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Regulation of osmotic stress transcription factor 1 (Ostf1) in tilapia (*Oreochromis mossambicus*) gill epithelium during salinity stress

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Summary

Mechanisms of induction of osmotic stress transcription factor 1 (Ostf1) were analyzed in gill epithelium of tilapia exposed to salinity stress. Experiments with primary cultures of gill epithelial cells revealed that hyperosmotic Ostf1 induction was independent of systemic factors. In addition, the synthetic glucocorticoid receptor agonist dexamethasone did not affect Ostf1 levels, arguing against cortisol being the signal for Ostf1 induction during hyperosmotic stress. Exposure of primary gill cell cultures to a hyperosmotic agent that is cell permeable and non-hypertonic (glycerol) did not trigger Ostf1 induction. However, when gill cells were exposed to hypertonicity (either in the form of NaCl or other forms) Ostf1 was rapidly and significantly induced. Analysis of hnRNA and mRNA levels revealed that Ostf1 upregulation in gill cells of intact fish and primary cultures of gill epithelial cells was mediated by transient mRNA stabilization. In addition to the initial transient mRNA stabilization a subsequent transcriptional induction of Ostf1 was observed. In cultured gill cells increase in Ostf1 mRNA synthesis was stable and very potent, whereas in gill cells of intact fish this increase was transient. This observation suggests positive feedback by Ostf1 or one of its targets and negative feedback by systemic factors on Ostf1 transcription.

We conclude that Ostf1 induction in gill epithelial cells of tilapia exposed to salinity stress (1) is independent of cortisol or other systemic factors; (2) depends on hypertonicity as the signal; and (3) is based on transient mRNA stabilization. Moreover, our data on primary cell cultures show that systemic signals are necessary to prevent sustained transcriptional induction of Ostf1 during hyperosmotic stress, indicating feedback regulation and a high degree of complexity of osmosensing and signaling networks in euryhaline fishes.

Key words: osmoregulation, teleost, stress signaling, salinity adaptation, gill epithelial cell, tilapia, *Oreochromis mossambicus*.

Introduction

Euryhaline teleosts are osmoregulators that maintain plasma osmotic homeostasis even in the face of fluctuating environmental salinity. During the acclimation of euryhaline fish from freshwater (FW) to seawater (SW) gill epithelium is extensively remodeled to account for altered requirements of ion transport and permeability. Remodeling includes changes in turnover of gill epithelial cells, altered differentiation patterns of gill epithelial cells, and modulation of expression and activity of many proteins, including ATPases, secondary active ion transporters, and structural proteins (Evans, 2002). In the gill epithelium numerous ion and water transporters are regulated at the mRNA level by salinity. Examples of this regulation include subunits of the Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter (Tipsmark et al., 2002), urea transporter (Mistry et al., 2001a), taurine transporter (Takeuchi et al., 2000) and aquaporin 3 water channel (Cutler and Cramb, 2002). In addition, other mRNAs such as 14-3-3 and C-type lectin are also regulated by osmolality in fish gills (Kültz et al., 2001; Mistry et al., 2001b). These responses could potentially be regulated by increases in the synthesis rate of the corresponding mRNAs and/or by mRNA stabilization mechanisms. Based on reports on mechanisms of osmotic regulation in mammalian cells it is likely that variations in mRNA abundance of these transporters result from transcriptional regulation (Burg et al., 1996).

Knowledge of transcriptome regulation during salinity adaptation of euryhaline fishes is still far from comprehensive but recent work in this field could shed light on mechanisms of osmosensory signal transduction. We demonstrated recently that Ostf1 (osmotic stress transcription factor 1) is early and transiently upregulated in gill epithelial cells from tilapia exposed to hyperosmotic stress at both the mRNA and protein levels (Fiol and Kültz, 2005). The function of this protein is not yet discerned, but, based on protein structure similarity studies, Ostf1 could be a transcriptional regulator. Tilapia Ostf1

contains DNA binding motifs such as Q-rich regions, as well as the signature motif of the TSC-22/GILZ/DSIPI protein family. Several members of this protein family have been characterized as transcriptional regulators (Mittelstadt and Ashwell, 2001; Shibanuma et al., 1992).

Tilapia Ostf1 has the highest sequence similarity to the mammalian glucocorticoid induced leucine zipper (GILZ) (Fiol and Kültz, 2005), suggesting that Ostf1 could also respond to glucocorticoids. In accordance with that notion, plasma cortisol levels increase with hyperosmotic stress (reviewed by Evans et al., 2005). Cortisol, referred to as the 'seawater-adapting' hormone, is involved in the upregulation of several hyperosmotic stress responsive genes (Mommsen et al., 1999). It is therefore possible that cortisol represents a signal for the induction of Ostf1 during hyperosmotic stress. Thus, we conducted the present study to discern the signals and mechanisms involved in the induction of Ostf1 in tilapia gill epithelial cells during salinity stress.

Materials and methods

Animals

Black tilapia (*Oreochromis mossambicus* Peters) were maintained in large (1 m×20 cm diameter) tanks supplied with flow-through, heated (26–28°C) Davis wellwater (=FW, Na^+=28 mg l^{-1}, K^+<5 mg l^{-1}, Ca^{2+}=33 mg l^{-1}, Mg^{2+}=36 mg l^{-1}, pH 8.0) at the Center for Aquatic Biology and Aquaculture (CABA) of the University of California, Davis, USA. Two days prior to treatments, the fish were transferred to 90.8 liter recirculation aquaria containing FW at 26–28°C to minimize handling stress during experiments. Experiments began when tilapia were transferred to aquaria containing either full-strength SW (1000 mOsmol kg^{-1}) or FW (handling stress control). Fish were sampled at the times indicated, gills were perfused, and gill epithelium was collected by scraping off from the cartilage of individual gill arches as described in previous work (Kültz and Somero, 1995). Gill epithelium was utilized for subsequent RNA or protein isolation.

Cell cultures

Experiments performed with epithelial gill cell primary cultures (EGCPC) were carried out using epithelial gill tissue scraped off the cartilage and dispersed into <1 mm^3 tissue pieces. FW acclimated fish were used as a source of EGCPC, which were seeded in plastic tissue culture dishes. Cells were cultured in minimum essential medium (MEM)–Eagle's medium (ATCC, Manassa, VA, USA) at 25°C and atmospheric oxygen and CO_2. Isosmotic medium had an osmolality of 300 mOsmol kg^{-1} and hyperosmotic media were prepared by the addition of the appropriate amount of NaCl to isosmotic medium yielding a medium osmolality of 600, 900 or 1100 mOsmol kg^{-1} of H_2O. When indicated, choline chloride, sodium gluconate, mannitol or glycerol was added instead of NaCl for hyperosmotic media preparation. Final osmolality of all media was verified with a micro-osmometer (Model 3300, Advanced Instruments, Norwood, MA, USA). Dexamethasone (DEX; D-4902, Sigma, St Louis, MO, USA) was added to a final concentration of 1 μmol l^{-1} when indicated. Controls with a vehicle (ethanol) were always run in parallel.

RNA isolation

Total RNA from gill epithelial cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer. RNA was treated with DNase (Turbo DNA free, Ambion, Austin, TX, USA). Purity and quality was confirmed and quantity determined by measuring absorbance of the samples at 260 and 280 nm with a Beckman DU520 spectrophotometer and by denaturing electrophoresis.

DNA synthesis and real-time qPCR

RNA (2 μg) was reverse-transcribed using Superscript III first strand synthesis reagents (Invitrogen) with a random hexamer:oligo(dT) mix (1:1) as primers. Abundance of selected transcripts was quantified by quantitative real-time PCR (qPCR) with a PRISM 7500 real-time thermal cycler (Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate using 20 μl reaction volumes with SYBR Green PCR Master Mix (Applied Biosystems) or Full Velocity SYBR Green Mix QPCR Master Mix (Stratagene), and 30 pmol of each primer. PCR conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were collected at 60°C. In the osmotic stress experiments, β-actin expression was the choice for normalization based on previous experiments (Fiol and Kültz, 2005; Fiol et al., 2006). In the mRNA decay experiments normalization was done using 18S rRNA levels, based on its high stability and higher reliability of obtaining remaining mRNA values. Relative expression ratios (referred to also as mRNA fold induction or mRNA relative abundance) were calculated using the method of Pfaffl (Pfaffl, 2001):

$$\text{Expression ratio} = E_{\text{target}}^{\text{CTtarget (control–treated)}} / E_{\text{normalizer}}^{\text{CTnormalizer (control–treated)}},$$

where $E=10^{(-1/\text{slope})}$, with the slope referring to the standard curve of the target genes (in Fig. 4D,E) or the normalizer gene (not shown, determined analogously E =1.90 for β-actin and E =1.94 for 18S).

qPCR primer sequences were: β-actin forward: CCACAGCCGAGAGGGAAAT, β-actin reverse: CCCATCTCCTGCTCGAAGTC, 18S forward: CGGACACGGAAAGGATTGAC, 18S reverse: CAACTAAGAACGGCCATGCA, Ostf1 forward: TGCGTGAGGAGGTGGAGATC, Ostf1 reverse: GTAACAGCACATCCGTCGGAAT, hnOstf1 forward: TTCAAAGTGACAATAGCCAGAAAGTT, hnOstf1 reverse: GCCTTTAGTTAAGAAATCCAGCAAA.

PCR and sequencing

PCR was performed using the Advantage HF-2 PCR kit (Clontech, Mountain View, CA, USA) in a MasterCycler (Eppendorf, Westbury, NY, USA) using the following cycling parameters: 94°C for 1 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and then 72°C for 5 min. PCR

products were extracted from agarose gels using GeneClean system (Q-BIO gene, Irvine, CA, USA) and then double-pass sequenced on an ABI 3730 automated DNA sequencer.

Bioinformatics and statistics

Sequence alignments were done using the ENSEMBL Blast search utility [www.ensembl.com (Hubbard et al., 2005)]. Statistical analyses for identifying significant differences from the control were performed using REST software (Pfaffl et al., 2002). REST uses the pair-wise fixed reallocation randomization test to calculate the significance of results. Differences at $P<0.001$ were considered significant. The REST software used for statistical analyses represents an established method for analyzing real-time PCR results directly. In addition, significant differences of relative increases between mature Ostf1 and hnOstf1 were assessed using unpaired *t*-test carried out with SigmaPlot 9.0 (Systat). Significance threshold was set at $P<0.05$ and data are presented as mean ± standard error of mean (s.e.m.).

Results

Ostf1 is upregulated in epithelial gill cell primary cultures

We showed previously that Ostf1 mRNA is early and transiently upregulated in gill cells from tilapia exposed to hyperosmotic stress. Thus, the suitability of epithelial gill cell primary cultures (EGCPC) as a model for studying hyperosmotic Ostf1 mRNA up-regulation was analyzed. Cells from FW acclimated tilapia were isolated and the primary cell cultures were exposed for 2 h to acute hyperosmotic stress by adding concentrated NaCl to increase medium osmolality from 300 mOsm kg^{-1} (isosmotic medium) to 600, 900 or 1100 mOsm kg^{-1}. Ostf1 mRNA levels were analyzed by qPCR and showed increases of 10.5±3-fold at 600 mOsm kg^{-1} and two- to threefold at 900 and 1100 mOsm kg^{-1} (Fig. 1A).

Considering the participation of cortisol in SW adaptation in fish, as well as sequence similarity between Ostf1 and the mammalian glucocorticoid responsive GILZ protein, we decided to test glucocorticoid involvement in the Ostf1 induction pathway. The synthetic glucocorticoid dexamethasone (DEX) was used in the following experiment as a potent cortisol receptor agonist. The concentration of DEX was optimized based on previous reports of its use as a glucocorticoid receptor agonist in fish gill cells (McCormick and Bern, 1989) and other tissues [e.g. rat hepatocytes (Wehner and Tinel, 1998)] under hypertonic stress. When media were supplemented with 1 μmol l^{-1} DEX, the induction pattern was the same as that of the controls (Fig. 1A), and were essentially identical to the vehicle treatments (not shown). Whereas no change was observed in isosmotic medium, Ostf1 mRNA was 13±3-fold induced at 600 mOsm kg^{-1}, and three- to fourfold increased at 900 and 1100 mOsm kg^{-1} when DEX was added to the medium (Fig. 1A). Thus, glucocorticoid receptor activation is not responsible for hyperosmotic OSTF1 up-regulation and did not significantly alter the hyperosmotic induction of Ostf1 in gill epithelial cells.

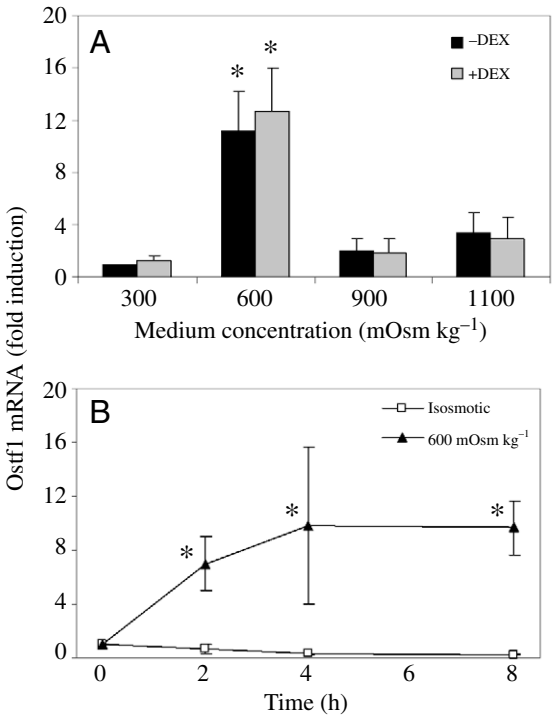

Fig. 1. Ostf1 mRNA response to hyperosmotic stress and dexamethasone (DEX) in EGCPC. (A) Primary cultures were exposed to isosmotic medium or different hyperosmotic media in the absence (black bars) or with the addition of 1 μmol l^{-1} DEX (grey bars). After 2 h treatment, cells were collected and mRNA quantified by qPCR. Relative Ostf1 mRNA abundances with respect to control (isosmotic medium, no DEX) and normalized to β-actin content are shown. Values are means ± s.e.m. (N=3). Asterisks indicate significant differences ($P<0.05$) relative to the control. (B) Primary cultures were exposed to isosmotic medium (open squares) or 600 mOsm kg^{-1} hyperosmotic medium (black triangles) prepared by addition of NaCl. At the indicated times cells were collected and mRNA quantified by qPCR. Relative Ostf1 mRNA abundances with respect to control (time zero) and normalized to β-actin content are shown. Values are means ± s.e.m. Asterisks indicate significant differences ($P<0.05$) relative to the control. N=3.

A time course experiment was performed in order to evaluate the dynamic nature of the response. EPCGC were exposed to either isosmotic medium or 600 mOsm kg^{-1} hyperosmotic medium with the addition of NaCl. Ostf1 mRNA was increased at comparable levels at 2, 4 and 8 h (Fig. 1B). Interestingly, this sustained increase differed from the time course previously obtained with whole fish exposed to hyperosmotic stress where the induction was transient, peaking at 2 h and reaching background levels after 6–8 h (Fiol and Kültz, 2005).

Ostf1 is up-regulated by hypertonic stress

Our results presented above show that gill epithelial cells are able to sense hyperosmolality independently of systemic stimuli such as cortisol and respond by increasing the Ostf1 mRNA steady state level. In order to assess the nature of the stimulatory signal more closely, different hyperosmotic media

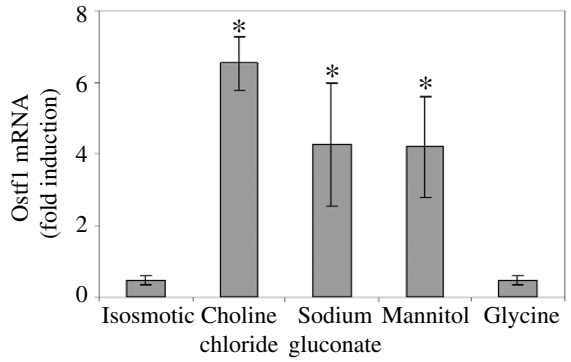

Fig. 2. Ostf1 mRNA response to different hyperosmotic media in EGCPC. Osmolality was increased from 300 to 600 mOsmol kg^{-1} by addition of the indicated compounds. After 2 h, cells were collected and Ostf1 mRNA quantified by qPCR. Relative mRNA abundance with respect to control (isosmotic medium) and normalized to β-actin content are depicted. Values are means ± s.e.m. (N=3 experiments). Asterisks indicate significant differences (P<0.05) relative to the control.

were tested for their ability to trigger Ostf1 upregulation. EGCPC were exposed for 2 h to acute hyperosmotic stress by adding either choline chloride, sodium gluconate, glycerol or mannitol to increase the osmolality from 300 mOsm kg^{-1} (isosmotic medium) to 600 mOsm kg^{-1}.

Only hypertonic media (prepared with the addition of choline chloride, sodium gluconate or mannitol) were able to induce Ostf1 upregulation (Fig. 2). Ostf1 induction was of similar magnitude for all hypertonic media (five- to sevenfold). On the other hand, hyperosmotic medium prepared with glycerol did not significantly alter the Ostf1 transcript level (Fig. 2).

Analysis of the mechanism of Ostf1 mRNA upregulation

Determination of intronic Ostf1 sequence and validation of qPCR quantification

In principle, steady-state Ostf1 mRNA levels can be upregulated either through an increase in the transcription rate or through a mRNA stabilization mechanism decreasing the degradation rate of the mRNA. In order to assess which of these mechanisms applies we decided to analyze Ostf1 mRNA synthesis rate during hyperosmotic stress.

We analyzed the effect of hyperosmotic stress on the transcription rate of the Ostf1 gene using the assay of Elferink and Reiners, Jr (Elferink and Reiners, Jr, 1996), which measures the quantity of nascent, unspliced transcript (hnRNA) from a particular gene using quantitative real-time RT-PCR, representing a surrogate measure of the gene transcription rate.

Knowledge of the sequence of unspliced mRNA is required to carry out this assay. Because tilapia Ostf1 nucleotide sequence was published as cDNA and the tilapia Ostf1

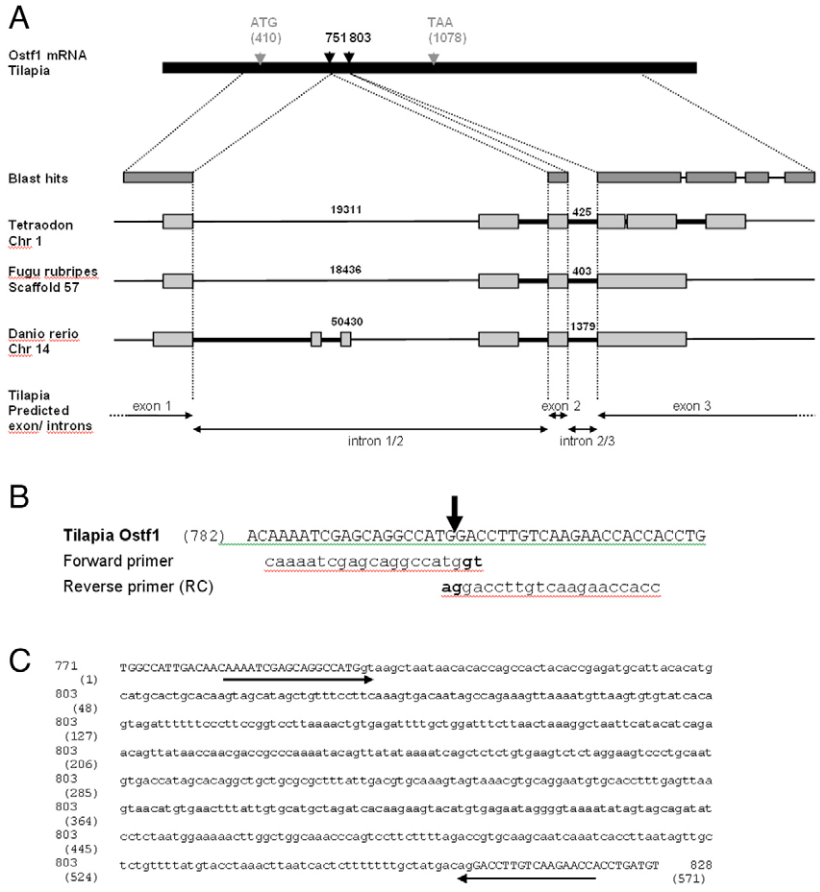

Fig. 3. Identification of intronic sequences of tilapia Ostf1. (A) Possible exon–exon junctions were determined through an analysis of synteny between *Tetraodron nigroviridis*, *Fugu rubripes* and *Danio rerio* genomic sequences based on homology to full-length tilapia Ostf1 mRNA (GenBank AY679524). Blast hits link regions of high similarity between Ostf1 mRNA and the three genomic sequences identified using BLAST. Gray boxes along the genomic representations indicate transcripts predicted using the Ensembl bioinformatics tool. Tilapia exon–exon junctions, predicted from the conserved gene structure of Ostf1 paralogs, are indicated by black arrows over tilapia Ostf1 mRNA. Lengths of predicted introns 1/2 and 2/3 are indicated in base pairs for each genomic representation. (B) Detail of exon 2–exon 3 junction region in tilapia Ostf1 mRNA. The arrow indicates the predicted junction site. PCR primers designed to amplify intron 2/3 are shown. Bold letters in the primer sequences correspond to conserved 3′ acceptor and 5′ donor consensus sites. (C) Genomic sequence of tilapia Ostf1 intron 2/3 (lowercase), flanked by exons (uppercase). Arrows indicate the position of PCR primers used for amplification. Numbers indicate the sequence position in Ostf1 mRNA and relative intron length (between parentheses).

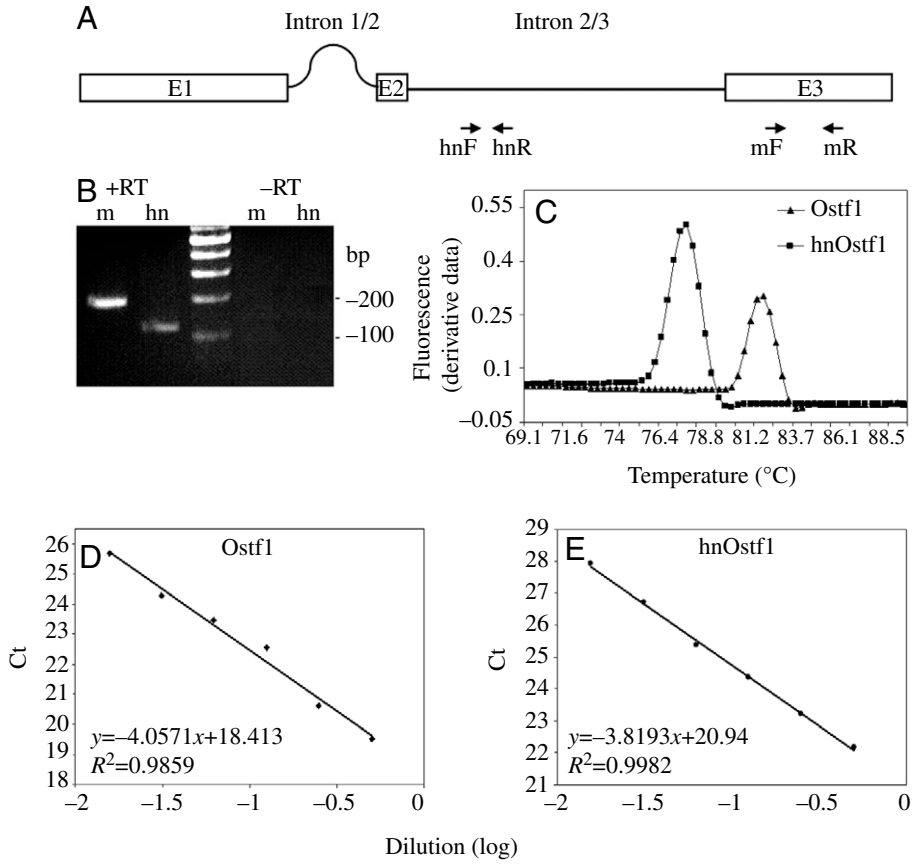

Fig. 4. Validation and specificity of qPCR primers and reactions. (A) Proposed partial intron/exon structure for tilapia Ostf1. Exons (boxes, E1–3) and introns (lines) are depicted. Location of qPCR primers for the mature (mF and mR) and nascent (hnF and hnR) mRNAs are indicated. (B,C) Gel electrophoresis of the qPCR products (B) and dissociation curve profiles (C). (D,E) Standard curves of qPCR reactions for Ostf1 mRNA (D) and hnRNA (E) obtained with serial dilutions of cDNA. The equation of the linear correlation and the correlation coefficient (*R*) are indicated.

genomic sequence was unavailable, we carried out a homology analysis between tilapia Ostf1 cDNA and genomic sequences of *Danio rerio*, *Fugu rubripes* and *Tetraodon nigroviridis.* Based on the structure of the orthologous loci we were able to determine possible exon-exon boundaries in the tilapia Ostf1 cDNA. Subsequent PCR-based cloning yielded an intronic Ostf1 sequence, not present in the mature mRNA (and cDNA). All three analyzed genomes predicted a transcript with an exon composition that matched the tilapia Ostf1 cDNA 3′ region. On the other hand, sequences homologous to the tilapia Ostf1 cDNA 5′ region were predicted by ENSEMBL as part of different transcripts. In these three genomes, sequences homologous to the 3′ part of tilapia Ostf1 cDNA were located quite distantly (~20 000 bp in *Fugu* and *Tetraodon* and ~50 000 bp in *Danio*) from the regions that are homologous to the 5′ part of tilapia Ostf1 cDNA.

Having identified the conserved sequence features among the genomes compared, we were able to predict that tilapia genomic Ostf1 constitutes at least three exons encompassing the entire ORF (Fig. 3A). The lengths of the introns located between the predicted exons were relatively conserved in the three fish genomes. Intron 1/2 was very long in every case (~20 000 bp in *Tetraodon and Fugu* and ~50 000 bp *Danio*) but intron 2/3 was shorter (403, 425 and 1079 bp in *Tetraodon*, *Fugu* and *Danio*, respectively). Exon 2 showed a high degree of sequence conservation, with a length of 52 bp in all three species, firmly supporting the prediction of tilapia Ostf1 exon–exon boundaries.

Based on the exon–exon boundary sites determined, we designed specific PCR primers flanking the exon2/exon3 boundary to amplify intron 2/3. PCR primers were designed based on the cDNA sequence plus the addition of two nucleotides of the 3′ acceptor and 5′ donor consensus sequences (5′-G/gt–ag/G-3′) in order to avoid amplification of the more abundant mature mRNA (Fig. 3B). A single PCR amplification product of ~700 bp was obtained and further purified and sequenced (Fig. 3C).

A schematic structure of Ostf1 unspliced mRNA showing the exonic and intronic regions identified by this analysis, is depicted in Fig. 4A. The intron 2/3 sequence was then used for the design of specific Ostf1 hnRNA qPCR primers. Validation of Ostf1 mRNA and hnRNA qPCR primers is shown in Fig. 4B–E. One specific band was detected in agarose gel electrophoresis of the PCR products, and a single peak was observed in the melting curve analysis of the qPCR for each reaction (Fig. 4B,C). No bands were detected in the minus RT samples (Fig. 4B), indicating the absence of genomic DNA contamination and hence validating the suitability of the method. Standard curves produced with serial dilutions of the cDNA templates are also shown (Fig. 4D,E). Different samples were used in each standard curve for the preparation of serial dilutions in order to overcome the relatively lower content of hnRNA compared to the mature mRNA (ΔCt 5.18±0.45, *N*=46). Ostf1 mRNA-specific primers are, as depicted in Fig. 4A, able to also amplify the hnRNA. However, given the much higher content of the mature RNA no corrections of the

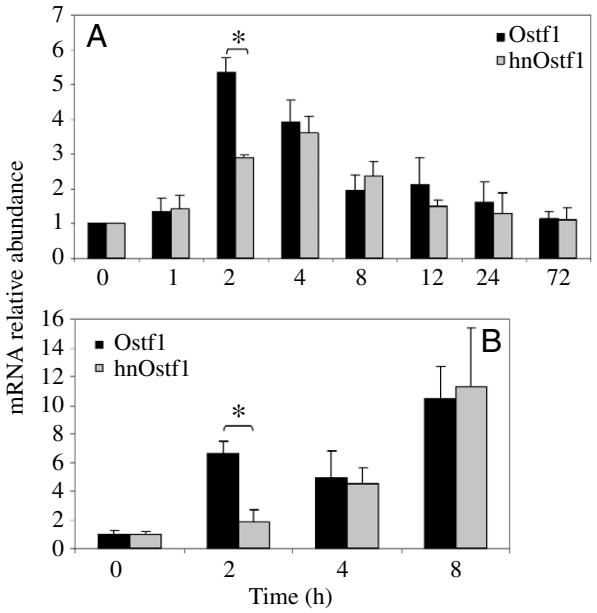

Fig. 5. Analysis of Ostf1 mRNA transcription rates. Intact fish (A) or EGCPC (B) were exposed to hyperosmotic stress (freshwater to seawater transfer for intact fish, and from 300 to 600 mOsmol kg^{-1} exposure for EGCPC). At the indicated times samples were collected and Ostf1 mature (Ostf1) and nascent (hnOstf1) mRNAs were quantified by qPCR. Relative mRNA abundance with respect to control (time 0) and normalized to β-actin content are depicted. Values are means ± s.e.m., *N*=3. Asterisks indicate significant differences between Ostf1 and hnOstf1 (*P*<0.05).

data were made because the error was not significant in the context of the biological and experimental variation (a ΔCt value of 5.18 implies a 36-fold difference in absolute amount between Ostf1 mature mRNA and hnRNA, amounting to an error of only 2.8%).

Quantification of hnRNA

Based on the work of Elferink and Reiners, Jr (Elferink and Reiners, Jr, 1996) the synthesis rate of a specific mRNA can be estimated through the quantification of the steady state of the immature hnRNA. Thus, we quantified hnOstf1 and Ostf1 mRNA levels using the validated specific qPCR primers in gill epithelial cells of fish exposed to hyperosmotic stress in a time course experiment. Significant differences between total mRNA and hnRNA increases were detected only after 2 h of exposure to hyperosmotic stress (Fig. 5A). Whereas total mRNA increased about fivefold, hnRNA only increased less than threefold. At later time-points, similar ratios of both kinds of transcripts relative to controls were detected. These results suggest that two mechanisms are responsible for Ostf1 mRNA increase. First, mRNA stabilization occurs very rapidly and early (within 2 h of hyperosmotic stress). Second, this is followed by an increase in the mRNA synthesis rate (from 2 to 6 h), perhaps as a result of autoregulatory feedback.

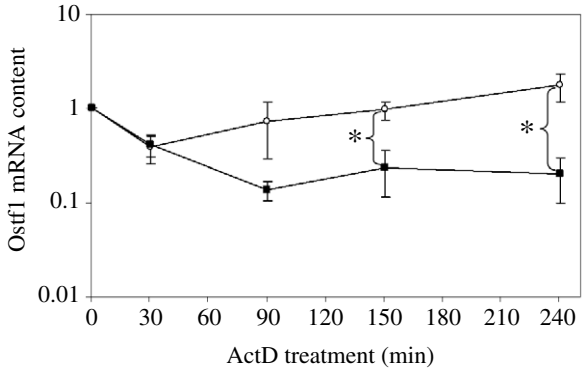

Fig. 6. Stability of Ostf1 transcripts. EGCPC were pre-incubated for 2 h with 5 μg ml^{-1} actinomycin D in isosmotic medium (300 mOsmol kg^{-1}). Treatments were initiated at time 0 for isosmotic controls (black squares) and cells exposed to hyperosmotic stress (600 mOsmol kg^{-1}, NaCl; open circles). Ostf1 mRNA levels were determined by quantitative real-time PCR and normalized to 18S RNA. Remaining mRNA relative to time 0 is shown on a logarithmic scale. Asterisks indicate significant differences between pairs (*P*<0.05). Values are means ± s.e.m., *N*=4.

When a similar analysis was done in EGCPC equivalent results were obtained (Fig. 5B). Differences between total mRNA and hnRNA were observed only after 2 h of exposure. At this time, total mRNA increased sixfold whereas hnRNA increased only twofold. At 4 and 8 h, comparable increases for both total mRNA and hnRNA were found. Results depict again a mRNA stabilization event early on (~2 h) followed by an increase in mRNA synthesis rate (Fig. 5B). Whereas timing of mRNA stabilization was identical in intact fish and isolated cells, the effect of hyperosmolality was more pronounced and sustained in cultured cells, suggesting negative feedback by systemic factors on Ostf1 transcription.

Analysis of mRNA stability of Ostf1

In order to further validate the mRNA stabilization component of the Ostf1 induction mechanism, we analyzed the response of Ostf1 mRNA to hyperosmotic stress in EGCPC incubated with the RNA synthesis inhibitor actinomycin D. In cells kept under isosmotic conditions, a decline in Ostf1 mRNA amount was observed, which is due to its degradation over time (Fig. 6). Based on the time course of mRNA degradation in the presence of actinomycin D a half-life shorter than 30 min was

Fig. 7. Localization of adenine-uridine rich elements (ARE) in the 3′ untranslated region of tilapia Ostf1 mRNA. Localization of Ostf1 open reading frame (ORF) and ARE are indicated (in base pairs) along full-length tilapia Ostf1 cDNA (AY679524).

estimated for Ostf1 mRNA, which is indicative of a typical immediate early gene. When EGCPC were exposed to hypertonic stress, Ostf1 degradation rate was significantly decreased. After the initial 30 min during which the values were the same as in isosmotic controls, Ostf1 mRNA levels remained significantly higher compared to isosmotic controls (Fig. 6). These results confirm that mRNA stabilization is involved in the rapid upregulation of Ostf1 mRNA during hyperosmotic stress.

Analysis of the Ostf1 mRNA structure

Messenger RNA stabilization is a mechanism generally associated with the binding of stabilizing proteins to the 3′ UTR of the mRNA (Guhaniyogi and Brewer, 2001). Messenger RNAs regulated by this mechanism usually have an extended 3′UTR containing specific sequences known as adenine/uridine-rich elements (AREs). These elements mediate the rapid turnover of several mRNAs encoding proteins involved in the regulation of cellular growth and responses to exogenous agents (Chen and Shyu, 1995). Ostf1 3′UTR is more than 2200 bp long and includes four Class I AREs, containing the motifs AUUUA or AUUUUA and one Class III ARE, constituting a U-rich region (Fig. 7). These structural characteristics support a rapid mRNA stabilization mechanism as observed in the hyperosmotic upregulation of Ostf1 mRNA abundance.

Discussion

In the present study we characterized the mechanisms of regulation of the hyperosmotic stress-specific putative transcription factor Ostf1. We also provided evidence that EGCPC represents a suitable and useful model for studying effects of hyperosmotic stress. The suitability of the EGCPC system has been established in previous experiments (e.g. Kültz, 1996) and many genes induced in gill cells *in vivo* are also induced in EGCPC, including Hsp70 (Kültz, 1996) and seven novel hyperosmotic stress responsive genes (Fiol et al., 2006). EGCPC showed only a substantial increase of Ostf1 mRNA at 600 mOsm whereas 900 and 1100 mOsm kg^{-1} were close to control. Apical membranes are generally less permeable than basolateral membranes of cells. Therefore, our data suggest that osmolalities significantly exceeding those seen at the basolateral side of gill cells *in vivo*, overwhelm the adaptive system of isolated EGCPC and prevent the adaptive increase in Ostf1.

Upregulation of Ostf1 was observed in EGCPC, indicating that gill epithelial cells respond to the hyperosmotic stress directly at the cellular level. This capability of gill cells suggests that exogenous systemic factors such as cortisol are not required for Ostf1 induction during hyperosmotic stress, despite strong homology of Ostf1 to mammalian glucorticoid-induced leucine zipper (GILZ) protein. Indeed, further experiments showed that gill epithelial cells do not induce Ostf1 in response to dexamethasone (a potent glucocorticoid receptor agonist) confirming that corticosteroids are not required for hyperosmotic Ostf1 induction. This finding was unexpected since cortisol is a corticosteroid hormone that is critical for seawater acclimation and glucocorticoid receptor is induced by hyperosmolality in euryhaline teleosts (Scott et al., 2004). It promotes adaptive changes in response to hyperosmotic stress, including chloride cell differentiation and proliferation in tilapia and salmonid gills (McCormick, 1990; Madsen, 1990a; Madsen, 1990b), and stimulates branchial Na^+/K^+-ATPase activity in a variety of fishes (Madsen et al., 1995; McCormick, 1995). However, cortisol proved unable to stimulate other adaptive responses such as active ion transport across tilapia opercular epithelium (Foskett et al., 1981).

Exploiting the advantages of the EGCPC model, we analyzed the nature of the Ostf1 induction signal by testing the effect of different hyperosmotic media. These experiments demonstrate that hyperosmotic medium *per se* (increased glycerol) is insufficient to elicit Ostf1 induction, which occurs only when hyperosmotic media are prepared with non-permeable solutes. Thus, hypertonicity is required to elicit Ostf1 induction. Our findings are in agreement with data on other cell types exposed to hyperosmotic stress. For instance, in mammalian renal medullary cells hypertonicity is also the signal for induction of mRNAs of the TonEBP transcription factor and of multiple genes involved in compatible osmolyte accumulation, and protein and DNA stabilization (Woo et al., 2000; Chakravarty et al., 2002). The molecular nature of the hypertonicity signal is not yet known. Hypertonicity is known to cause many secondary effects including cell shrinkage, macro- and micromolecular crowding, changes in the organization of cell membranes, altered water movements across cell membranes (osmosis), and stress on the cytoskeleton (Kültz and Burg, 1998). Such secondary effects are independent of the particular solutes responsible for hypertonicity, and indeed our results illustrate that there is no specific sodium or chloride ion requirement for Ostf1 induction. Therefore, we conclude that one or more of the above-mentioned secondary effects associated with hypertonicity are responsible for Ostf1 induction.

Using two different approaches (calculation of mRNA synthesis rate after hnRNA quantification, and measurement of mRNA decay after blocking mRNA synthesis) we found that Ostf1 upregulation in tilapia gill epithelial cells exposed to hyperosmotic stress is based initially on rapid mRNA stabilization. Ostf1 mRNA synthesis rate was quantified using a previously validated method (Elferink and Reiners, Jr, 1996). This method measures mRNA transcription rates *via* quantification of the steady state of nascent hnRNA. Transcriptional activities determined this way were previously validated by equivalent results obtained with nuclear run-on assays, representing an alternative method of transcription rate determination. However, compared to nuclear run-on assays, the method used in our study has the advantages of being more sensitive, easier to perform, and suitable for a broader range of target tissues compared with nuclear run-on assay.

In addition, analysis of the Ostf1 3′UTR revealed the presence of regulatory elements characteristic of mRNAs

regulated by mRNA stabilization. This initial mRNA stabilization is followed by augmentation of the mRNA synthesis rate. Both mechanisms, mRNA stabilization and transcriptional induction, are involved in upregulation of other hypertonicity-responsive genes. For instance, mRNA stabilization in response to hypertonicity is seen for GADD45 genes (Chakravarty et al., 2002), TonEBP transcription factor (Cai et al., 2005) and aquaporin (Leitch et al., 2001). Genes induced by transcriptional induction in response to hypertonicity include aldose reductase, *SMIT* and *BGT1* (Burg et al., 1997).

Stabilization of the mRNA is a regulatory mechanism involved in rapid responses to various forms of cellular stress, including heat shock (Andrews et al., 1987), UV irradiation (Wang et al., 2000; Westmark et al., 2005), hypoxia (Levy et al., 1998) and nutrient deprivation (Yaman et al., 2002). This mechanism permits a rapid increase of steady state mRNA levels by slowing degradation. It is characteristic of inducible transcription factors and other immediate early genes with high rates of mRNA turnover (Bakheet et al., 2001). Thus, stabilization of Ostf1 mRNA during hypertonicity supports a regulatory role of Ostf1 protein for reorganization of gill epithelium during salinity stress.

Of interest, we observed significant differences in Ostf1 mRNA upregulation profiles when comparing intact fish with cultured cells. Whereas Ostf1 mRNA increase is transient in intact fish, with a peak at 2 h and decline to normal levels within 6–8 h, cultured cells show a more sustained increase for at least 8 h. This difference suggests that negative feedback by systemic factors prevents sustained increases of Ostf1 mRNA in gill cells *in situ*, perhaps because sustained increases in Ostf1 may be detrimental to gill function.

We and others have previously used short-term cultures of gill epithelial cells for physiological and toxicological studies (Kültz, 1996; Sandbacka et al., 1999). This model system has many advantages but it also has the disadvantage that cultured cells are generally not exposed to osmotic gradients across their basolateral and apical surfaces. Generation of such gradients in culture is theoretically possible by growing cells on permeable supports but practically difficult because epithelial resistance of confluent EGCPC is different from that of gill epithelium *in situ*. Many responses in epithelia, such as ion transport phenomena, are dependent on an osmotic gradient and cell polarity and may be difficult to reproduce in the EGCPC system. Nevertheless, our data clearly demonstrate that this is not the case for Ostf1 upregulation, which is independent of transcellular osmotic gradients and cell polarity. Thus, it is possible that gill epithelial cells sense hyperosmotic stress directly *via* the apical membrane, or indirectly *via* subsequent increases in plasma osmolality *via* the basolateral membrane. Further experiments are necessary to find out which of these possible mechanisms applies.

In summary, we conclude that Ostf1 induction in gill epithelial cells of tilapia exposed to salinity stress is independent of cortisol or other systemic factors. Instead, it depends directly on hypertonicity as the primary induction signal and is based on rapid and transient mRNA stabilization followed by transcriptional induction. Systemic signals prevent sustained transcriptional induction of Ostf1 during hyperosmotic stress, indicating negative feedback regulation and possible detrimental effects of Ostf1 on gill function when its levels remain high for an extended period.

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