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Physiology

Expression of insulin-like growth factor I gene in osmoregulatory organs during seawater adaptation of the salmonid fish: Possible mode of osmoregulatory action of growth hormone

(gene regulation/RNA hybridization/gill/kidney/rainbow trout)

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ABSTRACT **Growth hormone has been shown to contribute to seawater adaptation of salmonid fishes. The growth influence of growth hormone is mediated largely by hepatic production of insulin-like growth factor I (IGF-I). To study the growth hormone–IGF-I axis in osmoregulation, we measured IGF-I mRNA in the liver, gill, and body kidney from rainbow trout by Northern analysis. The levels of IGF-I mRNA in all tissues increased significantly after injection of growth hormone. Transfer of trout from fresh water to 80% seawater evoked an increase in plasma growth hormone after 1 day. IGF-I mRNA was not altered significantly in the liver, but it was increased in the gill and body kidney after 1 and 8 days, respectively. These observations indicate that the IGF-I gene is expressed differently among these organs during seawater adaptation. Growth hormone may stimulate hypoosmoregulatory ability by inducing local expression of IGF-I in osmoregulatory organs, although the possibility that IGF-I expression might occur in part independently of growth hormone during seawater adaptation cannot be excluded.**

Insulin-like growth factor I (IGF-I), produced mainly in the liver, is an important mediator of the growth-promoting actions of growth hormone (GH) not only in mammals (1) but also in teleosts (2). GH regulates the levels of IGF-I peptide and mRNA in liver and other tissues, suggesting that GH is the primary (but not the sole) regulator of IGF-I production (3). Production of IGF-I in a variety of extrahepatic tissues is important for a wide variety of tissue processes mainly related to growth and differentiation (4, 5); however, circulating IGF-I of hepatic origin appears to be of primary importance for GH action on promotion of skeletal elongation in mammals (6).

In salmonid fishes, GH stimulates both growth and seawater adaptability (7–11). Bolton *et al.* (12) and Collie *et al.* (13) have demonstrated that the effect of GH on seawater adaptation of rainbow trout (*Oncorhynchus mykiss*) is independent of the growth-promoting actions of GH. Plasma levels and turnover rates of GH increase after transfer of several salmonids to seawater (14–19). Recently, we have shown that bovine IGF-I also enhances the hypoosmoregulatory ability of rainbow trout by inhibiting the increase in plasma osmolarity and sodium levels after transfer to seawater, independently of its growth-promoting effect (20). This seawater-adapting action of IGF-I has been confirmed in Atlantic salmon.† Furthermore, GH and/or IGF-I affect kidney function in mammals (2). Although these studies suggest that IGF-I is a possible mediator of the osmoregulatory actions of GH, it is not clear in which organ(s) IGF-I is produced during seawater adaptation and thus whether IGF-I may be an endocrine or an autocrine/paracrine osmoregulatory agent (or both). In the rainbow trout, GH receptors occur in osmoregulatory organs, such as the gills and body kidney (16). Thus, it is possible that GH exerts its effects directly on osmoregulatory organs by stimulating local production of IGF-I during seawater adaptation.

To assess the potential sites of IGF-I biosynthesis with regard to the seawater-adapting actions of GH in the trout, IGF-I protein production and/or IGF-I gene expression need to be examined in the liver and osmoregulatory organs. However, it is difficult to identify local production of IGF-I protein in tissues, as many tissues contain receptor-bound IGF-I protein of hepatic origin transported by the circulation. The possibility of contamination by blood-borne IGF-I during tissue preparation cannot be disregarded, as blood contains the highest concentration of IGF-I of all mammalian tissues (3). Furthermore, no specific assay is available to determine IGF-I protein levels in fishes since IGF-I protein has not been obtained from teleosts; only the cDNA of coho salmon IGF-I is known (21). Therefore, we examined the effect of GH on IGF-I mRNA levels in the liver, gill, and body kidney and their changes during seawater adaptation of rainbow trout, when GH levels are known to increase (14–19).

MATERIALS AND METHODS

Animals. Immature rainbow trout (*O. mykiss*), weighing 80–140 g, were obtained from a commercial dealer in Tokyo. Fish were kept in aerated dechlorinated fresh water in aquaria at 15°C for 3 weeks prior to the experiments. They were fed a ration of 1% wet body weight of trout pellets (Masu no. 6; Oriental Kobo, Tokyo) once daily. They were kept on this daily feeding regimen during the experiments.

To examine the effects of GH treatment on IGF-I expression, fish were anesthetized with 0.05% 2-phenoxyethanol and injected intraperitoneally every 2nd day with 0.25 μg of recombinant chum salmon GH (supplied by Tokyo Research Laboratories of Kyowa Hakko Kogyo) in a vol of 5 μl per g of body weight for a total of three injections. This injection protocol was chosen because our previous analysis of GH turnover in rainbow trout showed that the secretion rate of GH remained elevated for several days even though the increase in plasma GH levels was transient during seawater adaptation (18). Control fish received equivalent volumes of vehicle. Fish were killed 20 h after the last injection.

Effects of seawater acclimation were examined by transferring the fish from fresh water to 80% seawater (375 mM Na^+/436 mM Cl^-). Control fish were transferred to another

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Abbreviations: GH, growth hormone; IGF-I, insulin-like growth factor I.

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freshwater aquarium. Eight to 10 fish from each group were sacrificed 1, 2, 4, 8, 14, and 21 days after the transfer. There was no significant difference in mean body weight between the experimental and control groups in either experiment.

Tissue Preparation and RNA Isolation. After anesthetizing the fish with 0.02% tricaine methane sulfonate (Sigma), blood samples were obtained from the caudal vasculature and immediately centrifuged at 11,000 × *g* for 5 min. Plasma was stored in plastic microcentrifuge tubes at −70°C. Tissues were removed immediately, frozen in liquid nitrogen, and kept at −70°C.

Total RNA was extracted from frozen tissues by the method of Chomczynski and Sacchi (22). Tissues were homogenized in 10 vol of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol at room temperature using a Polytron blender (Brinkmann). A 1/10 vol of 2 M sodium acetate (pH 4) was added, and RNA was extracted with a 1:2 mixture of phenol/chloroform/isoamyl alcohol. RNA was precipitated twice with ethanol. The RNA pellet was washed in 75% ethanol and resuspended at 65°C in 0.5% SDS with gentle pipetting. Poly(A)$^+$ RNA was further purified by oligo(dT)-Latex (Oligotex-dT30; Roche, Tokyo) and eluted in sterile water at 65°C as described by Kuribayashi *et al.* (23). The quantity of poly(A)$^+$ RNA was determined by spectrophotometry.

Hybridization Probes. An IGF-I cDNA encoding the entire coding sequence for coho salmon (*Oncorhynchus kisutch*) IGF-I [1.1-kb insert (21)] was kindly provided by Stephen J. Duguay (University of Washington, Seattle). The cDNA probes for salmon IGF-I and for chicken β-actin (Oncor, Gaithersburg, MD) were labeled with [^{32}P]dATP and/or [^{32}P]dCTP (Amersham) by the random-primer method using a kit obtained from Amersham.

Northern Blot Analysis. Aliquots (1–20 μg) of poly(A)$^+$ RNA were denatured by incubation at 55°C for 15 min in gel running buffer [0.04 M 3-(*N*-morpholino)propanesulfonic acid/10 mM sodium acetate/1.0 mM EDTA, pH 7.0] with 50% formamide and 2.2 M formaldehyde, electrophoresed through a 1% agarose gel (FMC) containing 2.2 M formaldehyde, and transferred to a nylon filter (Nytran; Schleicher & Schuell, Dassel, F.R.G.) by capillary blotting (24). The RNA was covalently attached to the filter by UV cross-linking and was detected by hybridization to the ^{32}P-labeled cDNA probe (denatured by boiling). The RNA was hybridized for 16–20 h at 55°C in a solution containing 6× standard saline citrate (SSC; 1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate), 2× Denhardt's solution (25), 0.1% SDS, and 0.1 mg of sonicated, denatured calf thymus DNA per ml. The filters were washed in 2× SSC/0.1% SDS at room temperature for 20 min and then twice for 1 h each at 65°C in 0.1× SSC/0.1% SDS (for IGF-I cDNA probe) or twice for 1 h each at 55°C in 1× SSC/0.1% SDS (for β-actin cDNA probe). After analysis of messages corresponding to the IGF-I, the filters were dehybridized as described by the manufacturer and were rehybridized to the β-actin probe as a control.

Autoradiography was performed by exposing Kodak X-Omat AR film to the filters at −70°C for 1–7 days with Toshiba E-32 intensifying screens. Hybridization intensity was estimated by densitometric scanning with a chromatoscanner CS-910 (Shimadzu, Kyoto); peak areas were quantified with a digitizer. Known amounts of hepatic poly(A)$^+$ RNA pooled from freshwater trout, electrophoresed on the same gels as experimental poly(A)$^+$ RNA samples, were used as internal standards for comparison of the relative amounts of IGF-I mRNA and β-actin mRNA in different blots. The analytical procedure was validated by measuring the 3.9-kb IGF-I message and β-actin message in poly(A)$^+$ RNA samples pooled from each tissue. The data obtained by analyzing 1-, 2.5-, 5-, 10-, and 20-μg samples indicated linear dependence on the amount of RNA in the samples. Abundance of mRNA was assessed as autoradiographic intensity per μg of poly(A)$^+$ RNA, rather than as abundance per g of starting tissue or per μg of total RNA because of variability in poly(A)$^+$ RNA recovery among preparations. Molecular sizes were estimated relative to migration of RNA size standards (Life Technologies, Tokyo).

Other Assays. Plasma GH level was measured with a specific homologous radioimmunoassay (26). Plasma Na^+ concentrations were determined by using ion-selective electrodes (AVL 984-S; AVL Medical Instruments AG, Schaffhausen, Switzerland).

Statistical Analysis. Differences among groups were evaluated by analysis of variance followed by Duncan's new multiple-range test (27) and Student's *t* test.

RESULTS

Effect of GH. Levels of IGF-I mRNA were examined in potential target organs of the osmoregulatory action of GH in freshwater trout given injections of saline or GH (Table 1; Fig. 1). Northern blot analysis of poly(A)$^+$ RNA extracted from the liver, gill filaments, and body kidney of vehicle-injected control and GH-treated trout demonstrated a major band, hybridizing with coho salmon IGF-I cDNA at 3.9 kb; additional transcripts at 6.0, 2.8, and 1.8 kb were detectable in some experiments, but the nature of these additional mRNAs is uncertain. The position and relative intensity of bands compare well with the sizes of transcripts of the IGF-I genes in coho salmon (21). The specificity of the hybridization signal was supported further by the low degree of sequence identity between IGF-I and the other members of the insulin gene family (<65%). The low levels of hybridization seen in the gill and body kidney are not due to blood contamination, as no significant amount of RNA was extracted from blood. IGF-I transcripts were undetectable in poly(A)$^+$ RNA prepared from the head kidney in contrast to those from body kidney, although approximately equivalent amounts of intact RNA from head kidney and body kidney were loaded onto blots, as indicated by ethidium bromide staining of the gel and by β-actin hybridization (Table 1).

Although increases in IGF-I mRNA in the liver, gill, and body kidney were readily apparent after GH treatment (30, 4, and 17 times more than the values in the control trout, respectively), the changes were not statistically significant because of large individual variations (Table 1). According to Cao *et al.* (21), expression of the gene for the cytoskeletal protein β-actin was relatively unchanged in coho salmon injected with GH. In the present study, however, a significant increase in the level of mRNA for β-actin in the liver was observed following GH treatment (Table 1). This is possibly due, at least in part, to the increase in cell size and/or

Table 1. Effect of GH treatment on IGF-I mRNA and β-actin mRNA species in rainbow trout in fresh water

		3.9-kb IGF-I mRNA	β-Actin mRNA
Liver	Control	100 ± 46	100 ± 14
	GH	3030 ± 848	518 ± 59***
Gill	Control	2.8 ± 1.5	2330 ± 459
	GH	12.4 ± 5.4	2480 ± 900
Head kidney	Control	ND	1670 ± 19
	GH	ND	2711 ± 952
Body kidney	Control	9 ± 2	1500 ± 140
	GH	156 ± 53	2710 ± 952

Rainbow trout in fresh water were given three injections of GH (0.25 μg/g) or vehicle. Values represent means ± SEM (n = 3 or 4). For each mRNA species, autoradiographic intensity of Northern blot was calculated per μg of poly(A)$^+$ RNA and then expressed relative to the mean intensity of that species in the control liver. ***, $P <$ 0.001 for differences from control. ND, not detectable.

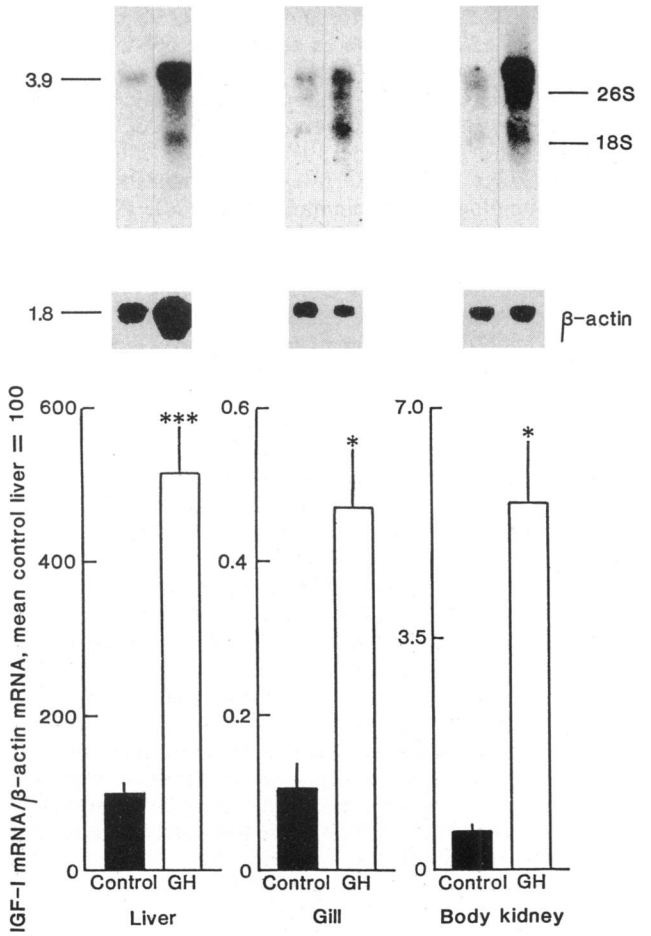

FIG. 1. Effect of GH treatment on IGF-I mRNA levels in tissues from rainbow trout in fresh water. (*Upper*) Typical results of Northern blots performed with poly(A)$^+$ RNA (left to right: liver, 2.5 μg; gill, 5 μg; body kidney, 5 μg) from GH-treated and vehicle-injected (control) trout. Blots were hybridized with the coho salmon IGF-I cDNA probe as described. Autoradiographic exposure was 1 day for liver, 3 days for gill, and 2 days for body kidney. Mobility of salmonid 18S and 26S rRNA is shown. Blots were washed and rehybridized with the chicken β-actin cDNA. Sizes of transcripts are indicated in kb. (*Lower*) Densitometric analysis of Northern blot hybridization. Results of analyses for 3.9-kb IGF-I mRNA were normalized with β-actin mRNA (Table 1). Scale is in arbitrary units with the average ratio obtained for livers from control trout set at 100. Values are expressed as means ± SEM (n = 3 or 4). Asterisks denote significant differences (*, $P < 0.05$; ***, $P < 0.001$).

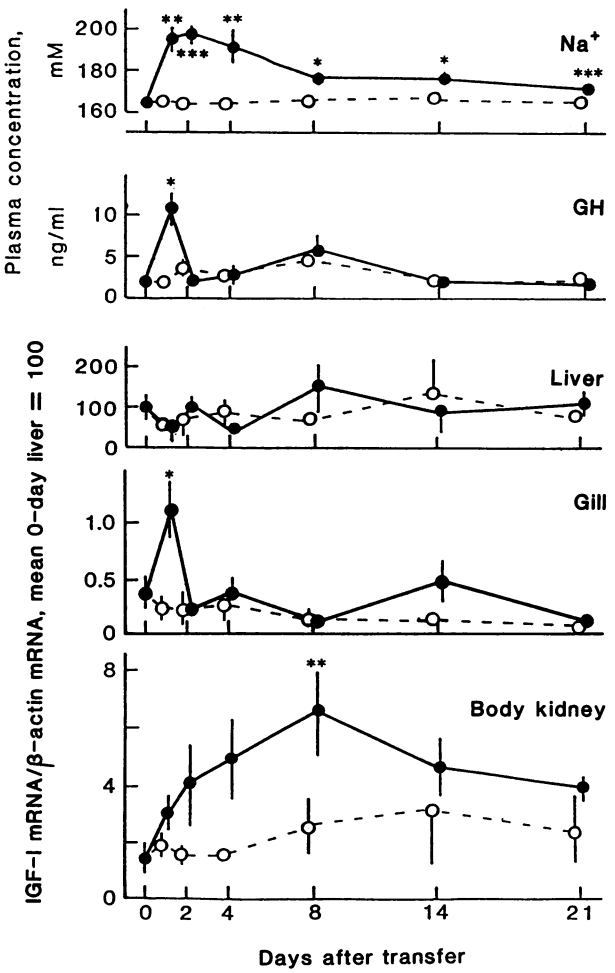

FIG. 2. Plasma concentrations of Na^+ and GH and IGF-I mRNA normalized to that of β-actin in liver, gill, and body kidney at various times after rainbow trout were transferred from fresh water to 80% seawater (●). Control fish were transferred from fresh water to fresh water (○). Poly(A)$^+$ RNA was analyzed and the results for 3.9-kb IGF-I mRNA were normalized to those of β-actin mRNA, as described for Table 1 and Fig. 1. Scale is in arbitrary units, with the average ratio obtained for the liver from day-0 trout set at 100. Each point represents mean ± SEM (n = 3–6). Asterisks denote values significantly different from the corresponding freshwater control values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

number, as liver weight of the GH-treated trout was 62% greater than that of the controls ($P < 0.05$). Fig. 1 shows the changes in IGF-I mRNA relative to β-actin mRNA after GH treatment, indicating significantly increased expression of IGF-I relative to β-actin in the liver, gill, and body kidney.

Transfer to Seawater. When rainbow trout were transferred to 80% seawater, plasma Na^+ concentrations increased significantly after day 1; the high levels were maintained for 4 days and then decreased to a level slightly but significantly higher than the freshwater level throughout the rest of the experiment. Plasma GH levels showed a significant increase 1 day after transfer and returned to the freshwater levels thereafter (Fig. 2).

Concomitant with the increase in plasma GH, gill IGF-I mRNA levels increased markedly 1 day after transfer to 80% seawater and remained elevated until the end of the experiment, although the increases were significant only after 1 and 4 days (data not shown). After transfer to 80% seawater, levels of β-actin mRNA in the gill increased gradually, reaching a maximum after 8 days (data not shown). When normalized to β-actin mRNAs, the relative IGF-I mRNA in the gill also increased significantly 1 day after transfer, with no significant change being seen thereafter (Fig. 2). The gill IGF-I mRNA levels of the day-0 trout were 4 times higher than those of the control of the first experiment (Fig. 1). This difference could result from a difference in physiological condition following the stress of injections.

The IGF-I message in the body kidney increased gradually after transfer to 80% seawater, reaching a level nearly 5 times greater than that of the control trout after 8 days. Although a significant elevation in IGF-I mRNA was seen in the body kidney on day 21 (data not shown), the expression of IGF-I relative to that of β-actin was significantly increased only 8 days after the transfer (Fig. 2).

Seawater transfer did not affect the levels of IGF-I message in the liver when normalized to either poly(A)$^+$ RNA (data not shown) or β-actin mRNA (Fig. 2).

DISCUSSION

Our previous work demonstrated that treatment of rainbow trout with GH or IGF-I accelerated seawater adaptability by

inhibiting the increase in plasma osmolarity and sodium levels after transfer to 80% seawater (20). The present study clearly demonstrates that IGF-I mRNA levels in the gill and body kidney increase significantly after transfer of rainbow trout to 80% seawater, accompanying an increase in plasma GH. These results indicate that not only GH secretion but also IGF-I gene expression are increased in response to increased environmental osmolality.

Despite simultaneous induction of IGF-I mRNA in the gill, body kidney, and liver after GH treatment of the freshwater trout, IGF-I mRNA was expressed differently among these organs during seawater adaptation, with no mRNA change in the liver, a rapid increase in the gill after 1 day, and a gradual increase in the body kidney until day 8. Since GH receptors have been found not only in the liver but also in the gill and body kidney of tilapia, coho salmon, and rainbow trout (16, 28–30), induction of IGF-I mRNA in the gill and body kidney is likely to be a consequence of increased plasma GH. However, GH does not seem to be the only factor that regulates the synthesis of IGF-I (31–34). Fagin and Melmed (35) observed an increase in IGF-I mRNA in hypertrophied kidneys of uninephrectomized rats in the absence of any changes in serum GH levels. Glucocorticoids have been shown to inhibit GH induction of IGF-I mRNA in the rat liver and tibia more than in the lung and kidney (36). A transient increase in cortisol during seawater adaptation of the trout (16, 37, 38) may inhibit GH induction of IGF-I mRNA in the liver and/or stimulate it in the gill and body kidney. In teleosts, GH and nutritional status are the only factors currently known to affect IGF-I gene expression (22, 39).

Little is known not only about the regulation of IGF-I biosynthesis but also about its role in osmoregulation. Although further experiments such as introduction of GH antibodies during seawater adaptation may lead to a clear understanding of the role of GH in IGF-I expression during seawater adaptation of the trout, our present observation coupled with previous studies suggests that locally expressed IGF-I in osmoregulatory organs may mediate the osmoregulatory action of GH in salmonid fish. This suggestion expands on studies on the role of GH and IGF-I in mammalian kidney and implies a possibly general role for these factors in vertebrate osmoregulation. Although nothing is yet known of the effect of GH and/or IGF-I on kidney function in teleosts, IGF-I may mediate some (or all) of the renal actions of GH in mammals (40, 41). GH treatment of hypophysectomized rats results in a 40% increase in kidney weight and a 2-fold increase in renal IGF-I mRNA (42). In addition to their potential effects on renal growth, GH and IGF-I stimulate renal plasma flow and glomerular filtration rate in humans (43).

Gill Na^+/K^+-ATPase activity and chloride cell differentiation are also stimulated by *in vivo* treatment of several salmonid species with GH (10, 11, 44–46). Pretreatment of coho salmon with GH results in increased sensitivity of isolated gill filaments to IGF-I, significantly stimulating Na^+/K^+-ATPase *in vitro*, although IGF-I was not effective in stimulating the enzyme in gill filament from untreated coho salmon *in vitro*.‡ In this context, the relatively early increase in gill IGF-I expression during seawater adaptation suggests that GH may stimulate differentiation of chloride cells (possibly through local production of IGF-I) and that systemic IGF-I may act largely on the differentiated cells. This suggestion is similar to the dual effector role of GH proposed in regard to promotion of tissue growth in mammals by Green *et al.* (47) and in teleosts (48), with GH stimulating undifferentiated cells to become responsive to IGF-I. Expression of the β-actin gene in the gill was also increased after seawater transfer, and this may be associated with differentiation of chloride cells (49).

On the other hand, IGF-I may not be the sole mediator of the hypoosmoregulatory actions of GH. Cortisol has previously been shown to be important in salmonid seawater adaptation by stimulation of gill Na^+/K^+-ATPase activity and chloride cell differentiation (11, 44, 50, 51). Although relatively little is known of the functional relationship between GH and cortisol, GH enhances the *in vitro* response of coho salmon interrenal to adrenocorticotropin, increasing cortisol production (52). In the present study, however, IGF-I transcripts were not detected in the head kidney containing the interrenal cells, although equivalent amounts of RNA from head kidney and body kidney were examined simultaneously. However, the proportion of interrenal cells in the lymphoid head kidney is small.

Increased IGF-I mRNA in the osmoregulatory organs during seawater adaptation suggests that IGF-I functions as an autocrine/paracrine factor to stimulate hypoosmoregulatory ability in salmonid fish, acting at or near the site of its synthesis. There is a need to clarify the cell type(s) that synthesizes IGF-I during seawater adaptation. The IGF-I gene was expressed locally in the rat kidney with cells of the thick ascending limbs; the renally synthesized IGF-I could reach distal tubules and collecting ducts (53). Blazer-Yost *et al.* (54) found that mammalian IGF-I stimulates sodium flux in toad urinary bladder, a model for studying the water and electrolyte movement across renal tubules, and that IGF-I receptors are present in the bladder. If this is also true in the salmonid kidney, IGF-I might affect monovalent ion and water reabsorption directly or indirectly through cell proliferation and differentiation. Focal expression of IGF-I in the gill chloride cells and/or accessory cells may also occur.

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