Biochemical and Biophysical Research Communications **255,** 175–181 (1999)
Article ID bbrc.1999.0166, available online at http://www.idealibrary.com on IDEAL®

Molecular Cloning of CLC Chloride Channels in *Oreochromis Mossambicus* and Their Functional Complementation of Yeast *CLC* Gene Mutant

Hiroaki Miyazaki,*,†,[1] Shinichi Uchida,* Yoshio Takei,† Tetsuya Hirano,† Fumiaki Marumo,* and Sei Sasaki*

**Second Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo, Tokyo 113-8519, Japan; and †Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo 164-8639, Japan*

Received January 6, 1999

We have cloned two members of the CLC chloride channel family (OmCLC-3 and OmCLC-5) from gill cDNA libraries of the euryhaline tilapia *Oreochromis mosammbicus*. At the amino acid level, OmCLC-3 is 90.5% identical to rat CLC-3 and OmCLC-5 is 79.2% identical to rat CLC-5. Ribonuclease protection assay revealed that OmCLC-5 was mainly expressed in the gill, kidney, and intestine in both freshwater- (FW) and seawater- (SW) adapted tilapia. Although the mRNA of OmCLC-3 was broadly expressed in tissues of FW- and SW-adapted tilapia, the most intense signals were observed in the gill, kidney, intestine, and brain. Injection of OmCLC-3 and OmCLC-5 cRNAs into *Xenopus* oocytes did not elicit chloride currents, but these clones did functionally complement the *gef1* phenotype of YPH250(*gef*), a yeast strain in which a single CLC channel (GEF1) has been disrupted by homologous recombination. These results clearly indicated that CLC channels closely related to the mammalian CLC-3, -4, and -5 subfamily exist also in tilapia and that OmCLC-3 and OmCLC-5 function as intracellular chloride channels. © 1999 Academic Press

Chloride channels are passive anion transport proteins involved in basic functions common to all cells such as regulation of cell volume and intravesicular pH. In mammals, chloride channels are important for transepithelial chloride transport in osmoregulatory organs and regulation of excitability of muscle and nerve. The CLC chloride channel family, originally established by the elasmobranch (*Torpedo*) electric organ (CLC-O) [1], is made up of members in bacteria [2], yeast [3], plants [4, 5], amphibian [6], and mammals. Nine *CLC* genes have been discovered so far in a single mammalian species. CLC-1 is established as the skeletal muscle chloride channel that controls the excitability of the muscle fiber [7]. The ubiquitously expressed CLC-2 can be activated by cell swelling in oocytes and possibly plays a role in the regulation of cell volume [8]. The CLC-K1 and CLC-K2 [9–11] channels are exclusively expressed in the kidney and involved in transepithelial chloride transport. CLC-3 [12], -4 [13], and -5 [14, 15] constitute a sub-branch of this gene family. CLC-3 is recently shown to be a volume-regulated chloride channel [16], and loss-of-function mutations of human CLC-5 were shown to result in X-linked recessive nephrolithiasis [17]. The only CLC homologue in yeast, *GEF1,* is most closely related to this sub-branch. Since disruption of this yeast gene results in reduced ability to utilize glycerol and ethanol in the absence of supplemented iron, *GEF1* is somehow involved in respiration and iron metabolism [3].

To further increase our knowledge of physiological function of this gene family in teleost, we sought to isolate CLC chloride channel in *Oreochromis mossambicus.* Teleost fishes maintain ionic composition and osmolality of the body fluid at levels different from external environments by adjusting the functions of osmoregulatory surfaces. In teleost, the gills, kidney and intestine are important osmoregulatory organs, creating ionic gradients between the body fluid and external environments. Among tilapia species, *Oreochromis mossambicus* is a suitable experimental model for studies of osmoregulatory mechanisms because of its strong euryhalinity [18, 19]. Accordingly, we chose the gill of tilapia for cloning CLC chloride channels in the teleost. We isolated two CLC chloride channels and named them OmCLC-3 and OmCLC-5 based on the sequence comparison with the previously cloned mammalian CLC chloride channels. A lack of functional

[1] To whom correspondence should be addressed at Second Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo, Tokyo 113-8519, Japan. Fax: 81-3-5803-0172. E-mail: hmiyazak@ori.u-tokyo.ac.jp.

0006-291X/99 $30.00

expression in *Xenopus* oocytes and functional complementation of the *gef1* phenotype suggested that OmCLC-3 and -5 function as intracellular chloride channels.

MATERIALS AND METHODS

Tissue preparation. Adult tilapia, *Oreochromis mossambicus*, were collected from a freshwater (FW) pond in northern Okinawa Island and maintained in circulating FW tanks at 25°C. To prepare seawater (SW)-adapted fish, some of the fish were transferred to 50% SW for 2 days and then to 100% SW for at least 30 days. Total RNA used for reverse transcription (RT)-PCR and construction of the cDNA library was extracted from SW- and FW-adapted tilapia gill tissue using TRIZOL Reagent (GIBCO BRL Life Technologies Inc.).

Reverse transcription PCR. For RT-PCR, we made degenerate PCR primers that corresponded to the conserved sequences in CLC families: sense strand, CCGGATCCTCNGGN(A/T/C)TNCCNGA(A/G)NTNAA; antisense strand, CCGAATTCTGNA(C/T)NA(G/A/T)NGGNCCN(A/T) (G/A/C) (C/T)TTNCC. One microgram samples of gill total RNA from FW- and SW-adapted tilapia were reverse-transcribed using avian myelopblastosis virus reverse transcriptase (Boehringer-Mannheim) at 42°C for 60 min and then heated at 94°C for 5 min. The synthesized cDNA was used for subsequent PCR in the following profile: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, 30 cycles. The PCR products were cut with *Eco*RI and *Bam*HI on both ends, ligated into *Eco* RI and *Bam*HI-cut pSPORT1 (GIBCO BRL Life Technologies Inc.), and then sequenced.

Library construction and screening. Oligo (dT)-primed directional tilapia gill (FW- and SW-adapted) cDNA libraries in λZipLox expression vector (GIBCO BRL Life technology Inc.) were constructed by the SUPERSCRIPT λSYSTEM for the cDNA Synthesis kit (GIBCO BRL Life Technology Inc.). Two PCR products that have 150 bp inserts homologous to mammalian CLC chloride channels screened these libraries. The full-length clones 11-2 and 11-6 (4.2 and 3.8 kbp, respectively) were obtained from the FW and SW cDNA libraries, subcloned into pZL1 (GIBCO BRL Life technology Inc.), and designated as OmCLC-5 and OmCLC-3, respectively. Nested deletion clones were prepared using the Erase-A-Base System (Promega), and then sequenced.

RNase protection assay. For the detection of OmCLC-3 and OmCLC-5 mRNA, PCR clones used for the library screening were linearized and used to prepare radiolabeled antisense RNA probes. These probes (1×10^5 cpm/sample) were mixed with 20 μg of total RNA from gill, kidney, intestine, liver, spleen, muscle, and brain of FW- and SW-adapted tilapia. After hybridization and RNase digestion, protected fragments were analyzed in 6% denaturing polyacrylamide gel (RPA II Kit, Ambion).

Generation of gef1 mutant. The *GEF1* gene disruption was introduced into the strain YPH250 (*ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2-Δ1*) [20]. Two synthetic oligonucleotides (57 mer) were prepared for amplification of the *HIS3* gene by PCR using pRS313 vector [20] as a template. For homologous recombination, the 40-nucleotide sequences corresponding to 750–789 bp and 1835–1874 bp of the *GEF1* open reading frame were incorporated into these primers at the 5′-ends. Accordingly, the PCR product contained *HIS3* gene flanked by 40 bp *GEF1* sequences. The cells transformed with this PCR product were selected on SD (yeast synthetic dropout)/-HIS plates, and genomic DNA from several transformants was analyzed to confirm homologous recombination by sequencing the mutated allele. Yeast phenotype was checked by the growth on a YPGE (glycerol and ethanol as non-fermentable carbon source) plate, a YPGE + 1 mM Ferrozine (iron-specific chelator) plate, and a YPG (galactose, 2%) plate [3, 21].

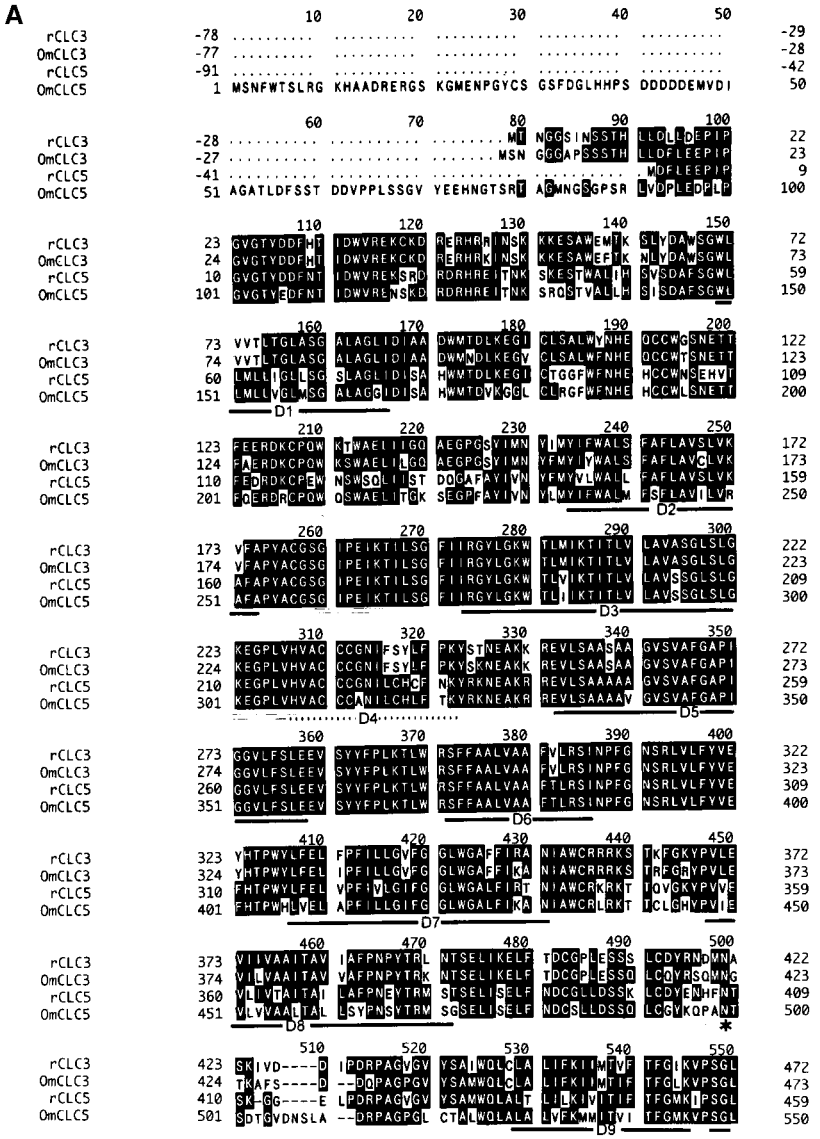
Functional complementation of gef1 yeast mutant. Open reading frames of OmCLC-3 and OmCLC-5 were subcloned into pVT102-U [22] expression vector and transformed into several *gef1* strains. Transformed cells were selected on an SD/-HIS/-URA plate and yeast phenotypes were checked on YPGE, YPGE + Ferrozine, and YPG plates.

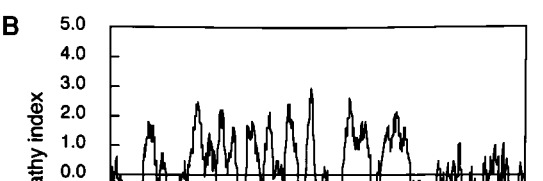
RESULTS

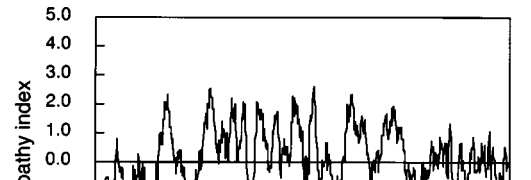
Cloning and sequence analysis of tilapia CLC chloride channels. RT-PCR was performed using tilapia gill RNA and degenerate primers designed from consensus sequences of several CLC chloride channels. Two PCR products (named 11-2 and 11-6) with the expected length (150 bp) were obtained. 11-2 and 11-6 were very homologous to rCLC-5 and rCLC-3, respectively. Using these PCR products as probes, we screened FW- and SW-adapted tilapia gill cDNA libraries and obtained 3.8- and 4.2-kbp clones designated as OmCLC-5 and OmCLC-3, respectively. Sequencing revealed that OmCLC-3 and OmCLC-5 were highly homologous to rCLC-3 (72.1% nucleotide identity) and rCLC-5 (74.1% nucleotide identity), respectively (Fig. 1A).

The open reading frame of OmCLC-3 was 2280 bp, resulting in a 760-amino-acid-protein with a molecular mass of about 85 kDa. OmCLC-5 encodes an 841-amino-acid protein with a molecular mass of about 92 kDa. Fig. 1B shows hydrophathy analysis of the predicted amino acid sequences of OmCLC-3 and OmCLC-5. The hydrophathy profiles show that at least 12 hydrophobic regions with similarities to those detected in other members of the CLC family [23]. In the amino acid sequence of OmCLC-3, Asn-422 located between hydrophobic regions D8 and D9 is a highly conserved glycosylation site among all CLC chloride channels. There are also consensus sequences for phosphorylation by cAMP-dependent protein kinase (PKA) [24] (Thr-360 and Thr-361) and protein kinase C (PKC) [25, 26] (Ser-52, Ser-363, Ser-647, Ser-693, and Thr-744). OmCLC-5 also has a potential N-glycosylation site at Asn-499 located between hydrophobic region D8 and D9. The potential phosphorylation site by PKA is Thr-441, and those by PKC are Ser-7, Ser-724, Thr-77, Thr-128, and Thr-218. To establish a possible evolutionary relationship among these CLC chloride channels, a phylogenic tree was

FIG. 1. Alignment of amino acid sequences of OmCLC-3 and OmCLC-5 with rat CLC-3 and rat CLC-5. (A) Amino acid sequence of OmCLC-3 and OmCLC-5. Conserved residues are filled in black blocks and putative transmembrane-spanning domains are underlined according to the original topology by Jentsch *et al.* (1). Potential *N*-linked glycosylation sites are indicated by asterisk. (B) Hydrophathy profile of OmCLC-3 and OmCLC-5. The mean hydrophathy index was computed according to the algorithm of Kyte and Doolittle.


A
rCLC3
OmCLC3
rCLC5
OmCLC5
D1
D2
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
D13


B
Hydropathy index
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
1
101
201
301
401
501
601
701
760
Amino acid
OmCLC-3


Hydropathy index
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
1
101
201
301
401
501
601
701
801
841
Amino acid
OmCLC-5

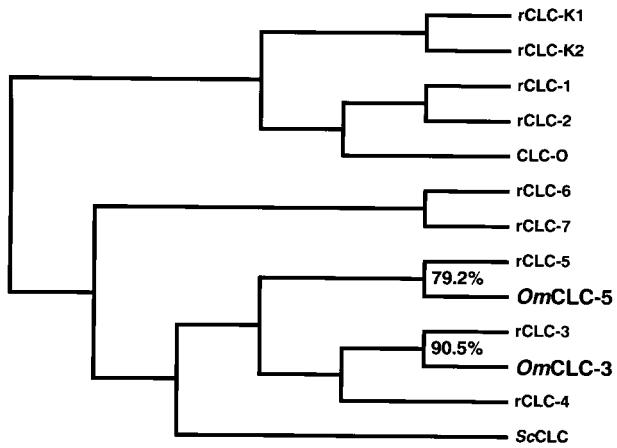


FIG. 2. Evolutionary relationship between the CLC chloride channels. The phylogenic tree shows the relationship among thirteen different members of the CLC family derived from yeast, *Torpedo*, tilapia, and rat. The phylogenic tree was constructed using the DNASIS computer software (Hitachi Software Engineering).

constructed using DNASIS computer software (Hitachi Software Engineering) (Fig. 2).

Tissue distribution of OmCLC-3 and OmCLC-5. Tissue distributions of OmCLC-3 and OmCLC-5 were determined by RNase protection assay using total RNA from FW- and SW-adapted tilapia tissues (Fig. 3). Protected bands were 150 bp. OmCLC-3 was expressed at a high level in the brain at moderate levels in the gill, kidney, and intestine, and at low levels in the liver, spleen, and muscle. There were no changes in expression patterns between FW and SW. OmCLC-5 was expressed at high levels in the gill, kidney, and intestine. The level of expression was much lower in the brain and spleen, and there was no detectable expression in either the liver or muscle.

Functional complementation of yeast gef1 mutant by OmCLC-3 and OmCLC-5. By homologous recombination, we generated a *gef1* mutant with phenotypes as described previously [3]. The phenotypes of *gef1* were checked by plating on a YPGE agar plate containing 1 mM Ferrozine. We obtained four clones that had a phenotype similar to the original *gef1* mutant. These clones were not able to grow on the agar plates containing the non-fermentable carbon source, the YPGE, or the Ferrozine (1 mM). The established *gef1* yeast strains YPH250(*gef*) were transformed with yeast expression vector pVT102-U containing OmCLC-3, OmCLC-5, or GEF1 cDNAs. OmCLC-3-, OmCLC-5-, and *gef1*-transformed cells were plated on YPD and YPG agar plates. Although cells transformed with empty vector could not grow on YPG, both OmCLC-3 and OmCLC-5-transformed cells were able to grow on YPG plate as *GEF1*-transformed cells (Fig. 4). Functional complementation of the *gef1* phenotype by OmCLC-3 and OmCLC-5 was also confirmed on the YPG-Ferrozine plate (data not shown).

DISCUSSION

The present study described the cloning of the CLC chloride channels from tilapia *Oreochromis mossambicus* and elucidated their specific function by functional expression in *S. cerevisiae.* The OmCLC-3 and OmCLC-5 are the first CLC chloride channels identified in a teleostean species. Among the CLC chloride channel family, they are most closely related to the mammalian CLC-3, -4, and -5 subfamily. OmCLC-3 and OmCLC-5 are highly homologous with rat CLC-3 (90.5% amino acid identity) and rat CLC-5 (79.2% amino acid identity), respectively. These results suggest that OmCLC-3 and OmCLC-5 may represent tilapia homologues of mammalian CLC-3 and CLC-5.

As in the case with the mammalian CLC-3 [12], OmCLC-3 is ubiquitously expression with the highest expressed in the brain. Accordingly, OmCLC-3 may have a function similar to that of the mammalian CLC-3. Duan *et al.* [16] recently proposed that CLC-3 is a swelling-activated chloride channel. Since OmCLC-3 is highly homologous with rat CLC-3, this contention might be correct, and OmCLC-3 might be involved in cell volume regulation in tilapia. However, the lack of expression in *Xenopus* oocyte and the functional complementation in *gef1* mutant suggest that it play a role in intracellular organelles rather than in the plasma membrane. Accordingly, it is less likely that OmCLC-3 is involved in cell volume regulation in tilapia. Further functional characterization and information about cellular localization OmCLC-3 will be necessary to elucidate its functional role.

Previous studies have shown that rat CLC-5 [15] and human CLC-5 [14] are predominantly expressed in the kidney, but that the mRNA is also detected in rat brain, liver, lung, and intestine. Recently, Lindenthal *et al.* [6] reported that *Xenopus* CLC-5 is highly expressed in oocytes, kidney, and intestine. In the present study, OmCLC-5 is highly expressed in the gill, kidney, and intestine. These results suggest that kidney and intestine are the main organs of CLC-5 expression from teleost to mammals. Recently, the mutation of human CLC-5 was the cause of low molecule proteinuria [27]. According to Gunther *et al.* [28], CLC-5 colocalizes with the H^+-ATPase (V-ATPas) in proximal tubules and intercalated cells of collecting duct of rat kidney. Colocalization of CLC-5 with H^+-ATPase suggested that CLC-5 is an endosomal chloride channel that regulates endocytosis in the proximal tubules. In tilapia larvae, V-ATPase immunoreactivity was present in the apical regions of pavement cells of the yolk-sac membrane in the FW-adapted larvae [29]. In an investigation of catfish gill epithelium, Laurent *et al.* [30] have identified vesicles which have a very similar morphology to the proton pump vesicles of toad urinary bladder and which are restricted to pavement cells and inserted into the apical membrane in re-

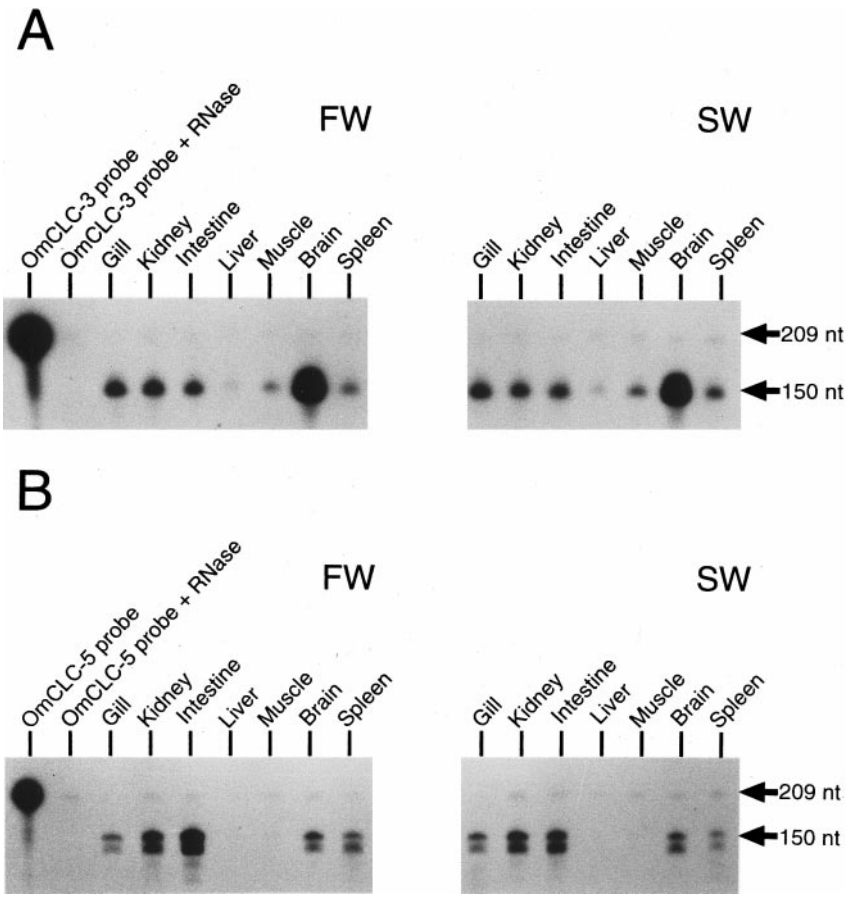


FIG. 3. Analysis of tissue distribution of OmCLC-3 and OmCLC-5 expression in *Oreochromis mosammbicus* by RNase protection assays. Probes were incubated with 20 μg of total RNA from yeast, and RNase protection analysis of 20 μg of total RNA isolated from gill, kidney, intestine, liver, muscle, brain and spleen of FW- and SW-adapted tilapia.

sponse to changes in the acid-base condition. Considering the co-localization of human CLC-5 and V-ATPase in the kidney, OmCLC-5 could be co-localized with the V-ATPase in the pavement cell in the gill. Functional complementation of *gef1* mutant with OmCLC-5 is consistent with the localization of OmCLC-5 in intracellular vesicles.

We were not able to express OmCLC-3 and OmCLC-5 in *Xenopus* oocyte. A small DIDS-sensitive current was induced in OmCLC-3 and OmCLC-5 cRNA-injected oocytes, but we could not characterize it further because of the low amplitude. There are several expressions for the lack of functional expression of OmCLC proteins. We cannot exclude that we have not yet identified the proper physiological stimulus for the channel activation, e.g., a second messenger pathway. It is possible that the oocytes, belonging either to the same or to a different gene family, lack the additional subunit necessary for their functional expression. However, it is more likely that the lack of expression in oocytes may be explained by their intracellular localization based on the functional complementation of the *gef1* mutant. In our complementation assay, we could not restore the growth of *gef1* mutant on the YPGE plate even by introducing wild-type GEF1 in our expression vector. This is probably due to the low promoter activity of our expression vector on YPGE plate. Accordingly, we performed a complementation assay on the YPG plate since the *gef1* mutant could not grow on the YPG plate, either. As in the case with GEF1, the introduction of OmCLC-3 and OmCLC-5 into the *gef1* mutant could restore the growth on YPG, suggesting that OmCLC-3 and OmCLC-5 may share the same function as GEF1 in the yeast. OmCLC-3 and OmCLC-5 could also restore the growth on the YPG + Ferrozine plate (data not shown), confirming the complementation of the *gef1* phenotype. Since GEF1 protein is present in late- or post-Goldi vesicles in yeast cells [21], the introduced OmCLC-3 and OmCLC-5 could also be localized to Goldi vesicles. But the growth of OmCLC-3- and OmCLC-5-transformed cells appeared slower than that of wild *GEF1* gene, suggesting that OmCLC-3 and OmCLC-5 may not have exactly the same function or localization in the yeast cells as GEF1. Identification of cellular local-

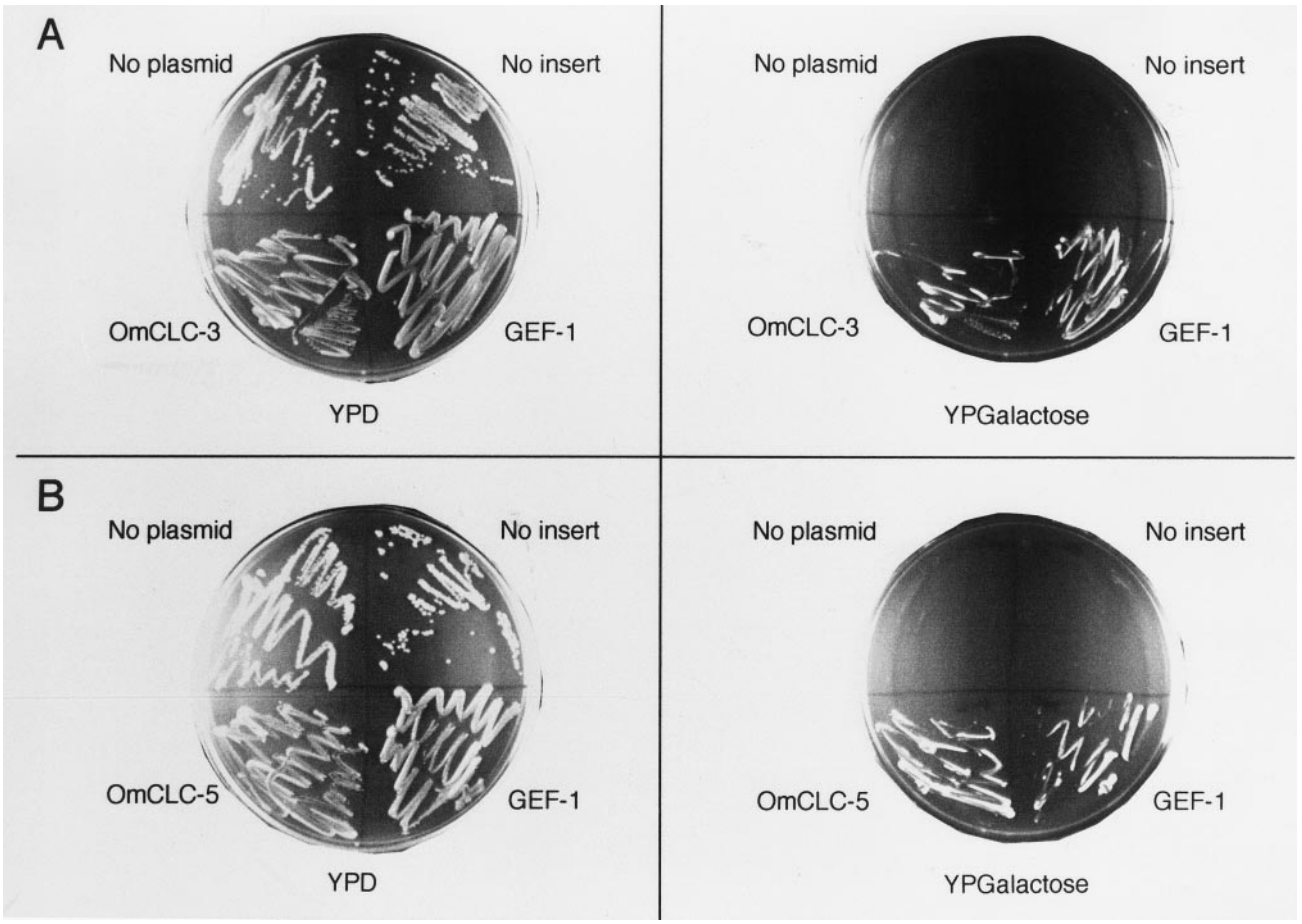


FIG. 4. Functional complementation of *gef1* yeast mutant by OmCLC-3 and OmCLC-5. YPH250(*gef*) was transformed with the expression vector containing either OmCLC-3 (A), OmCLC-5 (B), or the wild-type *GEF1* gene, or with the expression vector alone. Transformed cells and *gef1* mutant were then plated on the YPD or YPG agar plate. OmCLC-3- and OmCLC-5-transformed cells were able to grow on the YPG plate as well as the GEF1 transformed cells.

ization is the most important step to elucidate the physiological roles of OmCLC-3 and OmCLC-5.

In summary, two CLC chloride channels were cloned from *Oreochromis mosammbicus*. The isolation of two *CLC* genes will facilitate the identification of other members of this chloride channel family in teleost and provide an important step to further increase our understanding of the physiological roles of chloride channels in teleost.

ACKNOWLEDGMENTS

We thank to Professor Michihiro Kasahara, Laboratory of Biophysics, School of Medicine, Teikyo University, for supplying us with yeast strain and vectors. This research was supported in part by grants-in-aid for scientific research from Ministry of Education, Science, Sports, and Culture and the Fisheries Agency, Japan.

REFERENCES

1. Jentsch, T. J., Steinmeyer, K., and Schwarz, G. (1990) *Nature* **348,** 510–514.
2. Fujita, N., Mori, H., Yura, T., and Ishihama, A. (1994) *Nucleic Acids Res.* **22,** 1637–1639.
3. Greene, J. R., Brown, N. H., DiDomenico, B. J., Kaplan, J., and Eide, D. J. (1993) *Mol. Gene Genet.* **241,** 542–553.
4. Lurin, C., Geelen, D., Barbier-Brygoo, H., Guern, J., and Maurel, C. (1996) *Plant Cell* **8,** 701–711.
5. Hechenberger, M., Schwappach, B., Fischer, W. N., Frommer, W. B., Jentsch, T. J. and Steinmeyer, K. (1996) *J. Biol. Chem.* **271,** 33632–33638.
6. Lindenthal, S, Schmieder, S, Ehrenfeld, J., and Wills, N. K. (1997) *Am. J. Physiol.* **273,** C1176–C1185.
7. Steinmeyer, K., Ortland, C., and Jentsch, T. J. (1991) *Nature* **354,** 301–304.
8. Thiemann, A., Grunder, S., Pusch, M., and Jentsch, T. J. (1992) *Nature* **356,** 57–60.
9. Uchida, S., Sasaki, S., Furukawa, T., Hiraoka, M., Imai, T., Hirata, Y., and Marumo, F. (1993) *J. Biol. Chem.* **268,** 3821–3824.
10. Kieferle, S., Fong, P., Bens, M., Vandewalle, A., and Jentsch, T. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6943–6947.
11. Adachi, S., Uchida, S., Ito, H., Hata, M., Hiroe, M., Marumo, F., and Sasaki, S. (1994) *J. Biol. Chem.* **269,** 17677–17683.

12. Kawasaki, M., Uchida, S., Monkawa, T., Miyawaki, A., Mikoshiba, K., Marumo, F., and Sasaki, S. (1994) *Neuron* **12,** 597–604.
13. van Slegtenhorst, M. A., Bassi, M. T., Borsani, G., Wapenaar, M. C., Ferrero, G. B., de Conciliis, L., Rugarli, E. I., Grillo, A., Franco, B., Zoghbi, H. Y., *et al.* (1994) *Hum. Mol. Genet.* **3,** 547–552.
14. Fisher, S. E., van Bakel, I., Lloyd, S. E., Pearce, S. H., Thakker, R. V., and Craig, I. W. (1995) *Genomics* **29,** 598–606.
15. Sakamoto, H., Kawasaki, M., Uchida, S., Sasaki, S., and Marumo, F. (1996) *J. Biol. Chem.* **271,** 10210–10216.
16. Duan, D., Winter, C., Cowley, S., Hume, J. R., and Horowitz, B. (1997) *Nature* **390,** 417–421.
17. Lloyd, S. E., Gunther, W., Pearce, S. H., Thomson, A., Bianchi, M. L., Bosio, M., Craig, I. W., Fisher, S. E., Scheinman, S. J., Wrong, O., Jentsch, T. J., and Thakker, R. V. (1997) *Hum. Mol. Genet.* **6,** 1233–1239.
18. Stickney, R. R. (1986) *Progressive Fish-Culturist* **48,** 161–167.
19. Suresh, A. V., and Lin, C. K. (1992) *Aquaculture* **106,** 201–226.
20. Sikorski, R. S., Michaud, W. A., Tugendreich, S., and Hieter, P. (1995) *Gene* **155,** 51–59.
21. Gaxiola, R. A., Yuan, D. S., Klausner, R. D. and Fink, G. R. (1998) *Proc. Natl. Acad. USA* **95,** 4046–4050.
22. Vernet, T., Dignard, D., and Thomas, D. Y. (1987) *Gene* **52,** 225–233.
23. Jentsch, T. J., Gunther, W., Pusch, M., and Schwappach, B. (1995) *J. Physiol.* **482,** 19S–25S.
24. Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54,** 631–664.
25. Kemp, B. E., and Pearson, R. B. (1990) *Trends Biochem. Sci.* **15,** 342–346.
26. Kennelly, P. J. and Krebs, E. G. (1991) *J. Biol. Chem.* **266,** 15555–15558.
27. Morimoto, T., Uchida, S., Sakamoto, H., Kondo, Y., Hanamizu, H., Fukui, M., Tomino, Y., Nagano, N., Sasaki, S., and Marumo, F. (1998) *J. Am. Soc. Nephrol.* **9,** 811–818.
28. Gunther, W., Luchow, A., Cluzeaud, F., Vandewalle, A., and Jentsch, T. J. (1998) *Proc. Natl. Acad. USA* **95,** 8075–8080.
29. Hiroi, J., Kaneko, T., Uchida, K., Hasegawa, S., and Tanaka, M. (1998) *Zool. Sci.* **15,** 447–453.
30. Laurent, P., Goss, G. G., and Perry, S. F. (1994) *Arch. Int. Physiol. Biochim. Biophys.* **102,** 77–79.