# Localization of Na , K -ATPase in Tissues of Rabbit and Teleosts Using an Antiserum Directed Against a Partial Sequence of the α-Subunit

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**ABSTRACT**—A specific polyclonal antibody against Na+, K+-ATPase α-subunit was developed using a synthetic oligopeptide as antigen. By Western blot analysis under non-reducing conditions, this antibody recognized a protein band of approximately 150 kDa corresponding to the intact form (αβ-complex) of Na+, K+-ATPase in rabbit kidney. Furthermore, this antibody recognized a 150 kDa protein band corresponding to the intact form of Na+, K+-ATPase and some bands of about 60-65 kDa corresponding to fragments of the α-subunit in gill and kidney of masu salmon. This antibody did not recognize the α-subunit under reducing conditions. By immunohistochemical analysis, cells immunoreactive with this antibody were observed in renal tubular epithelial cells in kidney sections of rabbit, masu salmon, eel and rockfish. In addition, large spherical eosinophilic cells in gills of masu salmon, eel and rockfish were immunoreactive with the antibody. It is likely that these immunoreactive cells correspond to gill chloride cells. These data indicate that this antibody is a useful tool for studying changes in and the function of Na+, K+-ATPase during osmoregulatory adaptaion in a variety of fish species.

## INTRODUCTION

The enzyme sodium, potassium-dependent adenosine triphosphatase (Na+, K+-ATPase) is present in all animal cells and is responsible for maintaining high intracellular K+ and low intracellular Na+ concentrations, relative to the extracellular medium. Many transport systems utilize the potential energy of the resulting gradient to move a variety of substances across the cell membrane. Na+, K+-ATPase therefore confers upon the cell the ability to perform specialized functions such as propagation of nerve impulses, uptake and elimination of ions, etc (Geering, 1990).

In seawater, teleost fish are hyposmotic to the medium and compensate for the osmotic loss of water by drinking seawater. Na+ absorbed from the intestinal tract, or gained by diffusional influx via the gills, is eliminated by branchial Na+, K+-ATPase which is mainly located in chloride cells. In freshwater, the diffusional loss of ions and the osmotic influx of water are balanced by absorption of ions in the gills, kidney and other organs. Specifically, the activity of Na+, K+-ATPase in gill is down-regulated in fish in freshwater and

increased in fish adapted to seawater. Thus, one of the most important enzymes involved in the adaptation of fish to seawater is Na+, K+-ATPase (de Renzis and Bornancin, 1984). A large number of studies have been made on changes and control of Na+, K+-ATPase activity in order to understand the mechanism of osmoregulation in euryhaline teleosts, including salmonids and eel (Hoar, 1988). However, no reports have been published on the direct quantification of Na+, K+-ATPase protein in teleosts.

Na+, K+-ATPase is composed of two different protein subunits, α and β, both of which exist in several distinct isoforms (Shull *et al.*, 1986). The larger α subunit has a molecular weight of about 100 kDa and catalyses Na+/K+ exchange. The β subunit is a glycoprotein with a molecular weight of about 50 kDa. The function of this subunit is unclear, although it may have a role in the maturation and transport of the enzyme to the plasma membrane (Noguchi *et al.*, 1987). To date, much has been published on the purification and structure of Na+, K+-ATPase in many species (Jorgensen, 1982; Horisberger *et al.*, 1991). Moreover, several groups have produced polyclonal or monoclonal antibodies raised against the purified enzyme or its subunits in mammals (Siegel *et al.*, 1984; Sweadner and Gilkeson, 1985; Smith *et al.*, 1987). Recently, the amino acid sequence, encoding genes and cDNAs have been charac-

ized in a number of animals. At the amino acid level, the $\alpha$-subunit is highly conserved across species. In contrast, the sequence homology of the $\beta$-subunit decreases sharply when one compares mammals to fish (Horisberger *et al.*, 1991). Shyjan and Levenson (1989) produced antibodies against fusion proteins of $\alpha$- and $\beta$-subunits under cDNA sequences.

To increase the understanding of the mechanism and the endocrine control of osmoregulation in fish, we produced a specific antibody against a synthetic peptide corresponding to part of the highly conserved region of the Na$^+$, K$^+$-ATPase $\alpha$-subunit. Ultimately, we aim to utilize this antibody to examine quantitative changes in Na$^+$, K$^+$-ATPase at the protein level. In this study, we report on the method used to produce the antibody and results of tests on the specificity of the antibody using immunological techniques.

## MATERIALS AND METHODS

### Animals

Masu salmon (*Oncorhynchus masou*) used in this study were yearling smolts cultivated in fresh water. A group of these animals were adapted to seawater for 1 month. Japanese eels (*Anguilla japonica*) were purchased from a commercial supplier, and maintained in seawater for 2 weeks. Rockfish (*Sebastes schlegeli*) were captured off the coast of Hakodate, southern Hokkaido. New Zealand white rabbits were purchased from a commercial supplier.

### Peptide synthesis

The antigen used was: Val - Thr - Gly - Val - Glu - Glu - Gly - Arg - Leu - Ile - Phe - Asp - Asn - Leu - Lys - Lys - Cys. The amino acid sequence of the synthetic peptide was based on sequences of high homology and the areas of hydrophilicity of the $\alpha$-subunit of Na$^+$, K$^+$-ATPase in white sucker (*Catostomus commersoni*) (Schonrock *et al.*, 1991), electric ray (*Torpedo californica*) (Kawakami *et al.*, 1985), *Drosophila* (Lebovitz *et al.*, 1989), toad (*Xenopus laevis*) (Verrey *et al.*, 1989), chicken (Takeyasu *et al.*, 1988, 1991), rat (Shull *et al.*, 1986), sheep (Shull *et al.*, 1985) and human (Kawakami *et al.*, 1986).

The peptide chain was synthesized using a solid phase peptide synthesizer model 431A (Applied Biosystems, Forest City, CA) and Fmoc chemistry. The peptide was cleaved from HMP-resin with 6% phenol, 2% 1,2-ethanedithiol, 4% thioanisole in trifluoroacetic acid. After lyophilization, the oligopeptide was coupled to keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, MO) using N-($\varepsilon$-maleimidocaproyloxy) succimide (Dojindo, Japan).

### Immunization

A rabbit was immunized with 1 mg KLH conjugated synthetic peptide in complete Freund's adjuvant and was boosted at 2 weeks intervals by subcutaneous injection of 500 $\mu$g of the antigen. The rabbit was bled 10 days after the fourth injection. The antiserum was absorbed with an equal volume of phosphate-buffered saline (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.5; PBS) containing KLH (10 mg/ml). The specificity of the absorbed antibody was tested by Western blot and immunohistochemical analyses.

### Preparation of SDS-extracted microsome

SDS-extracted microsomal fractions were prepared by the method of Siegel *et al.* (1984). Tissues were removed immediately and frozen in liquid nitrogen. Tissues were homogenized with a ground glass homogenizer in four volumes of an ice-cold solution of 0.32 M sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA),

pH 7.4 adjusted with Tris, containing 1 mM PMSF. The homogenate was centrifuged at 1,000 x g for 20 min. The supernatant was then centrifuged at 10,000 x g for 20 min. The resulting supernatant was centrifuged at 100,000 x g for 1 hr to precipitate the microsomal fraction. All centrifugations were performed at 2°C. The microsomal fraction was suspended in a solution of 0.16 M sucrose, 1 mM EDTA, 3 mM Na$_2$ATP, 30 mM imidazole, pH 7.4 adjusted with Tris. The microsomal fraction was then incubated for 30 min at room temperature at a protein concentration of 4 mg/ml with 0.15% (w/v) SDS. The SDS was added slowly with stirring. After incubation, the suspension was recentrifuged at 100,000 x g for 1 hr and the supernatant, the SDS-extracted microsomal fraction, was removed and stored at −80°C until analysis. The concentration of protein was measured using a BCA Protein Assay kit (Pierce, Rockford, USA) with bovine serum albumin as the standard.

### Electrophoresis

Samples were dissolved in 1% SDS, 10% glycerol and 0.01% bromophenol blue. The mixture was heated in a boiling water bath for 2 min and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels (Laemmli, 1970). Gels were stained with 0.1% Coomassie brilliant blue R-250 in ethanol, acetic acid and distilled water (DW) (40 : 10 : 50). Gels were destained in ethanol, acetic acid, glycerol and DW (200 : 50 : 25 : 725). Molecular weights of proteins were estimated using the following marker proteins (Pharmacia, Uppsala, Sweden): myosin (212,000), $\alpha$-macroglobulin (170,000), $\beta$-galactosidase (116,000), transferrin (76,000) and glutamic dehydrogenase (53,000).

### Western blotting

Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA) using a Bio-Rad semidry trans-blot SD (Towbin *et al.*, 1979). The following procedures were done at room temperature. After blotting, the membrane was incubated with shaking for 1 hr in 5% (w/v) skim milk in 0.2 M Tris-HCl, pH 7.5, containing 0.5 M NaCl (TBS) to block free binding sites. The blocked membrane was immersed overnight in 2 ml skim milk (5%) containing the primary antiserum diluted 1 : 500 which had previously been co-incubated overnight at 4°C either with or without the synthetic oligopeptide (200 $\mu$g/ml). After washing twice with TBS containing 0.5% (v/v) Tween 20 (TTBS) and then with TBS, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad, South Richmond, CA, USA) diluted to 1 : 5,000 in TBS for 6 hr. After washing, HRP activity was visualized using a freshly prepared solution of 0.06% 4-chloro-1-napthol in TBS containing 0.06% H$_2$O$_2$. The color reaction was stopped by washing the membrane in tap water and DW.

### Immunohistochemistry

Tissues were removed and fixed for 48 hr in 4% parafolmaldehyde in 0.1 M phosphate buffer (pH 7.2) on ice. Tissue samples were dehydrated through a graded ethanol series, embedded in paraffin, and serial sections of 5 $\mu$m were mounted on gelatin coated glass slides. Sections were deparaffinized in xylene, hydrated in a graded ethanol series, and washed in PBS. The tissue sections were incubated for 30 min in methanol containing 1% H$_2$O$_2$ to block endogenous peroxidase activity and washed in PBS. To reduce nonspecific staining, sections were treated with 10% normal goat serum in PBS for 30 min at room temperature. Primary antiserum at 1 : 500 dilution was applied to sections overnight at 4°C. After washing with PBS, tissues were incubated with a solution of biotinylated goat anti-rabbit IgG at 1 : 400 dilution (Dako, Glostrup, Denmark) for 3 hr at room temperature. Finally, the streptavidin and biotinylated HRP complex (Dako) was applied for 30 min and the sections were then washed with 50 mM Tris-HCl (pH 7.6) (TB). The final reaction product was visualized using 3,3'-diaminobenzidine

tetrahydrochloride in TB containing 0.03% $H_2O_2$. The immuno stained sections and adjacent sections stained with hematoxylin-eosin were observed with a Zeiss Axiophot microscope.

## RESULTS

### SDS-PAGE and Western blot analysis

Electrophoretic patterns and Western blot analysis of SDS-extracted microsomal fractions from kidney of rabbit, and gill and kidney of masu salmon are shown in Figure 1. Under nonreducing conditions, many protein bands were stained with CBB (Fig. 1, panel A). In rabbit kidney, the antiserum reacted only with the 150 kDa band (Fig. 1, panel B, lane 1). In both gill and kidney of masu salmon (Fig. 1, panel B, lanes 2 and 3), the antibody recognized the 150 kDa and some bands around 60-65 kDa. The immunoreactivity of the kidney fraction was less than that of the gill fraction. Immunoreactivity disappeared after co-incubation of the antibody with the synthetic oligopeptide (Fig. 1, panel C).

### Immunohistochemistry

Immunohistochemical staining of kidney sections of rab-bit and freshwater masu salmon is shown in Figure 2 and 3, respectively. In the kidney of rabbit, the antibody stained the basolateral surface of proximal tubules (Fig. 2, panel C), distal tubules (Fig. 2, panel D) and collecting tubules (Fig. 2, panel E), while the glomerulus was virtually without staining. In the kidney of freshwater masu salmon, in addition to the basolateral surface, the luminal surface of distal tubules and collecting tubules was also stained (Fig. 3, pnel C and D).

Immunohistochemical staining of gill sections of fresh-water masu salmon is shown in Figure 4. Immunoreactivity was observed in large spherical eosinophilic cells situated between blood vessels and the free surface, and on lamel-lae. A large oval nucleus was located in the basal part of the immunoreactive cells.

Immunohistochemical staining of gill and kidney sec-tions of seawater-adapted masu salmon, eel, and rockfish is shown in Figure 5. In gills, most immunoreactivity was ob-served in large spherical eosinophilic cells situated on pri-mary lamellae. In kidneys, although the antibody stained the basolateral surface of proximal tubules, distal tubules and collecting tubules, immunoreactivity was weak. The glom-erulus was not stained with the antibody.
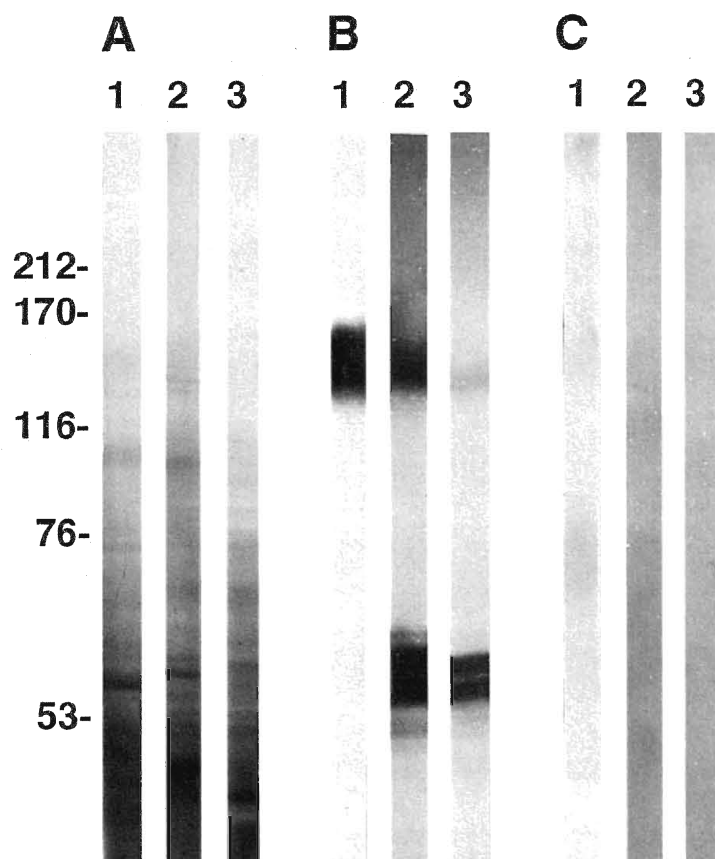


Fig. 1. SDS-PAGE gel stained with CBB (A) and Western blotting analysis (B, C) of SDS-extracted microsomes from rabbit kidney (lane 1), masu salmon gill (lane 2) and masu salmon kidney (lane 3). Panel C shows membranes exposed to antiserum by co-incubating with the synthetic oligopeptide. Positions of molecular weight markers, expressed in kilodaltons, are indicated on the left side of the figure.
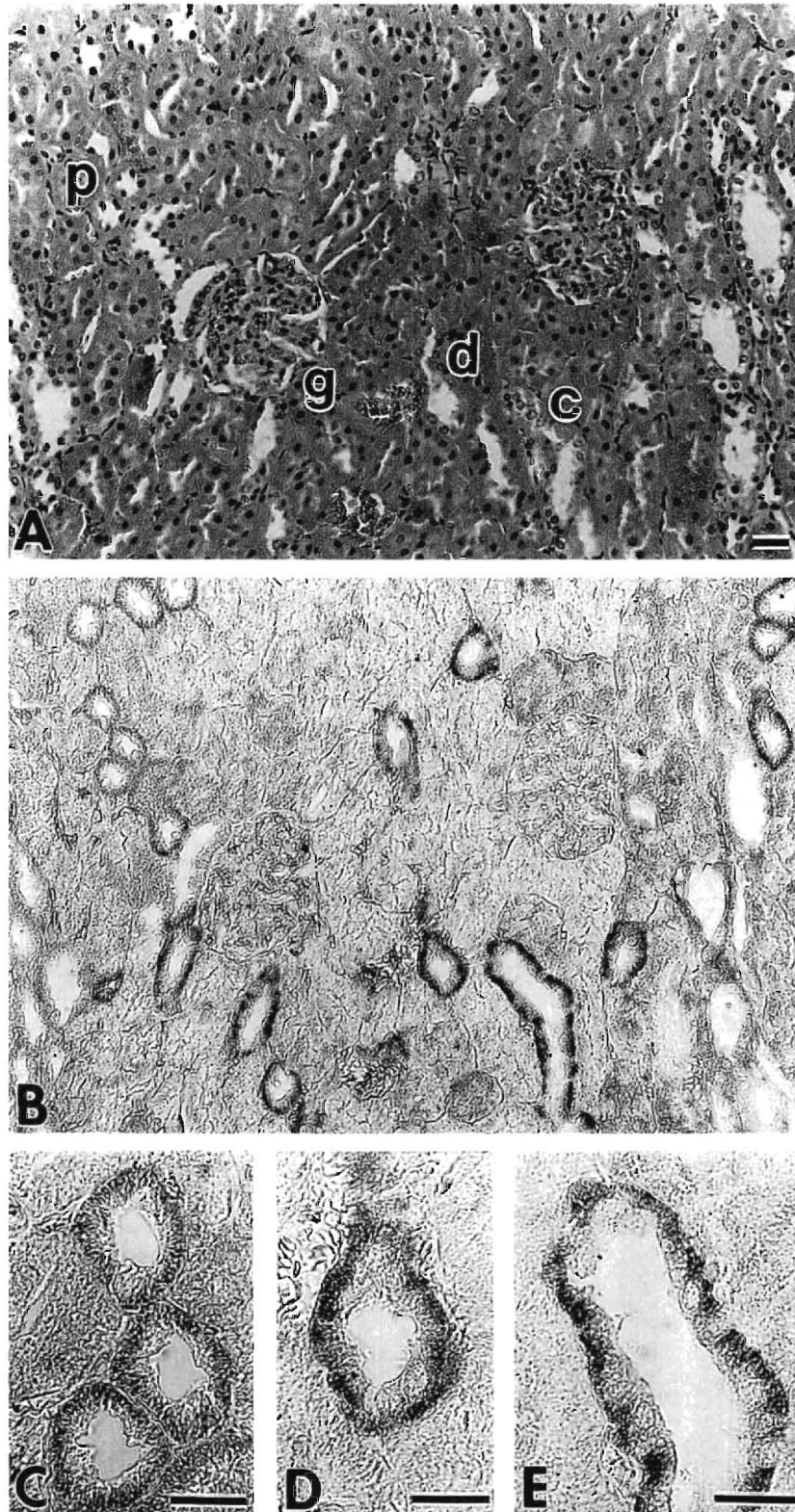
Fig. 2.   Adjacent sections of rabbit kidney stained with hematoxylin-eosin (A) and the antibody to $Na^+$, $K^+$-ATPase $\alpha$-subunit (B).   Panel C, D and E show proximal convoluted tubule, distal convoluted tubule and collecting tubule at high magnification, respectively.   g, glomerulus; p, proximal convoluted tubule; d, distal convoluted tubule; c, collecting tubule.   Bar 25 μm.
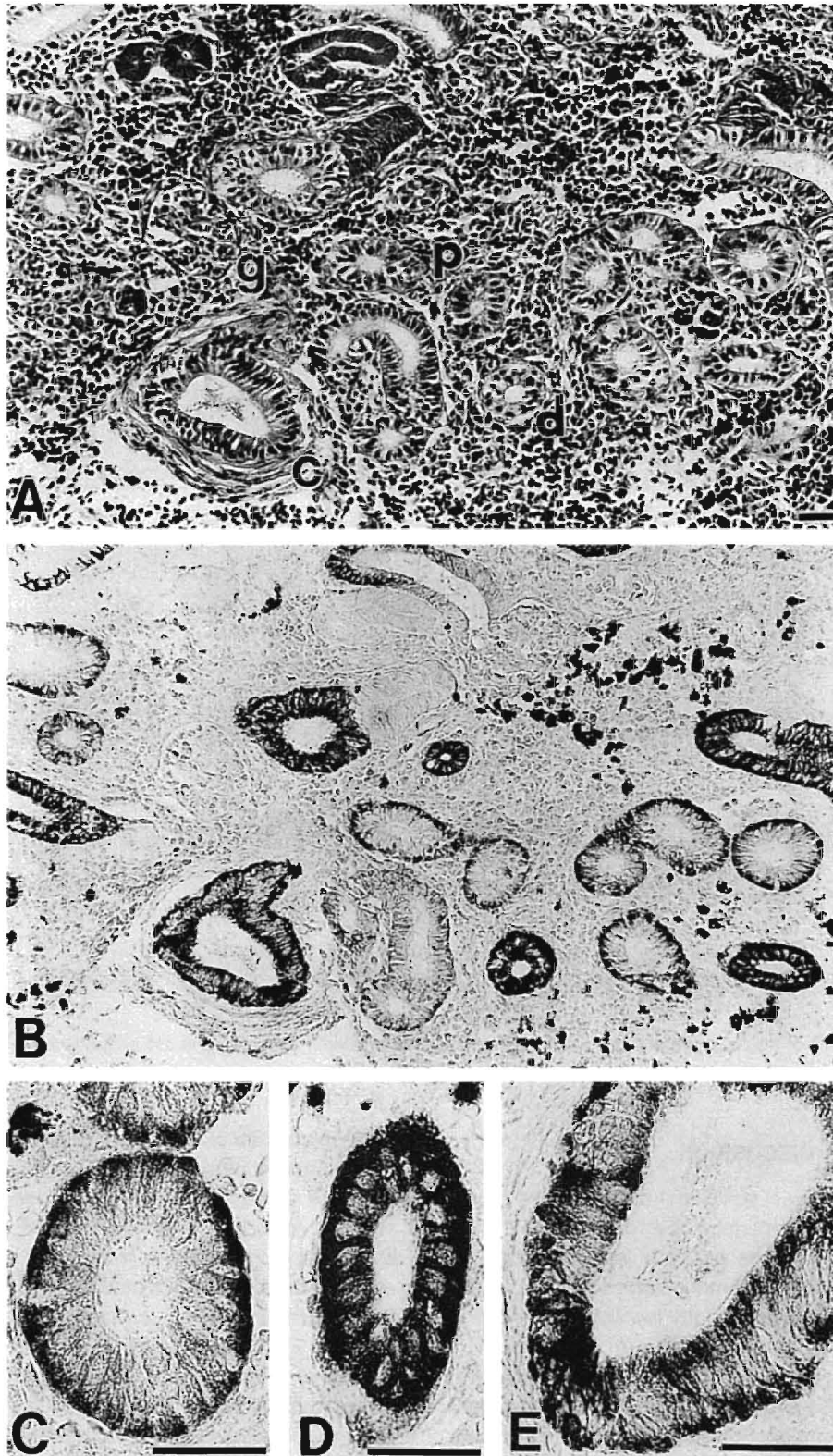
Fig. 3.   Adjacent sections of freshwater masu salmon kidney stained with hematoxylin-eosin (A) and the antibody to Na+, K+-ATPase α-subunit (B).   Panel C, D and E show proximal convoluted tubule, distal convoluted tubule and collecting tubule at high magnification, respectively.   g, glomerulus; p, proximal convoluted tubule; d, distal convoluted tubule; c, collecting tubule.   Bar 25 μm.
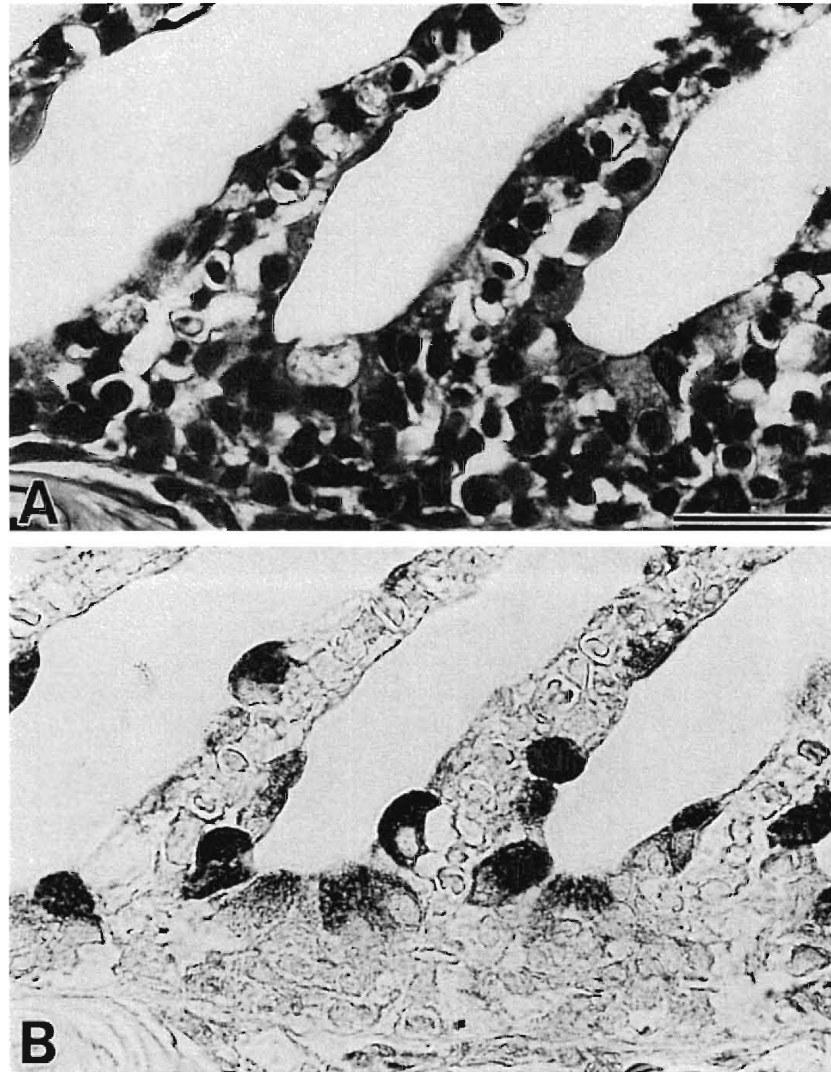
Fig. 4.   Adjacent sections of freshwater masu salmon gill stained with hematoxylin-eosin (A) and the antibody to $Na^+$, $K^+$-ATPase $\alpha$-subunit (B). Bar 25 μm.

## DISCUSSION

In the present study, a polyclonal antibody was raised against a synthetic oligopeptide of the $Na^+$, $K^+$-ATPase $\alpha$-subunit. The specificity of the antibody was evaluated by Western blot analysis and immunohistochemistry. Under non-reducing conditions, the antibody reacted with only one band of about 150 kDa from rabbit kidney microsomes. $Na^+$, $K^+$-ATPase is composed of two subunits, $\alpha$ and $\beta$, differing in size. Recent reports have estimated the molecular weight of each subunit by using antibodies against purified subunit proteins or fusion proteins in mammals (Siegel *et al.*, 1984, 1986; Shyjan and Levenson, 1989). The molecular weight of the $\alpha$-subunit was calculated to be about 100 kDa, while that of the $\beta$-subunit was calculated to be about 50 kDa. Thus, there is fairly general agreement that the molecular weight of the native enzyme is about 150 kDa. Therefore, these results strongly suggest that the 150 kDa immunostained band

is the intact form of $Na^+$, $K^+$-ATPase in rabbit kidney. In addition to this immunoblot analysis, in immunohistochemical analyses of rabbit kidney, the antibody specifically reacted with the epithelium of proximal, distal and the collecting tubules, but glomeruli were not stained. These findings are in agreement with previous studies localizing $Na^+$, $K^+$-ATPase in mammalian kidney by immunohistochemical methods (Kyte, 1976a, b; Siegel *et al.*, 1984; Felsenfeld and Sweadner, 1988). These results indicate that our antibody recognizes the intact form of $Na^+$, $K^+$-ATPase. However, this antibody did not recognize the band corresponding to the $\alpha$-subunit under reducing conditions (data not shown). We have had similar experiences using an antibody raised against synthetic oligopeptides derived from sequences of the $\beta$-subunits of masu salmon gonadotropin I and II (Ikeuchi *et al.*, 1995). It is possible that the conformation of the $\alpha$-subunit changes, or antigenic changes occur during treatment with 2-mercaptoethanol. Therefore, this antibody is not
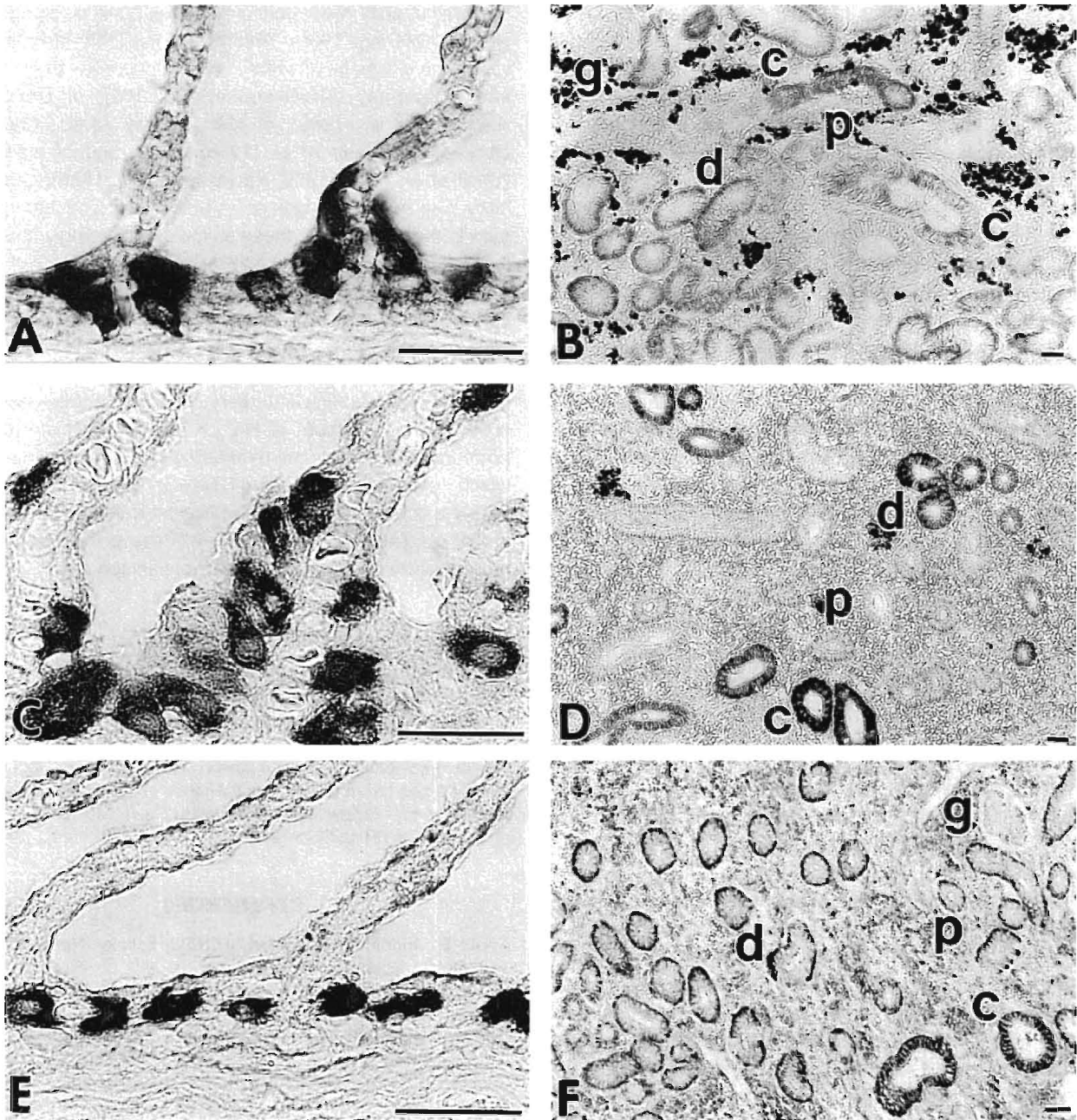
Fig. 5.   Localization of antigen recognized by the antibody in gill and kidney of seawater-adapted masu salmon (A and B), seawater-adapted eel (C and D) and rockfish (E and F).  Bar 25 $\mu$m.

useful for Western blot analysis under reducing conditions.

In gill and kidney of masu salmon, this antibody recognized a 150 kDa band and some bands around 60-65 kDa by Western blot analysis under non-reducing condition. Moreover, no positive bands were seen in any tissue after staining with antiserum co-incubated with the synthetic oligopeptide. Thus, this result indicates that this antibody was specific against the synthetic oligopeptide. Since Jorgensen (1974) has developed the SDS extraction procedure for the purification of Na⁺, K⁺-ATPase, this method has frequently been used for the purification of this enzyme from various tissues. Although SDS is useful for extraction of membrane-bound proteins, it denatures proteins. In gill and kidney of masu salmon, it is likely that the 150 kDa immunostained band is the intact form of Na⁺, K⁺-ATPase, and the positive bands of 60-65 kDa are proteolytic fragments of the $\alpha$-subunit arising from extraction with SDS. The selection of more appropriate detergents for preparation of Na⁺, K⁺-ATPase from tissues of teleost fish remains to be explored.

As in the rabbit kidney, the epithelium of proximal convoluted tubules, distal convoluted tubules and collecting tubules of teleost fishes were stained with the antiserum. Although a large number of studies have been made on the structure and activity of $Na^+$, $K^+$-ATPase in the kidney of fish, little is known about the localization of the enzyme. This is the first demonstration of the localization of $Na^+$, $K^+$-ATPase protein in the kidney of fish. In the kidney of mammals, $Na^+$, $K^+$-ATPase protein is localized in the basolateral surface of the nephron segment (Siegel *et al.*, 1984). In this study, however, not only the basolateral surface but also luminal surface of distal and collecting tubules were stained with this antibody in the kidney of freshwater masu salmon. Further work at the ultrastructural level is required to determine the precise localization of the enzyme. In the kidney of masu salmon, eel and rockfish in seawater, although the epithelium of proximal, distal convoluted and collecting tubules were stained with the antiserum, the immunoreactivity was less than that of kidney of fresh water masu salmon. This observation is compatible with previous results. In freshwater, glomerular filtration rate and renal reabsorption of $Na^+$ are higher than in seawater (Hickman and Trump, 1969), and $Na^+$, $K^+$-ATPase activity is lower in kidney of seawater-acclimated striped bass (*Morone saxatilis*) compared to that in freshwater fish (Madsen *et al.*, 1994).

In gill of masu salmon, immunoreactive cells were observed on the surface of primary lamellae and the proximal end of secondary lamellae. These cells are large spherical eosinophilic cells and appear to correspond to the "chloride secreting cells" of the eel described by Keys and Willmer (1932), (now "chloride cells" (Copeland, 1948, 1950)). At present, there is fairly general agreement that gill chloride cells have a predominant role in regulating plasma ions in teleosts. Many histological studies have localized that $Na^+$, $K^+$-ATPase in chloride cells, by cell isolation procedures (Kamiya, 1972; Sargent *et al.*, 1975), by autoradiography with $^3$H-ouabin (Karnaky *et al.*, 1976) and by enzymatic staining with $K^+$-NPPase (Hootman and Philpott, 1979). Chloride cells have a densely branched network of tubules (Pisam and Rambourg, 1991). Hootman and Philpott (1979) indicated clearly that the enzyme is located on the surface of tubules. Recent ultrastructural analyses have clearly distinguished two types of chloride cells, on the basis of their location, shape, and ultrastructural features (Pisam and Rambourg, 1991). In the present study, we do not know if all chloride cells were stained with the antibody, or where the enzyme is localized in the chloride cell. Further research at the ultrastructural level should answer these questions. Moreover, as in the rockfish gill, most chloride cells were shown to be located on the primary lamellae of gills of seawater-adapted masu salmon and eel. Similar observations were made following seawater acclimation of tilapia (*Oreochromis niloticus*) (Avella *et al.*, 1993).

In addition to the results described above, our antibody reacted with gill and kidney of various teleosts using immunohistochemical methods. Throughout the animal kingdom

the amino acid sequence of the *a*-subunit is highly conserved (Geering, 1990). About 80% similarity is found between the *a*-subunit of white sucker (Schonrock *et al.*, 1991) and electric ray (Kawakami *et al.*, 1985), of *Drosophila* (Lebovitz *et al.*, 1989), of toad (Verrey *et al.*, 1989), of chicken (Takeyasu *et al.*, 1988, 1991), and of mammals (Shull *et al.*, 1985, 1986; Kawakami *et al.*, 1986). Our antibody was produced against an amino acid sequence common to the *a*-subunit of these animals. Accordingly, this antibody cross-reacted across species.

$Na^+$, $K^+$-ATPase plays an essential role in adaptation of fish migrating from freshwater to seawater, such as salmonids and eels. While the function of $Na^+$, $K^+$-ATPase in ion and water absorption (intestine in seawater) and in ion elimination is reasonably well-understood, most studies have focused on measurment of $Na^+$, $K^+$-ATPase activity and its hormonal regulation. The availability of a specific antiserum which recognizes $Na^+$, $K^+$-ATPase in diverse species will permit biochemical and immunological analyses of the hormonal regulation of the $Na^+$, $K^+$-ATPase protein during osmoregulatory changes in a variety of fish species.

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