A homolog of the E3 ubiquitin ligase Rbx1 is induced during hyperosmotic stress of salmon

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Pan, Feng, Jacques Zarate, and Terence M. Bradley. A homolog of the E3 ubiquitin ligase Rbx1 is induced during hyperosmotic stress of salmon. Am J Physiol Regulatory Integrative Comp Physiol 282: R1643–R1653, 2002. First published February 14, 2002; 10.1152/ajpregu.00571.2001.—Juvenile salmon migrating from freshwater to the marine environment confront a marked change in environmental osmolality. Using differential display of mRNA expression, we cloned a 1.9-kb cDNA upregulated in isolated tissues of salmon exposed to the hyperosmotic stress associated with transition to the dehydrating marine environment. The cDNA codes for a 21-kDa protein, salmon hyperosmotic protein 21 (Shop21), with 98% identity to Rbx1, an E3 ubiquitin ligase; the protein also contains a novel 81-amino acid domain at the NH2 terminus not found in Rbx1. Moderate hyperosmotic stress (24 h at 550 mosmol/kg) increased Shop21 transcript 10-fold in branchial lamellae, whereas no upregulation was observed under more severe stress (≥800 mosmol/kg). Expression of the gene also was observed in heart and kidney. Replacement of NaCl with mannitol, but not glycerol, also elicited an increase in Shop21 mRNA. Inhibition of the mitogen-activated protein kinase and mitogen-activated extracellular regulated kinase kinase signal transduction pathways failed to blunt the Shop21 response during hyperosmotic stress. Shop21 mRNA also accumulated during thermal stress but to a lesser extent than heat shock protein 70 mRNA. The potential importance of Shop21 to the living animal is suggested by marked upregulation of the gene in salmon after transfer to seawater. The results of these investigations suggest that Shop21 may have a role in targeting selected proteins (e.g., in freshwater ionocytes) nonessential for adaptation to seawater for removal via the proteasome pathway.

ANADROMOUS SALMON MUST CONTEND with hyperosmotic stress during the transitional phase of the life cycle when juveniles migrate from the natal freshwater environment (typically <100 mosmol/kg) to the ocean (900–1,100 mosmol/kg). Plasma osmolality increases markedly from ~300 mosmol/kg to as much as 450–500 mosmol/kg and remains elevated until the fish are able to develop the requisite hyposmoregulatory mechanisms (19, 26). The onset of osmotic stress is more abrupt in aquaculture, where fish are directly transferred from freshwater hatcheries to seawater net pens (16). Juvenile salmon exposed to seawater outside the window of parr-smolt transformation (a series of physiological, biochemical, and morphological changes that prepare the animal for the dehydrating seawater environment) frequently die or fail to grow because of hyperosmotic stress (18, 19).

Several branchial and renal osmoregulatory mechanisms have been demonstrated to function in adaptation of salmon and other teleosts to the marine environment. The most well documented of these is the branchial Na⁺-K⁺-ATPase antiporter (22, 33, 52). Activity of this enzyme increases markedly during parr-smolt transformation and adaptation to seawater, facilitating branchial elimination of Na⁺ and restoration of osmotic homeostasis. More recently, the quantity of another transporter, the Na⁺-K⁺-2Cl⁻ cotransporter, has been observed to increase during parr-smolt transformation and adaptation to the marine environment (38). Previous studies in our laboratory indicate that exposure of salmon to hyperosmotic stress stimulates upregulation of heat shock protein 70 (Hsp70) and heat shock protein 90 in a tissue-specific and temporal manner (37, 48). Indeed, induction of heat shock proteins by mild thermal shock conferred protection against subsequent exposure to hyperosmotic stress (12). These transporters and heat shock proteins are the only proteins identified in branchial lamellae of salmon that appear to have a role in osmotic adaptation to seawater. Additional genes reported to be expressed in non-salmonid species during adaptation to hyperosmotic environments include a cDNA coding for the 14-3-3 protein that appears to be involved in osmosensory signal transduction (29) and the cystic fibrosis transmembrane conductance regulator gene (45) in the euryhaline mummichog (Fundulus heteroclitus) and a urea transporter (34) and an inward rectifier K⁺ channel (eKᵢ) (49) in the catadromous eel.

In contrast, a wide array of genes involved in adaptation of the mammalian kidney to hyperosmotic conditions have been uncovered, including heat shock protein 110 (41), osmotic stress protein 94 (27), GADD

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(30), tonicity-responsive enhancer binding protein (35), and transcription factors and transporters (7). The p38 mitogen-activated protein kinase (MAPK) and mitogen-activated extracellular regulated kinase kinase (MEK) signal transduction pathways also have been implicated in regulation of the cellular response to osmotic stress (6, 20, 36).

In the present study, we sought to identify and characterize additional genes that might function in adaptation of salmon to the marine environment. Analysis of differential expression of mRNA revealed tissue-, time-, and stressor-specific upregulation of a cDNA coding for a highly conserved 21-kDa protein containing a RING finger domain. Characterization, distribution, and putative identity of the gene product are presented.

METHODS

Fish and rearing conditions. Experiments were conducted with tissues from juvenile (1–2.5 yr of age) Atlantic salmon (Salmo salar) reared at the University of Rhode Island Aquaculture Center in 2-m-diameter fiberglass tanks provided with supplemental aeration and receiving single-pass water at ambient temperature (6–18°C). Illumination provided by fluorescent lights suspended ~1 m above the surface of the water was adjusted weekly to simulate natural photoperiod. Fish were fed a commercial formulated feed (Nelson Sterling Silver Cup, Murray, UT) to satiation three times daily. All husbandry practices and experimental procedures were approved by the University of Rhode Island Institutional Animal Care and Use Committee under Protocol A9596001 and fully conform with the American Physiological Society “Guiding Principles for Research Involving Animals and Human Beings.”

Tissue preparation. Fish were killed by a sharp blow to the head and exsanguinated by excision of the caudal fin to reduce contamination of tissues with erythrocytes. Three to four hemibranches of the gills were rapidly excised, and the liver, posterior kidney, and heart were removed through a ventral incision. Hemibranches were cut just above the septa to separate the lamellae, and the kidney, heart, and liver were diced into small cubes (2–4 mm³) with a scalpel blade. Approximately 0.8 g of tissue from each individual was rinsed in 10 ml of minimum essential medium with Earle’s salts (MEM; Sigma, St. Louis, MO), and 0.2 g of rinsed tissue was transferred to replicate 25-ml Erlenmeyer flasks containing 8 ml of MEM adjusted to pH 7.3 with a 7.5% solution of sodium bicarbonate and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Flasks containing the tissues in culture medium or medium supplemented with NaCl were gassed with 95% O₂-5% CO₂, capped with a rubber septum, placed on an orbital shaker (80 rpm), and incubated at ambient water temperature.

Total RNA was isolated from tissues according to the guanidinium-isothiocyanate method of Chirgwin et al. (10). The RNA concentration of each sample was quantified spectrophotometrically (absorbance at 260 nm), and preparations exhibiting a ratio of absorbance at 260 nm to absorbance at 280 nm >1.8 and no evidence of degradation, as indicated by clearly defined 28S and 18S rRNA bands on ethidium bromide-stained agarose gels, were analyzed further.

Differential display. One hundred micrograms of total RNA isolated from branchial lamellae incubated for 12 h in MEM or MEM supplemented with 125 mM NaCl were digested with 10 U of RNase-free DNase I (Promega, Madison, WI) for 30 min at 37°C, extracted with 3:1 phenol-chloroform, and precipitated with 3 M sodium acetate and ethanol. Two microliters of DNA-free RNA (0.1 mg/ml) were reverse transcribed using SuperScript II reverse transcriptase ( Gibco BRL, Grand Island, NY) and 2 μl of one of 12 different anchored oligo(dT) primers T₁₄ MV (M = A, G, or C; N = A, G, C, or T; Operon, Alameda, CA) at 2 μM.

cDNA prepared from two to four individuals exposed to control or osmotic stress conditions was used as the template for polymerase chain reaction (PCR) with each of 21 different arbitrary primer combinations (Operon). A 20-μl reaction volume for each primer set combination [one anchored oligo(dT) primer and one arbitrary primer] contained 6.5 μl of H₂O₂, 2 μl of 10 × PCR buffer, 2.4 μl of 25 mM MgCl₂, 1.6 μl of 25 μM dNTPs, 2 μl of arbitrary 10-mer primer (2 μM), 2 μl of T₁₄ MV anchor primer (2 μM), 2 μl of cDNA reaction mixture, 1 μl of [³²P]dATP (1:4 dilution of >2,000 Ci/mmol; ICN, Costa Mesa, CA), and 2.5 μl of Taq DNA polymerase (Promega). PCR amplification was conducted for 40 cycles at 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s followed by a final elongation step at 72°C for 5 min (33).

PCR amplicons from each reaction were resolved by electrophoresis through a 35 × 45-cm denaturing 6% polyacrylamide gel (19:1 acrylamide-bis-acrylamide) containing 7 M urea and 2 mM EDTA. Immediately before they were loaded onto the gel, 3.5 μl of PCR products were mixed with 2 μl of loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.09% xylene cyanole FF, 0.09% bromphenol blue), denatured by heating at 80°C for 2 min, and chilled on ice. Electrophoresis was conducted at 60-W constant power until the xylene cyanole FF dye approached the bottom of the gel. The gel was blotted onto a sheet of 3MM chromatography paper (Whatman, Maidstone, UK), dried at 80°C under vacuum for 1 h, overlaid with BioMax MS film (Kodak, Rochester, NY) and an intensifying screen, and stored at ~80°C for 3–4 days before it was developed. A needle was used to pierce the gel and film in the four corners to orient the autoradiogram on the gel.

Bands upregulated by exposure to osmotic stress were excised from the gel, transferred to a microfuge tube, and soaked in 100 μl of H₂O for 10 min, boiling for 15 min. The cDNA from the band was precipitated with linear polyacrylamide (Sigma) and amplified by a second round of PCR using the appropriate anchor and random primer pair and cycle conditions.

Reverse Northern dot-blot analyses were employed to verify that amplicons were upregulated. Amplicon DNA in 30 μl of a 100-μl PCR volume was denatured by boiling for 5 min in 2 N NaOH, neutralized with 5 μl of 3 M sodium acetate, and diluted to a volume of 105 μl with H₂O. Fifty microliters of the preparation were dot blotted onto duplicate charged nylon membranes using a Hybri-Dot vacuum manifold (Gibco BRL). Total RNA from lamellae (10 μg) incubated for 12 h in MEM or MEM supplemented with 125 mM NaCl was reverse transcribed with oligo(dT)₁₄ primers in the presence of [³²P]dATP (5,000 Ci/mmol; ICN) and used to probe the reverse Northern blot. Prehybridization, hybridization, and wash conditions were identical to those described in Northern blot analysis. After further verification by Northern blot, the upregulated amplicon was ligated into a pSTBlue vector and transformed into competent cells, as suggested by the supplier (Novagen, Madison, WI). Plasmid containing insert was amplified by growth of cells in Luria-Bertani medium containing carbenicillin (100 μg/ml) and isolated using silica column chromatography (Qiagen, Chatsworth, CA).

Cloning and sequencing full-length cDNA. The 250-nt cloned amplicon was radiolabeled with [α-³²P]dATP using DNA polymerase I (Klenow fragment; New England Biolabs,
Beverly, MA) and random primers (Ambion, Austin, TX) and used to screen a cDNA library constructed in a λ Uni-ZAP XR vector (Stratagene, La Jolla, CA) with cDNA prepared from salmon branchial lamellae incubated in MEM supplemented with 125 mM NaCl for 12 h (37). Approximately four to five plaques from each 150-mm plate (~50,000 plaques/plate) hybridized with probe. Several plaques were selected, subjected to two rounds of purification, and transformed to a SolR strain of Escherichia coli to generate the double-stranded phagemid pBluescript by in vivo excision. The size of selected clones was determined by PCR using primers to the T3 and T7 promoters flanking the multiple cloning site followed by electrophoresis of the amplicons in a gel of 1.0% agarose. Plasmid DNA was isolated from a clone containing a 1.9-kb insert and sequenced twice in each direction using a primer walking strategy with Tag FS DNA polymerase and fluorescent dideoxy terminators (ACGT, Northbrook, IL). Similarity of the contiguous cDNA with nucleic acid and deduced amino acid sequences in GenBank was determined using BLASTN (1). A 0.65-kb DNA fragment of salmon HSP70 and a 0.5-kb fragment of salmon actin were generated by PCR, as previously described (37, 48), for use as a probe for comparison with expression of salmon hyperosmotic protein 21 (Shop21).

Effect of stressors on gene expression. Isolated tissues were exposed to five different stressors. Osmotic stress was generated by incubation of branchial lamellae, kidney, heart, and liver in tissue culture medium supplemented with 0–450 mM NaCl. Supplementation with 125 mM NaCl provided a total osmolality of ~513 mosmol/kg, the maximum plasma osmolality of salmon transferred to seawater. NaCl at 450 mM generated a total osmolality (~1,108 mosmol/kg) slightly higher than that of seawater. Comparisons of the effects of nonionic solutes were made by substituting mannitol or glycerol for NaCl (550 mosmol/kg total osmolality). Osmolality was measured using a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, UT). To assess the effect of thermal stress, tissues were incubated at ambient (10°C) or elevated temperature (26°C) for 3 h before sampling, a regimen demonstrated to induce the major heat shock proteins of salmon (47). The effect of NaAsO2, another inducer of heat shock proteins, on gene expression was investigated by incubation of tissues in MEM made 100 μM in NaAsO2 for 4 h followed by an additional 8 h of incubation in fresh MEM lacking NaAsO2. Accumulation of Hsp70 mRNA also was assayed as a positive control, since Hsp70 is known to be induced by these stressors.

Signal transduction pathways. To determine whether expression of Shop21 might be controlled by the MEK or p38MAPK pathways, branchial lamellae were preincubated for 1 h in MEM containing the MEK or MAPK inhibitor PD-98059 or SB-202190 (CalBiochem, La Jolla, CA) at 5 and 10 μM. The concentration giving half-maximal inhibition is 350 nM for PD-98059 and 2 μM for SB-202190. Pretreatment was followed by an additional 12 h of incubation in MEM with or without supplemental NaCl (125 mM) and containing the respective inhibitors. The quantity of transcript after incubation was assayed by Northern blot analysis.

In vivo expression. Juvenile Atlantic salmon (mean length 18 cm) were transferred from the freshwater rearing conditions described above (13 mosmol/kg) to tanks receiving single-pass, filtered Narragansett Bay seawater (939 mosmol/kg). The temperature of freshwater and seawater was 16°C, and photoperiod was maintained at natural day length. Fish were offered a commercial salmon feed twice daily. At 0, 12, 24, 48, and 72 h after transfer, 6–10 individuals were randomly sampled and euthanized by a sharp blow to the head. Approximately 1 ml of blood was collected into a heparinized syringe by caudal venipuncture and centrifuged at 8,000 g for 5 min, and the plasma was collected and stored at ~50°C until determination of plasma osmolality with a vapor pressure osmometer. Branchial lamellae were collected from six individuals at each time point and processed for isolation of RNA as described below. Samples of kidney and heart also were collected at 0 and 24 h for assay of Shop21 expression.

Northern blot analysis. Aliquots of 20 μg of total RNA were resolved on a MOPS-formaldehyde-1% agarose gel (40) and transferred to a charged nylon membrane (Magnacharge, MSl, Westboro, MA) over 12 h using a Turboblotter (Schleicher & Schuell, Keene, NH). RNA was linked to the membrane by baking at 80°C for 1 h. Membranes containing RNA were incubated for 2 h at 42°C in prehybridization buffer [5× saline-sodium citrate (SSC), 5× Denhardt’s solution, 0.5% SDS, 100 μg/ml salmon sperm DNA], as previously described (48). The 1.9-kb cloned cDNA and a 0.65-kb ampiclon of salmon Hsp70 were labeled with [α-32P]dATP by random priming for use as probes, as described previously (37). Hybridization was conducted in buffer (5× SSC, 5× Denhardt’s hybridization solution, 50% formamide, 10% dextran sulfate, 0.2% SDS, 100 μg/ml salmon sperm DNA) containing radiolabeled probe (~1.5 × 106 cpm/ml) for 18–20 h at 42°C. Stringency washes were conducted in 2× SSC containing 0.5% SDS at 65°C for 30 min and then 2× SSC lacking SDS at room temperature for 5 min. Blots probed with the 1.9-kb clone and Hsp70 cDNAs were stripped in a solution containing 50% formamide and 3× SSC at 65°C for 30 min and reprobed with the 0.5-kb cDNA fragment of salmon actin to allow for determination of the relative quantity of mRNAs. Radiolabeling and hybridization conditions for the actin probe were as described above.

Quantification and statistical analysis. Northern blot images were captured, digitized, and analyzed using a Molecular Dynamics Storm 840 PhosphorImager and Imagequant image analysis software (Sunnyvale, CA). The sizes of the transcripts were determined by comparison with RNA markers coelectrophoresed on each gel, and the integrated optical density (IOD = optical density × area) of the 1.9-kb cDNA, Hsp70, and actin bands was quantified using this instrument. Relative concentrations of the 1.9-kb and Hsp70 transcripts were determined by normalizing the IOD of RNA bands to actin (target RNA-to-actin ratio). The use of actin provides an effective internal control for comparison of the effects of osmotic stress on expression of a stable constitutive gene (actin) and inducible genes (51). Statistical comparisons were performed on the normalized data using StatMost software (Datamost, Salt Lake City, UT). Differences in tissues incubated under control or treatment conditions were determined by analysis of variance followed by a Fisher’s protected least significant difference post hoc test if significant differences were detected. All differences are statistically different at P < 0.05.

RESULTS

Assay of differential expression of mRNA from isolated gill tissue incubated in MEM supplemented with 125 mM NaCl revealed marked upregulation of a 250-bp ampiclon that was isolated and cloned. Northern blot analysis indicated that a CDNA probe prepared from this clone hybridized with a 1.9-kb transcript upregulated in response to hyperosmotic stress. The probe subsequently was used to screen a salmon gill cDNA library, yielding 20 positive plaques in a popu-
lation of ~2 × 10^5 plaque-forming units. One clone contained a 1,933-bp insert that, when sequenced, was found to include an open reading frame from nt 1135 to nt 1704 coding for a protein 189 amino acids long (GenBank accession no. AY027936; Fig. 1). The deduced amino acid sequence reveals a 20.9-kDa protein with a theoretical pI of 7.62. The gene product was designated Shop21 on the basis of the molecular weight of the protein. Further analyses of the deduced amino acid sequence (www.expasy.ch; Swiss Institute of Bioinformatics) suggest that the estimated half-life of the protein is 30 h (14). The protein contains a putative endoplasmic retention signal (QKYG) in the COOH terminus, lacks an NH2-terminal signal peptide, and appears cytoplasmic in nature (k-NN prediction; http://psort.nibb.ac.jp). The predicted amino acid sequence suggests a high probability (0.90) of four serine phosphorylation sites (positions 7, 21, 99, and 146) and a single threonine phosphorylation site (position 90; NetPhos 2.0, www.cbs.dtu.dk).

A search of the protein databases using BLASTP revealed that the COOH terminus of Shop21 possesses a striking 98% identity with a 108-amino acid RING box protein, Rbx1, in humans and mice (GenBank accession nos. AF140598 and AF140599; Fig. 1). Shop21 contains an NH2-terminal domain of 81 amino acids, absent from the Rbx1 protein. The Shop21-specific domain contains a putative peroxisomal targeting signal (amino acid 44 RLKASADHL) and two of the four serine phosphorylation sites. The predicted stability index values based on dipeptide composition for Rbx1 and Shop21 are 38.61 and 51.78, respectively, suggesting that Rbx1 is the more stable of the two proteins (15).

Effect of osmolality on expression of Shop21. The level of hyperosmotic stress eliciting upregulation of Shop21 was determined by incubation of branchial lamellae in MEM supplemented with 0–450 mM NaCl for 12 h. Northern blot analysis revealed that accumulation of mRNA increased threefold after incubation in medium supplemented with 75 mM NaCl and more than fivefold in 125 mM NaCl (Fig. 2). Higher concentrations of NaCl failed to elicit an increase in Shop21 mRNA above control levels.
To assess the time course of expression of Shop21, branchial lamellae were incubated in hyperosmotic medium (125 mM) and sampled at selected time points up to 24 h during exposure to osmotic stress. Accumulation of mRNA increased 2- to 10-fold during exposure to hyperosmotic conditions (Fig. 3).

Expression of Shop21 was assayed in kidney, heart, and liver tissue incubated in medium supplemented with 125 mM NaCl for 12 h to assess tissue specificity. Accumulation of Shop21 in kidney was comparable to that in branchial lamellae. Shop21 transcript was barely detectable in liver tissue after osmotic stress, with levels only one-tenth of those observed in branchial lamellae (Fig. 4). Unanticipated was the high concentration of Shop21 mRNA in osmotically challenged cardiac tissue, nearly threefold greater than concentrations in branchial lamellae and kidney. Also of note was the presence of two transcripts in

Fig. 3. A: Northern blot analysis showing the time course of expression of Shop21 and actin in branchial lamellae exposed to medium supplemented with 125 mM NaCl for 0–24 h. B: histogram showing normalized levels of Shop21. Values are means ± SE for 3 individuals per time point. Values at the 1st time point (0) were arbitrarily set to 100. Different superscripts (a–d) signify statistical significance (P < 0.05).

Fig. 4. A: distribution of Shop21 mRNA in gill, liver, heart, and kidney incubated in medium supplemented with 125 mM NaCl for 12 h. B: histogram depicting normalized levels of Shop21. Values (means ± SE of 5 individuals) are relative to the quantity of transcript in branchial lamellae, which was arbitrarily set to 100. Different superscripts (a and b) signify statistical significance (P < 0.05).
cardiac tissue hybridizing with the actin probe under conditions of high stringency. The more prominent of the transcripts was ~1.7 kb, compared with the single 2.0-kb actin transcript observed in other tissues. The 1.7-kb transcript was used for normalization of Shop21 to actin mRNA. Multiple actin transcripts similar in size to those observed here have been observed in cardiac tissue of murine and bovine species (5, 25).

Effect of other solutes on expression. Exposure of mammalian kidney cell lines to nonionic solutes contributing to osmotic pressure has been demonstrated to elicit upregulation of heat shock proteins and osmolyte transporters. To assess the effects of dehydration and nonionic solute concentration on Shop21 accumulation, branchial lamellae were incubated for 12 h in medium made 550 mosmol/kg with NaCl, mannitol, or glycerol. Mannitol was employed to generate dehydration without influx of solute, and glycerol was used to increase intracellular solute content without disrupting electrical potential (2, 8). As anticipated from previous experiments, 550 mosmol/kg NaCl (~125 mM) stimulated a 5.5-fold increase in Shop21 transcript in branchial lamellae (Fig. 5). Similarly, mannitol elicited a threefold increase in Shop21. In contrast, the membrane-permeable solute glycerol failed to elicit a response. Exposure to these concentrations of solutes failed to increase accumulation of Hsp70 mRNA (Fig. 5).

Effect of thermal stress and NaAsO2. Previous investigations have demonstrated that hyperosmotic stress can induce heat shock proteins in kidney (3, 43) and branchial lamellae (37, 48). We sought to investigate whether Shop21 might be a novel heat shock protein, upregulated in isolated branchial lamellae by exposure to thermal stress or NaAsO2, both inducers of heat shock proteins. Incubation of branchial lamellae at elevated temperature (temperature change of 16°C) for 3 h and in medium made 100 μM with NaAsO2 resulted in 12- and 2-fold increases in Hsp70 mRNA, respectively (Fig. 6). By way of comparison, thermal stress stimulated accumulation of Shop21 mRNA ~2.5-fold, whereas exposure to NaAsO2 resulted in a decrease (Fig. 6).

Signal transduction pathways. Recent investigations indicate that the MAPK and MEK signal transduction pathways are involved in upregulation of a number of genes that respond to osmotic stress (4, 28). We sought to investigate whether transcription of Shop21 might be regulated through the MAPK or MEK pathways by incubating branchial lamellae in medium supplemented with specific inhibitors of these systems during osmotic stress. Northern blot analysis revealed that inclusion of 5 or 10 μM PD-98059 or SB-202190 in the medium failed to reduce the magnitude of upregulation of Shop21 in response to osmotic stress (Fig. 7). A small but statistically significant increase in accumulation of Shop21 was observed in the presence of SB-202190.

In vivo expression of Shop21. The effect of hyperosmotic stress on Shop21 expression in the living animal was investigated in juvenile salmon transferred to seawater and sampled at selected time points. Hyperosmotic stress of salmon after transfer to seawater was indicated by a marked increase in plasma osmolality (Fig. 8A). Plasma osmolality of fish in freshwater (304 ± 2 mosmol/kg) increased ~30% (403 ± 8 mosmol/kg)
kg) after 12 h in seawater and attained a peak value of 465 ± 10 mosmol/kg after 72 h in seawater. Similar to the results obtained with hyperosmotic stress in vitro, exposure of the living animal to seawater stimulated expression of Shop21 in branchial lamellae (Fig. 8B). Concurrent with the elevation in plasma osmolality, Shop21 mRNA increased almost fourfold within 24 h of transfer to seawater and remained elevated until 48 h, when the concentration was threefold greater than that in control animals. Although Shop21 was observed in isolated heart and kidney tissue incubated in culture medium made hyperosmotic with NaCl (Fig. 4), in vivo sampling demonstrated that the gene also is expressed in freshwater and not upregulated by seawater in these tissues (data not shown).

**DISCUSSION**

In contrast to the array of genes expressed in the mammalian kidney during response to hyperosmotic...
stress, only eight specific gene products have been reported to be upregulated in euryhaline fish challenged with the high osmolality of the marine environment. We identified a 1.9-kb transcript that was markedly upregulated in salmon tissues during adaptation to this hyperosmotic stress. The mRNA codes for a 21-kDa protein containing an evolutionarily conserved 108-amino acid domain possessing 98% identity with the human and murine RING box protein Rbx1 (24). Similar to Rbx1 and Mdm2, Shop21 contains a RING finger domain characteristic of the E3 ubiquitin family of proteins, consisting of eight cysteines and histidines with a cysteine at the fifth coordination site and forming two putative zinc binding sites (13, 24).

Fig. 8. Plasma osmolality (A) and Shop21 expression (B–C) in juvenile salmon transferred from freshwater (13 mosmol/kg) to seawater (938 mosmol/kg) and sampled at selected time points. A: values are means ± SE of 6–10 individuals per time point. Different superscripts (a–c) denote statistical significance (P < 0.05). B: RNA isolated from branchial lamellae and assayed by Northern blot for Shop21 and actin mRNAs. C: histogram depicting normalized levels of Shop21 mRNA. Values are means ± SE of 6 individuals per time point. *Significantly different (P < 0.05) from the control animals (0).

The E3 family of ubiquitin ligases is essential for selective degradation of proteins via the 26S proteasome complex (17). E3 ubiquitin ligases catalyze the third and final step of ubiquitination, in which an activated ubiquitin molecule is linked to the ε-amino group of a specific target protein for subsequent transport to and degradation by the proteasome. Investigation of a variety of proteins suggests that the presence of a RING fingerlike domain is the element conferring the ability to ubiquitinate specific proteins (50). Through selective targeting of an array of transcription factors and regulatory proteins for degradation, E3 ubiquitin ligases have been implicated as essential regulators of cell growth and proliferation, signal transduction, apoptosis, and response to environmental stimuli (13, 17, 23). Rbx1 has been demonstrated to be a component of the von Hippel-Lindau (VHL) tumor suppressor complex, conferring on the heteromeric protein complex the ability to ubiquitinate selected target proteins. Inactivation of the VHL complex is associated with most human kidney cancers (24). Shimura et al. (44) demonstrated that mutation of parkin, a 53-kDa E3 ubiquitin ligase, results in defective ubiquitination and accumulation of α-synuclein. The authors hypothesize that pathological accumulation of α-synuclein in the
human brain may be one of the primary causes of Parkinson's disease.

The 81-amino acid difference between Shop21 and Rbx1 is intriguing. Most RING box proteins are larger than Rbx1 and typically contain substrate recognition sites/domains, as with Ubr1 and Mdm2, which ubiquitinate p53 (13, 46). The relatively small size of Shop21 suggests that the protein is unlikely to function autonomously but, rather, is a component of a heteromeric complex similar to the VHL tumor suppressor or Skp1-Cdc53-F box protein complexes (21, 24). The additional domain of Shop21 might confer the ability to recognize specific consensus sites of target proteins or facilitate “docking” with other proteins to form a functional complex. Alternatively, the NH₂-terminal domain might suggest that Shop21 is a component of a different E3 ligase complex, perhaps unique to fish or ectotherms.

Tissue-specific upregulation of Shop21 was observed. Shop21 was present in kidney and heart, but accumulation of mRNA did not increase with exposure to hyperosmotic stress. In contrast, elevated levels of Shop21 transcript were detected in branchial lamellae during exposure of isolated tissue and the living animal to hyperosmotic conditions. Consistent with this finding, Shop21 accumulated in branchial lamellae incubated in mannitol, but not glycerol, suggesting that upregulation is stimulated by an increase in intracellular ion concentration or decrease in cell volume but not by an increase in nonionic solute concentrations. Branchial lamellae of salmon undergo significant structural and functional modification during adaptation to seawater. The most marked change is a proliferation of seawater ionocytes (α-chloride cells) located on the primary lamellae and a concurrent decrease in freshwater ionocytes (β-chloride cells), typically found on the secondary lamellae (32, 39). Apoptosis of freshwater ionocytes is likely, inasmuch as the fish synthesizes new structural and transporter proteins to contend with hyperosmotic conditions. Potential degradation of selected proteins, apoptosis, and signal transduction associated with this adaptive process suggest an essential role for ubiquitination of specific target proteins. The elevation of Shop21 in branchial tissue for ≥48 h supports a role for this protein in remodeling gill architecture/function, a process that may require several days to weeks (32). A recent investigation reveals that branchial Na⁺/K⁺-ATPase activity continues to increase for 4–11 days after transfer of salmon to seawater (11).

A previous report from our laboratory demonstrating increased accumulation of Hsp70 mRNA in tissues exposed to osmotic stress prompted investigation of whether Shop21 might be a heat shock protein. Exposure of lamellae to thermal stress elicited a 2.5-fold increase in Shop21 mRNA, albeit some 5-fold less than the 12.5-fold increase in Hsp70 exposed to the same conditions. In response to hyperosmotic conditions, Shop21 was upregulated most at ≥125 mM NaCl, whereas Hsp70 is induced only at >250 mM NaCl (48). Exposure to NaAsO₂ also stimulated induction of Hsp70, but not of Shop21. The selective response of Shop21 to denaturing conditions supports classification of this molecule as an E3 ubiquitin ligase, targeting specific proteins for degradation.

The decrease in expression of Shop21 at 250 and 450 mM NaCl might be related to the effect of high osmolality on transcription. In previous investigations, we observed a decrease in a variety of gene products at high osmolalities but continued expression of heat shock proteins (47). Alternatively, in the nonpolar tissue culture system employed in these investigations, the entire population of cells comprising the branchial lamellae is bathed in the hyperosmotic medium. Supplemental NaCl at >125 mM (>500 mosmol/kg) would exceed the level to which serosal cells and the basal portion of mucosal cells normally would be exposed. In the living animal, the apical portion of the lamellar epithelial cells is exposed to seawater (1,000 mosmol/kg), whereas the basal portion and serosal cells are in contact with extravascular fluid (<500 mosmol/kg). Elevated expression of Shop21 was observed in salmon transferred to seawater.

The absence of a negative effect of MAPK and MEK inhibitors on expression of Shop21 suggests that regulation of the gene is not through an osmotically driven element or promoter. Previous investigations demonstrated osmotically driven induction of mRNAs for HSP70 and the betaine transporter in Madin-Darby canine kidney cells by MAPK (42). Other investigators observed that blocking MAPK or MEK activity inhibited synthesis or activation of serum- and glucocorticoid-inducible kinase (Sgk) (4), aquaporins (20), and a variety of proteins (28). In contrast, Capasso et al. (9) observed that, in mIMCD3 cells, p38MAPK appears involved in acute, but not chronic, adaptation to hyperosmotic stress. Upregulation of Shop21 at chronic levels of hyperosmotic stress in the absence of MAPK activity is in agreement with these findings. Interestingly, incubation of branchial lamellae in hyperosmotic medium containing SB-202190 increased accumulation of Shop21 mRNA. Preliminary investigations demonstrated that a negative control compound (SB-202474) failed to stimulate Shop21 expression. Although speculative, inhibition of the p38MAPK pathway might impact expression of other genes involved in the osmoregulatory process. Subsequent premature turnover of proteins or apoptosis might stimulate expression of Shop21.

The present investigation is the first report of an E3 ubiquitin ligase in teleosts. The deduced protein has extensive homology with the human and murine Rbx1 E3 ubiquitin ligase but possesses an additional domain on the NH₂ terminus that might function in recognition of target proteins or in interactions with a heteromeric protein complex. mRNA coding for the protein markedly accumulated in response to hyperosmotic stress, suggesting a role for this protein in adaptation of salmon to the marine environment.

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UPREGULATION OF RBX1 BY HYPEROSMOTIC STRESS OF SALMON

The ubiquitin system.

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