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Isolation and Characterization of a Novel Copper-Inducible Metallothionein Gene of a Ciliate, *Tetrahymena tropicalis lahorensis*

Raheela Chaudhry and Abdul Rauf Shakoori*

School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan

ABSTRACT

The two isoforms of copper metallothionein (*CuMT*) gene of a copper resistant ciliate, *Tetrahymena tropicalis lahorensis* (*Ttl*), have been isolated and characterized. The molecular cloning and nucleotide sequencing of cDNAs coding for the two *CuMT* isoforms revealed that *TtlCuMT*1 gene has 300, while *TtlCuMT*2 has 327 nucleotides, both with ATG as the initiation codon and TGA as the translational termination codon. TAG codes for glutamine in *TtlCuMT*2 gene which is peculiar to *Tetrahymena*. The deduced or translated TtlCuMT1 and TtlCuMT2 peptide sequences contain 100 and 108 amino acid residues including 28 and 32 cysteine residues, respectively. The amino acid sequences of TtlCuMT1 and TtlCuMT2 have special features of two and three CXCXXCXCXXCXC intragenic tandem repeats with a conserved structural pattern of cysteine, respectively. The predicted tertiary structures of these two isoforms indicate two domains. Domain I and the initial part of domain II showed >98% homology with other *Tetrahymena* CuMT. On the basis of the differences in the domain II, the metallothionein subfamily 7b can be divided into two groups, one (TtlCuMT1) comprising >100 amino acids and the other (TtlCuMT2) comprising <100 amino acids. This is a novel finding of the present study as no such report on this type of classification exists at the moment. *TtlCuMT*1 has 95%, while *TtlCuMT*2 has 97% resemblance with the previously reported *CuMT* genes of *Tetrahymena* spp. SDS-PAGE analysis using fluorescent probe as well as Coomassie brilliant blue staining also confirmed the presence of metallothionein. J. Cell. Biochem. 110: 630–644, 2010.

KEY WORDS: METALLOTHIONEINS; HEAVY METALS; COPPER RESISTANCE GENE; *Tetrahymena tropicalis lahorensis*

Metallothionein (MT) constitute a superfamily of ubiquitous low molecular weight (<7–10 kDa), cysteine-rich proteins that bind heavy metal ions (mainly Cd, Zn, and Cu; typically 4–12 atoms/mole) via metal-thiolate clusters. Typically these proteins have 18–23 (23–33 mol%) highly conserved cysteine residues and lack aromatic amino acids and histidine [Coyle et al., 2002].

The widespread occurrence of MTs in nature suggests that they serve an important biological function but their primary biological role remains an enigma and is obscure. MTs have no enzymatic activity, nor do they perform any catalytic role in known metabolic processes [Gonzalez-Duarte, 2003]. MT fulfills protean functions, that is, a range of functions depending on the specific needs of the particular organism or tissue and its environmental circumstances. Some of the known functions of MTs [Coyle et al., 2002] include essential-metal homeostasis [Cousins, 1985], protection against heavy metal toxicity by sequestration [Liu and Klaassen, 1996], trapping reactive oxygen species [Viarengo et al., 2000], and protection against xenobiotics [Klaassen and Liu, 1998]. In mammals, they have also been implicated in protection against neurodegenerative diseases [Penkowa et al., 2000] and in development and cellular differentiation processes [Vidal and Hidalgo, 1993].

Although MTs from different organisms show identical functional properties, they are heterogeneous in terms of amino acid and nucleotide sequences and gene organization. However, the main feature of MTs is the distribution of conserved cysteine residues in the recurring structural motifs Cys-Cys, Cys-X-Cys or Cys-X-Y-Cys [Hamer, 1986]. Another interesting aspect of MTs is gene duplication. Cases of *MT* gene duplication associated with metal resistance have been described in *Drosophila melanogaster* [Maroni et al., 1987] and *Crassostrea gigas* [Tanguy and Moraga, 2001]. Also in *Tetrahymena pyriformis* and *T. pigmentosa*, the great similarity between the first and second halves of the MT-1 amino acid sequence suggests that this protein arose by gene duplication

*Correspondence to: Prof. Dr. Abdul Rauf Shakoori, Distinguished National Professor & Director, School of Biological Sciences, University of the Punjab, Lahore 54590, Pakistan.
E-mail: arshak@brain.net.pk, arshaksbs@yahoo.com, abdulrauf.shakoori@umassmed.edu
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[Piccinni et al., 1994, 1999]. The *Tetrahymena* MT gene promoters have also been used to build new genetic engineering tools [Shang et al., 2002; Boldrin et al., 2006]. Shang et al. [2002] introduced the first inducible-repressible promoter for driving high-level expression of heterologous genes was introduced.

Tetrahymena MTs constitute an excellent example of an MT subfamily that is considerably divergent from the vertebrate paradigm [Domenech et al., 2008]. At present, the purified and characterized metallothioneins in ciliated protozoa are only those from *Tetrahymena pyriformis*, *T. pigmentosa* and *T. thermophila* [Piccinni et al., 1990, 1994; Piccinni and Albergoni, 1996]. The first ciliate cadmium-binding MTs (CdMT) were isolated from *T. pyriformis* and *T. pigmentosa* [Piccinni et al., 1994] and their primary amino acid sequence determined. The corresponding gene of *T. pyriformis* (MT-1) was sequenced in 1999 [Piccinni et al., 1999]. Additional *Tetrahymena* MT sequences have since been reported: the MTT1, MTT2, MTT3, MTT4, and MTT5 genes of *T. thermophila* [Boldrin et al., 2002, 2008; Shang et al., 2002; Dondero et al., 2004; Santovito et al., 2007], the TpMT-2 gene of *T. pyriformis* [Fu and Miao, 2006], the MT-2 gene of *T. pigmentosa* [Boldrin et al., 2002] and TMCd1 gene of *Tetrahymena tropicalis lahorensis* [Shuja and Shakoori, 2007]. Recently, Guo et al. [2008] have reported novel CdMT gene (Tpig-MTT1) in *T. pigmentosa*, while Amaro et al. [2008] has reported isolation of two new MT genes (TrosMTT1 and TrosMTT2; Cd and Cu MT genes, respectively) from *T. rostrata*. Moreover, first cadmium metallothionein like gene (PMCdI) has also been isolated from *Paramecium* sp. [Shuja and Shakoori, 2009] which is the only metallothionein gene reported in any species of *Paramecium*. The highly complex gene/protein system in the *Tetrahymena* genus serves as an optimal model for the study of functional specificity and evolutionary divergence on a molecular level [Domenech et al., 2008].

Ciliate MTs have been assigned to MT family 7 [Binz and Kagi, 1999]. According to their phylogenetic relationships, the pattern of clustering of Cys residues, and which metal inducer they preferentially respond to, they were grouped into Cd-MTs (subfamily 7a) or Cu-MTs (subfamily 7b), showing little sequence identity with one another [Guo et al., 2008]. One group is most efficiently induced by cadmium (MT1, MTT1, TpMT-2, MTT-5), while the other responds best to copper (MT2, MTT2, and MTT4) [Dondero et al., 2004]. The Cd-induced MTs are unusually long and have an unblocked N-terminus [Piccinni and Albergoni, 1996], whereas, copper MTs are shorter and quite different from the ciliate Cd MTs [Santovito et al., 2001]. Cd-MTs have conserved CC, CCC, CXC, and CXYC motifs while Cu-MTs have conserved CKC, and CXCXYCXCXYCXC (where C is cysteine and X and Y, any other amino acid residue) motifs [Amaro et al., 2008]. The data of Diaz et al. [2007] and Amaro et al. [2008] also confirmed that Cd- and Cu-MTs fall in separate phylogenetic clades. The identification of a new MT gene isoforms in *Tetrahymena tropicalis lahorensis* (*T. t. lahorensis*) described in this paper allows more thorough phylogenetic study of the MT gene family in the ciliate, *Tetrahymena*. The genetic diversity of MT genes within and between *Tetrahymena* species may be related to their functional requirements, and in this respect, further research will provide more information.

MATERIALS AND METHODS

T. t. lahorensis copper metallothionein (CuMT) gene was isolated using both genomic DNA and cDNA as template.

CLONING OF *CuMT* GENE ISOFORMS FROM GENOMIC DNA

The isoforms of *CuMT* gene were amplified using genomic DNA as template. Genomic DNA was isolated from the vegetative growing *T. t. lahorensis* cells ($3–4 \times 10^4$ cells/ml). The culture was harvested; pellet was suspended in lysis buffer [42% urea, 0.3 M NaCl, 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 1% SDS] to which equal volume of phenol/chloroform (1:1) was added. After treating the aqueous phase with equal volume of chloroform, DNA was precipitated with twice volume of chilled absolute alcohol. DNA pellet was washed with 75% ethanol, air dried and dissolved in 50 μl of autoclaved deionized H_2O. Genomic DNA was quantified using spectrophotometer and its quality was observed by agarose gel electrophoresis.

For the amplification of one isoform of *T. t. lahorensis CuMT* gene, following primers were used:

Forward primer 5′-ATGGAYACIYARACIYARACIAA.
Reverse primer 5′-TCAGCATTTGCATTCAGCACA (Y = C + T; R = A + G and I = Inosine).

The genomic DNA, used as a template, was 450 ng. A total of 50 μl PCR reaction volume containing 1× Taq buffer with $(NH_4)_2SO_4$-$MgCl_2$ (Fermentas), 2 mM $MgCl_2$, 0.2 mM of each dNTPs, 200 pmol each primer and 0.5 U of Taq DNA polymerase (Fermentas) in a 200 μl PCR tube was incubated in a Brinkmann Eppendorf Mastercycler® Personal Thermal Cycler. The PCR reaction conditions comprised an initial denaturation step of 95°C for 5 min, followed by 30 cycles each of 1 min at 95°C, 1 min at 53°C and 1 min at 72°C; and a final extension step at 72°C for 10 min. The PCR product was resolved in 1.5% agarose gel stained with ethidium bromide.

The second isoform of *T. t. lahorensis CuMT* gene was amplified using 300–400 ng of genomic DNA as template and the following primers:

Forward primer 5′-CCATGGAYACIYARAC.
Reverse primer 5′-TCATTGCTTTTGGCAACAAGA (Y = C + T; R = A + G and I = Inosine).

Reaction mixtures (50 μl) containing 0.5 mM of each deoxynucleoside triphosphate (dNTPs), 100 pmol of each primer, 1× Taq buffer with $(NH_4)_2SO_4-MgCl_2$ (Fermentas), 2 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase (Fermentas) in 300 μl PCR reaction tube were incubated in a Brinkmann Eppendorf Mastercycler® Personal Thermal Cycler. An initial denaturation step at 95°C for 3 min was followed by 30 cycles, each of 94°C for 30 s, 52°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. The size of amplified DNA was determined by electrophoresis in 1.5% agarose gel.

The PCR amplified DNA fragments were purified from agarose gel in 1× TAE buffer, using DNA Extraction kit (Fermentas #K0513) by manufacturers given protocol, and ligated to pTZ57 R/T cloning vector (Fermentas Ligation kit #K1214) in 3:1 insert/vector ratio according to the given protocol. Competent cells of *E. coli* DH5α were transformed with the recombinant DNA fragments (i.e., two *CuMT* gene isoforms). The transformants were screened using X-Gal/IPTG and ampicillin selection. The plasmids were isolated from white colonies (those containing insert) and for confirmation of right size of insert, double digestion with *Eco*R1 (Fermentas #ER0272) and *Hind*III (Fermentas #ER0501) was done. Restricted fragments were analyzed on 1.5% agarose gel.

Plasmids for sequencing were prepared using QIAprep® Spin Miniprep Kit. Fluorescent sequencing of the positive clones was done by Macrogen, Korea.

CLONING OF *CuMT* GENE ISOFORMS USING CDNA

Isolation of total RNA. Total RNA was isolated from mid-log phase (5×10^5 cells/ml) growing *T. t. lahorensis* culture by TRIzol reagent (Sigma–Aldrich). Prior to RNA isolation, 100 ml culture of RT-1 was equally divided into two flasks. In one flask containing 50 ml *T. t. lahorensis* culture, 0.5 mM Cu^{2+} solution (~32 ppm) was added (copper treated) and in the other flask no metal ions were added (control). The cells were harvested after 1 h of copper stress in separate tubes from both the flasks. The pellet was suspended in 1 ml TRIzol reagent (ratio 1:3), vortexed to homogeneity and kept at room temperature for 5 min. Later chloroform (200 μl) was added, vigorously shaken, incubated at room temperature for 10 min and then centrifuged at 13,680*g* (MIKRO 200R Hettich refrigerated centrifuge) for 15 min at 4°C. The upper aqueous layer was removed and twice volume of chilled isopropanol was added to precipitate the RNA, incubated at room temperature for 5–10 min and then centrifuged at 6,080*g* (MIKRO 200R Hettich refrigerated centrifuge) for 15 min at 4°C. Then RNA pellets were washed twice with 75% ethanol (chilled), air dried and dissolved in sterile de-ionized water. The purity of RNA from both samples was determined by the $A_{260/280}$ absorbance ratio (1.7–1.8). Isolated RNAs were treated with 5 μl DNAase (Fermentas) for 30 min at 37°C and stored at −80°C, after checking the purity and integrity of 18S and 28S rRNA bands on denaturing agarose gel (1.5%) containing 2.2M formaldehyde [Sambrook and Russell, 2001].

cDNA synthesis and RT-PCR. cDNA of RNA of copper treated and control samples was synthesized using oligo $dT_{(18)}$ primer (Advantage™ RT-for-PCR Kit, Clonetech Laboratories, Inc.), using prescribed protocol. To 3 μl of each RNA, DEPC treated water was added to make up the volume up to 12.5 ml. One microliter of oligo $dT_{(18)}$ primer was added and heated at 70°C in Perkin Elmer (PE) GeneAmp® PCR system 9700 (Applied Biosystems, Singapore) for 2 min. Following reagents were added in sequence: 5× reagent buffer (4 μl); dNTP mixture (10 mM each−1 μl); Recombinant RNAase inhibitor (0.5 μl) and MMLV reverse transcriptase (1 μl). Contents were mixed by pipetting up and down and incubated at 42°C for 1 h. The mixture was heated at 94°C for 5 min to stop cDNA synthesis reaction and to destroy RNAase activity. The contents were shortly spun and dilutions were made. cDNA was stored in aliquots at −80°C till further use.

Amplification, cloning, and sequencing. Five microlitres of each cDNA (i.e., of control as well as copper treated samples) was used as a template in a total of 50 μl PCR reaction volume containing 1× Taq buffer with 5 μl $(NH_4)_2SO_4$-$MgCl_2$ (Fermentas), 2 mM $MgCl_2$, 0.2 mM of each dNTPs, and 1 U of Taq DNA polymerase (Fermentas). In order to amplify the two *CuMT* gene isoforms of *T. t. lahorensis*, one degenerate/inosinated forward primer and two different gene isoform specific reverse primers were used, that is, forward primer is the same for amplification of both isoforms while reverse primer (1) was used for the amplification of one isoform and reverse primer (2) for the amplification of second isoform, respectively. The concentration of each of the forward as well as reverse primer used in the PCR reactions was 200 pmol. The sequences of the primers are as follows.

Forward primer 5′-ATGGAYACIYARACIYARACIAA, (Y = C + T; R = A + G and I = Inosine).
Reverse primer (1) 5′-TCAGCATTTGCATTCAGCACA.
Reverse primer (2) 5′-TCATTGCTTTTGGCAACAAGA.

The PCR reaction conditions were the same as described previously in *CuMT* gene isoforms amplification using genomic DNA template. The cDNA amplified PCR fragments were purified from 1.5% agarose gel with DNA extraction kit (Fermentas #K0513) and ligated with pTZ57 R/T cloning vector by using T4 DNA ligase (Fermentas #K1214). White colonies containing the ligated insert were inoculated in LB medium (1% Tryptone, 0.5% Yeast extract and 0.5% NaCl) and plasmid DNA was extracted according to previously mentioned protocol. The cloned products were confirmed by double restriction with *Xba*1 (Fermentas #ER0681) and *Bam*HI (Fermentas #ER0051) restriction endonucleases. QIAprep® Spin Miniprep Kit purified plasmid DNA of the positive clones was sequenced in ABI PRISM 310 Automated DNA Sequencer (Applied Biosystems) using dye terminator and Taq FS in the facilities of School of Biological Sciences, University of the Punjab, Lahore.

SEQUENCE ALIGNMENT AND DATA ANALYSIS

The nucleotide sequences of *T. t. lahorensis CuMT* gene isoforms amplified from genomic as well as cDNA were aligned with other similar and closely related sequences from the NCBI Gene Bank data base. The program BLAST2 on the NCBI web site was used for calculating the identity over the entire length or between portions of the gene isoforms [Tatusova and Madden, 1999]. The amino acid sequences were deduced from their nucleotide sequences. The percentage homology and differences in the nucleotide as well as deduced protein sequences were determined. Protein sequences were aligned using the ClustalW Program [Thompson et al., 1994]. The program 123 Genomics was used for calculating the identity with previously reported *Tetrahymena* MT gene isoforms. The molecular weight, isoelectric points, hydrophobicity [Kyte and Doolittle, 1982], pK_a values of amino acid residues and structure of MT proteins of *T. t. lahorensis* was predicted with the help of computer software Vector NTI advance 10, Invitrogen, SCRATCH [Cheng et al.,

2005], and TRAMPLE [Fariselli et al., 2005] The phylogenetic tree was constructed using MEGA 3.1 [Kumar et al., 2004]. Genetic distances were calculated with the DNADIST program of the PHYLIP package, ver. 3.51c [Felsenstein, 1993] based on the Kimura 2-parameter model [Kimura, 1980]. To construct sequence identity matrix CLUSTALX program [Thompson et al., 1997] was used and this matrix showed percentage sequence similarity among isoforms of *CdMT* and *CuMT* gene sequences of different *Tetrahymena* species. A neighbor-joining phylogenetic tree is based upon alignment of the full length genes. The MTs used in analysis are listed in Table I.

cDNA SYNTHESIS USING RANDOM HEXAMER PRIMER

cDNA of RNA of copper treated and control samples were also synthesized using random hexamer primer (Advantage™ RT-for-PCR Kit, Clonetech Laboratories, Inc.). This served as a control experiment to check the quality and working efficiency of the isolated RNA. To 10 μl of each RNA (i.e., isolated from control and copper treated samples), 3 μl DEPC treated water, 4 μl Buffer (5×), and 1 μl dNTPs (10 mM each) was added. Pre-RT program comprises following steps: 65°C, 1 min; 4°C, 3 min; 25°C, 10 min; 37°C, 2 min; and 37°C, hold. Then 1 μl MMLV (200 U) was added to each reaction and RT program having following steps was run: 37°C, 50 min; 94°C, 5 min; and 4°C, hold. Contents were shortly spun, and cDNA was stored at −80°C till further use.

These cDNAs were used as template to amplify SS rRNA gene of *T. t. lahorensis* according to the PCR conditions explained elsewhere. The amplified product was checked on 1.5% agarose gel and the size was compared with the previously amplified SS rRNA fragment (data reported elsewhere).

ISOLATION AND SDS-PAGE ANALYSIS OF TOTAL PROTEINS

Total proteins were isolated from metal-treated and control *T. t. lahorensis*. In 250 ml conical flasks, Bold-basal salt medium (50 ml) supplemented with 0.1% glucose was inoculated with 10 μl culture of *T. t. lahorensis* containing 40–50 cells in log phase. The control and treated flasks were incubated at 25 ± 2°C for 24 h. In the treated set of flasks, copper at the non-toxic concentration (500 μM; ~32 ppm) was added. The ciliate cultures (4×10^5 cells/ml) were harvested at log phase of growth (24–48 h) by centrifugation at 9,803*g* (Beckman Coulter Allegra™ 25R Centrifuge) at 4°C for 10 min. Pellet (250–300 mg) was washed with 1.5 ml of 10 mM ice cold Tris–Cl (pH 7.5) and suspended in 50 μl of the same buffer. To the suspended pellet 200–500 μl of Triple Detergent lysis buffer [50 mM Tris–Cl (pH 7.5), 1% Triton X-100, 0.5% Na deoxycholate, and 5% SDS] containing 0.01% β-mercaptoethanol was added, mixed vigorously by vortex to obtain homogeneous mixture and heat shocked for 5 min in boiling water-bath. The tubes were immediately transferred to ice bucket, kept for 5 min and centrifuged at 9,803*g* (Beckman Coulter Allegra™ 25R Centrifuge)

TABLE I. Cd and Cu Metallothionein (MT) Gene Isoforms of Different Species of Tetrahymena

No.	Organisms	MT Gene Isoforms	Amino Acids	Authors	Accession Numbers
Cadmium metallothionein (CdMT) genes					
1	*T.* sp. B7	*MT1*	107	Piccinni et al. [2002][b]	AF479587
	T. pigm.			Boldrin et al. [2002]	AF509328
	T. pyri.			Piccinni et al. [1999]	AJ005080
2	*T. pigm.* HG2	*MT1*	118	Guo et al. [2008]	EU420056
3	*T. therm.*	*MTT1*	156	Dondero et al. [2004] [a]	AF537326
4	*T. therm.*	*MTT1*	162	Shang et al. [2002]	AY061892
	T. therm.			Diaz et al. [2007]	AY740525
	T. therm inbred B			Formigari et al. [2005][b]	DQ022186
	T. therm.			Gutierrez et al. [2007][b]	DQ517937
	T. therm SB210			Eisen et al. [2006a][c]	CH445533
5	*T. therm.*	*MTT3*	162	Diaz et al. [2007]	EF195744
6	*T. therm.* SB 1969	*MTT5*	99	Diaz et al. [2007]	DQ517936
	T. therm.			Santovito et al. [2007]	AY884209
	T. therm. SB 210			Eisen et al. [2006b][c]	CH445598
7	*T. therm.*	*Cd MT*	162	Miao et al. [2003][b]	AY273793
8	*T. pyri.*	*GLMT*	181	Fu and Miao [2006]	AY765220
9	*T. trop. lahorensis*	*TMCd1*	156	Shuja and Shakoori [2007]	EF185997
10	*T. rost.*	*MTT1*	113	Amaro et al. [2008]	EU627174
Copper metallothionein (CuMT) genes					
11	*T.* sp. B7	*MT*	96	Piccinni and Santovito [2005][b]	AY043281
12	*T. pigm.*	*MT2*	155	Boldrin et al. [2002]	AF479586
13	*T. pyri.*	*MT2*	96	Santovito et al. [2007]	DQ518910
14	*T. therm.* Cu428.1	*MTT2*	108	Boldrin et al. [2003] [b]	AY350738
	T. therm. Cu428.1			Boldrin et al. [2003]	AY204351
15	*T. rost.*	*MTT2*	78	Amaro et al. [2008]	EU627175
16	*T. therm.* Inbred B	*MTT4*	108	Bakshandeh et al. [2004][b]	AY660008
	T. therm. SB 210			Eisen et al. [2007][c]	CH445730
17	*T. trop. lahorensis*	*TtlCuMT1*	100	This study	FJ664125
18	*T. trop. lahorensis*	*TtlCuMT2*	108	This study	FJ664126

(T. pigm., Tetrahymena pigmentosa; T. pyri, Tetrahymena pyriformis; T. sp., Tetrahymena sp.; T. therm., Tetrahymena thermophila; T. rost., Tertahymena rostrata; T. trop. Lahorensis, Tetrahymena tropicalis lahorensis).
[a]partial cds.
[b]unpublished data.
[c]Genome sequence (hypothetical protein).

for 15 min at 4°C. Supernatants (containing the total protein extract) were immediately transferred to pre-ice cold eppendorf tubes, aliquot and stored at −80°C till further use. The pellets were discarded. As the metal proteins are temperature sensitive and labile, extra care was taken to process the sample as soon as possible, without letting sample stay outside ice. The protein quantification was done according to Lowry et al. [1951].

Fluorescent compound [Iodo-acetamido salicylic acid (IASA)] was used to locate the presence of metallothionein. To 40 μl of the protein extract, 4 μl IASA (1.40 g in 44.67 μl DMSO) was added and incubated at 37°C for an hour. To the mixture 50 μl of gel loading buffer (4% SDS, 30% glycerol and 20 mM β-mercaptoethanol) was added, loaded on polyacrylamide gel and run in 1× Tank buffer (25 mM Tris–Cl, 250 mM glycine and 0.1% SDS; pH 8.3). About 40 μg proteins were fractionated on 18% SDS-PAGE. The fluorescent bands were marked and the gel was stained in the protein staining solution [Coomassie brilliant blue 0.25% in Methanol/Water/Glacial acetic acid (45%:45%:10%)] for 2 h and then kept in destainer (150 ml Methanol + 50 ml acetic acid + 300 ml water) till clear bands appeared on the gel. The presence of copper metallothionein was checked at the marked position in treated culture.

Similarly, to the 40 μl of the protein extract, twice volume of gel loading buffer containing 0.002% bromophenol blue was added, boiled on water bath for 3 min and directly loaded on 18% polyacrylamide gel (SDS-PAGE). After staining and destaining the gel the presence of metallothionein was checked by comparing the proteins of treated culture with those of control culture.

TIME-COURSE OF CuMT EXPRESSION AFTER COPPER STRESS

In order to determine the time-course of CuMT expression, copper stress of non toxic concentration (500 μM) was given to vegetative growing log phase *T. t. lahorensis* culture (3–4 × 10^5 cells/ml). Samples (1.5 ml) were taken in pre-ice cold eppendorf tubes after 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, and 24 h and centrifuged at 9,803*g* (Beckman Coulter Allegra™ 25R Centrifuge) at 4°C for 10 min. Cell pellet was washed with 10 mM ice cold Tris–Cl (pH 8.5) and suspended in 50 μl of the same buffer. The cells were lysed with Triple Detergent lysis buffer [50 mM Tris–Cl (pH 8.5), 1% Triton X-100, 0.5% Na deoxycholate, and 10% SDS] containing 0.01% β-mercaptoethanol, mixed vigorously by vortex to obtain homogeneous mixture and kept on ice at −20°C.

To 30 μl of lysate equal volume of 2× gel loading buffer was added and boiled for 3 min in boiling water-bath. The mixture (40 μl) was loaded on 18% SDS containing polyacrylamide gel and run at 40 V for 35 min and then at 80V for 4–5 h in 1× tank buffer. The gel was stained in Coomassie brilliant blue containing protein staining solution for 2 h and then kept in destainer for 2–3 days till clear bands appeared on the gel.

RESULTS

ISOFORMS OF COPPER METALLOTHIONEIN GENE (*TtlCuMT*)

Approximately 300 bp PCR products of *TtlCuMT* gene isoforms amplified from genomic DNA were cloned in *E. coli* DH5α using T/A cloning vector (pTZ57 R/T). The clones were confirmed by double restriction using *Eco*RI and *Hin*dIII (Fig. 1A) and then sequenced.

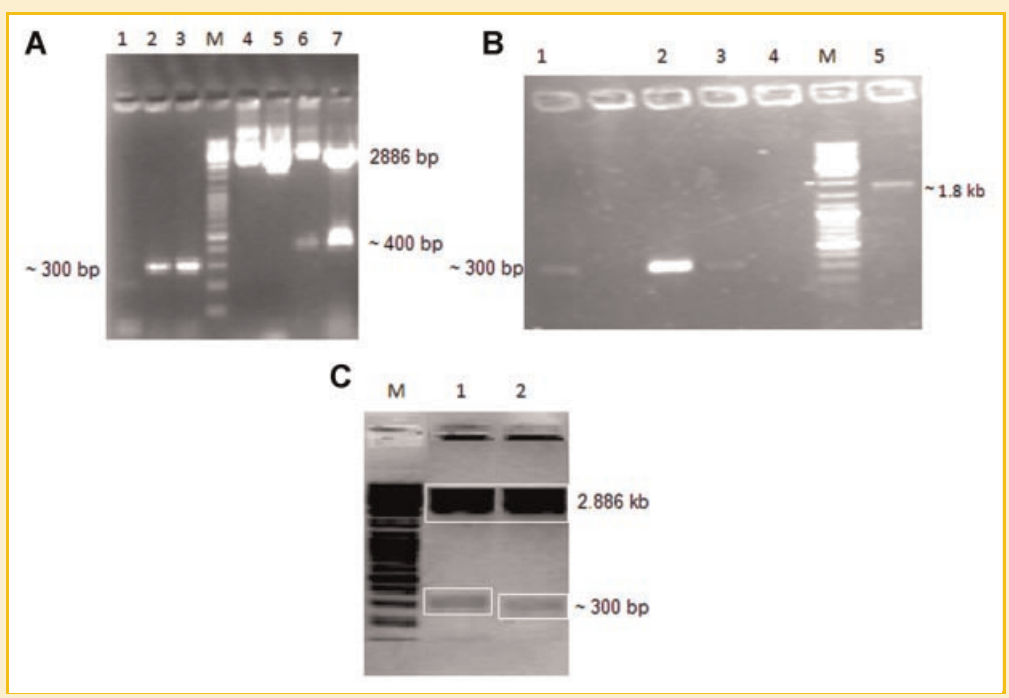

Fig. 1. Analysis of *T. t. lahorensis CuMT* gene isoforms PCR amplified from genomic DNA (A) and cDNA (B). In A: Lane 1, control without template; lane 2, *TtlCuMT*1 gene; lane 3, *TtlCuMT*2 gene; lanes 4 and 5, cloning vectors pTZ57R/T containing *TtlCuMT*1 gene isoform (4), and *TtlCuMT*2 gene isoform (5); lanes 6 and 7, restriction of positive clones of *TtlCuMT*1 gene (6) and *TtlCuMT*2 gene (7) with *Eco*RI and *Hind*III. The upper band in lanes 6 and 7 represent the restricted vector (2.886 kb), whereas the lower band represents insert. M represents marker lane.

Likewise RT-PCR amplification of *TtlCuMT*1 and *TtlCuMT*2 genes gave 300 and 327 bp products, respectively, which were of the same size as the ones amplified from genomic DNA (Fig. 1B). Thus both the gene isoforms were intronless. Moreover, no amplified PCR product was obtained when cDNA was prepared from an un-induced control culture. SS rRNA gene (~1.8 kb), which was amplified from cDNA library prepared by using random hexamer primer, confirmed the quality of RNA prepared from the copper treated culture and served as positive control. The cDNA of *TtlCuMT* gene isoforms were cloned in pTZ57 R/T vector and confirmed with double restriction using *Xba*1 and *Bam*H1 (Fig. 1C). The nucleotide sequencing of RT-PCR cloned fragment was found to be the same as that of the cloned *TtlCuMT* gene isoforms amplified from genomic DNA of *T. t. lahorensis*.

Figure 2 shows nucleotide sequences of the coding region of *TtlCuMT*1 gene (accession no. FJ664125) and *TtlCuMT*2 gene (accession no. FJ664126). As mentioned above, *TtlCuMT*1 consists of 300 nucleotides, while *TtlCuMT*2 has 327 nucleotides, both with ATG as the initiation codon and TGA as the translational termination codon. TAG which is also a stop codon in other eukaryotes codes for glutamine in *TtlCuMT*2 gene which is peculiar to *Tetrahymena*.

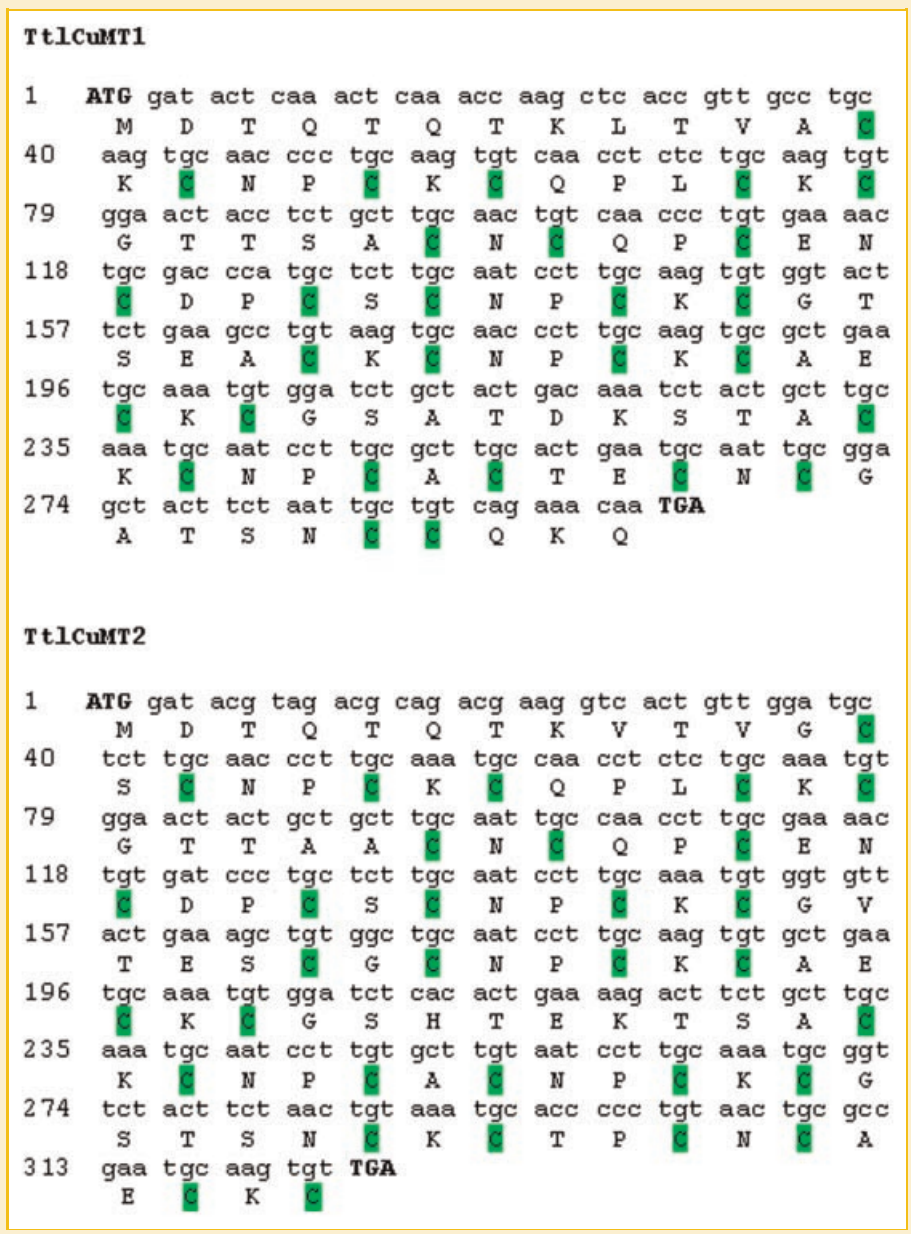

Fig. 2. Nucleotide sequence of *T. t. lahorensis CuMT* gene isoform 1, *TtlCuMT*1 (accession no. FJ664125) and gene isoform 2, *TtlCuMT*2 (accession no. FJ664126). The derived amino acid sequence is given below the nucleotides. The initiation codon ATG and translation termination codon TGA are shown in bold face. Cysteine residues have been highlighted. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DEDUCED MT PROTEINS

The deduced or translated TtlCuMT peptide sequences, TtlCuMT1 (accession no. ACN32472) and TtlCuMT2 (accession no. ACN32473), respectively, contain 100 and 108 amino acid residues including 28 and 32 cysteine residues, which make 28.0% and 29.63% of the total number of amino acids in the two isoforms, respectively (Fig. 3). Both isoforms lack aromatic amino acids, which is the key property of MTs. Moreover, one third of both the deduced proteins comprise sulfur containing amino acid residues, the aliphatic ones being the second major category (Fig. 3). Arg (R) and Ile (I) are absent, while seven negatively charged residues (Asp + Glu) and eleven positively charged residues (Arg + Lys) are present in both the isoforms. The codified proteins have calculated molecular weights of 10,415 and 11,174 Da and theoretical (computed) isoelectric point (pI) is 7.54 and 7.48, respectively.

HYDROPHOBICITY OF TtlCuMT ISOFORMS

Figure 4 shows the hydrophobicity of *T. t. lahorensis* copper MTs. In Kyte and Dolittle Hydropathy plot, hydropathic regions achieve a positive value. For finding putative surface-exposed regions, setting

A

		TtlCuMT1			TtlCuMT2		
Protein Length		100 amino acids			108 amino acids		
Mol. Mass (Da)		10415			11174		
Predicted pI		7.54			7.48		
Amino acid		Count	%mol/mol	%w/w	Count	% mol/mol	% w/w
A	Ala	8	8.00	5.84	6	5.56	4.08
C	Cys	28	28.00	27.78	32	29.63	29.56
D	Asp	3	3.00	3.27	2	1.85	2.03
E	Glu	4	4.00	4.82	5	4.63	5.61
F	Phe	0	0.00	0.00	0	0.00	0.00
G	Gly	4	4.00	2.46	6	5.56	3.44
H	His	0	0.00	0.00	1	0.93	1.18
I	Ile	0	0.00	0.00	0	0.00	0.00
K	Lys	11	11.00	13.17	11	10.19	12.26
L	Leu	2	2.00	2.15	1	0.93	1.00
M	Met	1	1.00	1.22	1	0.93	1.14
N	Asn	8	8.00	8.66	9	8.33	9.07
P	Pro	7	7.00	6.60	9	8.33	7.90
Q	Gln	6	6.00	7.18	4	3.70	4.46
R	Arg	0	0.00	0.00	0	0.00	0.00
S	Ser	6	6.00	5.17	7	6.48	5.61
T	Thr	11	11.00	10.73	11	10.19	9.99
V	Val	1	1.00	0.96	3	2.78	2.68
W	Trp	0	0.00	0.00	0	0.00	0.00
Y	Tyr	0	0.00	0.00	0	0.00	0.00

B

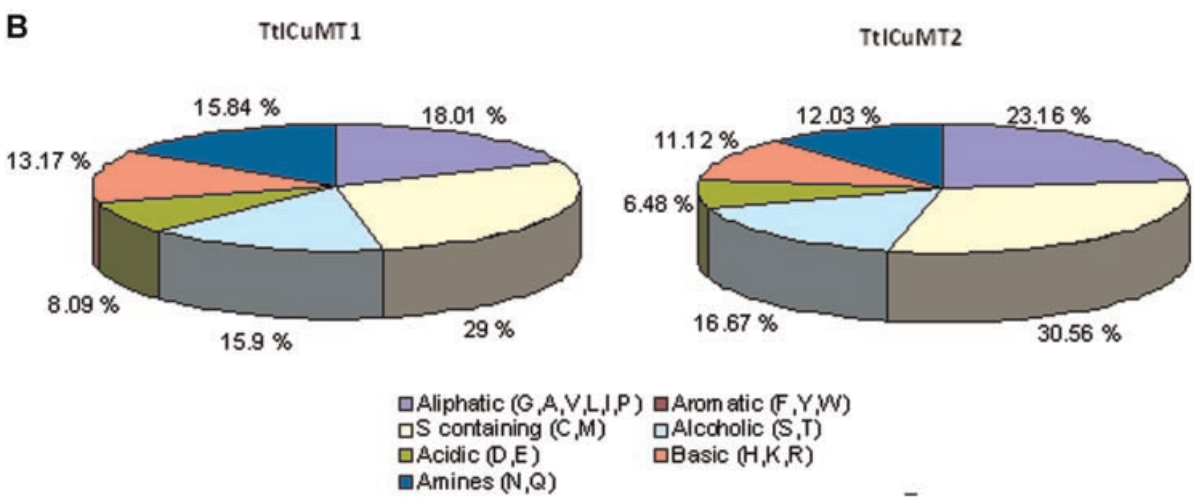

Fig. 3. (A) Amino acid composition (percentage of different amino acid residues) in codified *T. t. lahorensis* CuMT isoforms, TtlCuMT1 and TtlCuMT2. (B) Pie with 3D visual effect to display the contribution of each group of amino acids in TtlCuMT1 and TtlCuMT2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

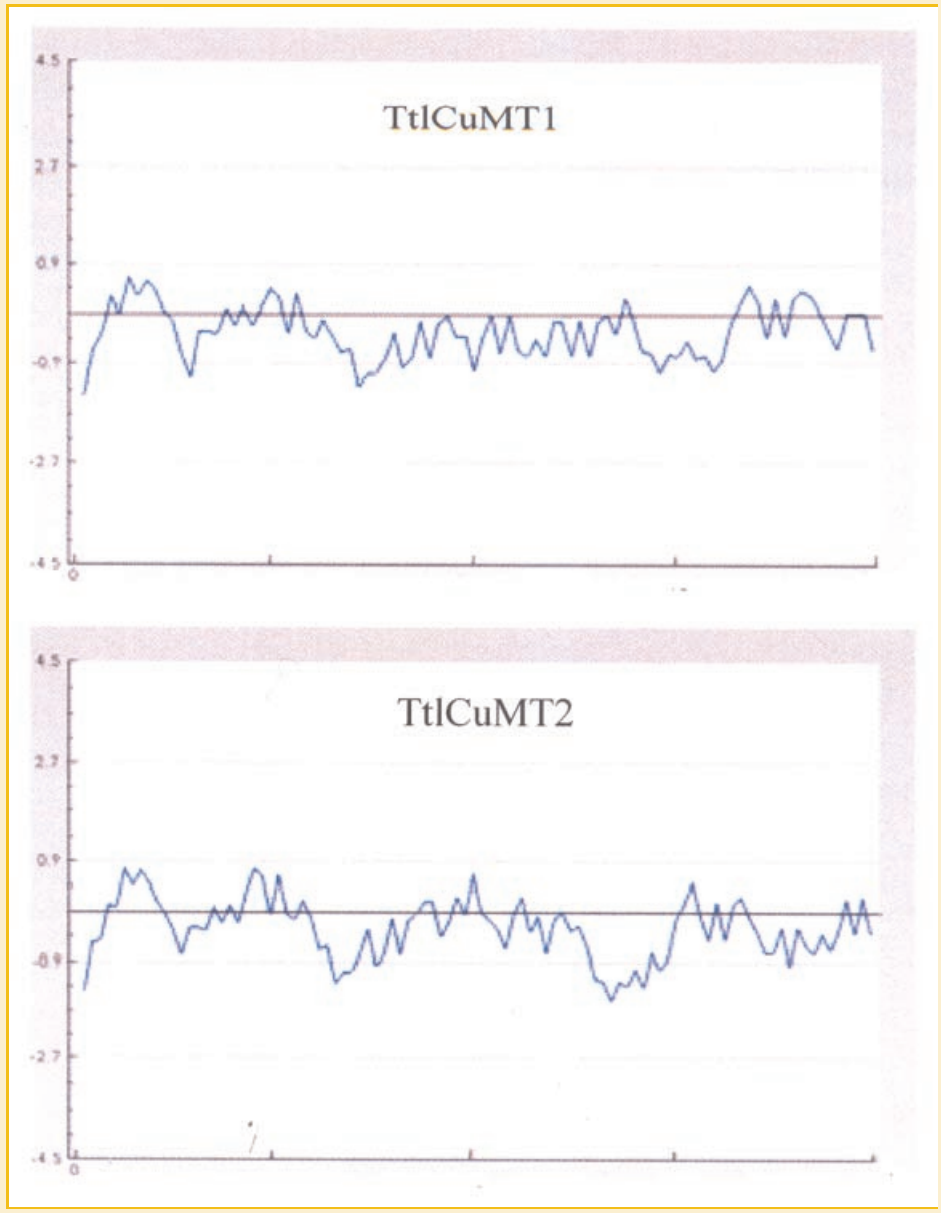

Fig. 4. Graphic display of hydrophobicity (Kyte and Doolittle Hydropathy plot with window size 9) of deduced *T. t. lahorensis* CuMT isoforms, TtlCuMT1 and TtlCuMT2. The *y* axis represents the hydrophobicity scores and the x axis represents the position in the protein sequence. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

window size 5–7 is suggested to be a good value. Strong negative peaks indicate possible surface regions of globular proteins when the window size is 9. Each amino acid was given a hydrophobicity score between −4.5 and 4.5 (score of 4.5 is the most hydrophobic and −4.5, the most hydrophilic). Then a window size is set. A window size is the number of amino acids whose hydrophobicity scores will be averaged and assigned to the middle amino acid in the window.

CONSERVED CYSTEINE MOTIFS IN TtlCuMT ISOFORMS

The deduced amino acid sequences of *T. t. lahorensis* CuMT isoforms (TtlCuMT1 and TtlCuMT2) contained the cysteine residues which are highly conserved and are organized in specific motifs. Both the isoforms have CXCXXCXC, CXC, CXCXXCXXCXXCXXCXC motifs once in the deduced amino acid sequences while CCX motif was present only in isoform 1 (X is any other amino acid). The amino acid sequences of TtlCuMT1 and TtlCuMT2 have special features of two and three CXCXXCXCXXCXC intragenic tandem repeats with a conserved structural pattern of cysteine, respectively (Fig. 5).

STRUCTURE OF TtlCuMT ISOFORMS

The predicted secondary structure of the deduced amino acid sequences indicate the absence of α helices or β sheets in both the *T. t. lahorensis* CuMT isoforms. Instead, the amino acids tend to form turns and extended coils. Figure 6 shows the predicted amino acid

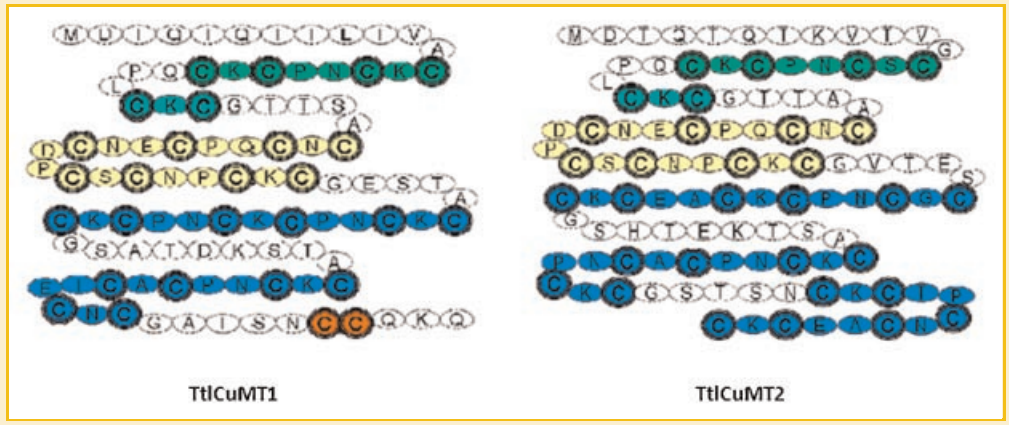

Fig. 5. Model of distribution of cysteine residues in codified *T. t. lahorensis* CuMT isoforms, TtlCuMT1 and TtlCuMT2. The conserved cysteine residues are organized in the form of recurring structural motifs that are shown in colors (green, Motifs 1, 2; yellow, motif 3; blue, motif 4; red, motif 5). Motifs in blue indicate intragenic tandem repeats.

domains in TtlCuMT1 (Domain 1: 1–47 and Domain 2: 48–100) and TtlCuMT2 (Domain 1: 1–55 and Domain 2: 56–108) in the tertiary structure (Fig. 6). The position of cysteine containing recurring structural motifs has been shown in the predicted tertiary structure (Fig. 7).

DISULFIDE BONDS

As the deduced *T. t. lahorensis* copper MT isoforms are cysteine-rich, the formation of disulfide bonds can be predicted. In TtlCuMT1, 28 cysteines form 14 bonds. The following positions are predicted to form the disulfide bond: 13, 15, 18, 20, 24, 26, 32, 34, 37, 40, 43, 45, 48, 50, 56, 58, 61, 63, 66, 68, 78, 80, 83, 85, 88, 90, 96, and 97. The total number of cysteines in TtlCuMT2 is 30, which are predicted to form 15 disulfide bonds at the following positions: 13, 15, 18, 20, 24, 26, 32, 34, 37, 40, 43, 45, 48, 50, 56, 58, 61, 63, 66, 68, 78, 83, 85, 88, 96, 98, 101, 103, 106, and 108. Table II shows the predicted cysteine pairs.

PHYLOGENETIC RELATIONSHIPS WITH CuMTs

BLAST result indicate that *T. t. lahorensis* CuMT gene isoforms 1, *TtlCuMT*1, has 95% resemblance with *Tetrahymena pyriformis* copper-inducible metallothionein gene MT-2 (DQ518910), *Tetrahymena* sp. B7 copper-induced metallothionein gene (AY043281) and *Tetrahymena pigmentosa* copper-induced metallothionein gene (AF2587795) while, with *Tetrahymena pigmentosa* metallothionein gene MT-2 (AF479586), it is 94% identical.

Similarly, BLAST result of *T. t. lahorensis* CuMT gene isoforms 2, *TtlCuMT*2, shows very high level of resemblance (97–95%) with following previously reported Cu metallothionein genes from different species of *Tetrahymena*: *Tetrahymena thermophila* strain Inbred B copper-inducible metallothionein (MTT4) gene (AY660008, 97%), *Tetrahymena thermophila* strain CU428.1 copper-induced metallothionein (MTT2) gene (AY204351, 95%) and *Tetrahymena thermophila* strain CU428.1 metallothionein (MTT2) gene (AY350738, 95%).

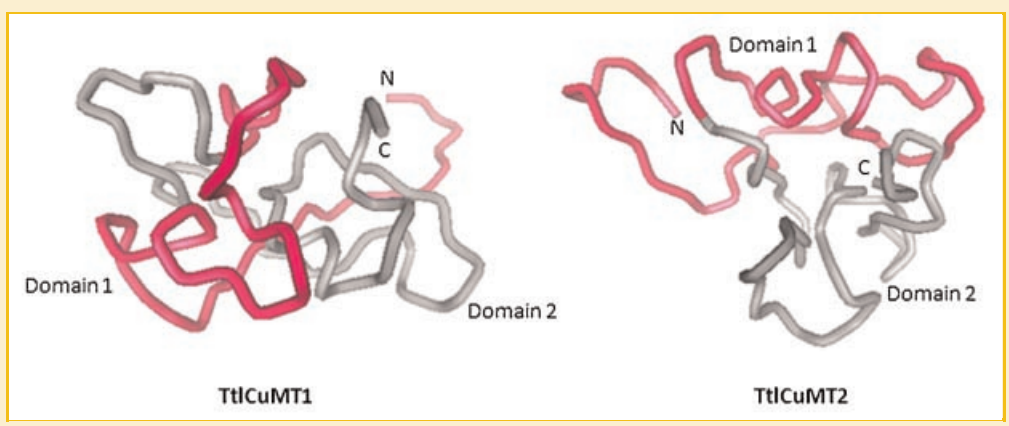

Fig. 6. Tertiary structure of *T. t. lahorensis* CuMT isoforms, TtlCuMT1 and TtlCuMT2 showing two domains in separate colors (Domain 1, red and Domain 2, gray). C and N terminals of the deduced protein are also indicated.

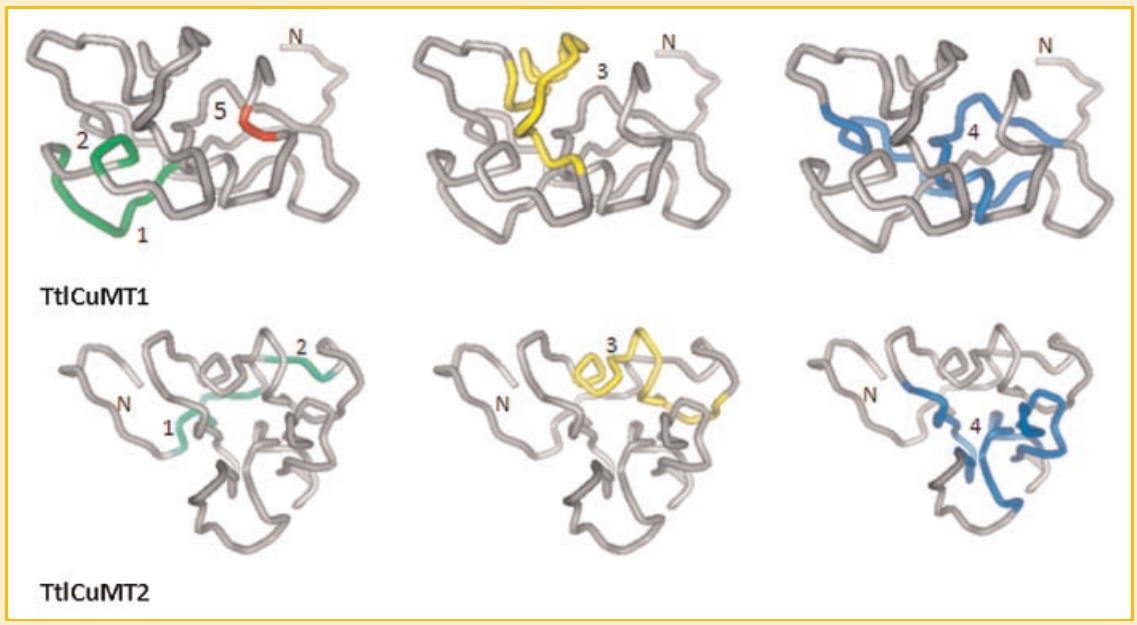

Fig. 7. Position of different motifs in the ribbon model tertiary structure of *T. t. lahorensis* CuMT isoforms. The motifs are indicated by numerals. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 8 shows the alignment of TtlCuMT1 and TtlCuMT2 with all the previously reported CuMT of *Tetrahymena* spp. (Table III). It is clearly visible that the first domain of all the CuMT has high similarity, whereas the differences were present in the second domain. The conserved CXC motif, which is the hallmark of CuMTs, was also indicated in the figure by blocks. The color key represents different classes of amino acids in these MTs.

SDS-PAGE ANALYSIS OF TOTAL PROTEINS

Figure 9 shows the SDS-PAGE pattern of total proteins of copper tolerant *Tetrahymena tropicalis lahorensis* in the absence (control) and presence (treated) of 500 μM copper. Fluorescent compound, IASA, was used to locate the position of cysteine rich proteins (metallothioneins, etc.) containing regions. It was noted that both the treated and untreated culture had fluorescent regions. After marking those regions on the gel, staining with Coomassie blue also indicated the appearance of low molecular weight (~10–11 kDa) protein band at the marked positions (Fig. 9A).

When total proteins of untreated and copper treated *T. tropicalis* were resolved on 18% SDS-PAGE, the bands were seen in the same pattern and on same size (Fig. 9B) as in the gel on which fluorescent probe treated samples were resolved. The bands in the fluorescent regions (low molecular weight) in both the gels were, however, more robustly expressed under copper stress.

TABLE II. Predicted Disulfide Bonds (Cysteine Pairs) Ordered by Probability in Descending Order in *T. t. lahorensis* CuMT Isoforms

	TtlCuMT1		TtlCuMT2	
Bond index	Cys 1 position	Cys 2 position	Cys 1 position	Cys 2 position
1	15	24	85	96
2	63	78	63	78
3	85	96	15	24
4	45	56	50	66
5	50	66	68	83
6	40	48	98	108
7	20	37	20	32
8	18	34	40	56
9	68	83	48	58
10	88	97	18	34
11	26	43	103	106
12	61	80	45	61
13	32	58	88	101
14	13	90	26	43
15	–	–	13	37

TIME-COURSE STUDY OF COPPER INDUCED MT EXPRESSION

The expression of copper-induced MT was determined after giving copper stress of non-toxic concentration (500 μM) to *T. t. lahorensis*. Total proteins were isolated after 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, and 24 h. Equal quantity of proteins was loaded and resolved on 18% SDS containing polyacrylamide gel (Fig. 10). A gradual increase in the MT expression with time was recorded which expressed maximally 1 h of copper stress. After 24 h, the expression was almost comparable to the un-induced culture. The expression at 30 min, 1 and 1.5 h of copper stress was 10-fold higher as compared with that of un-induced culture or after 15 min of copper stress.

Some proteins of 35 kDa were also induced in copper treated samples of *T. t. lahorensis* and their expression also became high up to 1 h, and then gradually decreased with time. The 14 kDa protein in Cu-induced *T. t. lahorensis* is closely related to the calculated molecular weight of *TtlCuMT* genes.

DISCUSSION

MOLECULAR CHARACTERIZATION OF TtlCuMT ISOFORMS

This study describes the cloning, sequencing and molecular characterization of novel copper inducible MT gene isoforms

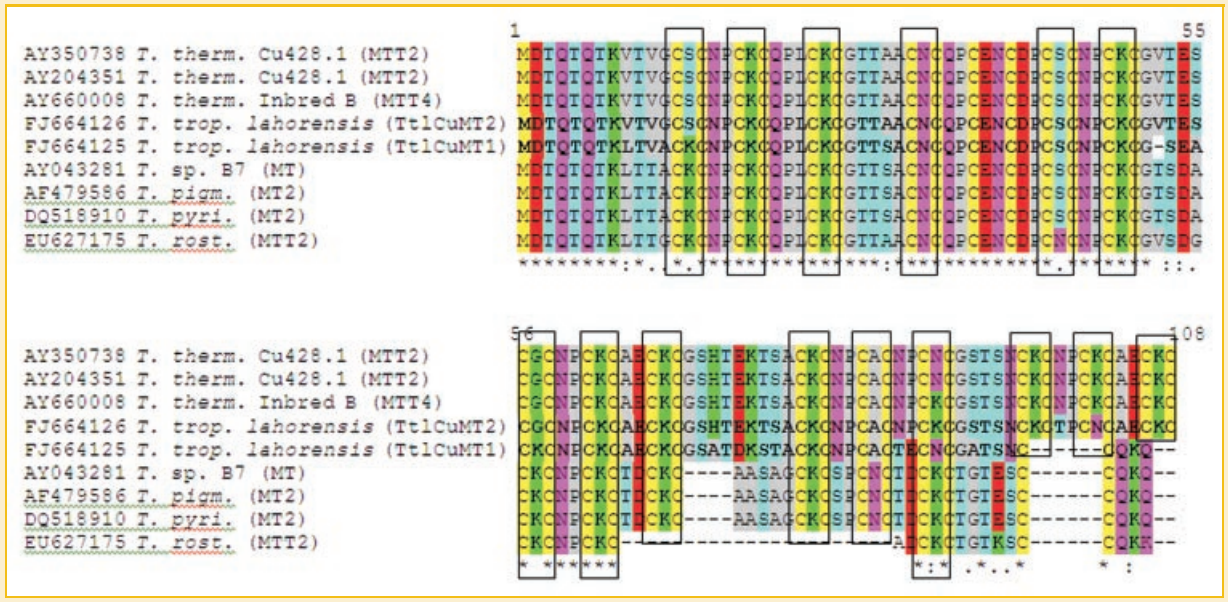

Fig. 8. Comparison of translated *T. t. lahorensis* CuMT gene isoforms, *TtlCuMT*1 and *TtlCuMT*2 with previously reported CuMT genes by Clustal W multiple sequence alignment program. The *TtlCuMT*1 and *TtlCuMT*2 sequences are in bold format. The conserved motif CXC is shown in blocks. The amino acids are in color according to the nature of their R-group while, S-containing amino acids are colored separately (sulfur containing, acidic, amines, aliphatic, alcoholic, and basic). Clustal consensus [(*) identical, (:) strongly similar, and (.) weakly similar] have also been shown.

(*TtlCuMT*1 and *TtlCuMT*2) from *T. t. lahorensis*. TMCd1 (ABM 74559), Cd-metallothionein, was previously reported from *T. t. lahorensis* [Shuja and Shakoori, 2007] whose homology of amino acid sequence with other Cd inducible MTs of *Tetrahymena* spp. varied from 78% to 45%. TtlCuMT1 and TtlCuMT2 are the small *Tetrahymena* MTs (100 and 108 amino acids, respectively), but their cysteine residues percentage (28.0% and 29.63%, respectively) is not the lowest among *Tetrahymena* MTs. Cysteine percentage of TtlCuMT1 is similar to one group of CuMTs (*T.* sp. B7 MT1; *T. pigm.* MT2; *T. pyri.* MT2) and that of TtlCuMT2 to the other group (*T. therm.* MTT2 and *T. therm.* MTT4). The cysteine percentage of TtlCuMT1 is lower than that of *T. pyri.* MT1 and *T. rost.* MTT2 (28.9% and 28.2%, respectively), while cysteine percentage of both isoforms is higher than that of *T. therm.* MTT5 and *T. therm.* MTT3 (24.2 and 25.9, respectively).

TABLE III. Distribution of Cysteine Clusters Among *Tetrahymena* CuMTs (Subfamily 7b)

	Cluster type			Total cysteine residues
	CCX	CXC	XXCXX[a]	
T. t. lahorensis FJ664125 (TtlCuMt1)	1	12	2	28
T. t. lahorensis FJ664126 (TtlCuMt2)	0	15	2	32
T. therm. AY350738 (MTT2)	0	15	2	32
T. therm. AY204351 (MTT2)	0	15	2	32
T. therm. AY660008 (MTT4)	0	15	2	32
T. rost. EU627175 (MTT2)	1	9	2	22
T. sp. AY043281 (MT)	1	12	2	28
T. pigm. AF479586 (MT2)	1	12	2	28
T. pyri. DQ518110 (MT2)	1	12	2	28
All (Total C)	10	234	18	262
All (%C)	3.82	89.31	6.87	

Numbers in "total" and "%" rows or columns refer to cysteine residues counts. The other numbers refer to cysteine cluster counts.
[a]Un-clustered cysteine.

Analysis of coding region of *TtlCuMT1* and *TtlCuMT2* show that the proteins contain methionine and aspartic acid at the N-terminus just as has been reported in all *Tetrahymena* MTs [Piccinni et al., 1999; Boldrin et al., 2002; Shang et al., 2002; Fu and Miao, 2006; Diaz et al., 2007; Santovito et al., 2007; Shuja and Shakoori, 2007; Amaro et al., 2008; Guo et al., 2008] except MTT3 [EF195744; Diaz et al., 2007] in which methionine is followed by glutamic acid (E). TGA is the translational termination codon. TGA, apparently, is the only stop codon used in *Tetrahymena* [Harper and Jahn, 1989] and some other ciliates such as *Stylonychia*, *Oxytrichia*, and *Paramecium*, whereas in *Euplotes crassus* TAA is the translation termination codon [Harper and Jahn, 1989]. Thus it has been generally assumed that all the ciliates use non-universal genetic code in which TGA (rarely TAA) acts as sole termination codon. The universal stop codon TAG of eukaryotes encodes glutamine here in *T. t. lahorensis* and is at codon 4 of *TtlCuMT2* gene. It is reported to code for cysteine in pheromone 3 of *Euplotes octocarinatus* [Meyer et al., 1991] and *Euplotes adiculatus* [Kervestin et al., 2001]. Keeping in view all the results reported for other ciliates, Meyer et al. [1991] suggested that the role of the classic termination codons had not yet been established when the ciliates started to diverge from other eukaryotes.

The predicted secondary structure of both the *T. t. lahorensis* CuMT isoforms lacks α helices and β sheets, while the predicted tertiary structure indicates two domains. Domain I and initial portion of domain II (up to 68 amino acid residues) show very high homology (>98%) in all the *Tetrahymena* CuMTs reported to date. The differences are present in the remaining portion of the domain

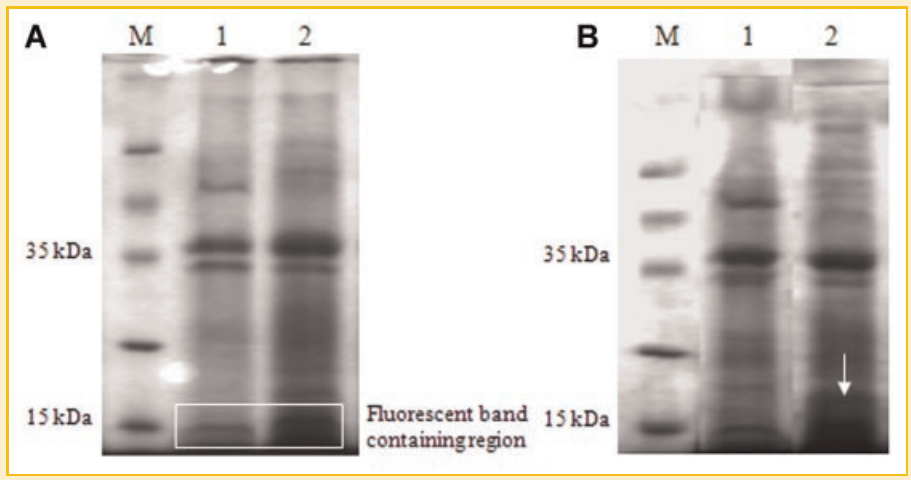

Fig. 9. SDS-PAGE (18%) profile of *T. t. lahorensis* proteins using fluorescent probe IASA (A), and Coomassie brilliant blue staining (B). Lane 1, untreated control; lane 2, copper treated. M represents protein molecular weight marker.

II, on the basis of which the subfamily 7b can be divided into two groups (Fig. 8). *T.* sp. B7 MT1, *T. pigm.* MT2, *T. pyri.* MT2, and TtlCuMT1 form one group comprising of >100 amino acids and *T. therm.* MTT2 and *T. therm.* MTT4, *T. rost.* MTT2 and TtlCuMT2 form the other group containing <100 amino acids. This is the novel finding of the present study as no such report on this type of classification exists at the moment.

COMPARATIVE STRUCTURAL ANALYSIS

Based on their structural pattern, *Tetrahymena* MT genes possess some typical and peculiar characteristics. Like other *Tetrahymena* MTs [Piccinni et al., 1999; Santovito et al., 2001; Shang et al., 2002; Boldrin et al., 2003, 2008; Fu and Miao, 2006; Shuja and Shakoori, 2007] both the gene isoforms, *TtlCuMT1* and *TtlCuMT2*, too are intronless, which is confirmed by the comparative analysis of the coding regions of the full-length cDNA and gene sequences.

A general feature of classic MTs is that they tend to lack aromatic amino acids and/or histidine [Coyle et al., 2002], but as it has been previously reported both *T. thermophila* Cd and CuMTs [Diaz et al., 2007] and *T. t. lahorensis* TMCD1 [Shuja and Shakoori, 2007] present some aromatic amino acid and/or histidine. This atypical feature is also present in some MTs from other organisms, for example, cyanobacteria MTs [Olafson et al., 1988] and several plant type 1 MTs [Cobbett and Goldsbrough, 2002]. As it occurs in the majority of classic MTs, in both TtlCuMT1 and TtlCuMT2 aromatic amino acids are absent in both molecules, whereas, one histidine is present in TtlCuMT2.

Another general feature of MTs is the extreme asymmetry in the ratio of the positively charged amino acids viz., lysine (K) and arginine (R). According to Amaro et al. [2008], among all seven, at present reported *Tetrahymena* CdMTs (subfamily 7a), the K/R ratio value is 124/1 (the only arginine residue is in MTT3), likewise, among all four *Tetrahymena* CuMTs (subfamily 7b) the K/R ratio is 45:0. In both, TtlCuMT1 and TtlCuMT2, arginine is absent and lysine is the only preferred positively charged amino acid (11 K in each of the two isoforms; Fig. 3). Thus, the preference to use exclusively or mainly lysine instead arginine seems to be a general feature among MTs, excepting in some rare cases [Amaro et al., 2008] such as ZnMT class I of *Zea mays* (P43401) or some prokaryotic putative MTs (*Nitrosococcus oceani* YP344861 or *Pseudomonas entomophila* YP608349).

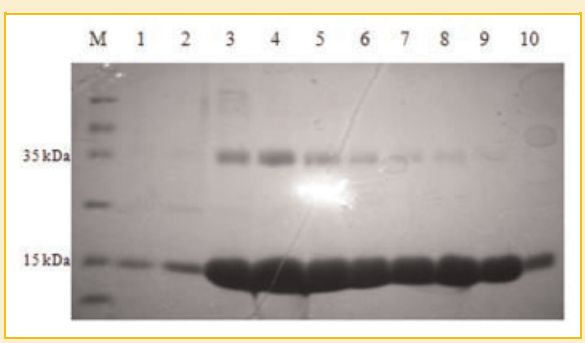

Fig. 10. Time-course SDS-PAGE (18%) analysis of *T. t. lahorensis* treated with 500 μM copper for different periods of time. Lane 1, 0 h; lane 2, 15 min; lane 3, 30 min; lane 4, 1 h; lane 5, 1.5 h; lane 6, 2 h; lane 7, 2.5 h; lane 8, 3 h; lane 9, 4 h; lane 10, 24 h. M represents protein molecular weight marker.

PHYLOGENETIC ANALYSIS

It has been previously reported [Diaz et al., 2007] that alignment of all *Tetrahymena* CdMTs (subfamily 7a) revealed remarkably regular and hierarchical modular organization. These modules appear to have evolved through the combinatorial accumulation of repeat units of increasing length and complexity, built from two elementary types of motifs; CKCXXCKCCK (consensus) and C2X6 [Amaro et al., 2008]. With only minor modifications, these motifs have been successively combined into sub-modules, modules, and finally MTs [Diaz et al., 2007]. TtlCuMT1 and TtlCuMT2 were identified as *Tetrahymena* CuMT isoforms, including them into

subfamily 7b [Diaz et al., 2007], based on their similarity to other *Tetrahymena* CuMT (Fig. 11), their modular structure or cysteine clustering and their gene expression pattern under copper exposure. Both have the usual length of *Tetrahymena* CuMT isoforms and high level of internal similarity as well. They differ from other animal MTs in their gene organization, a characteristic which is in accordance with the other ciliate MTs. *Tetrahymena* CuMTs (subfamily 7b) do not present a clear modular structure like CdMTs, but from their alignment (Fig. 8), a structural organization based on CKCX2-5CKC repeats (where K may be substituted in minority by other amino acid viz., G, N, S) can be derived. It shows that an evolutionary history based on intra-gene duplications might be possible in these *Tetrahymena* MTs also [Amaro et al., 2008].

The phylogenetic tree (Fig. 11), after addition of the two new CuMT isoforms of *T. t. lahorensis*, corroborates the previous analysis [Diaz et al., 2007; Amaro et al., 2008; Gutierrez et al., 2009], which divides the *Tetrahymena* MT gene family 7 [Binz and Kagi, 1999; http://www.expasy.ch/cgi-bin/lists?metallo.txt] into two subfamilies—7a for CdMTs and 7b for CuMTs.

The ciliate MTs have been classified as family 7 under the classification system of the MT superfamily, and it has been divided into two subfamilies. The subfamily 7a is composed of eight CdMTs and superfamily 7b is constituted, until this finding, by four CuMTs. Both subfamilies differ in the induction patterns by heavy metals (Cd/Zn or Cu) and the pattern of Cys residue clustering [Boldrin et al., 2002; Shang et al., 2002; Fu and Miao, 2006; Diaz et al., 2007; Amaro et al., 2008; Guo et al., 2008].

The CCX, CXC clusters (any group of contiguous residues in which cysteines are grouped with or are separated from one another by at most one non-cysteine residue; every cysteine belongs to a single cluster) and un-clustered cysteine residues are the common cysteine distribution in classic MTs, and likewise the only cysteine

Fig. 11. Phylogenetic tree of all, at present, *Tetrahymena* spp. metallothioneins. Two well separated subfamilies (7a or CdMTs and 7b or CuMTs) are distinguished. Moreover, two separate groups in subfamily 7b are also indicated. The numbers at the nodes represent the bootstrap percentages of 1,000. Bar = 0.1 expected changes per site. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

organization in *Tetrahymena* CuMTs or subfamily 7b MTs [Amaro et al., 2008]. In contrast to subfamily 7a CdMTs, CCC clusters are completely missing and CCX are very rare in subfamily 7b CuMTs. TtlCuMT1 has twelve CXC and one CCX cluster along with two un-clustered cysteines, whereas fifteen CXC were the only clusters found in TtlCuMT2 with two un-clustered cysteins (Table III).

Another genetic feature that separates both the superfamilies and probably has the phylogenetic implications is the codon usage for glutamine residues. Ciliates use particular genetic code, the two codons TAA and TAG, which are universal stop codons in nuclear genes, code Gln, and TGA is the only stop codon used by these eukaryotic microorganisms [review by Gutierrez et al., 2009]. Both the isoforms of CuMT of *T.t. lahorensis* use TGA as the translation termination codon. Moreover, TAG in codes for Gln *TtlCuMT2* isoform, although TAA and TAG have been reported to be preferentially used as codons for CdMTs.This asymmetry in the codon usage for Gln residues again corroborate that *TtlCuMT1* and *TtlCuMT2* isoforms have evolved divergently from other *Tetrahymena* species.

Contrary to the views of Gutierrez et al. [2009] we believe that Cd MTs are more ancient proteins compared with CuMTs, and of the two isoforms TtlCuMT1 evolved earlier than the other isoform TtlCuMT2.

PROTEIN ANALYSIS

In order to check the effect of copper on the protein expression, a comparison was carried out among control and copper treated *T. t. lahorensis* through polyacrylamide gel electrophoresis. In treated culture certain low molecular weight proteins (≈10–15 kDa) were induced under copper stress which appeared in the SDS-PAGE profile. Presence of such low molecular weight, metallothionein like proteins in *T. t. lahorensis* under copper stress indicates that this ciliate is adapted to produce such proteins during high copper stress conditions.

Widely defined MTs include all of the metal-thiolate polypeptides which have low molecular weight, cysteine richness, metals binding ability and/or resemble equine renal MT. These MTs play important role in the regulation of essential metals and detoxification of the unusual entry of the essential as well as non-essential metals [Rigby et al., 2006].

The presence of metallothionein was checked on SDS-PAGE using fluorescent probe as well as Coomassie brilliant blue staining. The isolation of metallothionein in the absence (control) and presence of copper stress (treated) was investigated using the recently developed method that combined fluorescent tagging of thiol-rich peptides by iodo-acetamido salicylic acid (IASA) with SDS-PAGE. The alkalyting properties of IASA are comparable to that of bromobimane which is classically used as a fluorescent probe to detect sulfur rich proteins and alkylates thiol groups replacing the H with a fluorescent tag ($\lambda_{emission} = 478$ nm). This method allowed a fast and simultaneous assay of both phytochelatins and metallothionein-like proteins [Paul et al., 1986].

The bands of untreated and copper treated *T. tropicalis* total proteins, resolved on 18% SDS-PAGE, were seen in the same pattern and on same size as in the gel on which fluorescent probe treated samples were resolved. This result confirmed the basal expression of MTs [Bonneton et al., 1996]. However, the bands in the fluorescent regions (low molecular weight) in both the gels were more robustly expressed under copper stress. A gradual increase in the MT expression with time was recorded which expressed maximally (10-fold higher as compared with that of un-induced culture) 1 h of copper stress.

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