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Specific Characteristics of the Conserved Region of Partial cDNA Clone of Transferrin Gene of an Air-breathing Snakehead, *Channa gachua* (Hamilton)

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Abstract

By immunoblotting against rabbit anti-TF antisera, a clone showing sequence homology with N- and C- lobes of conserved region of teleost transferrin genes has been isolated from cDNA library constructed from liver mRNA of air-breathing murrel, *Channa gachua*. The sequence, though partial, exhibits several features characteristic to this murrel, which belongs to a highly specialized group of teleosts. The cloned insert is a 483-nucleotide fragment with a deduced amino acid sequence of 161 residues. The primary sequence displays species specific positioning of two cysteine residues and a stretch of 30 tandem asparagine residues. The asparagine stretch suggests the existence of an unstable region, unique to *C. gachua* and unreported for a fish serotransferrin. In addition, the sequence is also more AT-rich than any of the accessed sequences of the teleost TF. Some differences between the polar, hydrophobic and acidic interactions are also indicated. Percentage of identity and positives of *C. gachua* clone in TF sequences of several other fish taxons suggests a descent from specialized lineage.

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Introduction

Vertebrate transferrin (TF) is a single polypeptide folded into two homologous globular N- and C-lobes, each possessing one iron-binding site (Welch and Skinner 1989). Some 40% of the residues between N-lobe (residues 1-336) and C-lobe (residues 337-678) are identical (MacGillivray et al. 1983). cDNA cloning and sequencing of TF genes have revealed a wide distribution among quite distantly related vertebrates and invertebrate species (Moskaitis et al. 1990; Jamroz et al. 1993; Bartfeld and Law 1990). An interesting aspect of synthesis of TF variants is that they may be synthesized in non-hepatic tissues of some vertebrates (Baldwin et al. 1990).

The TF genes of a number of commercially important teleosts such as Atlantic salmon (Kvingedal et al. 1993), medaka (Hirono et al. 1995), coho salmon (Lee et al. 1995), Atlantic cod (Denovan-Wright et al. 1996), Japanese flounder (Kim et al. 1997) and rainbow trout (Tange et al. 1997) as well as some laboratory fishes have been cloned and sequenced. In addition to detailed nucleotide sequences of TF genes, cloning data has also provided molecular explanation to its polymorphism in some fishes (Yang et al. 2004). More importantly, molecular aspects of phylogeny of fish transferrins are now better understood. The data on salmonids (Ford et al. 1999; Ford 2001) extended credence to the proposal of Kirpichnikov (1981) that fish TF genes evolved by gene duplication through polyploidy and subsequent natural selection.

Information on molecular evolution of TF genes of air-breathing fishes is scarce; though air-breathing has been one of the most important events in vertebrate evolution. As a specialized group of accessory air-breathers, genus *Channa* occupies an important position in evolutionary hierarchy. It is intriguing that even morphologists have ignored the phylogeny of this important group. Chandy (1955), on the basis of morphometric estimates, inferred that air breathing in channids evolved due to persistent hypoxia in marshy habitats of Yunnan region in China. Extant species despite wide geographical distribution from China to Iran including Indian subcontinent and entire Southeast Asia dwell ecologically similar habitats (Banerjee et al. 1988).

The C-value and polyploidy of *C. gachua* (Manna and Prasad 1973; Sharma and Agarwal 1981) in relation to diploid karyotype of two other channids has been discussed by Banerjee et al. (1988). Chromosomal DNA of channids has not been investigated any further. The present work was

initiated to address phylogenetic relationships of genus *Channa* at gene level. *Channa gachua* was selected because: (i), it is a polyploid (Sharma and Agarwal 1981) and thus has a unique evolutionary history within the genus; and (ii), TF gene is a proven choice to trace phylogenetic relationships among polyploid fish species.

This preliminary report documents sequence of a cDNA clone that shows identity with conserved region of known transferrin genes. Despite being a partial clone it does reveal a few important features of the structural gene of *C. gachua* TF.

Materials and Methods

Specimens of *C. gachua* were obtained from local fish markets of Aligarh (Uttar Pradesh). Liver from an adult female following anaesthetizing with tricane-methosulphate, was carefully dissected out and immediately processed for total RNA extraction. Total RNA was prepared from liver of *C. gachua* using Trizol reagent containing kit (GIBCO BRL, USA). Poly $(A)^+$ mRNA was isolated from total RNA by chromatography on oligo(dT)-cellulose spun columns (Pharmacia Biotechnology). Double stranded cDNA was synthesized using oligo(dT) primer essentially according to the manual of Promega Universal RiboClone cDNA Synthesis Kit. The cDNA library was constructed using synthesized cDNA in a pBluescript II phagemid (QIAGEN Inc.) after ligating cDNA to EcoRI adaptor. The cDNA library for recombinants was scored by the method of Sambrook et al. (1989). *Escherichia coli* DH5α infected with pBluescript cDNA library was cultured on agar plates containing 50 μg ml^{-1} Ampicillin, 40 μg ml^{-1} X-gal and 0.5 mM IPTG and incubated overnight at 37°C. White colonies were picked up and kept as glycerol stocks at –20°C. The transferrin cDNA was screened from the cDNA library employing immunological detection according to Helfman et al. (1983). Briefly, the nitrocellulose membranes containing transformants were incubated primarily with rabbit anti-TF *C. gachua* antisera, which was preabsorbed with boiled lysates of DH5α and then with goat anti-IgG antiserum coupled with horseradish peroxidase. The cross-reactivity was recorded on ultrasound film using chemiluminiscent substrate (LumiGLO). Small-scale plasmid preparations for sequencing were made of the positive clones using QIAprep miniprep kit employing the alkaline lysis procedure. The selected cDNA clones were sequenced using an automated DNA sequencer (Model: ABI

377 Prism). To monitor size of plasmids, electrophoresis was carried out in 0.8 % agarose gel.

Reported sequences of previously cloned transferrin genes of rainbow trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*), Japanese flounder (*Paralichthys olivaceus*) and salmon (*Salmo trutta*) were accessed from GenBank database with the Accession Numbers: BAA84103, BAA10901, AAF33233 and BAA84102, respectively. Sequence alignment was made with TF sequences of other fish species accessed from NCBI GenBank database. The source in determining percent identity and positives was also NCBI GenBank.

Results and Discussion

The fluorescence of immuno-cross reactivity against anti-TF antisera of *C. gachua* could be recorded in three positive colonies only after overnight exposure of the film to colony blot. From the purified plasmid preparations of the three positive clones (Fig. 1), one clone with the large insert (lane 2, Fig. 1) was finally taken as partial clone of TF on the basis of sequence comparison with accessible sequences of other teleost TFs. The clone of *C. gachua* showed maximum sequence similarity with evolutionarily most conserved region of vertebrate transferrin gene. The insert was a 483-nucleotide fragment with a deduced amino acid sequence of 161 residues (Fig. 2). The molecular weight of the polypeptide was calculated as 17.9808 kDa that has an isoelectric point of 7.81.

The percentage values of bases in the insert were: 179 A (37.06%), 71 G (14.70%), 143 T (29.61%) and 90 C (18.63%), indicating its AT rich composition, with percent A+T ratio (66.67 %) being just double of its own percent C+G ratio (33.33 %). In this respect, the nucleotide sequence of the clone differs from the accessed sequences of TF of four teleosts used for comparison. While AT:GC ratio is almost equal in TF gene of *Salmo trutta, Oryzias latipes* and *Paralicthys olivaceus*, the conserved sequence of *Oncorhynchus mykiss* TF gene is GC rich. It is obvious that AT rich characteristic of cloned sequence of *C. gachua* will facilitate easier melting during replication and transcription.

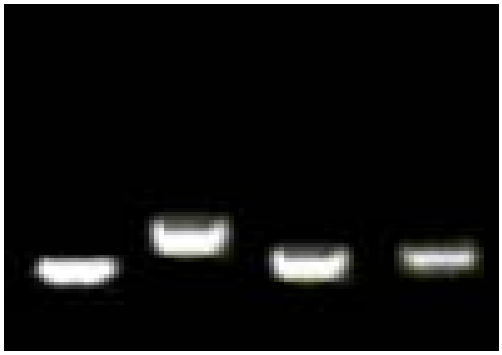

Fig 1: Agarose gel (0.8%) electrophoresis of plasmid DNA purified from clones selected after immunological detection. From left to right: (1) pBluescript II vector without insert and (2 to 4) recombinant clones. Sequence of clone insert of lane 2 was identified as partial cDNA clone of TF gene of *C. gachua*.

GGC	AGT	GAC	CCT	GCA	TTC	AGA	ATG	TTC	AGC	30
G	S	D	P	A	F	R	M	F	S	10
TCT	CAA	GGA	GGA	AAG	AAC	CTT	CTC	TTC	AAA	60
S	Q	G	G	K	N	L	L	F	K	20
GAC	TCC	ACT	AAA	TGT	CTC	CAG	GAG	GTT	CAA	90
D	S	T	K	C	L	Q	E	V	Q	30
GCT	GGA	ACA	AAC	TAT	GTA	CAG	TTT	TTG	GGA	120
A	G	T	N	Y	V	Q	F	L	G	40
ACA	AAT	TAT	ATG	AAT	GCC	ATG	AAT	TCA	CTC	150
T	N	Y	M	N	A	M	N	S	L	50
AGA	CAG	TGC	AGT	GAA	ACT	GCT	CCA	GGT	TTG	180
R	Q	C	S	E	T	A	P	G	L	60
TCT	TTC	TCA	AAG	CTA	CAT	CAA	GAC	GAA	CAT	210
S	F	S	K	L	H	Q	D	E	H	70
AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAT	240
N	N	N	N	N	N	N	N	N	N	80
AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAT	270
N	N	N	N	N	N	N	N	N	N	90
AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAT	AAC	AAT	300
N	N	N	N	N	N	N	N	N	N	100
GTA	TTC	TTG	TCA	GCC	TCA	GAT	AAA	TCA	CAT	330
V	F	L	S	A	S	D	K	S	H	110
ATT	ACA	AAA	ATA	AGT	GGC	TGC	TCT	GCA	TTC	360
I	T	K	I	S	G	C	S	A	F	120
TTA	AGA	TTA	AAT	TTG	CTC	TTT	TCT	TTT	TTT	390
L	R	L	N	L	L	F	S	F	F	130
CCC	CAA	TCA	GAT	GTG	GGA	GAA	ATA	CTG	CAC	420
P	Q	S	D	V	G	E	I	L	H	140
TTT	CCA	TTC	CTG	TCA	ACA	AAA	AAA	CTA	GTG	450
F	P	F	L	S	T	K	K	L	V	150
GTG	ACA	CTG	GAC	CAC	ATG	CCT	CAG	CTG	TCT	480
V	T	L	D	H	M	P	Q	L	S	160
TGC	A									483
C										161

Fig. 2. Nucleotide sequence of partial cDNA of the conserved region of TF gene of *Channa gachua* Ham. one alphabet standard abbreviation below the nucleotide triplets (codons) shows the deduced amino acid sequence.

Figure 3 compares the deduced amino acid sequence of the partially cloned conserved TF region of *C. gachua* and selected accessible TF sequences of the four teleosts domain-wise. The sequence of the partial TF clone between residues 6 and 56 shows percent homology in the "N" domain to the extent of 60% identity with *O. mykiss* between 281-333 residues, 69% with *O. latipes* between 280-329 residues, 54% with *P. olivaceus* between 281-329 residues and 39% with *S. trutta* between 284-334. Residues 2-60 of the partial clone share 41% identity with "C" domain of *O. mykiss*, between residues 619-678, 40%, with *O. latipes* between residues 619-678, 38%, with *P. olivaceus* between residues 619-675 and 61% with *S. trutta* between 619-678. The primary sequence of clone of *C. gachua* shows greater similarity with the sequence of *O. latipes* TF than with any of the other three teleosts.

The cysteine residues involved in the disulfide bridge formation are well conserved in the transferrin family and their locations have been important markers in determining phylogeny of TFs (Williams 1982). There are 14-19 disulfide bridges in the previously cloned vertebrate transferrins and lactoferrins. Cloned sequence of *C. gachua* shows the presence of 4 Cys residues at positions 25, 53, 117 and 161, out of which 2 residues at positions 25 and 53 are at different positions from other four advanced teleost sequences compared here. In addition, tandem placement of 30 Asn residues at 71 to 100 is also characteristic of the primary structure of the cloned sequence. At high temperatures and pH values, the amide (asparagine) is deaminated converting it to corresponding acid (aspartic acid) resulting in easy unfolding of the polypeptide. According to the primary sequence of the cloned insert, 76 amino acid residues are polar and uncharged; 59 hydrophobic or non-polar; 16 basic and 10 residues are acidic (Fig. 2). As compared to other accessed sequences, *C. gachua* sequence has the highest total number of polar amino acids followed by hydrophobic residues, while basic and acidic residues are lesser than them. The differences indicate relative instability in this region of *C. gachua* transferrin molecule.

By BLAST search, the primary sequence of *C. gachua* clone was also used to score 'positives' and 'percent identities' within TF genes of 17 fish species of different phylogenetic origins (Fig. 4). Ford (2000; 2001) and Yang et al. (2004) have demonstrated the evolutionary importance of synonymous-to-synonymous substitutions in transferrin genes of salmonids and goldfish. Alignment as % identity and unchanged locations of amino acid residues (positives) is a comparison that includes synonymous substi-

N-LOBE

C. gachua	FRMFSSQ--GGKNLLFKDSTKCLQEVQAGTNYVQFLGTNYMNAMNSLRQCSET	6-56
O. mykiss	F +FSS G KNL+FKDST+L ++ T+ +LG YM+ + SL + T	281-333
O. latipes	F +FSS+ KNL+FKDST+L ++ T+ +LG YM+ + SL++	280-329
P. olivaceus	F +FSS GGKNL+FKDST L ++ ++ +LG +Y++ + +L++	281-329
S. trutta	+FSS G KNL+FKDS +L ++ T+ +LG YM+ + SL++ T	284-334

C-LOBE

C. gachua	SDPAFRMFSSQGGKNLLFKDSTKCLQEVQAGTNYVQFLGTNYMNAMNSLRQCSETAPGL	2-60
O. mykiss	GSD +F MF S GKN LFKDSTKCLQE+ +GT + FLG YM AM SLR+CS + L	619-678
O. latipes	GSDP+F++F SQ G NLLFKDSTKCLQEV AGT+Y QFLG+ YM AM SLR+CS+TA L	619-678
P. olivaceus	S+ +F+MF+S G+NLLFK STKCLQE+ A +Y FLG YM M+SLR C E+ L	617-675
S. trutta	GSD +F+MF S KNLLFKDSTKCLQE+ GT Y FLG YM AM SLR+CS++ L	619-678

Fig. 3. Identity of the deduced amino acid sequence of partial cDNA of TF gene of *C. gachua* with the sequences of rainbow trout (*O. mykiss*), medaka (*O. latipes*), Japanese flounder (*P. olivaceus*) and the salmon (*S. trutta*). (+ Sign indicates high probability of conserved sequences as indicated by NCBI GenBank BLAST search).

tutions. Almost parallel trend of changes was shown by both of the selected parameters (Fig. 4). Only the species of genus *Oncorhynchus* displayed a range of minimal changes in % identity and positives, suggesting that species with close phylogenetic relationships display minimal differences within the conserved sequence compared. Minimum % identities and positives existed in the Tf sequences of *Paralichthys olivaceus* and *P. flesus*, the flat fishes (order: Pleuronectiformes) and the maximum with *O. latipes* (Fig. 4), which belongs to Beloniformes. Rest of the compared TF sequences belong to species of the order Perciformes and show ~5% variations in the identity or positives. A major difference in any case is the typical occurrence of a stretch of 30 Asn residues in clone of *C. gachua*.

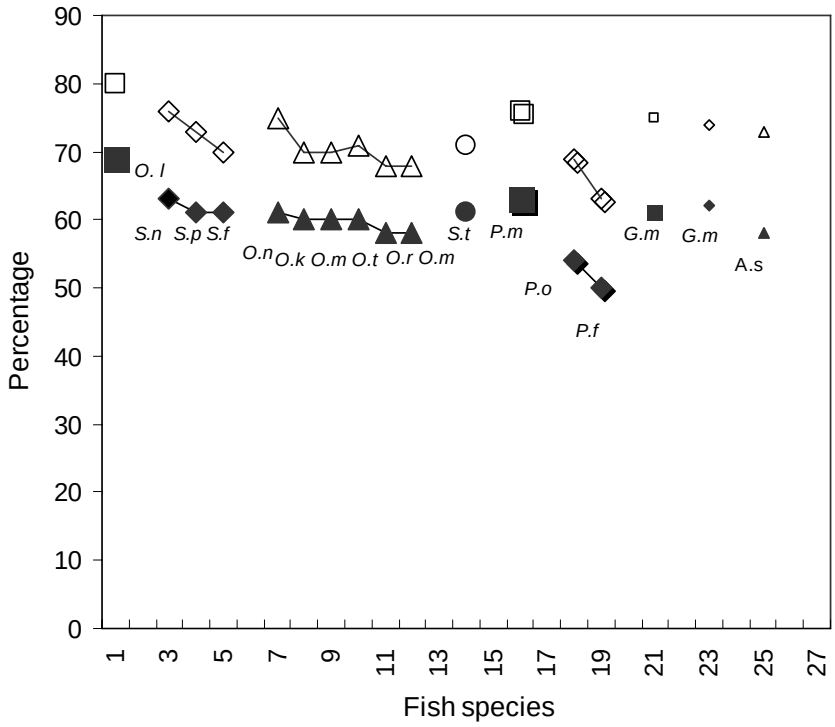

Fig. 4. Chart showing the extent of percent identity of deduced amino acid sequence of partial cDNA clone of conserved region of C. gachua TF gene with the TF sequences of 17 other teleosts. The percent identity values were accessed from NCBI GenBank BLAST database. (Teleosts taken into consideration are: *Oryzias latipes*, *Salvelinus namaycush*, *S. pluvius*, *S. fontinalis*, *Onchorhynchus nerka*, *O. kisutch*, *O. mykiss*, *O. tshawytscha*, *O. rhodurus*, *O. masou*, *Salmo trutta*, *Pagrus major*, *Paralichthys olivaceus*, *Platichthys flesus*, *Gadus morhua*, *Gillichthys mirabilis* and *Acanthpagrus schlegelii*).

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Although some authors have included channids under Perciformes (Pethyagoda 1991), the above comparison supports that evolutionary descent of *C. gachua* is different from the advanced teleosts of this group (Chandy 1955). This is also consistent with the anatomical data of Liem and Greenwood (1981) that disclosed primitive nature of pharyngeal-parasphenoid bite in Channiformes along with Nandidae.

Conclusions

The partial cDNA clone of air-breathing snakehead *Channa gachua* showed 41-71% identity with the conserved regions of both N- and C-lobes of teleost TF. Being AT-rich, the 483 nucleotide long sequence appeared easy to melt. Characteristic features of its deduced primary sequence were the specific positioning of two cysteine residues and an unstable unique region of 30 tandem asparagine residues. These characteristics of the partial clone and its percent 'identity' or 'positives' with the accessed 17 fish TF sequences suggested evolution of *C. gachua* TF gene from a specialized lineage unrelated to the compared species.

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