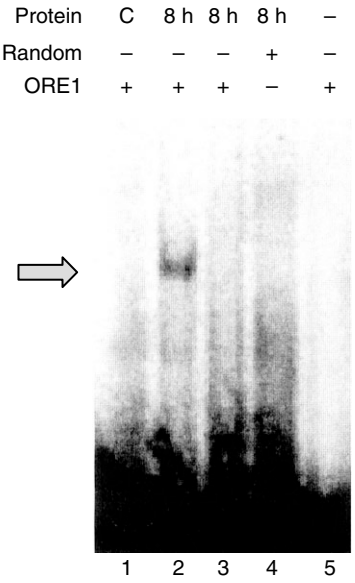

Fig. 3. Specific binding of nuclear proteins to ORE. The specificity of nuclear extracts to radiolabeled ORE1 binding under different experimental conditions is shown. Control (not challenged; column 1) and 8 h post *in vivo* hypo-osmotic stress (column 2); competitive displacement in the presence of a 100-fold excess of unlabeled ORE-1 oligonucleotide (column 3); formation of a nonspecific complex using a random oligonuclotide (column 4), and control without nuclear extract (column 5). The arrow shows the putative OREBP–ORE complex.

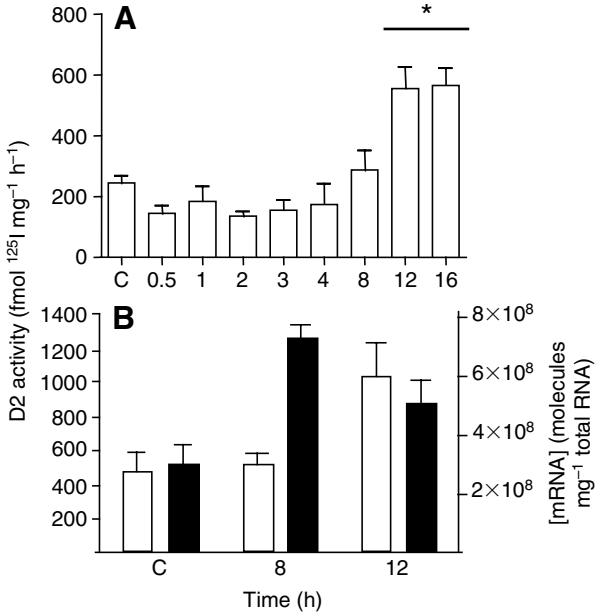

Fig. 4. Up-regulation of D2 mRNA and enzyme activity after hypo-osmotic stress. (A) D2 enzymatic activity from the *in vivo* time-course experiment. The increase in D2 activity became significant only 12 h after the osmotic challenge. (B) A separate set of experiments showing mRNA concentrations (filled bars) and D2 activity (open bars). The increase in mRNA precedes the corresponding rise in D2 activity, and attained maximum values 8 h after osmotic stress. For each panel, values are means ± s.e.m. (N=10) of two separate experiments, each in triplicate. *P<0.05 *vs* control (C).

4 h. Fig. 6 shows that genistein blocked both OREBP-DNA binding and D2 activation. These data suggest the direct participation of a tyrosine kinase pathway in the putative OREBP activation and in the subsequent increase in D2 activity.

Discussion

This work confirms and extends previous preliminary results (Orozco et al., 1998) showing that killifish hepatic D2 increases during hypo-osmotic stress. Indeed, the present studies demonstrate that to cope with a sudden drop in salinity, the liver of killifish triggers a homeostatic response in which a putative OREBP kinase-activated pathway stimulates D2 transcription and enzymatic activity. Furthermore, the participation of this putative signaling pathway is strongly supported by the complete blockade of the response by a tyrosine kinase inhibitor.

In mammals it is well known that hyperosmotic stress activates diverse transcription factors, which in turn regulate key genes that maintain hydrosmotic homeostasis (Ferraris et al., 1999; Gatsios et al., 1998). However, in teleosts, less is known about this transcriptional regulation (Fiess et al., 2007). Moreover, despite the fact that euryhaline fish are constantly coping with osmoregulatory demands, available information about hypo-osmotic stress is even scarcer. Our results clearly demonstrate that in response to hypo-osmotic stress, at least one nuclear protein is recruited into the ORE sequence present in *FhDio2*. Accordingly, we call this protein a 'putative OREBP'. Studies regarding OREBP activation under hypotonicity have been scanty. Recently, *in vitro* studies have shown that the nucleocytoplasmic traffic of human OREBP is a dynamic, bi-directionally functional process. Whereas under isotonic conditions, OREBP is detected in nuclear and cytoplasmic compartments, it accumulates exclusively in the nucleus or cytoplasm when cells are subjected to hypertonic or hypotonic challenges, respectively (Tong et al., 2006). Previous results from our group agree with these findings, since they suggest the opposite pattern in the recruitment of putative OREBPs when rainbow trout (Orozco et al., 2002b) and tilapia (L.-B., unpublished observations) are transferred to seawater. These results, together with new findings (Tong et al., 2006), and the present results, support the proposal that the functional motifs

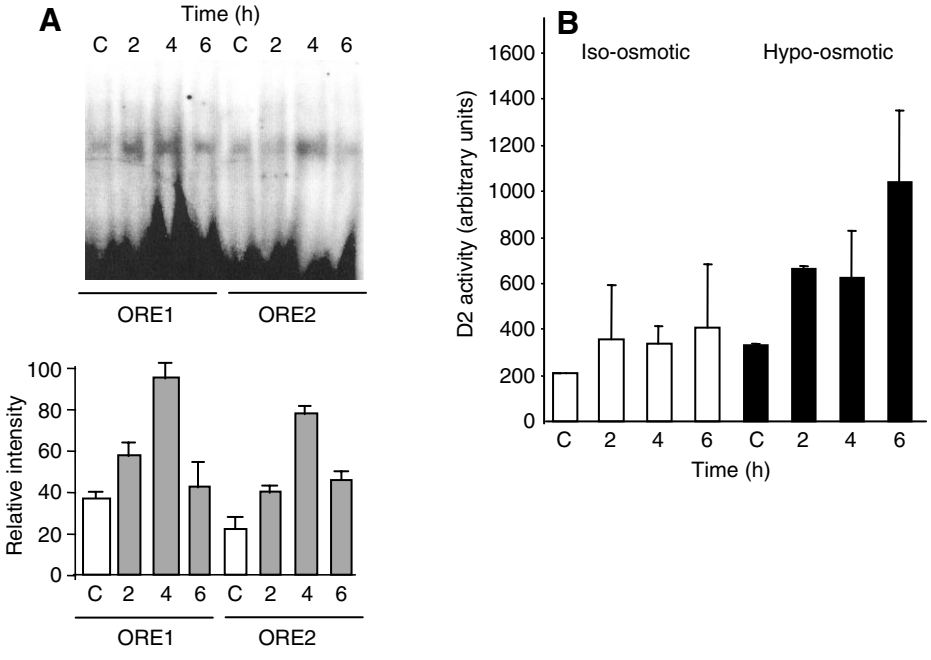

Fig. 5. *In vitro* temporal course of putative OREBP nuclear binding and D2 activity after hypo-osmotic challenge. To assess whether liver cells could detect and respond directly to osmotic changes, liver explants from seawater-adapted killifish were pooled and randomly distributed into iso-osmotic (control, C) or hypo-osmotic L-15 medium. (A) Temporal course of recruitment of putative OREBP into the nuclear compartment. Significant recruitment can be observed as early as 2 h after challenge and peaks at 4 h post-stress. The lower portion of A shows the quantification (relative intensity) of this response. (B) The associated increment in hepatic D2 activity (filled bars). Values are means ± s.e.m. of four separate experiments, each in triplicate. *P<0.05 *vs* control (open bars). Normalized D2 activity is expressed in arbitrary units (see Materials and methods).

contained within the OREBP allow its bi-directional shuttling in response to extracellular osmolarity. It should be noted that at this stage, in contrast to mammals, our studies indicate that putative OREBP translocates into the nucleus during hypo-osmolarity. Clearly, further studies are necessary to fully elucidate the intimate mechanisms that regulate osmotic homeostasis in the different species, especially in those continuously exposed to environmental osmotic demands.

In contrast to the rapid osmolyte movement and the immediate gene transcription that characterize the acute response to osmotic stress (Pasantes-Morales et al., 2006), in the present study the course of events extends for 12 h after challenge. Thus, the time frame of this response is relatively slow, but it coincides with the critical period for adaptation to a salinity change in the killifish (Marshal et al., 1999). Furthermore, within this time period, the present results document a series of events that culminate in providing hepatocytes with active TH. Indeed, both *in vivo* and *in vitro* experiments showed a sequential correlation between the peak in translocation of putative OREBP to the nucleus, the increase D2 transcription, and the subsequent rise in enzymatic activity.

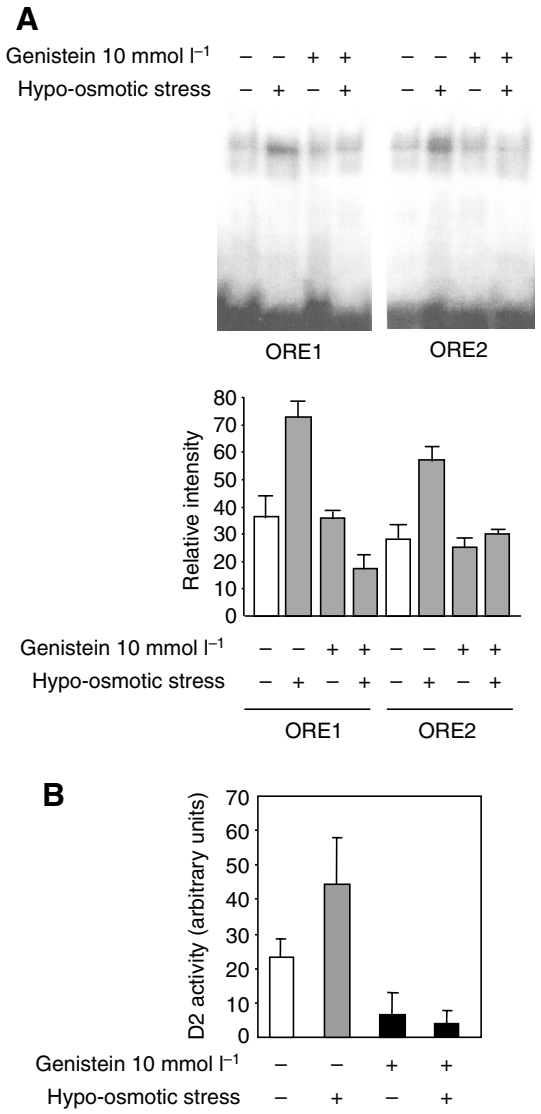

Fig. 6. (A) Genistein *in vitro* blocks nuclear translocation of putative OREBP. The drug prevents both the translocation of OREBP and the associated increase in D2 activity. Notice that even in those explants that were not challenged, genistein reduced both protein–DNA binding and enzyme activity. Lower portion of A shows the corresponding quantification (relatively intensity) of the complexes. (B) The associated D2 activity (means ± s.e.m. of three separate experiments, each in triplicate). Normalized D2 results are expressed in arbitrary units.

Recently, the mammalian liver has been recognized as an important osmosensing and osmosignaling organ. Mammalian hepatocyte swelling or shrinkage triggers an array of intracellular transduction signals that are integrated with those that are hormone- and metabolic-dependant (for a review, see Schliess and Haussinger, 2006). In elasmobranch osmoregulation, the liver is the main provider of organic osmolytes including urea (Hazon et al., 2003). The osmoregulatory role of the teleostean liver has been less documented (Fiess et al., 2007). Osmoregulation is a highly expensive physiological process in terms of metabolic energy. In fish it has been suggested to require from 20% to 50% of the total energy expenditure, and is greater in freshwater than in seawater (Boef and Payan, 2001; Fiess et al., 2007). In addition to supplying glucose and organic osmolytes (Fiess et al., 2007), fish liver contains the largest pool of glutamine, which is the major source of ammonia as well as an important blood carrier for this nitrogenous waste product (for reviews, see Wood, 1993; Haberle et al., 2006). Two key enzymes involved in glutamine and ammonia metabolism are glutamine synthetase and glutamate dehydrogenase, which interestingly, at least in mammals, are both TH-dependent (Doulabi et al., 2002). In this context it is paradoxical that the current dogma considers the role of TH in osmoregulation to be indirect. Indeed TH have been thought to support long-term adaptive responses mediated by growth hormone, prolactin and cortisol, among other classical osmoregulatory messengers (Sakamoto and McCormick, 2006). However, previous studies from our laboratory (Orozco et al., 1998; Orozco et al., 2002b) and recent studies in the seabream gill (Klaren et al., 2007), strongly suggest a more direct involvement of TH in the hydro-osmotic balance in fish. Furthermore, our present results support the suggestion that a putative hypo-osmotic, OREBP-mediated increase in hepatic D2 activity could be an important endocrine component for the maintenance of hydro-osmotic homeostasis in fish. Thus, we hypothesize that the local intra-hepatic T_3 increase that follows D2 activation during hypo-osmotic stress may be instrumental for promote the hepatic synthesis of ammonia, providing ammonium as a counter-ion for sodium absorption in the gill (Salama et al., 1999).

In summary, this study examined the possible transcriptional regulatory role played by the ORE motifs in the 5′ promoter region of *FhDio2* during hypo-osmotic stress. Together, the present results provide strong evidence for the involvement of a putative OREBP kinase-dependant pathway as an important regulatory signal for *FhDio2* transcription. To our knowledge, this is the first report that associates a response to hypo-osmotic stress with an ORE-regulated gene. The fact that this gene corresponds to the enzyme responsible for the local supply of intracellular T_3 strongly suggests that, in addition to their

permissive action, TH may play a more direct role in osmoregulatory homeostasis in fish, possibly by participating in hepatic ammonia metabolism. These findings may provide a clue to understanding the physiological function of TH in hydro-osmotic homeostasis in fish.

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