Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part C 147 (2008) 232–240
CBP
www.elsevier.com/locate/cbpc

MTT2, a copper-inducible metallothionein gene from *Tetrahymena thermophila*

Francesco Boldrin [a,*], Gianfranco Santovito [a], Alessia Formigari [a], Yelena Bisharyan [b], Donna Cassidy-Hanley [b], Theodore G. Clark [b], Ester Piccinni [a]

[a] *Department of Biology, University of Padova, 35100 Padova, Italy*
[b] *Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*

Received 2 August 2007; received in revised form 11 October 2007; accepted 11 October 2007
Available online 25 October 2007

Abstract

Metallothioneins (MTs) are ubiquitous, cysteine-rich, metal-binding proteins whose transcriptional activation is induced by a variety of stimuli, in particular heavy metals such as cadmium, copper and zinc. Here we describe the sequence and organization of a novel copper-inducible metallothionein gene (*MTT2*) from *Tetrahymena thermophila*. Based on its deduced sequence, the gene encodes a protein 108 amino acids, containing 29 cysteine residues (30%) arranged in motifs characteristic of vertebrate and invertebrate MTs. We demonstrate that the 5′-region of the *MTT2* gene can act as an efficient promoter to drive the expression of heterologous genes in the *Tetrahymena* system. In the latter case, a gene for a candidate vaccine antigen against *Ichthyophthirius multifiliis*, a ubiquitous parasite of freshwater fish, was expressed at high levels in transformed *T. thermophila* cell lines. Moreover, the protein was properly folded and targeted to the plasma membrane in its correct three-dimensional conformation. This new copper-inducible MT promoter may be an attractive alternative to the cadmium-inducible *MTT1* promoter for driving ectopic gene expression in *Tetrahymena* and could have a great impact on biotechnological perspectives.

Keywords: Heavy metals; Metallothioneins; Recombinant protein expression; *Tetrahymena thermophila*

1. Introduction

Metal ions play critical roles in a variety of metabolic pathways across a wide range of phyla. Nevertheless, even essential metals such as copper can have toxic effects when present at elevated levels. As a result, dedicated mechanisms for regulating uptake, distribution, and detoxification of copper and other heavy metals have evolved (Peña et al., 1998). Detoxification pathways rely heavily on metallothioneins (MTs), a group of low molecular weight proteins that can bind group Ib and IIb transition metals, forming tetra-metal-thiolate clusters with their numerous cysteine residues. One of the major properties of MTs is their ability to be transcriptionally induced by heavy metals and other physiological stressors (Kägi, 1993).

MTs are widely distributed in nature (Kojima et al., 1999) and have a wide range of possible functions associated with metal absorption/excretion, metal homeostasis and metabolism, free radical scavenging, metal detoxification, apoptosis, and modulation of the intracellular redox balance. Generally, multiple isoforms encoded by separate genes are present in most organisms, and different cells express distinct MT isoforms with varying levels of expression and inducibility (Miles et al., 2000). The existence of multiple isoforms within the same organism would clearly suggest that these proteins have specialized functions depending on the circumstances and cell types in which they are expressed.

In the ciliates, MT genes have been actively studied in *Tetrahymena*. Two tetrahymenid species, namely, *T. pyriformis* and *T. pigmentosa* have been shown to contain genes for MT-1 and MT-2 MT isoforms that are differentially expressed in response to cadmium (Cd) and copper (Cu), respectively (Santovito et al., 2001; Boldrin et al., 2002; Boldrin et al., 2003). More recently, an orthologous gene for the cadmium-inducible isoform (*MTT1*) was

* Corresponding author. Department of Biology, Via Ugo Bassi 58/B, 35100 Padova, Italy. Tel.: +39 049 827 6310, fax: +39 049 827 6300.
E-mail address: francesco.boldrin@unipd.it (F. Boldrin).

1532-0456/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2007.10.002

identified in a third species, namely, *Tetrahymena thermophila* (Shang et al., 2002). The presence of *MTT1*, and the existence of additional metallothionein isoforms in other tetrahymenid species suggested that a copper-inducible metallothionein gene might be present in *T. thermophila* as well, and a preliminary report on the existence of such a gene (designated, *MTT2*) has recently appeared (Boldrin et al., 2006).

Here we describe the *MTT2* gene and corresponding transcript in detail, including its isolation, nucleotide and deduced amino acid structures, and response to copper. Sequence comparisons clearly indicate that this gene is orthologous with the copper-inducible *MT-2* genes of other tetrahymenid species, and is nearly identical in its coding region with *MTT4* (GenBank accession no. AY660008), yet another metallothionein gene recently identified within the *T. thermophila* macronuclear genome (Santovito et al., 2007; Boldrin et al., unpublished).

The scientific community has recently taken into great consideration the use of *T. thermophila* for the production of recombinant proteins for both biotechnological perspectives and as research tools, since no system (e.g., bacteria, fungi, insect and mammalian cell lines) is universally suitable for the expression of foreign genes (Gaertig et al., 1999). *T. thermophila* has been used successfully to express membrane proteins from parasitic protozoa (including Apicomplexa) and may be of general use for the expression of genes from organisms with AT-rich genomes since these tend to be unstable in conventional systems (Gaertig et al., 1999; Peterson et al., 2002). Moreover, *T. thermophila* has been proposed as experimental model system for functional and structural characterization of proteins and production of vaccines (Turkewitz et al., 2002). In this paper we evaluate the upstream promoter region of *MTT2* for its ability to drive foreign gene expression in *T. thermophila* using the gene for a candidate vaccine antigen from *Ichthyophthirius multifiliis* as a model for heterologous protein expression.

2. Materials and methods

2.1. Strains and culture conditions

T. thermophila wild-type strain CU428.1 and paclitaxel-sensitive strain CU522 were grown in Neff medium (0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, and 0.033 mM $FeCl_3$) with shaking at 30 °C. All *Tetrahymena* cultures were supplemented with 100 U penicillin/mL, 100 μg streptomycin/mL and 0.25 μg Fungizone/mL. *T. thermophila* CU522 was used as a transformation host; this line carries a Lys350Met substitution in the gene beta-tubulin 1 (*BTU1*) that confers sensitivity to growth in the microtubule-stabilizing agent paclitaxel (Gaertig et al., 1994), which was utilized as a selective agent. Cultures at mid-log phase (5×10^5 cell/mL) were treated with either 500 μM Cu (as $CuSO_4$) or 11 μM Cd (as $CdCl_2$), unless otherwise indicated.

2.1.1. Quantification of copper and MT in cell-free extracts

Cells were harvested by centrifugation at 2000×*g* for 15 min at the indicated times, then homogenized as previously described (Piccinni et al., 1990). Homogenates were centrifuged at 48,000×*g* for 50 min at 4 °C, and the resulting supernatants were used for metal and MT quantifications. Cu analyses were performed using an atomic absorption spectrophotometer (Perkin-Elmer mod. 4000). MT concentrations were determined by the silver saturation method (Scheuhammer and Cherian, 1991). Total protein concentrations were assayed by the Folin phenol reagent method (Lowry et al., 1951). All results are reported as means±standard deviation (SD). Each experiment was performed at least 3 times. Linear regression was carried out to estimate correlations between MT and metal concentrations.

2.2. Genomic DNA extraction and MTT2 gene cloning

Extraction of whole-cell DNA was carried out as described by Gaertig et al. (1994).

Based on the amino acid sequence of the *T. pigmentosa* MT-2 (Santovito et al., 2001), two degenerate/inosinated oligonucleotides were designed in order to PCR amplify a 0.3 kb product encoding a fragment of *MTT2* coding sequence. The oligonucleotide sequences were: MTT2FW (5′-ATGGAYACIYARACIYARACIAA-3′); MTT2RE (5′-TCIGCRCAKTTRCAIGGIKWRCAYTT-3′). PCR reactions (50 μL) contained 1 μM of each primer, 400 ng genomic DNA, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1× PCR buffer (Promega) and 2.5 U Taq DNA polymerase (Promega). Reactions were performed in a Progene thermocycler (Techne) using the following protocol: 95 °C for 5 min, then 95 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min for 30 cycles and 72 °C for 10 min.

2.3. RACE techniques for generating full-length cDNA MTT2 sequence

Total RNA was isolated from mid-log cells ($3–5\times10^5$ cells/mL) using TRIzol reagent (Invitrogen) and its purity monitored by the $A_{260/280}$ ratio. RNA integrity was determined by visualization of rRNAs in ethidium bromide-stained gels. First-strand cDNA was reverse transcribed at 42 °C for 1 h using 1 μg of total RNA in a 20 μL reaction mixture containing 1 μL of ImProm II Reverse Transcriptase (Promega) and 0.5 μg of Oligo $d(T)_{18}$ Adaptor Primer 5′-GTTTTCCCAGTCACGAC$(T)_{18}$-3′ (Takara). For 3′ end analysis (3′RACE) of *T. thermophila MTT2*, the cDNA pool was primed with the Adaptor Primer (5′-GTTTTCCCAGTCACGAC-3′) and the primer MTT2-3RACE (5′-ATGGAYACIYARACIYARACIAA-3′) (Takara). The PCR reaction was performed according to the following protocol: 94 °C for 2 min, then 94 °C for 30s, 48 °C for 40s, 72 °C for 45s for 30 cycles, 72 °C for 10 min.

Amplification of 5′ end of *T. thermophila MTT2* cDNA was performed with 5′RACE System for Rapid Amplification of cDNA Ends (Invitrogen). From 2 μg of total RNA, the MTT2-5RACE primer (5′-TCAGCATTTGCATTCAGCACA-3′) was used to generate a first-strand cDNA that was subsequently tailed with oligo-dC. The 5′ end of *MTT2* cDNA was amplified from the oligo-dC-tailed single-stranded cDNA using the nested primer MTT2-5RACENEST (5′-TACAGTTGGAAGTAGAACCGCAAT-3′) and an oligo-dG-containing anchor primer, following the manufacturer's protocol. The amplified products were cloned and sequenced.