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NOTE

Metallothionein Gene from *Tetrahymena thermophila* with a Copper-Inducible-Repressible Promoter

Francesco Boldrin,[1]* Gianfranco Santovito,[1] Jacek Gaertig,[2] Dorota Wloga,[2] Donna Cassidy-Hanley,[3] Theodore G. Clark,[3] and Ester Piccinni[1]

Department of Biology, University of Padova, Padova, Italy 35100[1]; Department of Cellular Biology, University of Georgia, Athens, Georgia 30602[2]; and Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853[3]

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We describe a novel metallothionein gene from *Tetrahymena thermophila* that has a strong copper-inducible promoter. This promoter can be turned on and off rapidly, making it a useful system for induction of ectopic gene expression in *Tetrahymena* and enhancing its applications in cell and molecular biology, as well as biotechnology.

The fresh-water ciliate *Tetrahymena thermophila* is a highly evolved cell with a functional complexity that rivals that of metazoa. Moreover, it combines ease of growth with the availability of advanced genetic tools, which has made *T. thermophila* a key model for studies of cell and molecular biology (13). More recently, *Tetrahymena* has shown considerable promise within the biotechnology arena, in particular as a system for the expression of heterologous membrane proteins (4, 5). In 2002, the first inducible-repressible promoter for driving high-level expression of heterologous genes was introduced (11). This promoter belongs to a Cd-inducible metallothionein gene (*MTT1*) from *T. thermophila*, and it could be rapidly activated or deactivated by simply adding or depleting cadmium in the growth medium. Metallothioneins (MTs) are ubiquitous, low-molecular-weight, cysteine-rich metal-binding proteins that can be transcriptionally induced by a plethora of stimuli, the most effective being heavy metals, in particular cadmium, zinc, and copper (6). Usually multiple MT isoforms are present in one organism. Indeed, in other *Tetrahymena* species (*T. pyriformis* and *T. pigmentosa*) MT-1 and MT-2 isoforms, induced by Cd and Cu, respectively, have been identified (2, 3, 7, 8, 9, 10).

Based on the coding region of the *Tetrahymena pigmentosa MT-2* gene (GenBank accession no. AF479586), degenerate PCR was used to amplify the *MTT2* coding region of *T. thermophila*.

MTT2 gene expression was examined by Northern blotting analysis of total RNA extracted from wild-type *T. thermophila* strain CU428.1 grown in the presence or absence of copper. *MTT2* transcript levels were extremely low in the absence of Cu but increased rapidly within 30 min of treatment (Fig. 1A). This increase was followed by a transient down regulation and then a second increase over the next hour. Transcript levels then slowly declined, reaching a plateau at roughly 4 h (Fig. 1A). A rapid decline occurred when copper was withdrawn from the medium. Following overnight growth in 630 μM Cu, no *MTT2* transcripts were detectable in cells 30 min after transfer to medium without Cu (Fig. 1B).

To assess whether the 5′ region of *MTT2* could act as a promoter to drive the expression of other genes, a region 1,456 bp upstream of the start codon in *MTT2* was amplified using a genome-walking technique (Clontech). This region was juxtaposed with the coding sequence of a chimeric gene specifying the endogenous NIMA (never in mitosis in *Aspergillus nidulans*) kinase, NRK2, linked to green fluorescent protein (designated *NRK2-GFP*). *MTT2^P-NRK2-GFP* was biolistically introduced into the β-tubulin 1 (*BTU1*) locus of *T. thermophila* strain CU522 by homologous recombination followed by growth in the microtubule-stabilizing drug paclitaxel (5). Expression of chimeric *NRK2-GFP* gene products in transformed cell lines was investigated by Northern blotting and fluorescence microscopy. As shown by Northern blotting using an *NRK2*-specific probe, transcripts from the chimeric gene accumulated rapidly, reaching maximal levels 1 h after the addition of Cu to the growth medium and then gradually declining (Fig. 1C). At the protein level, GFP fluorescence was readily visible within transformed cells 2 h after their exposure to Cu (Fig. 2). NRK2-GFP fluorescence was present in the basal bodies and at the tips of cilia (mainly in the anterior region), with the label appearing more evident in dividing cells (Fig. 2A and B). Furthermore, the affected cilia underwent shortening, presumably due to kinase activity associated with the NRK-GFP fusion protein (D. Wloga, unpublished data). Control cells showed only a weak autofluorescence of cytoplasmic granules and vesicles (Fig. 2C). Taken together, these results argue that the region upstream of the start codon of *MTT2* can act as a functional promoter when introduced at an ectopic site in the *Tetrahymena* genome.

* Corresponding author. Mailing address: Department of Biology, Via Ugo Bassi 58/B, 35100 Padova, Italy. Phone: 39-049-827-6310. Fax: 39-049-827-6300. E-mail: francesco.boldrin@unipd.it.

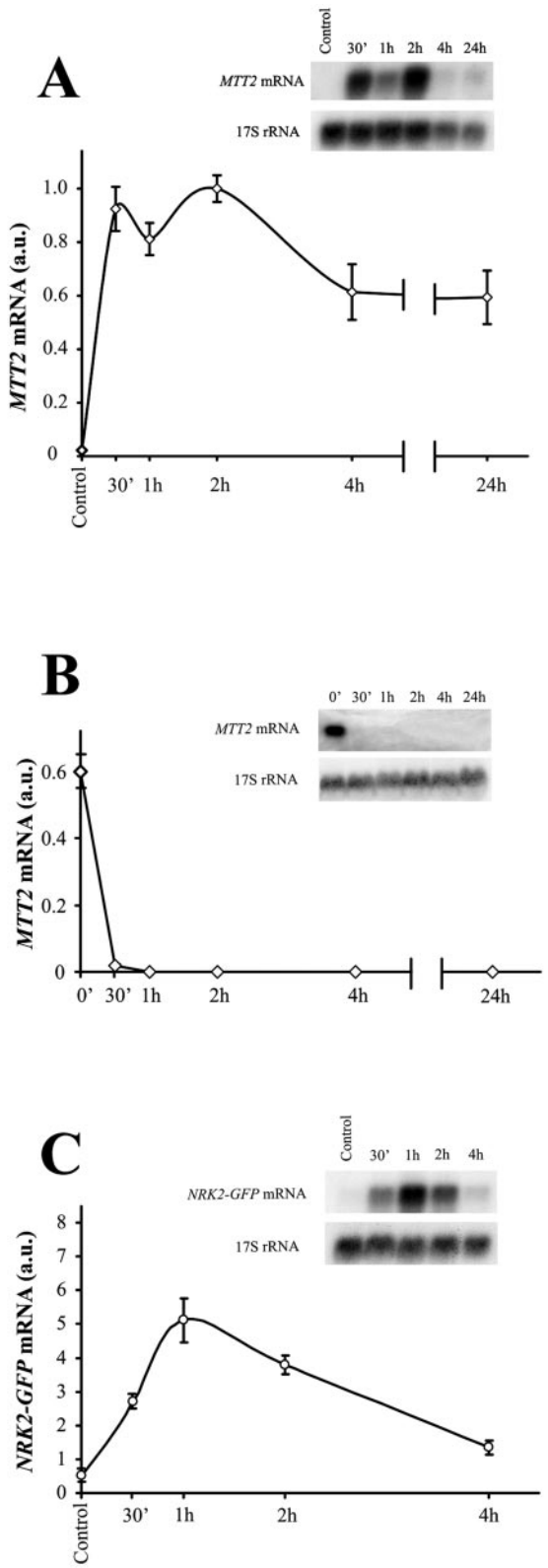

FIG. 1. Northern blot analyses of *MTT2* and *NRK2-GFP* mRNAs in *T. thermophila*. *T. thermophila* wild-type strain CU428.1 was grown in SPP (1% proteose peptone, 0.8% glucose, 0.1% yeast extract, 0.003% EDTA ferric sodium salt) medium to log phase (3×10^5 to 5×10^5 cells per ml), and cells were exposed to 630 μM Cu. (A) Time course of induction of *MTT2* mRNA. Total RNA (20 μg) from control cells (harvested at 24 h without Cu) and cells treated with Cu for various periods of time was hybridized with a [α-^{32}P]dATP-labeled, randomly primed *MTT2*-specific cDNA probe. The nylon membranes were then stripped and rehybridized with a [γ-^{32}P]dATP end-labeled oligonucleotide probe against *T. thermophila* 17S rRNA (12). (B) Cells were treated with 630 μM Cu overnight, washed twice with 10 mM Tris-HCl (pH 7.5), resuspended in SPP medium without Cu, and harvested at the times indicated. (C) Northern blot analysis of *NRK2-GFP* mRNAs. The plasmid replacement vector pMTT2-NRK2-GFP was biolistically introduced into strain CU522, and macronuclear transformants were selected based on paclitaxel resistance resulting from loss of function at the *BTU1* locus. The MTT2-NRK2-GFP strain was grown in SPP medium and exposed to 630 μM Cu. Samples of total RNA (20 μg) were hybridized with a [α-^{32}P]dATP-labeled, randomly primed *NRK2-GFP* probe and rehybridized with a [γ-^{32}P]dATP end-labeled *T. thermophila* 17S rRNA oligonucleotide probe. Controls were harvested at 24 h; treated cells were harvested after the indicated times. Northern blots were scanned, and densitometer readings for *MTT2* and *NRK2-GFP* mRNAs, expressed as arbitrary units (a.u.), were normalized against those for *T. thermophila* 17S rRNA.

The overall kinetics of *MTT2* gene expression, including the transient decrease following induction by Cu (Fig. 1A), is similar to that described for many organisms, including other *Tetrahymena* species (10). However, it should be noted that a second Cu-inducible MT isoform encoded within the *T. thermophila* genome has recently been described (*MTT4*; GenBank accession no. AY660008) (http://www.tigr.org/tdb/e2k1/ttg/). Because the cDNA sequences of *MTT2* and *MTT4* are nearly identical and because the probe used in Northern blots would not be expected to distinguish them, it is entirely possible that the transcript levels shown in Fig. 1A represent the combined mRNAs of both genes.

From a practical standpoint, use of the *MTT2* promoter to drive inducible, high-level expression of heterologous genes in *T. thermophila* is quite significant. In the case of potentially toxic gene products such as the endogenous NIMA-related kinase NRK2, controlled expression is often a prerequisite for functional assays, as well as for localization studies of expressed proteins. At the same time, overexpression of foreign genes has considerable importance from a biotechnological perspective. We are currently focusing on two broad categories of proteins for overexpression in the *Tetrahymena* system: (i) vaccine antigens and potential drug targets against protozoan pathogens of humans and animals (in particular, apicomplexans) and (ii) eukaryotic membrane and secretory proteins that are difficult to express in more conventional systems. To date, these studies have utilized the Cd-sensitive *MTT1* promoter for gene expression (11). While this promoter is a powerful tool in basic research, it requires the addition of a highly toxic metal to the growth medium, which must be disposed of during the production process (1). Clearly, the availability of alternative promoters for driving inducible expression of foreign genes in *T. thermophila* would be useful. To our knowledge, only two such promoters exist. The first is a temperature-sensitive promoter from the *hsp70* gene of *T. thermophila* (C. Miceli, personal communication), whose relative strength has yet to be determined. The second is the Cu-dependent promoter from *MTT2* reported here.

In conclusion, we have described a new MT gene from *Tetrahymena thermophila* that has a strong promoter induced by an essential metal, Cu. This promoter can be turned on and

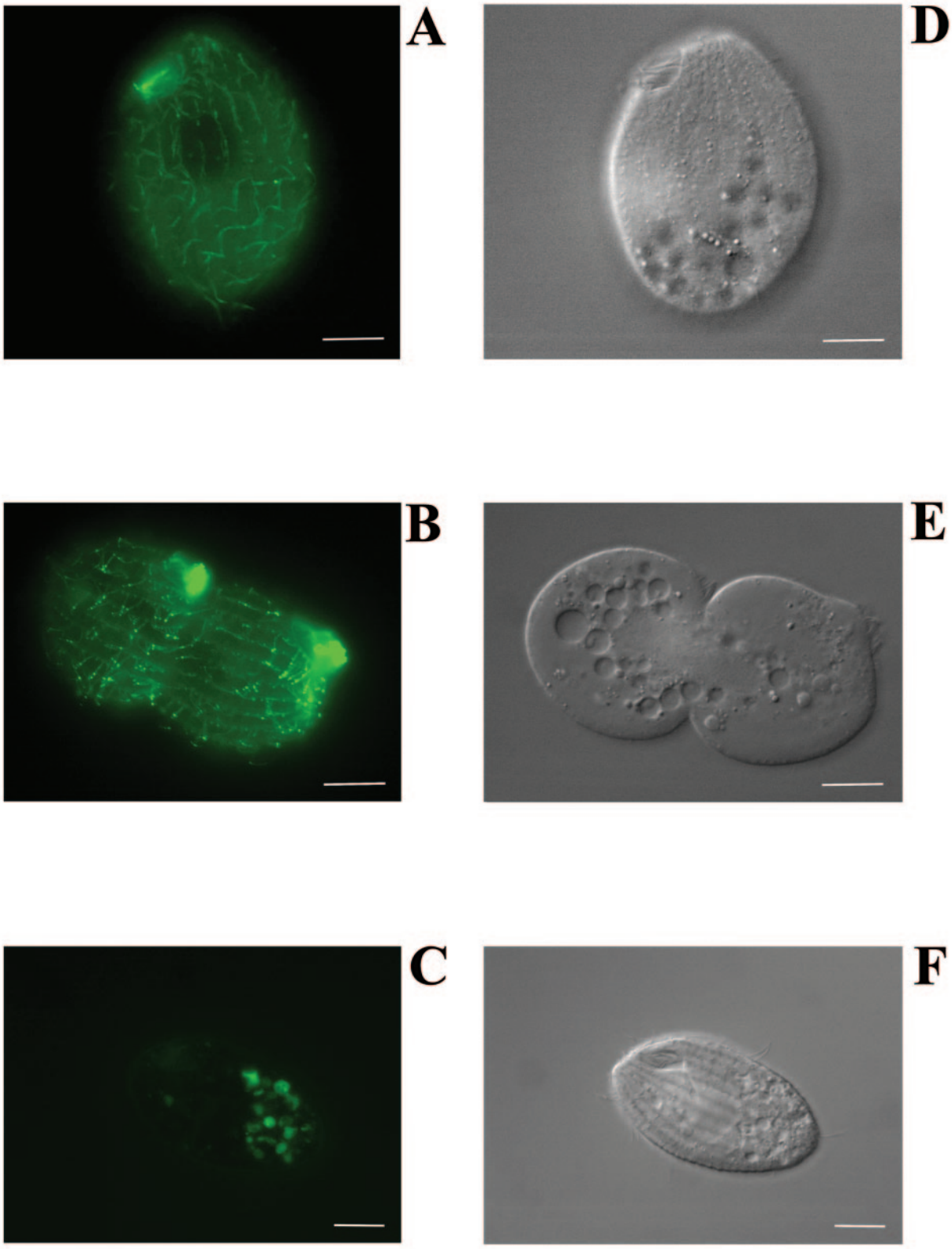

FIG. 2. Fluorescence localization of the chimeric protein NRK2-GFP in transgenic cells. Living MTT2-NRK2-GFP cells were observed by fluorescence (A to C) and Nomarsky contrast (D to F) microscopy. (A, D) Single cells grown in SPP medium and exposed to 630 μM Cu for 2 h; (B, E) treated cells in division phase; (C, F) single untreated cells. Bars, 10 μm.

off rapidly, making it a good system for induction of ectopic gene expression in *Tetrahymena* and enhancing its applications in cell and molecular biology, as well as biotechnology. Because *MTT2* gene expression requires Cu rather than Cd, its promoter may offer an attractive alternative to the previously described *MTT1* promoter for driving foreign gene expression in *T. thermophila*. Although this will depend to some extent on its relative strength as a promoter, preliminary studies (not shown) clearly suggest that the upstream region of *MTT2* can direct high-level expression of heterologous genes.

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