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P. PRUNET, M. PISAM, J. P. CLAIREAUX, G. BOEUF, AND A. RAMBOURG
Laboratoire de Physiologie des Poissons, Institut National de la Recherche Agronomique, Campus de Beaulieu, 35042 Rennes Cedex; Département de Biologie, Centre d'Etudes Nucléaires de Saclay, 91191 Gif-Sur-Yvette; Institut Français de Recherche sur la Mer, Centre de Brest, 29280 Plouzane, France

Prunet, P., M. Pisam, J. P. Claireaux, G. Boeuf, and A. Rambourg. Effects of growth hormone on gill chloride cells in juvenile Atlantic salmon (*Salmo salar*). *Am. J. Physiol.* 266 (*Regulatory Integrative Comp. Physiol.* 35): R850–R857, 1994.—Experiments were performed to investigate the effects of ovine growth hormone (oGH) on both the ultrastructural features of chloride cells and the ability of gills to extrude Na^+ after transfer into seawater. February presmolts and June parrs of the Atlantic salmon (*Salmo salar*) were implanted with oGH. In such animals, spontaneously showing a poor ability to adapt themselves to seawater life, GH significantly increased gill Na^+-K^+-adenosinetriphosphatase activity as well as gill sodium efflux into seawater. When examined by electron microscope, two types of chloride cells (α- and β-types) were identified in control parrs and presmolts. GH treatment induced an increase in size and number of α-cells that displayed an extensive tubular system, while the β-cells, thought to be specific to freshwater life, decreased in number. There was, concomitantly, an increase in number of accessory cells associated with the apical portion of the α-cells and, as a result, the formation of extensive shallow junctions between these cell types. Such functional and ultrastructural modifications that mimicked those naturally occurring during the last steps of the smoltification strongly suggest that GH stimulates the differentiation of freshwater chloride cells toward a seawater type.

sodium extrusion; parr-smolt transformation

IN TELEOST FISH, gill chloride cells are primarily responsible for ion secretion in seawater and active branchial ion absorption in hypoosmotic environment (for reviews see Refs. 10, 13). Recent ultrastructural analyses have clearly distinguished in freshwater euryhaline fish two types of chloride cells, the so-called α- and β-cells, on the basis of their location, shape, and ultrastructural features (reviewed in Ref. 32). During adaptation to seawater, the β-cells disappeared, while the α-cells increased in size and became associated with accessory cells to form multicellular complexes in which cells were interconnected by numerous short junctions (8, 43). This complex multicellular organization has been shown to be the major site of active ion extrusion when fish were placed in a hyperosmotic environment (12).

During parr-smolt transformation salmon in fresh water undergo a series of morphological and physiological modifications that preadapt the fish to seawater life (14), and one of the most characteristic features of the smolt is its enhanced seawater adaptability (11, 24).

Recent studies of the endocrine control of smoltification in salmonids indicated that growth hormone (GH) was involved in such preadaptation of smolt to seawater (see review in Ref. 42). Indeed, in vivo treatment of several salmon species with GH inhibited the increase in plasma ion levels observed after direct transfer from fresh water to seawater, while there was a stimulation of the gill Na^+-K^+-adenosinetriphosphatase (ATPase) activity associated with an increase in size and number of the chloride cells (1, 4, 6, 21, 27, 39). Such a role of GH in the processes of smolt preadaptation to seawater was also confirmed by the increase in plasma GH levels currently observed during salmon smoltification and after transfer to seawater (5, 38, 41, 49).

Yet, despite the increasing number of studies confirming the hypoosmoregulatory role of GH in salmonids, no extensive investigation has been so far performed on the effect of GH on the ultrastructure of chloride cells, which are primarily responsible for the net ion excretion in seawater. This is, thus, the purpose of the present study: to analyze in the juvenile Atlantic salmon the effect of GH on both the ultrastructural features of chloride cells and the ability of gills to extrude Na^+ after transfer into seawater.

MATERIALS AND METHODS

Experimental animals. Juvenile Atlantic salmons (*Salmo salar*) of a Norwegian strain were obtained from a freshwater hatchery (Le Conquet, Brittany, France). Fish were reared in Ewos tanks (4 m^2) supplied with fresh water (Na^+: 1.48 meq/l; Ca^{2+}: 0.65 meq/l) and fed on manufactured dry pellets by automatic feeders (2% body wt/day). The photoperiod and water temperature were natural during the experimental period.

In January-February, parrs and presmolts were identified in the same population according to their length, following the technique described by Thorpe et al. (47) and Boeuf and Prunet (3). Briefly, a histogram of the length of the fish in the fish population showed a classical bimodal distribution and it was demonstrated that fish belonging to the lower mode consisted of parrs, while those in the upper mode corresponded to presmolts. Presmolts will undergo a complete smoltification the following spring, whereas in parrs smoltification will only be observed the following year. Both populations were reared separately.

Hormonal treatment. GH treatment was applied on presmolts in February or on parrs in June, since previous results have shown that the hormonal treatment was most efficient on these fish at these particular periods (2, 4). Fish were intraperitoneally implanted either with a pellet of compacted cholesterol powder (sham implanted) or with a pellet containing 250 μg of ovine GH (NIADK-oGH-14). The oGH-containing pellet released GH for a duration of 3–4 wk (P.-Y. Le Bail, unpublished results). After implantation, fish were kept in the same tank during 14 days under normal conditions. On *day 11*, feeding was stopped until the end of the experimental period (*day 14*), at which time fish were sampled either for ultrastruc-

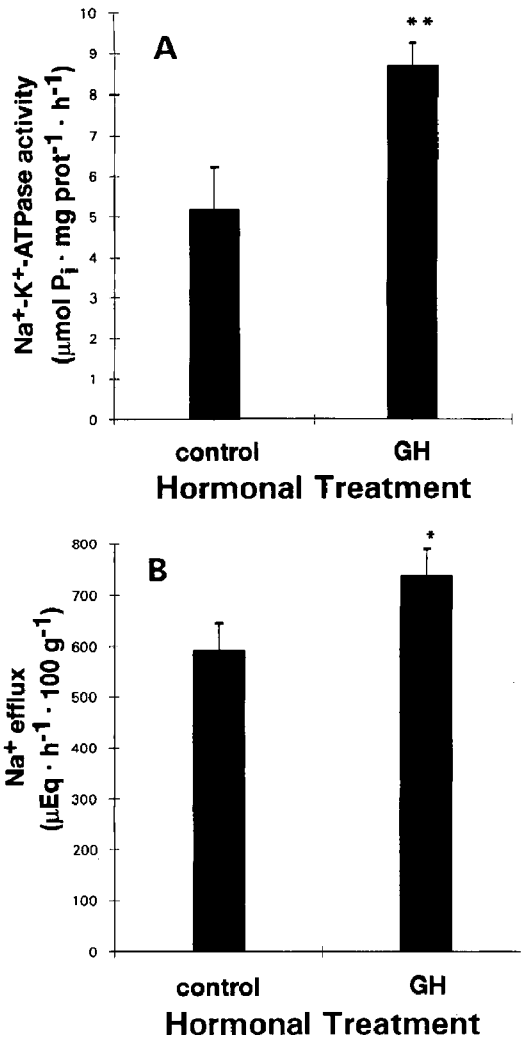

Fig. 1. Effects of in vivo GH treatment (14 days) on gill Na^+-K^+-ATPase activity (*A*) and on gill Na^+ efflux (*B*) in Atlantic salmon presmolt in February. Values are means + SE ($n = 8$). Different from control: $^*P < 0.05$ and $^{**}P < 0.01$.

tural analysis or transferred into seawater (35‰) for Na^+ efflux measurement.

Gill Na^+ efflux determination. Na^+ effluxes ($J^{out}_{Na^+}$) were determined in vivo in fish that had been transferred into full strength seawater for 4 h. Assuming that fecal and urinary losses were negligible, branchial effluxes in seawater were measured according to the technique described by Potts et al. (36): fish were injected into the peritoneal cavity with a known quantity of $^{22}Na^+$. After 30 min, during which time the sodium was distributed throughout the fish sodium space, the animals were transferred into a small aerated box (total volume: 200 ml); immediately after transfer, the radioactivity of the external medium was measured every minute for 10 min by collecting an aliquot of 1 ml. Control and GH-treated salmon were analyzed simultaneously and distributed at random in the experimental box. At the end of the experiment, a plasma sample was collected from every fish and analyzed: $^{22}Na^+$, in the external medium or in the plasma, was measured by liquid scintillation, and plasma Na^+ was determined by atomic absorption spectrophotometry. The efflux $J^{out}_{Na^+}$ (in μeq/h × 100 g) was calculated as

$$J^{out}_{Na^+} = \frac{V_{ext} * Q/t * 6{,}000}{(Q_{int}/Na^+) * W}$$

where V_{ext} is the external medium in milliliters, Q/t is the slope of the regression line between the radioactivities (disintegrations per minute) in the external medium and the time expressed in minutes, Q_{int}/Na^+ the specific activity of the plasma in disintegrations per minute per micromole sodium, and W the fish weight in grams.

Na^+-K^+-ATPase activity. Gill lamellae were rinsed with 0.25 M sucrose (pH 7.4) and immediately stored in liquid nitrogen. Measurements of gill Na^+-K^+-ATPase activity were performed according to the method described by Lasserre et al. (19).

Ultrastructural studies. Gills were fixed in 2% glutaraldehyde in 0.08 M sodium cacodylate (pH 7.5). Fragments were treated with the MnPb technique (31) and postfixed in potassium ferrocyanide-reduced osmium (17). After dehydration in ethanol, fragments were embedded in resin (Epon).

Thin sections running parallel to the long axis of the primary lamella and perpendicular to the secondary lamella were stained for 2 min with lead citrate and examined at 60 kV with a Philips CM 12 electron microscope.

The surface areas of chloride cells were measured on sections running through the nucleus. Area was calculated

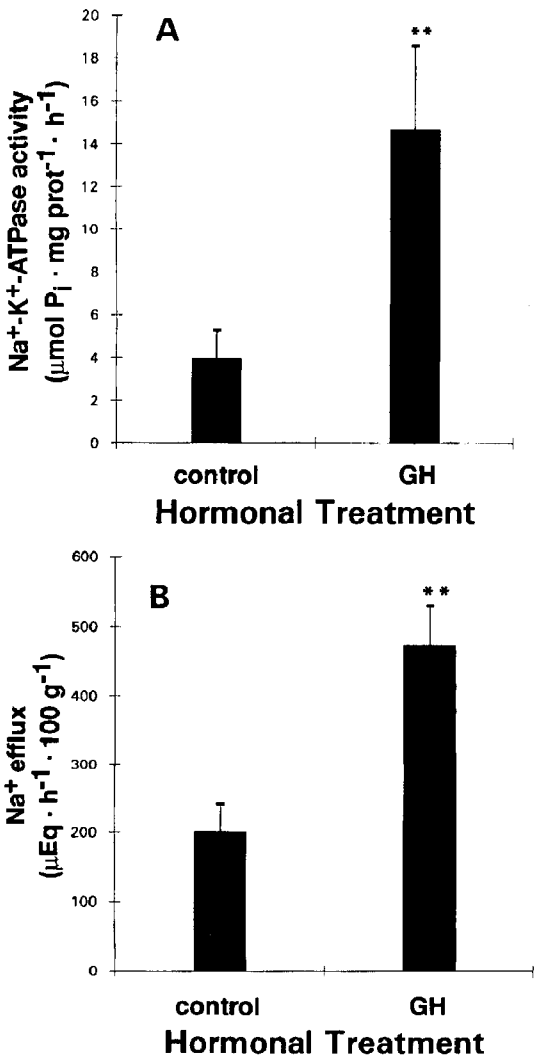

Fig. 2. Effects of in vivo GH treatment (14 days) on gill Na^+-K^+-ATPase activity (*A*) and on gill Na^+ efflux (*B*) in Atlantic salmon parrs in June. Values are means ± SE ($n = 8$). **Different from control value ($P < 0.01$).

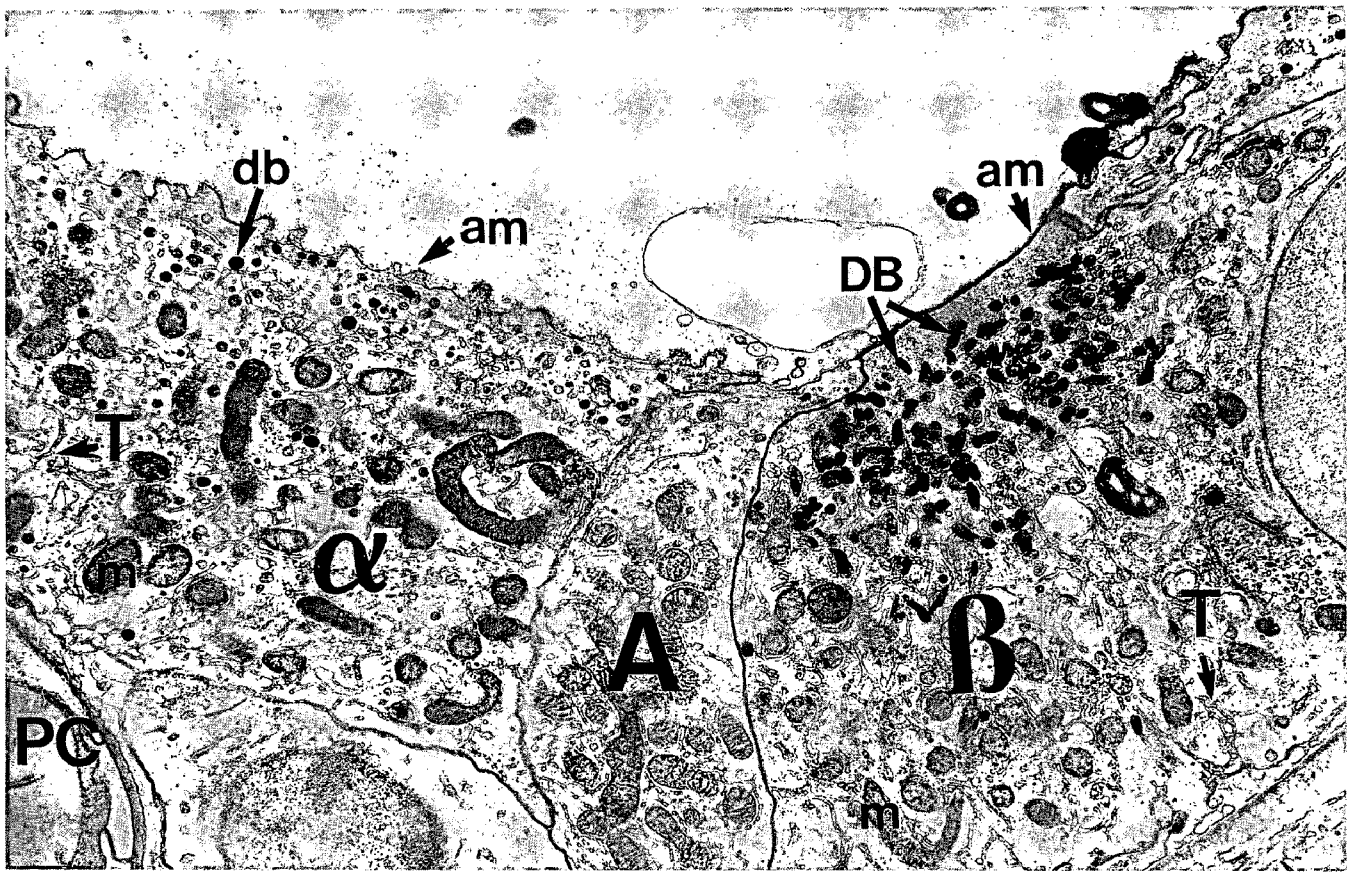

Fig. 3. Chloride cells in a parr gill epithelium. The α-chloride cell, associated with an accessory cell (A) is always found exclusively at the base of a secondary lamella in contact with the pillar capillary (PC). Its apical membrane (am) is endowed with short microvilli, while a few darkly stained bodies (db) are encountered in the apical portion of its cytoplasm. A β-chloride cell, located in the interlamellar region, displays an unfolded apical membrane and an apical region filled with dense bodies (DB) of various sizes and shapes. m, Mitochondria; T, tubular system.

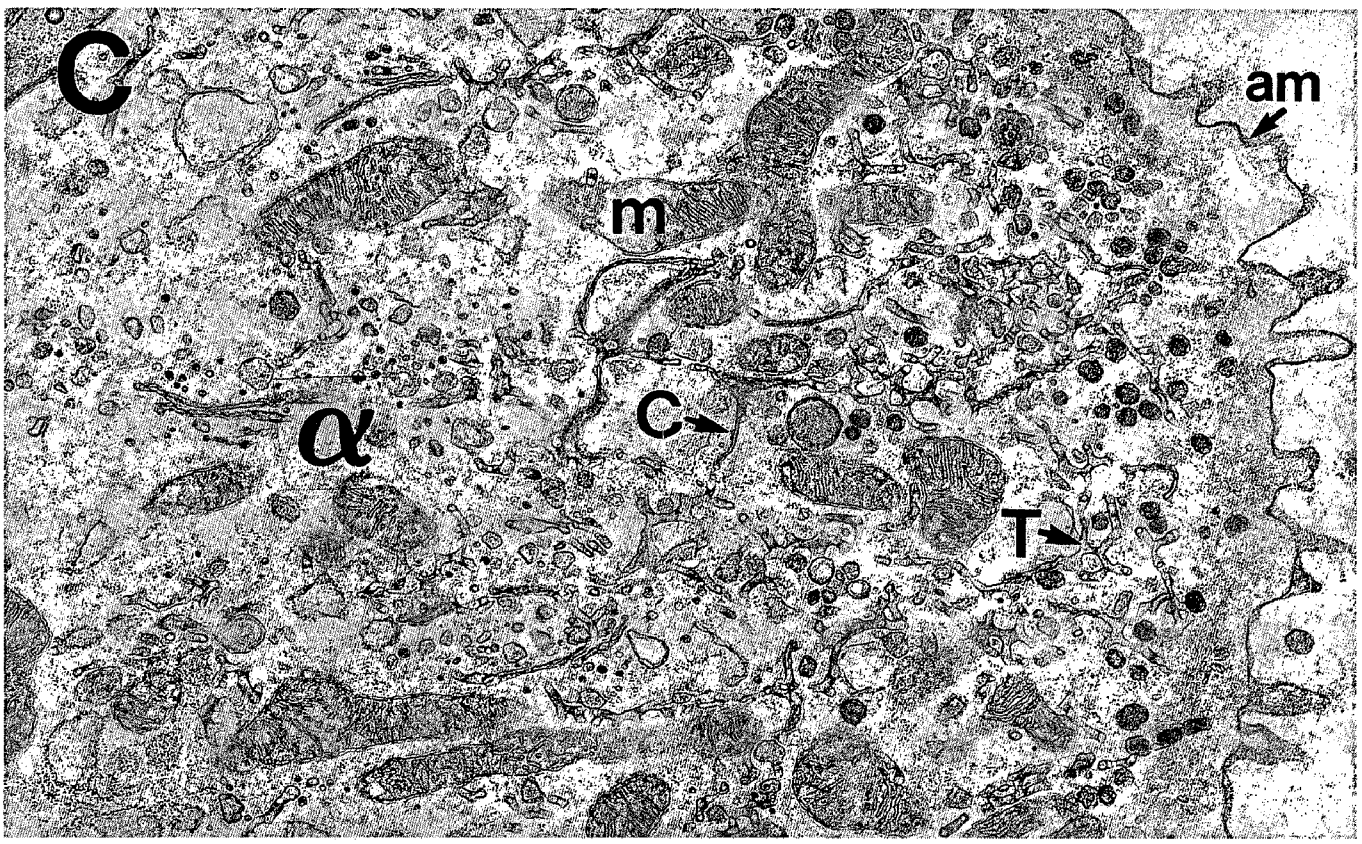

Fig. 4. Apical portion of an α-chloride cell in control animal (C). The tubular system is heterogeneous and consists of membranous tubules (T) in the apex and of loosely anastomosed cisternae (c) in the rest of the cytoplasm. m, mitochondria; am, apical membrane.

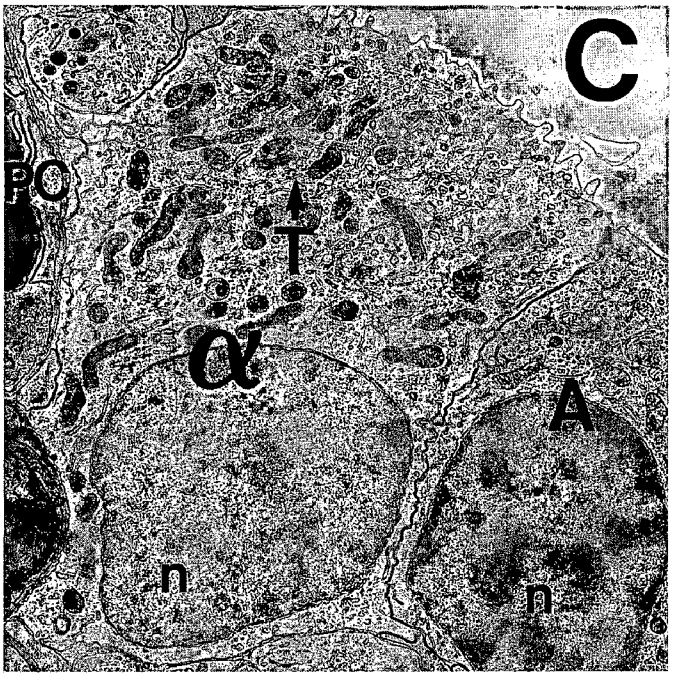

Fig. 5. α-Chloride cell in control animal (C) observed in close contact with a pillar capillary (PC). n, Nucleus; A, accessory cell.

using a Kontron image analyzer on micrographs enlarged at a final magnification of ×3,000.

Data analysis. The statistical significance of the differences in means between groups was determined using the Mann-Whitney nonparametric test. Differences between groups were considered significant if $P < 0.05$.

RESULTS

Effects of GH on gill Na^+ extrusion capacity. When applied in February on Atlantic salmon presmolts, GH treatment significantly stimulated both gill Na^+-K^+-ATPase activity (Fig. 1*A*; $P < 0.01$) and gill sodium efflux measured 4 h after transfer of the fish into seawater (Fig. 1*B*; $P < 0.05$). Such a treatment did not significantly increase these parameters in the February parrs (data not shown). Yet, when GH was given to parrs 4 mo later, there was also a significant ($P < 0.01$) stimulation of these two parameters characteristic of gill Na^+ transport (Fig. 2, *A* and *B*).

Ultrastructural studies. Only fish that responded to GH stimulation, i.e., presmolts in February and parrs in June, were selected for ultrastructural studies of gill chloride cells. Furthermore, since the ultrastructural modifications observed in presmolts or in parrs were similar, the results were pooled together and will now be described in a single section.

In control animals, i.e., in parrs and presmolts not stimulated with GH, two types of chloride cells, the α- and β-cells, were currently found in the gill epithelium.

The α-cells were located at the base of the secondary lamellae near the pillar capillary (Fig. 3). Their apical surface was endowed with short microvilli, while the apical portion of their cytoplasm displayed a few darkly stained spherical bodies (100–250 nm in diam). Their tubular system, that is, the membranous system in continuity with their basolateral plasma membrane, consisted in the apical region of irregularly anastomosed membranous tubules, whereas in the rest of the cell this system was made up of loosely anastomosed cisternae (Fig. 4). They were occasionally associated with an accessory cell (Figs. 3 and 5).

The β-cells, in contrast, were commonly found in the interlamellar region (Fig. 3). Their apical surface was plane, and they showed in their apex an accumulation of large dense bodies variable in size (200–1,000 nm) and shape. In contrast to what was observed in α-cells in which the tubular system was heterogeneous in structure, in β-cells the latter system consisted exclusively of irregularly and loosely anastomosed tubules (Fig. 3).

In GH-implanted animals, the size of the α-cells (Fig. 6) as well their percentage with regard to the total

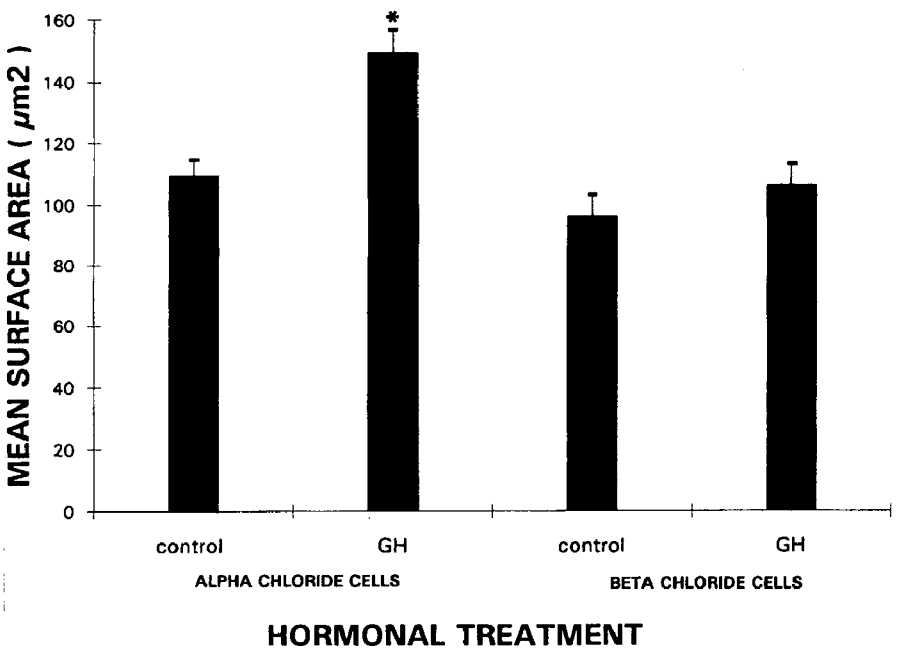

Fig. 6. Effects of in vivo GH treatment on mean surface area (in μm^2) of α- and β-chloride cells in section of the primary gill epithelium from Atlantic salmon parr and presmolt treated in June and February. Values are means + SE (n = 5–6). * Different from control value ($P < 0.05$).

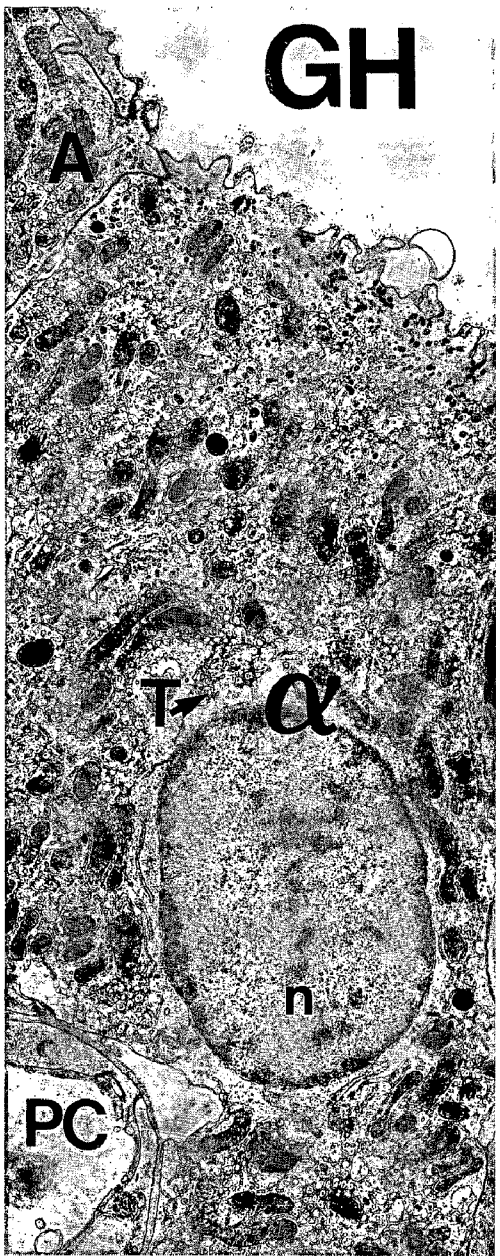

Fig. 7. α-Chloride cell after growth hormone (GH) treatment increases in size and its tubular system (T) is clearly more developed than in control (C) (Fig. 5). A, accessory cell; PC, pillar capillary; n, nucleus.

chloride cell population (73.5 ± 4.8%, $n = 6$ instead of 52.5 ± 3.2%, $n = 5$ in controls) increased significantly ($P < 0.05$). Their tubular system developed extensively to form a tight network of anastomosed membranous tubules (Figs. 7 and 8). The percentage of α-cells associated with accessory cells was significantly higher (49.3 ± 3.7%, $n = 6$) than in control animals (19.4 ± 2%, $n = 5$) ($P < 0.05$) and the length of the apical junction binding both types of cells was shorter: 60–90 nm in length (Fig. 9) as compared with 150–200 nm in control animals (Fig. 10).

As for the β-cells, they decreased in number but neither their size (Fig. 6) nor their ultrastructural features were significantly modified.

DISCUSSION

The heterogeneity of the chloride cell population has been reported to occur in the branchial epithelium of euryhaline and stenohaline freshwater teleosts (see review in Ref. 32). In the present study, a careful analysis of the ultrastructural features of chloride cells allowed us to clearly identify in parrs and presmolts two cell types that obviously correspond to the α- and β-cells previously described in several freshwater fish species (31, 33, 34). The presence of these two cell types and, more specifically, the presence of β-cells is possibly related to the limited ability of these juvenile salmons to withstand direct transfer to seawater (4, 30).

Data obtained in the present study clearly demonstrate, on the one hand, that GH induces important physiological and morphological changes in the gill epithelium that occurred at the level of chloride cells. Elevated plasma GH levels, on the other hand, have been found during smoltification in coho, Atlantic, and masu salmon (29, 38, 46, 49), while GH treatment stimulated the seawater adaptation of juvenile salmons (4, 6, 27). It is therefore likely that this hormone might play an important role in the development of hypoosmoregulatory ability in salmon smolt.

Indeed, several arguments support such an interpretation.

1) In agreement with previous results obtained in salmon or in trout (4, 20, 21, 27, 39), GH treatment applied to presmolts or parrs stimulates gill Na^+-K^+-ATPase activity. A high level of Na^+-K^+-ATPase activity has been specifically localized on the basolateral membrane of chloride cells (15, 44) and it has been shown that this enzyme was involved in salt extrusion by chloride cells in seawater (7, 22, 45). Thus the stimulatory effect of GH on Na^+-K^+-ATPase activity is likely to indicate important changes in the salt secretion ability of chloride cell.

2) Morphological analysis of the chloride cells located on the primary lamellae demonstrates that GH treatment induces ultrastructural modifications known to be associated with seawater adaptation. There was an increase in size and number of the α-cells that displayed an extensively developed tubular system, while the β-cells, thought to be specific of freshwater life (32), decreased in number. Such an observation indicates that the stimulation of gill Na^+-K^+-ATPase activity is likely to be the functional counterpart of the development in chloride cells of the tubular system where this enzyme is known to be located (16). Furthermore, since there was also a concomitant increase in number of accessory cells associated with the apical portion of the α-cells, the capacity of gill to extrude ions in a hyperosmotic environment is presumably facilitated by the formation of extensive shallow junctions between these cell types (12, 32, 43) and would lead to an increase of the outward diffusion of Na^+ through paracellular pathways.

3) It has been suggested that the hypoosmoregulatory role of GH in salmonid would involve stimulation of salt secretion (4, 20, 21). In the present study, GH appears to

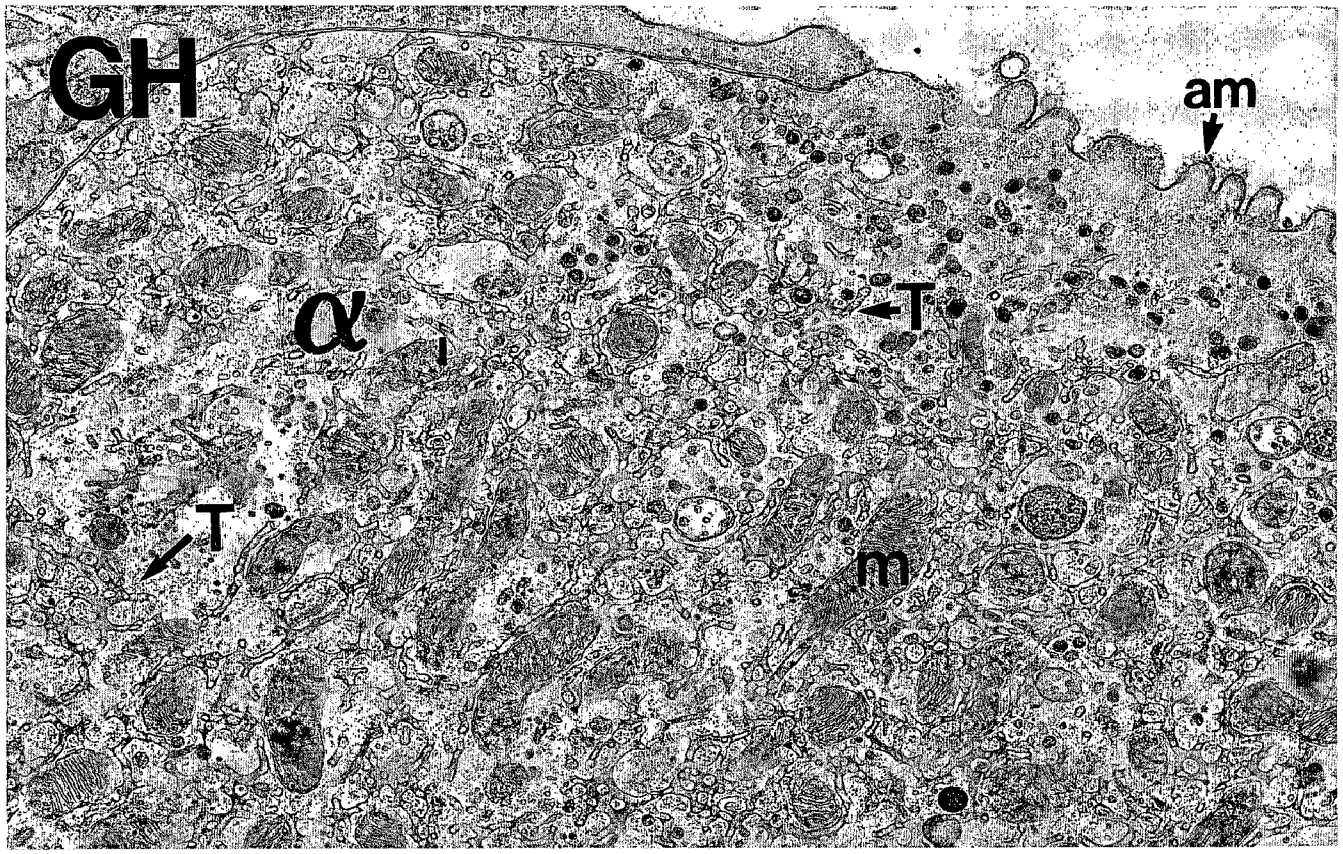

Fig. 8. Apical portion of an α-chloride cell after GH treatment (GH). Tubular system consists mostly of a network of tightly anastomosed membranous tubules (T). m, mitochondria; am, apical membrane.

stimulate in Atlantic salmon presmolts and parrs gill Na^+ efflux, measured immediately after transfer into seawater. Such an efflux may be attributed partly to exchange diffusion, partly to active excretion, and partly to a passive outward diffusion of sodium. The impossibility to measure in vivo gill membrane potential did not allow us to differentiate active Na^+ excretion from passive diffusion. Yet, as mentioned above, the GH effect on Na^+-K^+-ATPase activity suggests that the active component of the Na^+ efflux may be stimulated by the hormonal treatment. Such a conclusion would in fact agree with previous studies showing high plasma GH levels in Atlantic salmon smolts (38). Indeed, in vitro measurements of gill Na^+ fluxes in these fish (28) have shown the development of an active Na^+ excretion mechanism at the level of the primary lamellae immediately after seawater transfer, a characteristic never observed in nonsmolt fish in which GH plasma levels always remained at low values (28, 38). However, it should be pointed out that the GH effects on the apical junctions between chloride and accessory cells observed in the present study suggest in addition a likely stimulation by the hormone of a passive component of the Na^+ efflux, a result in keeping with the increase of transepithelial Na^+ permeability previously reported in Atlantic salmon smolts (36, 37). Thus, in presmolts and parrs, GH stimulated the gill Na^+ efflux at the level of both the active excretion and passive diffusion of sodium to reach Na^+ efflux values close to those measured in seawater adapted salmonids (9, 36).

The appearance of morphological and physiological changes at the level of gill chloride cells after in vivo GH treatment raises the question of the mode of action of this hormone. GH receptors have been characterized in the gill of rainbow trout (40) and juvenile Atlantic salmon (J. Almendras, B. Auperin, and P. Prunet,

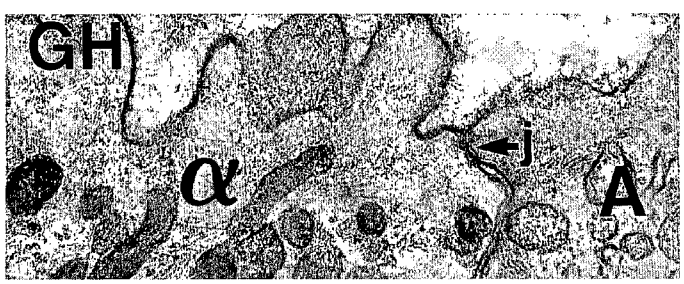

Fig. 9. Apical junction (j) between an α-chloride cell (α) and accessory cell (A) after GH treatment. Junction is shallower than in control.

Fig. 10. Apical junction (j) between an α-chloride cell (α) and an accessory cell (A) in control animal (C).

unpublished data), a finding that indicates that the gill may be a target organ of GH osmoregulatory actions. However, McCormick et al. (25) recently showed that recombinant bovine insulin-like growth factor I (IGF-I) was more potent in promoting hypoosmoregulatory ability in rainbow trout than ovine GH. This suggests that IGF-I produced by the liver or in the osmoregulatory organs might mediate at least part of the seawater-adapting action of GH, since a similar dual regulation by IGF and GH of kidney function has also been proposed in mammals (18). Finally, cortisol has also been found to play an important role in seawater adaptation of several salmonid species (20, 23, 26, 39). Thus, after in vitro observation of an enhancement of interrenal response to adrenocorticotropic hormone in presence of GH (48), it has been suggested that cortisol might mediate some of the osmoregulatory effects of GH (25, 42). Yet in previous studies on the osmoregulatory actions of GH in Atlantic salmon (Ref. 2, P. Prunet, M. Avella, and G. Boeuf, unpublished data), it was impossible to observe any stimulatory effect of cortisol on seawater adaptability or gill ion excretion. Clearly then, further studies are needed to clarify the functional relationship between GH and cortisol during salmon smoltification.

In conclusion, the present study demonstrates that GH treatment stimulates the differentiation of freshwater chloride cells toward the seawater type, as indicated by the morphological changes of these cells, which are reflected by an increased gill Na^+ efflux in seawater. Such modifications are clearly responsible for the strong enhancement of hypoosmoregulatory ability of the GH-treated juvenile Atlantic salmon previously described by Boeuf et al. (4), since they obviously mimicked those naturally occurring during Atlantic salmon smoltification (28, 35). Thus, at least through its effects on gill chloride cells, GH is likely to play a major role in Atlantic salmon smoltification.

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Address for reprint requests: M. Pisam, Département de Biologie, Centre d'Études de Saclay, 91191 Gif-Sur-Yvette cedex, France.

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