DISCUSSION
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Commonly known as a snakehead or the murrel, *Channa gachua* Hamilton is a small sized (<20 cm) hardy obligate air-breathing fish of morphological features described under the identification key reproduced on pages 16-17. It has a wide distribution not only in Indian subcontinent but also in several other Asian countries as well as in some Middle East countries and the mainland China.

Among vertebrates, transferrin is one of the most abundant protein in sera and numerous tissues, which include liver and several other non-hepatic tissues (Baldwin *et al.*, 1990). Due to its vital function of inter-tissue transport of free iron and clinical significance, it has been subjected to intensive investigations in mammals including humans (Djeha *et al.*, 1992). As already pointed out under Introduction, the information on the functional and clinical aspects of fish Tfs or other members of this multigene family is scarce (Winter *et al.*, 1980; Hirono and Aoki, 1995 and Yano, 1996).

*The nucleotide sequence of Tf-like cDNA clone of C. gachua:*

Percent homology of nucleotide sequence of Clone-2 cDNA with the sequences of other fish Tfs indicated that it is a partial clone of
structural gene of Tf of *C. gachua*. Since the cDNA clone was not a complete sequence, it was preferred to call it a clone of Tf-like protein, despite the homologies with four accessed complete sequences; namely: *Oncorhynchus mykiss, Oryzias latipes, Paralichthys olivaceus* and *Salmo trutta*. Poly(A) 3'-tail in mRNA lies towards C-terminus of the polypeptide and cDNA was synthesized using oligo(dT) primer (first strand synthesis 3'-5'), the partial clone has to exhibit more homology with C domain of teleost Tfs. The sequence of *C. gachua* exhibits such a homology with C and N domains of *S. trutta* to the extent of 61% and 39%, respectively.

The sequence of partial clone of *C. gachua* Tf-like protein also shows two major differences with the sequence of *S. trutta* Tf, *i.e.* the difference in number and position of Cys residues. Whereas, sequence of *C. gachua* shows the presence of 4 Cys residues, 2 residues each occur at different positions in *S. trutta* and *O. mykiss*. It is noteworthy that locations of disulfide bridges have been an important marker in determining phylogeny of Tfs (Williams, 1982). In the absence of any additional data on *C. gachua*, the numerical and positional differences in Cys residues suggest the evolutionary distinctions of N-lobes of the
compared Tf isoforms. The part of sequences showing general homology in both N and C lobes of vertebrate Tfs has been the most conserved sequence during evolution (Baldwin, 1993).

The cDNA sequence of *C. gachua* is AT rich, being two times of its own GC contents. In this respect the DNA sequence of the clone differs from those of four accessed sequences. Out of them, AT:GC ratio is almost equal in case of *Salmo trutta*, *Oryzias latipes* and *Paralichthys olivaceus*. On the other hand, *Oncorhynchus mykiss* exhibits an inverse AT:GC ratio. It is well known that AT rich regions melt more easily during molecular processes such as replication and transcription, as well as at elevated temperatures. The extent of relative unstability of the nucleotide sequence is also supported by amino acid sequence, where 30 asparagine residues at a stretch are present in the primary sequence of the cloned part of polypeptide. High temperatures and pH values deaminate the amide (asparagine) converting it to corresponding acid causing unfolding of protein. As for the general picture of the amino acid percentage of the deduced primary sequence, *C. gachua* Tf has highest total number of polar amino acids followed by hydrophobic residues while basic and acidic residues are lesser than the
values in this region of other accessed sequences. This may again have a bearing on the pH or thermostability of this part of the polypeptide chain.

**Biochemical Characteristics:**

On the basis of three staining criteria (CBB, Nitroso-R and negative identification with FENTA) and Western blotting applied to detect and identify Tf bands in native PAGE patterns, the occurrence of two phenotypes was confirmed. As of now, it appears that genetics of Tf in *C. gachua* is under control of two codominant alleles designated here as TfA<sub>cg</sub> and TfB<sub>cg</sub>. Each allele encodes two identical isoforms, giving rise to one banded pattern represented by homozygous phenotype AA (that is yet to be discovered) and homozygous BB, respectively. Two AA isoforms co-stack (as one homozygous phenotype), because they have identical electrophoretic mobility, though slower than that of the other two costacking isoforms BB (fast migrating homozygous phenotype). So far the occurrence of AA and BB phenotypes is concerned, our results support the existence of two banded pattern (homozygotes: AA/BB) reported by Sahoo and Khuda-Bukhsh (1989) in a population of quite distant location. Occurrence of
one banded pattern (phenotype BB only) is a new finding. It is also puzzling that no heterozygous individuals are detected neither within the populations around Aligarh nor in Bengal.

Due to polyploidy, *C. gachua* is a special case among channids and may be a model species to explore the possible implications of the origin of Tf polymorphism by gene duplication. Kirpichnikov (1973) has suggested that function of transferrins is so vital that additional loci are lost during the course of evolution.

Most of the studies have so far mainly dealt with polymorphism of fish Tfs with the objective of applying the information to genetic identification of stocks and races (see for example, Lacy, 2000). In the present attempt, therefore, the attention was directed to cover biochemical and functional characterization of Tf of *C. gachua* and hitherto unattended aspect of cloning its structural gene. Whereas within genus *Channa*, specificity of *C. gachua* is polyploidy, an attainment of relatively large size (~70cm) is the characteristic of *C. striatus* and *C. marulius*. Since relevant information on some of these aspects of *C. punctatus* was already available (Nabi, 1999), the
remaining two species were included for a broader understanding of Tf molecules of the genus as a whole.

Several workers have recommended the use of rivanol fractionation to remove non-Tf proteins of sera (Sutton and Karp, 1965; Sahoo and Khuda-Bukhsh, 1989). In our lab also *C. punctatus* Tf has been purified by this method; but experiments with *C. gachua* sera showed that ammonium sulfate fractionation gives better yield. Even the number of bands in liver extracts could be minimized and Tf-like band concentrated by this method. The results of initial monitoring of such preparations by PAGE were satisfactory. However, in view of the possibility of tissue specific isoforms, further characterization may be necessary to make a final statement about using liver as the source of pure transferrins. Heart extracts have been used by some workers, but, in fish species of the size of *C. gachua* or *C. punctatus* heart is too small a tissue for this purpose.

In fish species, Tf polymorphism of varying degrees and magnitudes has been discovered. Among the highest recorded so far, mirror carp has been shown to have 7 Tf alleles (Valenta *et al.*, 1976) and an Amazonian fish *Plagioscion squamasiissimus six* (Teixeira *et al.*, 1989).
2002). Similarly, 4-allele system in coho salmon (Van-Doornik et al., 1996) and 3-allele systems in tuna (Fujino and Kang, 1968) have also been recorded. In *C. punctatus*, a sister species of *C. gachua*, Tf polymorphism based on the 3-allele system has been recorded (Nabi et al., 2003). Since the polymorphism in Tfs owes its origin to gene duplication, beside other genetic applications, it would be of phylogenetic interest to determine the evolutionary response of polyploidy of *C. gachua* (*2n* = 72). However, the number of Tf alleles recorded for this species is only 2, the lowest recorded so far (Fig. 9-10). Coincidently, screening of the population inhabiting locations >1300 km. apart also showed the total lack of any heterozygote (Sahoo and Khuda-Bukhsh, 1989).

Though some fish transferrins may be totally devoid of carbohydrates (Stratil et al., 1985), most of them are glycoproteins. The resolution of glycoproteins is retarded in other SDS-PAGE systems. Porous gel electrophoresis protocol of Doucet and Trifaro (1988) has been essentially designed to separate glycoproteins. Electrophoresis of purified transferrins of *C. gachua* and other channid species in widely adopted system of Laemmli (1970) produced smears. It was taken as an
initial indication of their glycoprotein nature and subsequent runs were made exclusively in the system Doucet and Trifaro (1988). As typically shown under Results in Fig. 15, following SDS-PAGE in this system, Tf isoform B of *C. gachua* migrates slightly faster than pure human serum transferrin giving the approximate molecular weight values of ~71.8 kD in comparison with 80 kD of human Tfs. There appear no apparent intrageneric differences if the value obtained for *C. gachua* Tf is compared with those of the Tfs of other three species.

Fishes constitute the largest group among the vertebrates. They include primitive forms such as the cyclostomes as well as a number of specialized teleosts such as the air-breathing fishes. Therefore, certain variations in the molecular weight of transferrins of different groups have been recorded (Stratil et al., 1983). The lowest value of 44 kD has been reported for Tfs of the primitive hagfish (Palmour and Sutton, 1971). Employing several criteria, the molecular weight of brook trout and carp Tfs has been calculated as 78 and 70 kD respectively (Hershberger, 1970; Valenta et al., 1976). In comparison with this data, somewhat high value of 80 kD obtained by SDS-PAGE (Hara, 1984). Molecular weight values of transferrin isoforms of *C. gachua* and Tfs
of three other species of the genus are in reasonable agreement with the general range of 70-81 kD determined for fish as well as mammalian Tfs (Stratil et al., 1983) and do not show an anomalous behavior reported for evolutionarily distinct cyclostomes (Palmour and Sutton, 1971).

There, however exist unstability and conformational differences between Tf isoform of *C. gachua* and Tfs of other three channid species. A comparison of Fig. 14b and 16 supports this statement. In spite of identical conditions applied in preparing protein samples for SDS-PAGE, subsequent analysis in this system and for washing out SDS, only *C. gachua* and *C. punctatus* Tf bands exhibited immunocross-reactivity as chemiluminiscent signals. These results categorize Tfs of genus *Channa* in two conformational groups: 1), the immunologically detectable **group-1** constituted by *C. gachua* and *C. punctatus*; and 2), immunocross-reactivity lacking **group-2**, represented by Tfs of *C. striatus* and *C. marulius*. It is obvious that in Tf isoforms of **group-1**, some of the epitopes assume a conformation that permits immunological detection. Such a conformation was not assumed by the **group-2**, *i.e.*, by Tfs of *C. marulius* and *C. striatus*. Therefore, *C.
gachua and C. punctatus Tfs (group-1) appear to be relatively stable proteins against thermal, SDS and acidic treatments (given during washing of gels to remove SDS). No published evidence is available to compare these results. However, inter species differences in thermostabilities have been reported in case of several mammalian Tfs (Welch, 1990).

Tf of C. gachua as well as those of three other channids are glycoproteins containing sialic acid. This is supported by the results in Fig. 16 (lanes designated D), which reveal that upon 24 hour long digestion with neuraminidase, following which Tfs of C. gachua as well as other three species are gradually converted into a major band of approximately 69.2-70.5 kD. Therefore, this value represents mass of only the protein whereas the remaining >2 kD is constituted by carbohydrate moiety, of which sialic acid is an integral component. In this respect Tfs of C. punctatus are similar to those of brook trout (Hershberger, 1970) and differ from those of carp, which are devoid of any sialic acid, while in some other fish transferrins other carbohydrates happen to be the major contributors (Stratil et al, 1983). The reduction in Mr of human Tf is half of the values noted for fish Tfs (Fig. 15, Table
3). It is already established that human Tf is a glycoprotein with two Asn linked bifurcated glycan chains. One sialic acid each is borne upon by each terminus making the total four (Harris and Aisen, 1989). The results presented in this work are not sufficient to speculate about the nature and number of sialic acid residues or the presence of other carbohydrates. However, the change in all of the four fish Tfs is similar, but of different magnitude as compared to that of human Tf.

Similarly, fish transferrins also exhibit a wide range of characteristic $pI$ values, which were on higher side being 9.0 and 8.9 for Tfs of petromyzon and a shark, respectively (Boffa et al., 1966); but 5.0 for carp (Valenta et al., 1976) as well as mammalian Tfs (Welch, 1990; Baldwin, 1993). For human Tf, a $pI$ value of 5.1 calculated here (Fig. 17, Table 4) is in good agreement with already published value of 5.35 (Welch, 1990). $pI$ values for channid Tfs obtained in the present study show them to be more acidic with only minor differences between their $pI$ values in the range of 4.5-4.7 (Fig. 17, Table 4). It is worth mention that similar to the distinct immunocross-reactivity behaviour after SDS and acid denaturation (Fig. 14b), Tfs of C. gachua and C. punctatus are recognized as one category or group-1 in terms of $pI$ values also (Table
4). Tfs of *C. striatus* and *C. marulius* belonging to immunologically undetectable group-2 are slightly more acidic in their *pI* values. The differences in *pI* values are also an indication of the differences in their primary structure or/and amino acid composition.

Stoichiometry of iron binding of *C. gachua* and other channid Tfs were typically vertebrate in pattern, showing an absorption maximum at 470 nm and total bound iron being 2.1-2.3 atoms/molecule of Tf. No published evidence is available on iron binding properties of fish Tf except that on brook trout (Hershberger, 1970) and unpublished results of this lab on *C. punctatus* Tf (Nabi, 1999). Results obtained by the latter worker could be reproduced during the present investigations also. Tfs of *C. gachua* and three other channid species differ in their iron binding capacity as indicated by the starting points (initial values) of each curve (Fig. 18), though final amount of bound iron was the same in each case.

Welch (1990) categorized the pH dependent iron release from diferric Tfs into three categories. 1, clear biphasic release as in case of the reptile turtle and some mammals; 2, coupled pattern of two slightly different gradients as in human Tf; and, 3, monophasic steep decline as
in snake Tf. Iron binding curve of human Tf obtained during the present analysis is similar to the published one. For fish Tf's also, a pattern that shows a coupling of two gradients is obtained with the distinction that 20-30% iron still remains bound even at pH 2.5 a value at which human diferric Tf releases all bound iron. Again, a similarity of iron releasing gradient was observed for diferric Tf's of *C. gachua* and *C. punctatus* (group-1), whereas the behaviour of *C. striatus* and *C. marulius* (group-2) was nearly identical. Differences in release of iron from diferric Tf's have been attributed to differences in pH stabilities of N and C domains (Antonio and Brock, 2001). That such variations, might to some extent exist, is supported by the sequence specifications of the partial cDNA clone of Tf-like protein of *C. gachua* (Fig. 6 and 7).

Iron binding capacities of fish Tf's have a crucial role in determining post-hatching viability of progenies of various phenotypes (Hershberger, 1970) and the results obtained here somehow reflect the differences in interspecies survivability (relative hardiness) of the four channid species, in general.
Genetic Significance of Transferrin isoforms of Channa gachua Ham.

1. Utilization of the partial cDNA clone of Tf-like protein:

   It is for the first time that the construction of a cDNA library from *C. gachua* mRNA was attempted. Positive identification of a partial clone as that of Tf-like isoform offers the opportunity to use it as a probe to search out more extended sequences from the library. Since the cloned sequence represents one of the most conserved portions of Tf molecule, in general, the probe may be reasonably suitable to screen cDNA libraries of the remaining fish species of this genus or other genera.

2. Negative Identification by FENTA:

   Keeping in view the vastness of India’s fisheries resources, the information on *Biochemical Genetics* of fish fauna is inadequate. Simple experimental procedures, which can yield information employing non-hazardous (*e.g.* non-radioactive) means, are likely to promote the efforts by those laboratories which are spread across the country and carry out research with limited means. Though Nitroso-R staining for transferrin bands in electropherograms was in use for nearly four decades, it has the disadvantage of staining a few non-Tf bands
also. Purification and subsequent detailed biochemical investigations were the only alternative to verify their chemistry beyond doubt. Negative identification of Tf bands by incubation in FENTA, as applied here, can unambiguously verify Tf nature of bands in superimposed or duplicate gels run under identical conditions. There are no previous reports of the Negative Identification by FENTA.

3. The probable number of loci encoding isoforms of Tf:

The observed polymorphism in *C. gachua* isoforms is a 2-allele (A and B) system. In a system of co-dominant inheritance, typical of Tfs, one isoform each would be synthesized by the allele of each locus. Therefore, the slower migrating phenotype is genotypically homozygous AA and the faster one homozygous BB. Co-stacking isoforms B, constituting genotype BB, were purified and used for biochemical characterization.

4. Level of Polymorphism:

In the present study, biochemical characterization employing several criteria, has conclusively established Tf nature of the bands identified in fish sera samples as those of transferrin. The occurrence of two banded Tf phenotype among *Channa gachua* populations has been
reported from Bengal (Sahoo and Khuda-Bukhsh, 1989). However, there is no previous report from any other part of India, and the present report is not only the first from this region but it also reports the occurrence of BB phenotype for the first time. If confirmed by the population analysis of the larger habitats in both regions, then those inhabiting Western Uttar Pradesh (Aligarh and around) would appear more heterogeneous than those of Eastern India (Bengal). Both, the report on populations inhabiting a location in Bengal (Sahoo and Khuda-Bukhsh, 1989) as well as the present investigations share the limitations of sample size. The differences in genetic composition are, however, apparent.

5. Significance of functional properties of Tf isoforms in determining genetic composition of fish populations:

It has been previously proposed (Hershberger, 1970) that iron binding capacity of fish Tfs may be crucial in determining post-hatching viability of the progeny. A survey of *C. punctatus* populations inhabiting a large area of Rohilkhand plains (in Uttar Pradesh) also supported the key role played by the above mentioned functional property of transferrin isoforms (Nabi, 1999, Nabi *et al.*, 2003). During the present investigations, functional properties of only isoform B of *C.
gachua were worked out. Additional data will be required for intraspecies comparison between iron binding capacities of different isoform of C. gachua. The information presented in this work, however, demonstrates that isoform B of C. gachua, exhibits differences with isoforms of other three species, both in iron binding as well as pH dependent release. The magnitude of differences in these properties of C. gachua Tf is lesser than those of C. punctatus Tf; whereas higher than those of the C. marulius and C. striatus Tf isoforms.

It is significant that no heterozygous phenotypes were discovered at neither of the two localities, though the investigated area around Aligarh (present results) and those located in Bengal (Sahoo and Khuda-Bukhsh, 1989) are >1300 km away from each other. The total lack of heterozygotes is unlikely, which suggests that the population data should be further expanded. It is worth mention that in case of C. punctatus also, at least one homozygote (BB) was in excess of heterozygote AC and homozygote AA was very rare (Nabi, 2003).