ELSEVIER

Biochimica et Biophysica Acta 1464 (2000) 219–230

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

A hyperosmotic stress-induced mRNA of carp cell encodes Na^+- and Cl^--dependent high affinity taurine transporter[1]

Kazuharu Takeuchi, Haruhiko Toyohara *, Morihiko Sakaguchi

Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo, Kyoto 606-8502, Japan

Received 18 August 1999; received in revised form 29 December 1999; accepted 14 January 2000

Abstract

A cDNA clone encoding a Na^+- and Cl^--dependent high affinity taurine transporter was isolated from a common carp cell line, *Epithelioma papulosum cyprini* (EPC), as a hyperosmotic stress-inducible gene by RNA arbitrarily primed PCR. The clone contained a 2.5-kb cDNA fragment including an open reading frame of 1878 bp encoding a protein of 625 amino acids. The deduced amino acid sequence of carp taurine transporter shows 78–80% identity to those of cloned mammalian taurine transporters. The functional characteristics of the cloned transporter were analyzed by expression in COS-7 cells. Transfection with the cDNA induced Na^+- and Cl^--dependent taurine transport activity with an apparent K_m of 56 μM. The Na^+/Cl^-/taurine coupling ratio for cloned transporter was estimated as 2:1:1. Uptake of radiolabeled taurine was inhibited by excessive cold taurine, hypotaurine, β-alanine and γ-aminobutyric acid but not by α-alanine, glycine, leucine or glycinebetaine. Taurine transporter mRNA is ubiquitously distributed in carp tissues, and its level decreases in the order: kidney > spleen > heart > fin > eye ≈ intestine ≈ gill > skin > brain > muscle > hepatopancreas. Taurine transporter mRNA level increased up to 7.5-fold on raising the ambient osmolality from 300 to 450 mosmol/kgH_2O. These data suggest the significant role of taurine as an osmolyte in carp cells. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Taurine transporter; Hyperosmotic stress; Fish cell

1. Introduction

Plasma osmolarity of both freshwater and seawater teleosts is kept constant at about one third of that of seawater. Osmoregulatory functions of gill, intestine and kidney are coordinately regulated by the endocrine system and achieve homeostasis of plasma osmolarity against the osmotic gradient between plasma and the environment [1]. While this osmoregulation helps protect the individual as a whole from changes in tonicity, cells of the epidermis, gill, intestine and kidney epithelium are exposed to an anisosmotic environment. In addition, cells of fish embryos are directly exposed to the osmotic pressure of the environmental water, because the osmoregulatory organs are not well developed. It is essential for these cells to protect themselves from the osmotic gradient.

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazinenthanesulfonic acid; DTT, dithiothreitol; MMLV, Moloney murine leukemia virus; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; EF1α, elongation factor 1α; MAP kinase, mitogen-activated protein kinase; HSP, heat shock protein

* Corresponding author. Fax: +81-75-753-6446;
E-mail: toyohara@kais.kyoto-u.ac.jp

[1] The sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank database with accession no. AB006986.

0005-2736/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(00)00158-9

To understand the cellular response of fish cells to osmotic stress, we employed *Epithelioma papulosum cyprini* (EPC) cells [2] as a model and studied their growth under various osmolalities [3]. EPC cells are able to survive in media of 150–500 mosmol/kgH_2O. The growth of EPC cells exposed to hypoosmolality in a medium of 150 mosmol/kgH_2O or hyperosmolality in a medium of 450 mosmol/kgH_2O was suppressed for several hours and then recovered [3]. During this period of suppression, cells seemed to adapt to the environmental tonicity by various processes including changes in gene expression. In this study, we employed RNA arbitrarily primed polymerase chain reaction (RAP-PCR) [4] to screen mRNAs specifically induced by hyperosmotic stress and isolated a cDNA encoding Na^+- and Cl^--dependent high affinity taurine transporter from EPC cells.

Cells of various organisms have an osmolyte system to adapt to hypertonicity or water stress. Cells exposed to hypertonicity lose their water along the osmotic gradient and accumulate various osmolytes such as polyol, methylamine and amino acids to maintain their volume and intracellular condition [5]. Mammalian renal medullary cells exposed to hypertonicity accumulate sorbitol, glycinebetaine, *myo*-inositol, taurine and glycerophosphocholine as osmolytes [6]. Sorbitol is synthesized from glucose by the action of aldose reductase, and glycinebetaine, *myo*-inositol and taurine are accumulated by specific transmembrane transporters. Hypertonicity induces the expression of these proteins involved in the accumulation of osmolytes [6].

Taurine is a non-protein-constitutive β-amino acid found in high concentration in various organisms in the animal kingdom. Marine invertebrates, fishes, birds and mammals contain large amounts of taurine in their tissues [7]. Common carp contains taurine in muscle and liver at more than 10 mmol/kg of tissue water [8]. Taurine is suggested to act as an osmolyte in fish tissues. Intracellular taurine content is mainly regulated via transmembrane transport [7]. Increases in taurine uptake and diffusion in response to hypertonicity and hypotonicity, respectively, have been reported in teleost erythrocytes [9]. The release of taurine is mediated via a Na^+-independent system associated with anion exchanger band 3 [10]. The accumulation of taurine is mediated by Na^+- and Cl^--dependent taurine transporter [11–18]. The taurine transporter has been cloned from Madin–Darby canine kidney (MDCK) cells [11], rat brain [12], mouse brain [13], mouse retina [14], pig kidney (LLC-PK1) cells [15], human FRTL-5 thyroid cells [16], human JAR placental choriocarcinoma cells [17] and human retinal pigment epithelium (HRPE) cells [18]. Taurine transporter mRNA and taurine transport activity of MDCK cells are up-regulated by medium hypertonicity [11]. Taurine transporter contains 12 transmembrane helices, which are typical of the Na^+- and Cl^--dependent transporter gene family [11–18]. The substrates of this transporter family include taurine, betaine, creatine, serotonin, dopamine, noradrenaline, γ-aminobutyrate (GABA), glycine and proline [19,20].

While the properties of taurine transport of teleost erythrocytes have been reported [9], the molecular cloning of teleost taurine transporter has not. In this report, we describe the structure, function and hyperosmotic response of carp taurine transporter.

2. Materials and methods

2.1. Cell culture

EPC cells were grown in Eagle's minimum essential medium (BRL) supplemented with 10% fetal bovine serum, 25 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin at 30°C. The medium was made hypertonic by adding NaCl to an osmolality of 450 mosmol/kgH_2O. COS-7 cells were grown in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum, 10 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 air at 37°C.

2.2. RNA arbitrarily primed polymerase chain reaction

Total RNA was prepared from EPC cells cultured in isotonic or hypertonic medium for 24 h by the guanidine isothiocyanate/cesium chloride method, then digested with RNase-free DNase. Poly(A)$^+$ RNA was isolated using oligo(dT) latex beads (Takara). To obtain arbitrarily primed cDNAs as PCR templates, each poly(A)$^+$ RNA was reverse-tran-

scribed with 10 different arbitrary primers (Stratagene). For first-strand cDNA synthesis, a mixture of 1 μl of 25 μM arbitrary primer, 2 μl of 50 ng/μl poly(A)$^+$ RNA, 4 μl of 5×first-strand synthesis buffer, and 8 μl of H_2O was heated at 70°C for 10 min, then chilled on ice. Then 2.5 μl of 10 mM dNTP, 2 μl of 100 mM DTT, 1 μl of 20 units/μl RNase inhibitor and 0.5 μl of 200 units/μl MMLV reverse transcriptase (BRL) were added, and the mixture was incubated at 42°C for 60 min. The reaction was terminated by heating the mixture at 95°C for 5 min, then samples were stored at −20°C. The cDNA was amplified by PCR as follows. The stored cDNA solution was diluted 1/10 with distilled water, and 5 μl of this solution was mixed with 1 μl of 25 μM primer, 2.5 μl of 10×PCR buffer, 1 μl of 2.5 mM dNTP, 1.5 μl of 25 mM $MgCl_2$, 0.1 μl of 10 μCi/μl [^{32}P]dCTP (3000 Ci/mmol, Amersham), 0.1 μl of 5 units/μl *Taq* DNA polymerase (Perkin–Elmer) and 13.8 μl of H_2O. The primer used was the same as that used for reverse transcription. Amplification was performed in 1 cycle of low-stringency reaction (94°C, 1 min; 36°C, 5 min; 72°C, 5 min), followed by 40 cycles of high-stringency reaction (94°C, 1 min; 50°C, 2 min; 72°C, 2 min). The reaction products were denatured in formamide sequencing dye and resolved on 6% acrylamide, 7 M urea, sequencing gels. The gels were dried on 3MM paper (Whatman) and exposed to X-ray film.

2.3. Cloning and sequencing of differentially amplified products

Differentially displayed bands were excised from the gel and placed into a microcentrifuge tube with 100 μl of TE buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA). The tube was incubated for 1 h at 60°C, then left at room temperature overnight. The sample was centrifuged, and the supernatant was used as a template for PCR reamplification with the original primer. The reamplified product was ligated with pGEM-T vector (Promega) and used to transform *E. coli* DH5α. Several clones containing the insert were selected and sequenced using the dye terminator cycle sequencing kit and ABI 373A automated sequencer (Perkin–Elmer).

2.4. cDNA cloning and sequencing

Poly(A)$^+$ RNA was prepared from EPC cells exposed to hypertonic medium as described above and used to construct a cDNA library in λZAPII vector (Stratagene). A cloned PCR product containing a sequence similar to that of mammalian taurine transporters was labeled by random priming using [^{32}P]dCTP (ICN) and used to screen the cDNA library. A positive clone with a 2.5-kb cDNA insert was selected, and nested deletion clones were prepared to sequence both strands. The clones were sequenced as described above.

2.5. Northern blot analysis

Total RNA was prepared from EPC cells, carp liver, gill, kidney, gut, heart, brain, fin, spleen, eye, muscle and skin by the guanidine isothiocyanate/cesium chloride method. RNA samples (10 μg) were electrophoresed on a 1% agarose/2 M formaldehyde gel, then transferred to a nylon membrane (Gene Screen Plus, Dupont/NEN). A cloned PCR product or *Bam*HI-excised cDNA fragment was ^{32}P-labeled as described above, and the blots were hybridized at 42°C for 24 h in 50% formamide, 5×standard saline citrate, 6×Denhardt's solution, 1% SDS and 100 μg/ml shared salmon sperm DNA. The blots were washed at 65°C with 2×standard saline citrate containing 0.1% SDS. Electrophoresed RNA samples were normalized by rehybridizing the same membrane with ^{32}P-labeled medaka EF1α cDNA fragment [21]. Radioactivity of the bands was determined by scanning using a BAS 2000 bio-imaging analyzer (Fuji photo film).

2.6. Transfection and uptake measurement in COS-7 cells

The cDNA fragment containing a whole open reading frame was subcloned into pcDNA3 expression vector (Invitrogen). The plasmid (0.5 μg) was purified by CsCl ultracentrifugation, then introduced into 1–2×10^5 COS-7 cells in a well of a 24-well plate by liposome-mediated transfection (Lipofectamine, BRL). Uptake was measured 48 h after transfection. The cells were rinsed twice with uptake solution (10 mM HEPES–Tris (pH 7.4), 150 mM NaCl, 5 mM

KCl, 2 mM $CaCl_2$ and 1 mM $MgSO_4$), then incubated with 500 μl of the same solution containing 0.5 μCi/ml [^{3}H]taurine (NEN) at 37°C. Since the taurine uptake was almost linear during at least the first 15 min of incubation (data not shown), we used a 10-min uptake incubation period for the experiments. After incubation, cells were rinsed three times with stop solution (5 mM HEPES–Tris (pH 7.4) and 160 mM choline chloride). The cells were solubilized with 0.2 ml of 0.25 N NaOH, and radioactivity was measured by liquid scintillation counting.

3. Results

3.1. Cloning of carp taurine transporter cDNA

To screen hypertonicity-induced mRNAs, poly-$(A)^+$ RNAs prepared from EPC cells cultured in isotonic and hypertonic media were reverse-transcribed with an arbitrary primer, and PCR was performed with the same primer. Use of the primer A3 5′-AATCTAGAGCTCTCCTGG-3′ generated a band significantly enhanced under the hypertonic condition (Fig. 1A). This band was reamplified and cloned into pGEM-T vector. Ten selected clones were sequenced, of which nine were revealed to be the same. These clones are 456 bp long and their nucleotide sequence is highly homologous to those of reported mammalian taurine transporters. One clone, A3–7, was selected for Northern blot analysis, and hypertonicity responsiveness of the target gene was verified. This clone was shown to hybridize to a 4.5-kb mRNA species, which was up-regulated 4.4-fold in EPC cells by 24 h of hypertonic treatment (Fig. 1B). A probe derived from clone A3–7 was used to screen the EPC cDNA library, and 19 positive clones were isolated. One of these clones, designated cTAUT15, contained a 2.5-kb cDNA fragment including an open reading frame of 1878 bp that encodes a protein of 625 amino acids with a calculated molecular mass of 69 905 (Fig. 2). The sequence of the putative initiator codon flanking region exhibits a strong consensus site for translation initiation [22]. The Kyte–Doolittle hydropathy profile of the clone predicts 12 transmembrane helices and a large hydrophilic loop between helices 3 and 4 (data not shown).

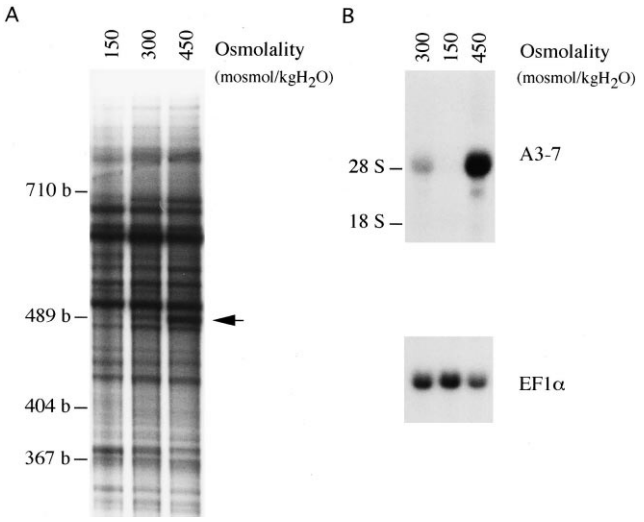

Fig. 1. Hyperosmotic stress-induced mRNA of EPC cells. (A) A RAP-PCR product of EPC cells induced by hyperosmotic stress. Poly $(A)^+$ RNAs were isolated from EPC cells exposed to hypoosmotic (150 mosmol/kgH_2O), isoosmotic (300 mosmol/kgH_2O) and hyperosmotic (450 mosmol/kgH_2O) medium for 24 h. These RNAs were reverse-transcribed and amplified by PCR with an arbitrary primer. Arrow indicates the band induced by hyperosmotic stress. (B) Hyperosmotic induction of mRNA hybridized with the cloned cDNA fragment. Total RNAs (20 μg) isolated from EPC cells exposed to hypoosmotic (150 mosmol/kgH_2O), isoosmotic (300 mosmol/kgH_2O) and hyperosmotic (450 mosmol/kgH_2O) medium for 24 h were separated on formaldehyde–agarose gel and blotted onto nylon membrane. The blot was hybridized with ^{32}P-labeled A3-7 probe. The same blot was rehybridized with medaka EF1α probe to normalize RNA abundance of each lane.

Table 1
Na^+ and Cl^- dependence of cloned taurine transporter expressed in COS-7 cells

Uptake medium	Taurine uptake (pmol/10 min per 10^6 cells)	
	pcDNA3	pcDNA3–cTAUT
NaCl	313.4 ± 23.3 (100)	1417.0 ± 65.5 (100)
Na^+ free (choline chloride)	5.5 ± 1.0 (1.8)	5.4 ± 0.6 (0.3)
Cl^- free (sodium gluconate)	9.2 ± 0.9 (2.9)	27.1 ± 3.6 (1.9)

Control vector (pcDNA3) and cDNA construct (pcDNA3–cTAUT) were introduced to COS-7 cells, and 10 μM [^{3}H]taurine (0.5 μCi/ml) uptake was measured. For Na^+-free medium, NaCl was replaced by choline chloride. For Cl^--free medium, NaCl, KCl and $CaCl_2$ were replaced by sodium gluconate, potassium gluconate and calcium gluconate, respectively. Each value is mean ± standard deviation of four samples from a single experiment. The values in parentheses represent percentage uptake compared with those of NaCl medium.

```
1    GATAATGGGAAGAGTGTCTGTCGGCTAACAGTAAGTGATAGTAGCTATTAAAGATTTTAA   60
61   TGAGGAGGTAAGCTGCTTCTTCTTTCAAAGAGCAAATCACAAAGCACAATACAGAAGGGA  120
121  CAAGACAGTTTCAAAGGAATTAAAGGAAATAAACAATAAAGCATGGCTCAGAAGGAGAAA  180
                                          M  A  Q  K  E  K
181  CTCCAGTGTCTGAAGGACTTCCACAAAGATATTCTGAAACCCTCTCCAGGAAAGAGCCCA  240
      L  Q  C  L  K  D  F  H  K  D  I  L  K  P  S  P  G  K  S  P
241  GGCACAAGGCCGGAGGACGAAGCCGAGGGAAAACACCCTCAAAGGGAAAAGTGGGCTAGC  300
      G  T  R  P  E  D  E  A  E  G  K  H  P  Q  R  E  K  W  A  S
301  AAGCTTGACTTTCTTCTGTCAGTCGCCGGGGGCTTCGTTGGTTTAGGGAACGTTTGGCGG  360
      K  L  D  F  L  L  S  V  A  G  G  F  V  G  L  G  N  V  W  R
361  TTCCCGTACCTCTGCTACAAAAATGGCGGAGGTGCTTTTCTCATCCCATACTTCATTTTC  420
      F  P  Y  L  C  Y  K  N  G  G  G  A  F  L  I  P  Y  F  I  F
421  CTGTTCGGTGGAGGACTGCCTGTGTTCTTCCTGGAGGTGGCACTCGGCCAGTTCACCTCT  480
      L  F  G  G  G  L  P  V  F  F  L  E  V  A  L  G  Q  F  T  S
481  GAGGGTGGGATCACCTGTTGGGAAAAACTTTGCCCCATATTTACAGGTATTGGCTACGCG  540
      E  G  G  I  T  C  W  E  K  L  C  P  I  F  T  G  I  G  Y  A
541  TCCATCGTGATCGTTTCTCTGCTGAACATCTACTACATTGTGATTCTTGCCTGGGGTTTG  600
      S  I  V  I  V  S  L  L  N  I  Y  Y  I  V  I  L  A  W  G  L
601  TACTACCTGTTCCAGTGCTTTCAGCCAGAGCTCCCCTGGGCAAGCTGCAACAACAAATGG  660
      Y  Y  L  F  Q  C  F  Q  P  E  L  P  W  A  S  C  N  N  K  W
661  AACACAGAAAATTGCATTGAGGATACTCTCCGCAAGAACAAGACACTCTGGGGTGCTGTG  720
      N  T  E  N  C  I  E  D  T  L  R  K  N  K  T  L  W  G  A  V
721  AATGCCACTAACTTCACTTCTCCCGTCACTGAGTTCTGGGAGCGAAATGTCCTCAGCATC  780
      N  A  T  N  F  T  S  P  V  T  E  F  W  E  R  N  V  L  S  I
781  TCCGATGGTATCGAGGACGTGGGTCATGTTAAATGGGACCTGGCTCTGTGTCTACTCGCT  840
      S  D  G  I  E  D  V  G  H  V  K  W  D  L  A  L  C  L  L  A
841  GTGTGGGTCATCTGCTTCTTCTGTATCTGGAAAGGTGTTAAGTCCACTGGGAAGGTTGTG  900
      V  W  V  I  C  F  F  C  I  W  K  G  V  K  S  T  G  K  V  V
901  TACGTCACGGCAACATTCCCGTTTGTCATGTTAATTGTCCTGCTTGTCCGTGGTGTCACT  960
      Y  V  T  A  T  F  P  F  V  M  L  I  V  L  L  V  R  G  V  T
961  CTTCCTGGGGCAGCCGAGGGCATCAAATTTTATCTGTACCCAAATTTGACTCGCCTTGGA 1020
      L  P  G  A  A  E  G  I  K  F  Y  L  Y  P  N  L  T  R  L  G
1021 GACCCAGAGGTGTGGATCGATGCAGGGACACAGATTTTCTTTTCCTACGCCATCTGTCTT 1080
      D  P  E  V  W  I  D  A  G  T  Q  I  F  F  S  Y  A  I  C  L
1081 GGAGCCATGACGTCACTGGGAAGCTACAACAAGTACAAATACAACTGCTACAGGGACTGT 1140
      G  A  M  T  S  L  G  S  Y  N  K  Y  K  Y  N  C  Y  R  D  C
1141 TTGCTGCTGGGAGGCCTGAACAGCGCTACCAGTTTTGTGTCTGGCTTCGCAATATTTTCC 1200
      L  L  L  G  G  L  N  S  A  T  S  F  V  S  G  F  A  I  F  S
1201 GTCCTGGGCTTCATGGCACAAGAGCAAGGGGTGGACATAGCCGATGTGGCAGAGTCAGGT 1260
      V  L  G  F  M  A  Q  E  Q  G  V  D  I  A  D  V  A  E  S  G
1261 CCTGGCTTGGCCTTCATTGCTTACCCTAAAGCCGTGTCTATGATGCCACTGCCTACGTTT 1320
      P  G  L  A  F  I  A  Y  P  K  A  V  S  M  M  P  L  P  T  F
1321 TGGGCCATTCTTTTCTTCATCATGCTTCTGCTGTTGGGCCTCGACAGTCAGTTTGTCGAA 1380
      W  A  I  L  F  F  I  M  L  L  L  L  G  L  D  S  Q  F  V  E
1381 GTGGAAGGGCAGATCACATCCCTAGTGGATCTGTATCCATCCTTCCTACGGAAGGGTTAT 1440
      V  E  G  Q  I  T  S  L  V  D  L  Y  P  S  F  L  R  K  G  Y
1441 CGGCGTGAAATCTTCATAGCTATAGTCTGTTTTTTGAGCTACCTGTTGGGACTTACCATG 1500
      R  R  E  I  F  I  A  I  V  C  F  L  S  Y  L  L  G  L  T  M
1501 GTCACCGAGGGTGGCATGTATGTATTTCAACTCTTTGACTACTATGCAGCCAGTGGCGTG 1560
      V  T  E  G  G  M  Y  V  F  Q  L  F  D  Y  Y  A  A  S  G  V
1561 TGCCTTTTATGGGTTGCATTCTTTGAATGTATTGCTGTAGCCTGGGTTTATGGTGCTGAT 1620
      C  L  L  W  V  A  F  F  E  C  I  A  V  A  W  V  Y  G  A  D
1621 AATTTCTATGATGCGATTGAAGAGATGATTGGTTACAGACCAAATCCCTGGATGAAGTGG 1680
      N  F  Y  D  A  I  E  E  M  I  G  Y  R  P  N  P  W  M  K  W
1681 AGTTGGACTGTAATTACACCTTTTCTGTGTGTGGGATGCTTTATCTTCTCATTGGTCAAG 1740
      S  W  T  V  I  T  P  F  L  C  V  G  C  F  I  F  S  L  V  K
1741 TACACGCCCCTGAGATACAACAAAGTCTACGAGTACCCGGACTGGTCCATCGGAGTCGGC 1800
      Y  T  P  L  R  Y  N  K  V  Y  E  Y  P  D  W  S  I  G  V  G
1801 TGGACCCTCGCTCTGGCGTCCATGATCTGCATACCTATGGTGGTTGTGATCAAAATCATT 1860
      W  T  L  A  L  A  S  M  I  C  I  P  M  V  V  V  I  K  I  I
1861 CAATCAGATGGACCCCTCATCGAGAGGATCAAAGCCGTGGCGGCCCCTGTGAGGGGTGGG 1920
      Q  S  D  G  P  L  I  E  R  I  K  A  V  A  A  P  V  R  G  G
1921 GCCAGTTCTTGCCCCCCAGAGTACCAGCCCAAGAGCAACGAGCTGGCGCAGCCGCTGGAT 1980
      A  S  S  C  P  P  E  Y  Q  P  K  S  N  E  L  A  Q  P  L  D
1981 CCCAACTGGAACGGAGGCTTGACGAAGCCAACACACACCATCGTAGAAACTATGATGTGA 2040
      P  N  W  N  G  G  L  T  K  P  T  H  T  I  V  E  T  M  M  *
2041 ACGCTCTCTGTGAGAGGATTCAAATGACCTGCTTTTCCATTATATATATATATATGTATA 2100
2101 TATATATATGTGATTATATATACATATATTAAAATATACTCTGTAACTTTTGACATAGTC 2160
2161 GTAGGACCAGGTTTACATTGCTACGCATCTGCACTAGGAGTCCTTGTTTTGTTTTGTTTT 2220
2221 TCTACAGAGGAAGTTACATTTTTCTGTCTGTATTTTTAATATTTTTATCATAATACTATT 2280
2281 ATATTACTATTATCGTCGCTGTTATTAGACAGAGAACAGATCGCAACCAGCAGTATCATG 2340
2341 AACGTAGAACCATGCCCGTTCTATTTTTGTAACTTTTATCTTGTACATATATTAAAATCG 2400
2401 AAATTGAGCTGAGCTTATTTTTAGACAAGATTCATTGGGGAGGGGGGACAACAAAAAAAC 2460
2461 AAAGGGCTCCGTAAACATGAATCGTAAAAAGTAACATTGGTGTACAAAGATTTAAAAAAT 2520
2521 ACCACCCTCATCCTCGCCCATCGTGATGTTCCTGGATAGTTGTGTCCCAACAAACTGTTT 2580
2581 AGACTGGAGATTGTGCAGTCCGGAAGAATCGAGCCGTTAGCCCTGCTTGTTTTCAGTGCA 2640
2641 CTATACAACCTAGGACACTAGAATTTACACAATGGTGCTCCCTTAGTTTAAAAGGACAGT 2700
2701 TTGCGC                                                       2706
```

Fig. 2. Nucleotide and deduced amino acid sequence of cTAUT. The sequence corresponding to the RAP-PCR product is underlined.
←

The second and third extracellular loops contain three and one potential *N*-glycosylation sites, respectively (Fig. 3). The predicted amino acid sequence of cTAUT15 shows 78–80% identity to those of canine, rat and human taurine transporters [11,12,18].

3.2. Identification of the clone as a Na^+- and Cl^--dependent taurine transporter

The function of cTAUT was analyzed by a transient expression in COS-7 cells. Taurine uptake activity of cells transfected with pcDNA3–cTAUT vector and control mock vector was measured. The cDNA-transfected cells showed a marked increase in the taurine transport activity. The initial uptake rate in cDNA-transfected cells was about 4.5-fold higher than that in mock vector-transfected cells.

Table 1 shows the Na^+- and Cl^--dependence of taurine uptake in cTAUT cDNA-transfected and control COS-7 cells. Uptake of taurine by cDNA-transfected COS-7 cells decreased by $>97\%$ when Na^+ was replaced by choline or when Cl^- was replaced by gluconate. Inhibition of taurine uptake observed in control cells as well as in cTAUT cDNA-transfected cells indicates that endogenous transport activity of COS-7 cells also requires Na^+ and Cl^-.

Fig. 4 shows the kinetics of taurine transport induced by cTAUT in COS-7 cells. Eadie–Hofstee plots revealed an apparent K_m of 56 μM for taurine (Fig. 4B). The K_m value for the endogenous taurine transporter was 15 μM (data not shown). Thus the affinity of endogenous transporter of COS-7 cells for taurine is higher than that of the cloned transporter.

To analyze the substrate specificity of the cloned transporter, various agents were examined for their ability to inhibit the transport of [^{3}H]taurine by cTAUT cDNA-transfected COS-7 cells and control cells (Fig. 5). Uptake of [^{3}H]taurine in cDNA-transfected cells was markedly inhibited by unlabeled taurine, hypotaurine and β-alanine. GABA also inhibited the uptake to a smaller extent than the β-amino acids. α-Alanine, glycine, leucine and glycine-

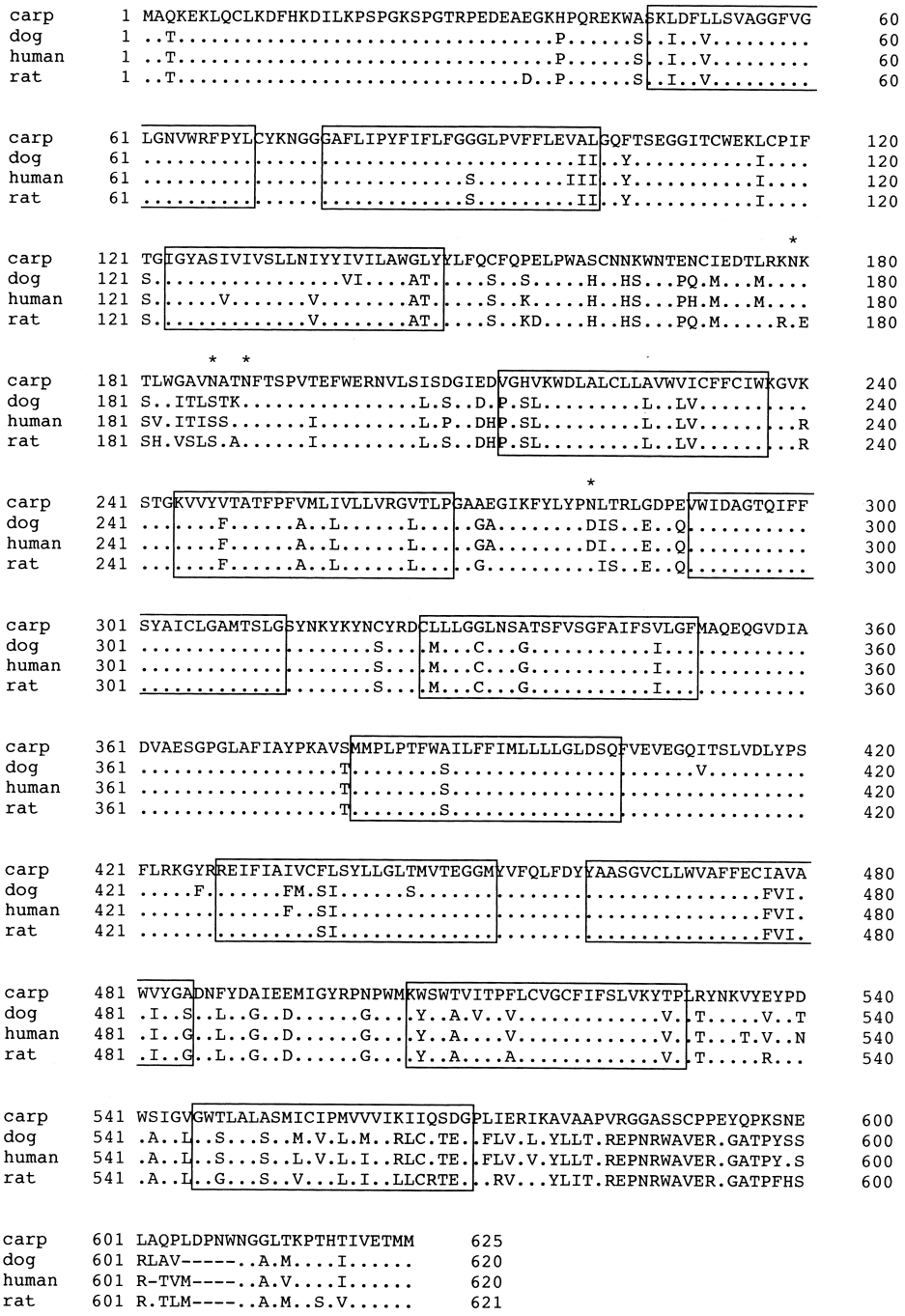

Fig. 3. Amino acid sequence alignment of cTAUT with mammalian taurine transporters. Dots indicate amino acids identical with those of cTAUT. Hyphens indicate gaps. Boxes indicate the 12 putative membrane-spanning domains. Asterisks show the potential *N*-glycosylation sites of cTAUT.

betaine had no effect on taurine uptake. Similar specificity was also observed in control cells.

The Na^+ and Cl^- stoichiometry of taurine uptake induced by cTAUT was estimated by the 'activation method' (Fig. 6). To estimate the Cl^- stoichiometry, cTAUT-specific taurine uptake rate was measured at various Cl^- concentrations with a constant Na^+ concentration. The relationship between taurine uptake

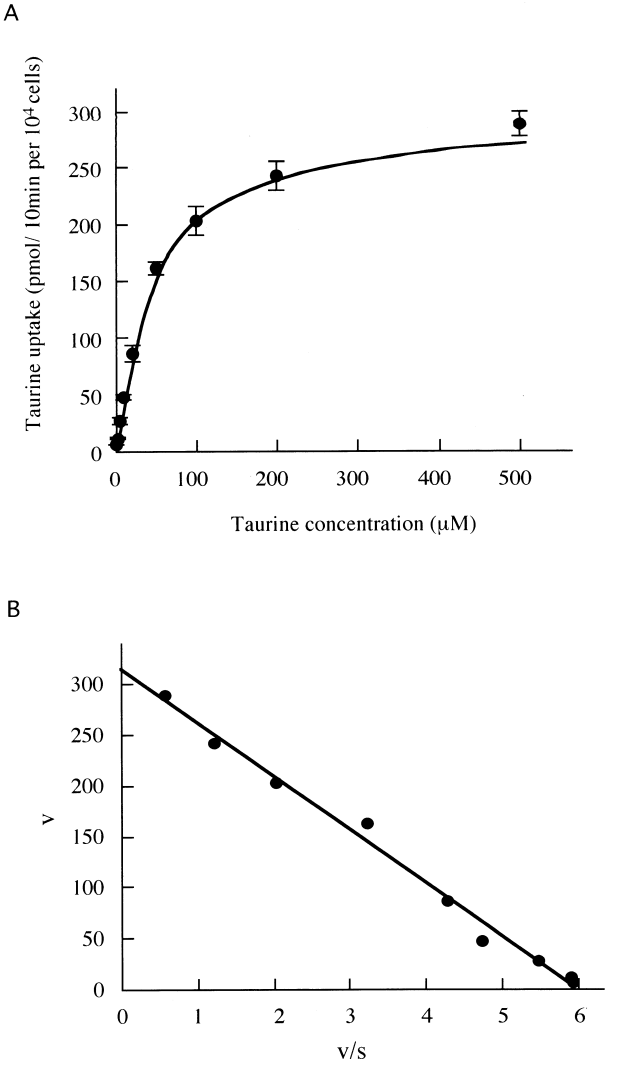

Fig. 4. Kinetics of taurine uptake in cTAUT-transfected COS-7 cells. (A) cTAUT construct and control vector were introduced into COS-7 cells. For each pool of cells, taurine uptake was measured at taurine concentration from 1 to 500 μM. Radioactivity of the uptake medium was kept constant at 0.5 μCi/ml. Values of cTAUT-transfected cells were adjusted by subtraction of those of control vector-transfected cells and plotted. Each value is the mean of four samples from a single experiment. Error bars represent the standard deviation. (B) Eadie–Hofstee plot of the data. Linear fitting was used to determine the kinetic constant.

rate and Cl^- concentration was hyperbolic (Fig. 6A). Hill-type analysis of the data revealed a Hill coefficient of 1.01 with a K_{50} value of 82 mM Cl^- (Fig. 6C). These data suggest the involvement of one Cl^- ion per transport of one taurine molecule.

To estimate the Na^+ stoichiometry, cTAUT specific taurine uptake rate was measured at various Na^+ concentrations with a constant Cl^- concentration. In this case, the uptake rate exhibited a sigmoidal dependence on Na^+ concentration, suggesting involvement of more than one Na^+ ion per transport of one taurine molecule (Fig. 6B). Further analysis of the data according to the Hill equation revealed a Hill coefficient of 2.22 with a K_{50} value of 116 mM, suggesting the involvement of two Na^+ ions per transport of one taurine molecule (Fig. 6D).

3.3. Tissue distribution of taurine transporter mRNA

Northern blot analysis with RNA isolated from various carp tissues revealed a major transcript of 5.4 kb and a minor transcript of 7.6 kb (Fig. 7). The major 5.4 kb transcript was present at levels decreasing in the order: kidney > spleen > heart > fin > eye ≈ gut ≈ gill > skin > brain > muscle > hepatopancreas. The transcript of 7.6 kb was detected in all these tissues except muscle and hepatopancreas.

3.4. Regulation of taurine transporter mRNA abundance by hypertonicity in EPC cells

Time course of mRNA induction by hypertonicity was analyzed by Northern blot analysis. As shown in Fig. 8B, cTAUT mRNA level began to rise after 1 h of hyperosmotic stress, reached a maximal level (7.5-fold of control) at after 6 h of stress, then decreased slightly.

4. Discussion

This is the first report of molecular cloning and the hyperosmotic stress response of teleost taurine transporter. The expression analysis of cTAUT revealed that its functional features are almost identical to those of cloned mammalian taurine transporters [11–18]. Transport activity of expressed cTAUT in COS-7 cells exhibited complete dependence on Na^+ and Cl^-, and specificity to β-amino acids including taurine, hypotaurine and β-alanine. The K_m value for taurine transport induced by cTAUT in COS-7 cells was 56 μM, and those for taurine transport of MDCK cells [11], human placenta [17], mouse brain [13] and rat brain [12] taurine transporters are 9.1

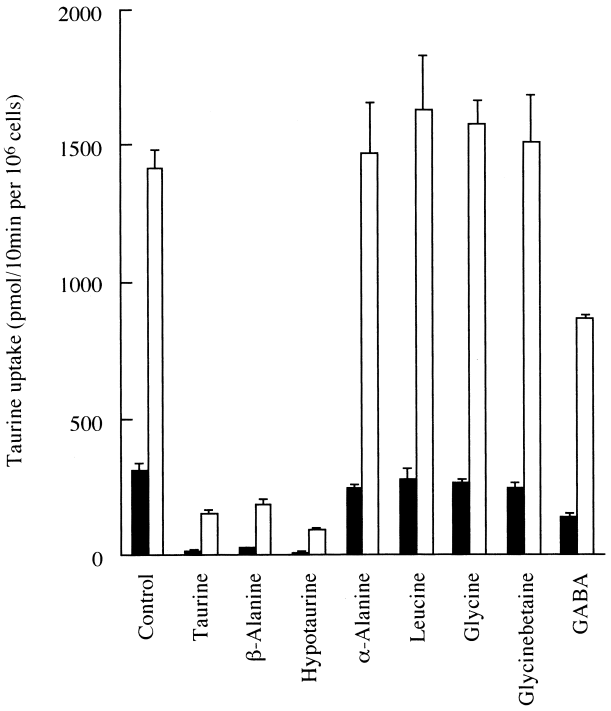

Fig. 5. Competition analysis of taurine uptake in cTAUT-transfected COS-7 cells. Uptake of 10 μM [^{3}H]taurine (0.5 μCi/ml) in cTAUT- and control vector-transfected COS-7 cells was measured in the presence of 1 mM unlabeled compounds. Right columns represent the uptake of cTAUT-transfected cells and left columns represent that of control vector-transfected cells. Each column represents the mean of four samples. Error bars represent the standard deviation.

μM, 5.9 μM, 4.5 μM and 40 μM, respectively. Of these, the K_m values of EPC cells and rat brain taurine transporters are relatively high, suggesting lower affinity for taurine. This difference in affinity might derive from either a difference in primary structure of the proteins or a difference in the expression systems used. In the case of EPC cells and rat brain taurine transporters, COS cells were employed for measurement of transport activity [12]. On the other hand, MDCK cells and mouse brain taurine transporters were expressed in *Xenopus* oocytes [11,13] and human placental taurine transporter was expressed in vaccinia-virus-infected HeLa cells [17]. Na^+/Cl^-/taurine stoichiometry for cloned transporters has been determined for human placental [17] and mouse retinal [14] taurine transporters. In both cases, at least two Na^+ ions and one Cl^- ion are required for transport of one taurine molecule, as in the case of EPC cells. The primary structure of cTAUT exhibits the consensus structural feature of the Na^+- and Cl^--dependent transporter gene family [19]. The amino acid sequence of the 1st extracellular loop, which has been reported to be common among Na^+- and Cl^--dependent transporters, is completely conserved. This domain is thought to be involved in interaction with Na^+ and Cl^-, and thus its complete conservation is consistent with the Na^+- and Cl^--dependency of the taurine transport activity of cTAUT. Although the overall amino acid sequence of cTAUT is highly homologous to those of cloned mammalian taurine transporters, the second extracellular domain and the intracellular domain of the carboxyl terminal show considerable variation. Since the functional features of cTAUT are virtually identical to those of mammalian taurine transporters, these variable domains are unlikely to be involved in specific transport activity.

In EPC cells, hyperosmotic stress induced an increase in taurine transporter mRNA level, strongly suggesting the role of taurine as an osmolyte. In common carp, all the tissues examined contained the taurine transporter mRNA in detectable amounts by Northern blot analysis. The ubiquitous tissue distribution of taurine transporter mRNA suggests the significant role of taurine as an osmolyte. In the hepatopancreas, taurine synthesis from cysteine and methionine might compensate for the lower level of taurine transporter expression [23].

The cellular response to hyperosmotic stress and the role of compatible organic osmolytes have been studied intensively in mammalian renal medullary cells [6]. Cells exposed to hyperosmotic medium shrink due to loss of water along the osmotic gradient. Generally such cells quickly recover their volume by uptake of inorganic ions. This process, called regulatory volume increase (RVI), leads to high intracellular ionic concentrations, which perturb the function of intracellular macromolecules, and which must be reduced in order to restore this function. The accumulation of osmolytes in cells would help decrease the intracellular concentration of inorganic ions without changing cell volume. In this study, hyperosmotic stress induced an increase in taurine transporter mRNA in EPC cells. This, in turn, would lead to an increase in the number of taurine transporter molecules per cell and activation of the cellular uptake of taurine. The resulting rise in intracel-

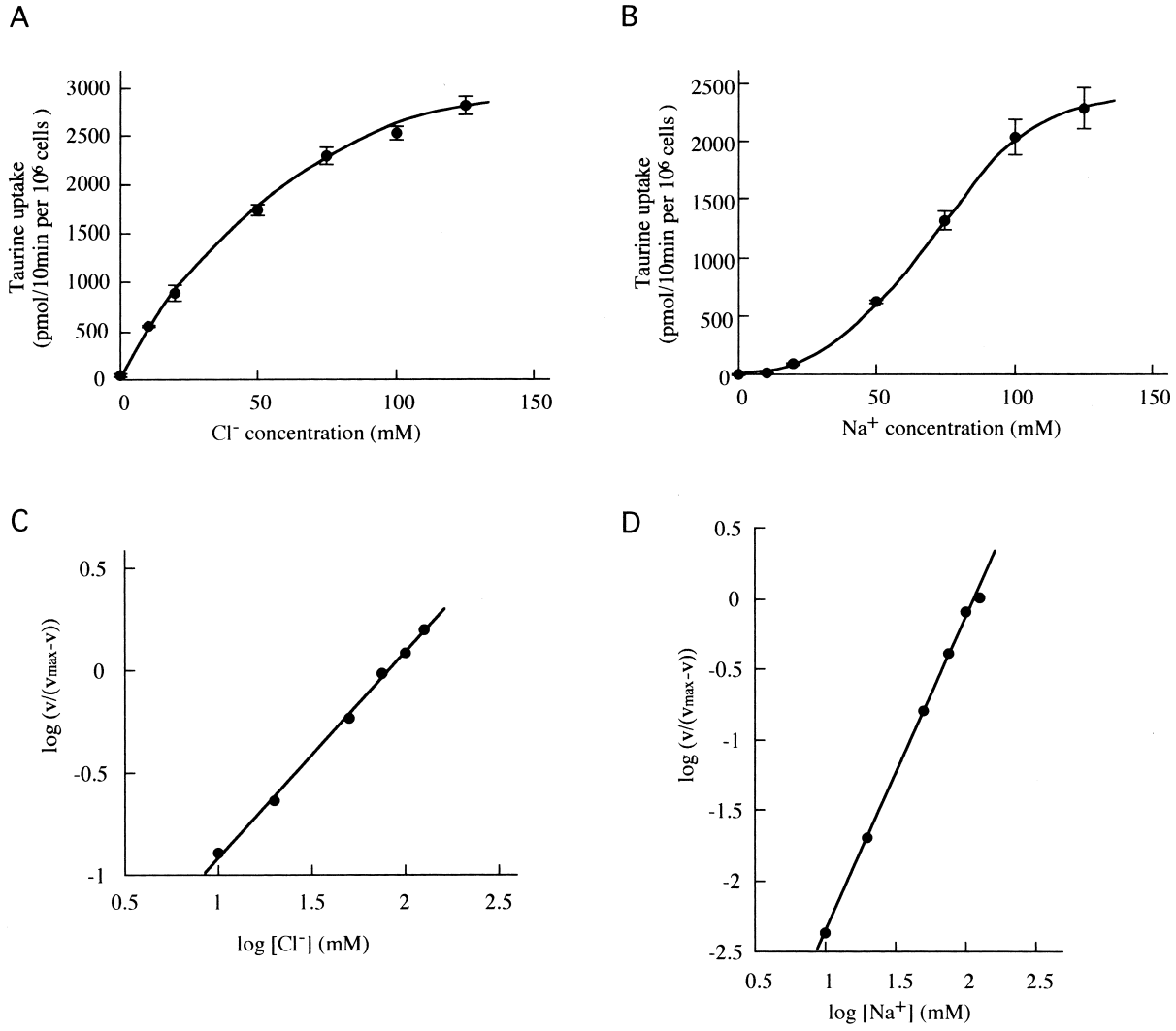

Fig. 6. Na^+ and Cl^- stoichiometry of taurine uptake in cTAUT-transfected COS-7 cells. Uptake of 10 μM [^{3}H]taurine (0.5 μCi/ml) in cTAUT- and control vector-transfected COS-7 cells was measured at various Cl^- (1–125 mM) concentrations and constant Na^+ concentration (150 mM) (A) or at various Na^+ (1–125 mM) concentrations and constant Cl^- concentration (150 mM) (B). Isotonicity of medium was maintained with sodium gluconate or choline chloride. Values of cTAUT-transfected cells were adjusted by subtraction of those of control vector-transfected cells and plotted. Each value is the mean of four samples from a single experiment. Error bars represent the standard deviation. Hill coefficient and K_{50} were estimated by the linear fitting of Hill plots (C,D).

lular taurine concentration would help decrease the intracellular ionic strength and eventually protect cells from hyperosmotic stress. Once the accumulation of osmolyte is complete, further expression of taurine transporter gene would be suppressed. This mechanism is consistent with the time course of taurine transporter mRNA abundance in EPC cells.

Accumulation of taurine in cells in vivo depends on both biosynthesis and transmembrane uptake from plasma. Efficiency of taurine accumulation by uptake depends on plasma taurine level. Many teleosts, including common carp, contain large amounts of taurine in various tissues [24–26]. Although taurine would not be the only organic osmolyte in the cells of common carp, it is one of the most readily available substances for cells and thus would play an important role in cellular response to hyperosmotic stress. Although the epidermal cells of common carp, from which EPC cells originated [3], are not usually exposed to such large changes in extracellular tonicity under natural conditions as mammalian renal cells, EPC cells intrinsically have the hyperosmotic

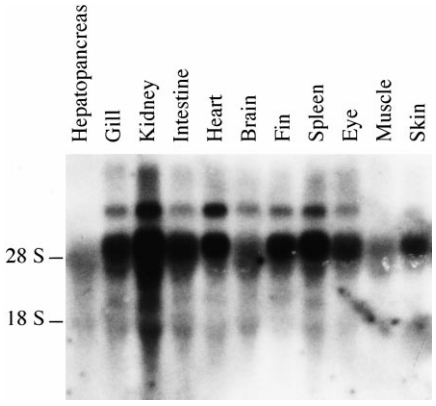

Fig. 7. Tissue distribution of taurine transporter mRNA in common carp. Total RNAs (10 μg) prepared from carp tissues were separated on a formaldehyde–agarose gel, blotted on a nylon membrane and hybridized with ^{32}P-labeled cTAUT probe.

stress response system as shown in the present study. Probably all types of teleost cells are potentially equipped with such a system. This cellular mechanism of osmotic stress response would be especially important in the embryonic stage, because teleost embryos usually develop ex vivo and their osmoregulatory organs are not yet fully developed.

There are several reports on the inhibition of taurine transporters by activation of protein kinase C (PKC) [14,27–30]. It is suggested that an intracellular peptide segment of taurine transporter participates in inactivation of taurine transport modulated by PKC phosphorylation [30]. There are three potential sites of phosphorylation by PKC in cTAUT, and these sites are also present in cloned mammalian taurine transporters. At least one of them must be essential for the inhibition of taurine transport activity of cTAUT by PKC.

How the expression of the genes involved in the osmolyte-accumulating system is regulated has been partially elucidated [6]. The minimal essential osmotic response elements of several osmotic responsive genes, human [31] and rabbit [32] aldose reductases, canine betaine GABA transporter [33] and canine sodium/*myo*-inositol cotransporter [34], have been identified. Gel mobility shift assays of nuclear extracts revealed that the exposure of cells to hypertonicity results in activation of enhancer binding protein [32–36]. Recently, a tonicity-responsive enhancer binding protein was identified [37]. However, no tonicity-responsive element has yet been identified for the taurine transporter gene. Presumably, the hyperosmotic stress response of taurine transporter gene expression is controlled by similar enhancer and *trans*-acting factor(s). Recently p38 kinase, a MAP

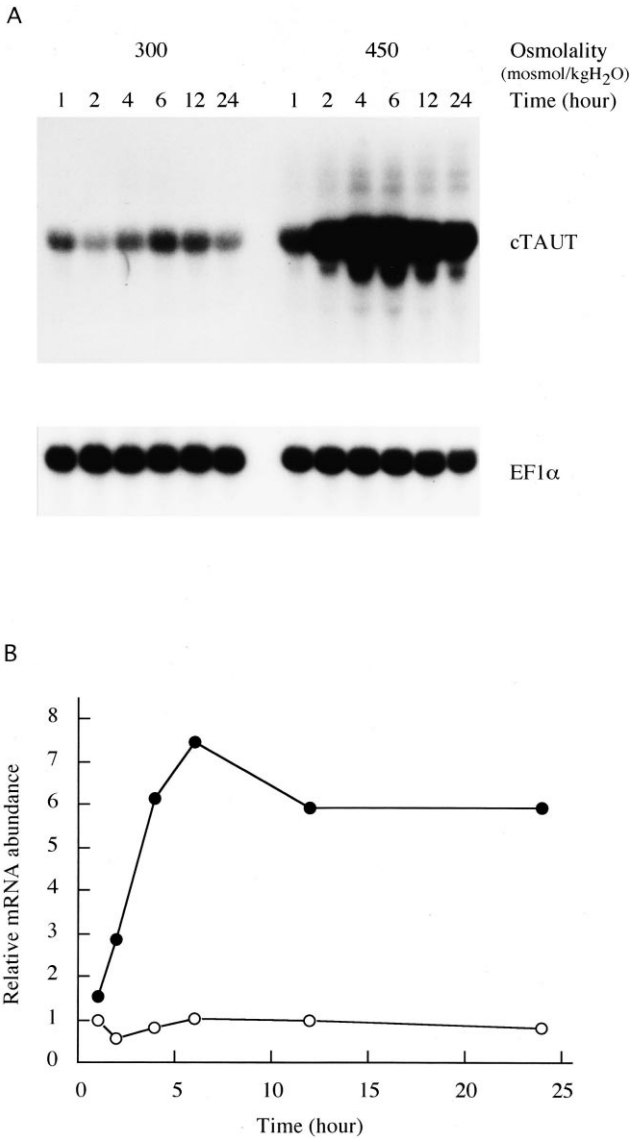

Fig. 8. Time course of taurine transporter mRNA abundance in EPC cells exposed to a hyperosmotic medium. (A) Culture media of EPC cells were replaced with fresh isotonic (300 mosmol/kgH_2O) or hypertonic (450 mosmol/kgH_2O) media at time 0. After 1–24 h, total RNAs were prepared, and 10 μg of each RNA was separated on a formaldehyde–agarose gel, blotted on nylon membrane and hybridized with ^{32}P-labeled cTAUT probe. The same blot was rehybridized with medaka EF1α probe to normalize the signal intensity. (B) Radioactivity of the each band was quantified by use of a BAS 2000 bio-imaging analyzer, and cTAUT/EF1α ratio was plotted. The value under the isotonic condition at 1 h was assigned as 1.

kinase involved in intracellular stress signaling pathways, was reported to be essential for osmotic induction of mRNAs of HSP70 and betaine GABA transporter in MDCK cells [38]. These signaling pathways could also mediate the induction of mRNA of taurine transporter. This assumption is consistent with the proposed concept of coordinate regulation of genes for organic osmolyte accumulation [6]. However, what triggers the activation or inactivation of the signaling remains unknown.

Acknowledgements

This work was supported in part by a Grant-in-Aid for JSPS Research Fellows from the Ministry of Education, Science, Sports and Culture, Japan and from the Research for the Future Program of the Japan Society for the Promotion of Science (97L00902).

References

[1] D.H. Evans, Osmotic and ionic regulation, in: D.H. Evans (Ed.), The Physiology of Fishes, CRC Press, Boca Raton, FL, 1993, pp. 315–341.
[2] N. Fijan, D. Sulimanovic, M. Bearzotti, D. Muzinic, L.O. Zwillenberg, S. Chilmonczyk, J.F. Vautherot, P. de Kinkelin, Some properties of the *Epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio*, Ann. Virol. (Inst. Pasteur) 134E (1983) 207–220.
[3] H. Hashimoto, K. Takeuchi, Y. Matsuo, Y. Yokoyama, H. Toyohara, M. Sakaguchi, Suppression of apoptosis of fish cells by acclimation to hypertonicity, Fisheries Sci. 65 (1999) 145–149.
[4] J. Welsh, K. Chada, S.S. Dalal, R. Cheng, D. Ralph, M. McClelland, Arbitrarily primed PCR fingerprinting of RNA, Nucleic Acids Res. 20 (1992) 4965–4970.
[5] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Living with water stress: evolution of osmolyte systems, Science 217 (1982) 1214–1222.
[6] M.B. Burg, E.D. Kwon, D. Kltz, Regulation of gene expression by hypertonicity, Annu. Rev. Physiol. 59 (1997) 437–455.
[7] R.J. Huxtable, Physiological actions of taurine, Physiol. Rev. 72 (1992) 101–163.
[8] S. Abo Hegab, W. Hanke, The significance of the amino acids during osmotic adjustment in teleost fish – II. Changes in the stenohaline *Cyprinus carpio*, Comp. Biochem. Physiol. 74A (1983) 537–543.
[9] D.A. Fincham, M.W. Wolowyk, J.D. Young, Volume-sensitive taurine transport in fish erythrocytes, J. Membr. Biol. 96 (1987) 45–56.
[10] B. Fivet, N. Gabillat, F. Borgese, R. Motais, Expression of band 3 anion exchanger induces chloride current and taurine transport: structure–function analysis, EMBO J. 14 (1995) 5158–5169.
[11] S. Uchida, H. Moo Kwon, A. Yamauchi, A.S. Preston, F. Marumo, J.S. Handler, Molecular cloning of the cDNA for an MDCK cell Na^+- and Cl^--dependent taurine transporter that is regulated by hypertonicity, Proc. Natl. Acad. Sci. USA 89 (1992) 8230–8234.
[12] K.E. Smith, L.A. Borden, C.D. Wang, P.R. Hartig, T.A. Branchek, R.L. Weinshank, Cloning and expression of a high affinity taurine transporter from rat brain, Mol. Pharmacol. 42 (1992) 563–569.
[13] Q.R. Liu, B. Lpez-Corcuera, H. Nelson, S. Mandiyan, N. Nelson, Cloning and expression of a cDNA encoding the transporter of taurine and β-alanine in mouse brain, Proc. Natl. Acad. Sci. USA 89 (1992) 12145–12149.
[14] S. Vinnakota, X. Qian, H. Egal, V. Sarthy, H.K. Sarkar, Molecular characterization and in situ localization of a mouse retinal taurine transporter, J. Neurochem. 69 (1997) 2238–2250.
[15] X. Han, A.M. Budreau, R.W. Chesney, Molecular cloning and functional expression of an LLC-PK1 cell taurine transporter that is adaptively regulated by taurine, Adv. Exp. Med. Biol. 442 (1998) 261–268.
[16] S.M. Jhiang, L. Fithian, P. Smanik, J. McGill, Q. Tong, E.L. Mazzaferri, Cloning of the human taurine transporter and characterization of taurine uptake in thyroid cells, FEBS Lett. 318 (1993) 139–144.
[17] S. Ramamoorthy, F.H. Leibach, V.B. Mahesh, H. Han, T. Yang-Feng, R.D. Blakely, V. Ganapathy, Functional characterization and chromosomal localization of a cloned taurine transporter from human placenta, Biochem. J. 300 (1994) 893–900.
[18] Y. Miyamoto, G.I. Liou, T.J. Sprinkle, Isolation of a cDNA encoding a taurine transporter in the human retinal pigment epithelium, Curr. Eye Res. 15 (1996) 345–349.
[19] N. Nelson, Porters and neurotransmitter transporters, J. Exp. Biol. 196 (1994) 213–228.
[20] G.R. Uhl, P.S. Johnson, Neurotransmitter transporters: three important gene families for neuronal function, J. Exp. Biol. 196 (1994) 229–236.
[21] M. Kinoshita, T. Nakata, K. Adachi, T. Yabe, Y. Yokoyama, H. Toyohara, T. Hirata, E. Takayama, S. Mikawa, N. Kioka, M. Sakaguchi, cDNA cloning of polypeptide chain elongation factor 1α from medaka *Oryzias latipes*, Fisheries Sci. 65 (1999) 133–137.
[22] M. Kozak, An analysis of 5′-noncoding sequence from 699 vertebrate messenger RNAs, Nucleic Acids Res. 15 (1987) 8125–8148.
[23] S. Divakaran, S. Ramanathan, A.C. Ostrowski, Endogenous production of taurine in two teleost fish: *Coryphaena hippurus* and red hybrid tilapia, Comp. Biochem. Physiol. 101B (1992) 321–322.

[24] T. Suzuki, T. Hirano, T. Shirai, Distribution of extractive nitrogenous constituents in white and dark muscles of freshwater fish, Comp. Biochem. Physiol. 96B (1990) 107–111.
[25] J. Van der Boon, G.E.E.J.M. Van den Thillart, A.D.F. Addink, Free amino acid profiles of aerobic (red) and anaerobic (white) skeletal muscle of the cyprinid fish, *Carassius auratus L.* (goldfish), Comp. Biochem. Physiol. 94A (1989) 809–812.
[26] M. Sakaguchi, M. Murata, Distribution of free amino acids, creatine, and trimethylamine oxide in mackerel and yellowtail, Bull. Jpn. Soc. Sci. Fish. 52 (1986) 685–689.
[27] M. Brandsch, Y. Miyamoto, V. Ganapathy, F.H. Leibach, Regulation of taurine transport in human colon carcinoma cell lines (HT-29 and Caco-2) by protein kinase C, Am. J. Physiol. 264 (1993) G939–946.
[28] G.C. Tchoumkeu-Nzouessa, G. Rebel, Regulation of taurine transport in rat astrocytes by protein kinase C: role of calcium and calmodulin, Am. J. Physiol. 270 (1996) C1022–1028.
[29] D.D.F. Loo, J.R. Hirsch, H.K. Sarkar, E.M. Wright, Regulation of the mouse retinal taurine transporter (TAUT) by protein kinase in *Xenopus* oocytes, FEBS Lett. 392 (1996) 250–254.
[30] X. Han, A.M. Budreau, R.W. Chesney, Role of conserved peptide in taurine transporter inactivation modulated by protein kinase C, J. Am. Soc. Nephrol. 7 (1996) 2088–2096.
[31] B. Ruepp, K.M. Bohren, K.H. Gabbay, Characterization of the osmotic response element of the human aldose reductase gene promoter, Proc. Natl. Acad. Sci. USA 93 (1996) 8624–8629.
[32] J.D. Ferraris, C.K. Williams, K.Y. Jung, J.J. Bedford, M.B. Burg, A. Garca-Prez, ORE, a eukaryotic minimal essential osmotic response element, J. Biol. Chem. 271 (1996) 18318–18321.
[33] M. Takenaka, A.S. Preston, H.M. Kwon, J.S. Handler, The tonicity-sensitive element that mediates increased transcription of the betaine transporter gene in response to hypertonic stress, J. Biol. Chem. 269 (1994) 29379–29381.
[34] J.S. Rim, M.G. Atta, S.C. Dahl, G.T. Berry, J.S. Handler, H.M. Kwon, Transcription of the sodium/*myo*-inositol cotransporter gene is regulated by multiple tonicity-responsive enhancers spread over 50 kilobase pairs in the 5′-flanking region, J. Biol. Chem. 273 (1998) 20615–20621.
[35] B.C.B. Ko, B. Ruepp, K.M. Bohren, K.H. Gabbay, S.S.M. Chung, Identification and characterization of multiple osmotic response sequences in the human aldose reductase gene, J. Biol. Chem. 272 (1997) 16431–16437.
[36] H. Miyakawa, S.K. Woo, C.P. Chen, S.C. Dahl, J.S. Handler, H.M. Kwon, *Cis*- and *trans*-acting factors regulating transcription of the BGT1 gene in response to hypertonicity, Am. J. Physiol. 274 (1998) F753–761.
[37] H. Miyakawa, S.K. Woo, S.C. Dahl, J.S. Handler, H.M. Kwon, Tonicity-responsive enhancer binding protein, a Rel-like protein that stimulates transcription in response to hypertonicity, Proc. Natl. Acad. Sci. USA 96 (1999) 2538–2542.
[38] D. Sheikh-Hamad, J. Di Mari, W.N. Suki, R. Safirstein, B.A. Watts III, D. Rouse, p38 kinase activity is essential for osmotic induction of mRNAs for HSP70 and transporter for organic solute betaine in Madin-Darby canine kidney cells, J. Biol. Chem. 273 (1998) 1832–1837.