In adult teleosts, chloride cells in the gill epithelia are important osmoregulatory sites for maintaining ionic balance.1 Using a vibrating probe technique, Foskett and Scheffey were the first to demonstrate that chloride cells function as a salt-secreting site in seawater fish.2 Recently, localization of various ion-transport proteins in chloride cells has been increasingly clear in fish adapted to freshwater and seawater, suggesting that chloride cells play a significant role in the adaptation to hypo- and hyperosmotic environments.3,4 Although the gills of teleosts are not yet developed or fully functional during the early life stages, a rich population of chloride cells have been demonstrated in the yolk-sac membrane and other body surfaces in several teleosts, such as Mozambique tilapia Oreochromis mossambicus,5–7 chum salmon Oncorhynchus keta,8 killifish Fundulus heteroclitus,9 and Japanese eel Anguilla japonica.10 Those extrabranchial chloride cells undergo drastic changes in their morphology when transferred from fresh water to seawater, and vice versa. In the embryos and larvae of Mozambique tilapia, for instance, chloride cells in the yolk-sac membrane become larger after the fish have transferred from fresh water to seawater.5 The chloride cells form multicellular complexes together with adjacent accessory cells.5 This morphological activation by chloride cells in seawater suggests that they play a significant role as a salt-secreting site in place of gill chloride cells in adult fish. However, direct evidence for this is still lacking. In the present study, a chloride test and X-ray microanalysis were used to localize chloride-secreting sites in the yolk-sac membrane of seawater-adapted Mozambique tilapia larvae.

Fertilized eggs were obtained by removing them from the mouth of brooding females reared in fresh water.3 Eggs typically hatch approximately 5 days after fertilization at 25°C. At 2 days before hatching, they were separated into two groups: half of the embryos were maintained in fresh water, and the other half was transferred directly to seawater. Following 3 days' incubation in their respective media, fresh water and seawater larvae at 1 day after hatching were used for the experiment.

To determine the chloride-secreting site in the yolk-sac membrane, fresh water and seawater larvae were subjected to the chloride test, which is based on the principle reported by Copeland.11 The embryos were first rinsed with distilled water (DW) three times (for 1 min each) to remove Cl– on the body surface, and immersed in 0.25% AgNO3 in DW for 1 min. During the period of incubation in AgNO3 solution, newly secreted Cl– reacts with Ag+ to form photosensitive AgCl. After a brief rinse with DW, the embryos were placed in glass vials containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and exposed to sunlight for 30 min. The sunlight exposure causes the reduction reaction of AgCl to form a Ag precipitate. After fixation in the same fixative in the dark overnight, the samples were transferred to phosphate-buffered saline (PBS), and the yolk-sac membrane was carefully removed using sharp-pointed forceps under a dissecting microscope. The membrane was then mounted onto a glass slide, a cover slip applied with PBS, and the slide observed using a microscope equipped with a Nomarski’s differential interference contrast (DIC) device.
Using this device, chloride cells in the yolk-sac membrane were readily identified as large, round cells when the device was focused on the subsurface plane (Fig. 1).7 In accordance with previous reports,5–7 chloride cells in the yolk-sac membrane were consistently larger in seawater than in fresh water. A brown deposit, representing the presence of \( \text{Cl}^- \), was detected in the apical pits of chloride cells in seawater-adapted larvae (Fig. 1a), whereas the deposit was not associated with chloride cells in fresh water larvae (Fig. 1b). These findings indicate that \( \text{Cl}^- \) is secreted from the apical pits of chloride cells in seawater larvae, but not in fresh water larvae.

To reconfirm the chloride-secreting activity of chloride cells in seawater larvae, the seawater samples were examined using X-ray microanalysis. The seawater larvae, which were subjected to the chloride test and fixed as described previously, were dehydrated in ethanol, immersed in \( \text{t}-\text{butyl alcohol} \), and dried using a freeze-frying device (JFD-300; JEOL Co. Ltd, Tokyo, Japan). The larvae were mounted on an aluminum specimen stub, coated with gold in an ion sputter (JFC-1100; JEOL Co. Ltd), and examined with a scanning electron microscope (SEM) (S-2150; Hitachi, Tokyo, Japan) that was equipped with an energy-dispersive X-ray microanalyzer (EMAX-5770; Horiba, Kyoto, Japan) and a Super Xerophy X-ray detector (Horiba). The elemental profile of Ag was examined by detecting the X-ray characteristic of Ag at 2.986 keV (L\( \alpha_1 \)). For mapping the Ag profile, the X-ray signals were accumulated for 60 s. Figure 2 shows the distribution image of Ag, together with the actual SEM image corresponding to the same area. From
these images, it is evident that the Ag distribution is confined to the apical pit of the chloride cell and its adjacent area. This finding indicates that Ag is the reaction product of the chloride test, and that chloride cells definitely secrete Cl\(^-\) through the apical pit in seawater tilapia larvae.

Chloride cells in the yolk-sac membrane of tilapia embryos and larvae developed in seawater are characterized by the formation of cellular complexes with adjacent accessory cells.\(^6\) It is also indicated that single chloride cells in fresh water enlarge and are indented by newly differentiated accessory cells to form multicellular complexes during seawater adaptation.\(^7\) Thus, the chloride cells, which are larger in seawater than in fresh water as observed in the present study, are considered to represent the formation of multicellular complexes. Taken together, the findings demonstrate for the first time that seawater-type chloride cell complexes in the yolk-sac membrane have the definitive function of Cl\(^-\) secretion through their apical pits. The present study also provides direct evidence for the ion transporting function of chloride cells in the yolk-sac membrane of fish embryos and larvae, when gill chloride cells are not yet fully functional.

REFERENCES

2. Foskett JK, Scheffey C. The chloride cell: definitive identifi-

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