Identification of Two Nickel Ion-Induced Genes, NCI16 and PcGST1, in Paramecium caudatum

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Here, we describe the isolation of two nickel-induced genes in Paramecium caudatum, NCI16 and PcGST1, by subtractive hybridization. NCI16 encoded a predicted four-transmembrane domain protein (~16 kDa) of unknown function, and PcGST1 encoded glutathione S-transferase (GST; ~25 kDa) with GST and glutathione peroxidase (GPx) activities. Exposing cells to cobalt chloride also caused the moderate upregulation of NCI16 and PcGST1 mRNAs. Both nickel sulfate and cobalt chloride dose dependently induced NCI16 and PcGST1 mRNAs, but with different profiles. Nickel treatment caused a continuous increase in PcGST1 and NCI16 mRNA levels for up to 3 and 6 days, respectively, and a notable increase in H2O2 concentrations in P. caudatum. NCI16 expression was significantly enhanced by incubating cells with H2O2, implying that NCI16 induction in the presence of nickel ions is caused by reactive oxygen species (ROS). On the other hand, PcGST1 was highly induced by the antioxidant tert-butyldihydroquinone (tBHQ) but not by H2O2, suggesting that different mechanisms mediate the induction of NCI16 and PcGST1. We introduced a luciferase reporter vector with an ~0.42-kb putative PcGST1 promoter into cells and then exposed the transformants to nickel sulfate. This resulted in significant luciferase upregulation, indicating that the putative PcGST1 promoter contains a nickel-responsive element. Our nickel-inducible system also may be applicable to the efficient expression of proteins that are toxic to host cells or require temporal control.

Nickel is used extensively for electroplating metals, alloys such as cupronickel, and rechargeable batteries. Occupational exposure to nickel occurs in industrial workers, in particular those involved in mining, smelting, and refining, the production of steel and other metals, and electronic devices (1). Nickel compounds are released into the environment from power plants that burn oil, trash incinerators, wastewater from nickel mines, and industries that manufacture nickel products for industrial and consumer use. Nickel has toxic and carcinogenic effects on most microorganisms and animals and is considered to impose an industrial health hazard (2, 3). Nickel compounds such as nickel sulfide (NiS2) are potent carcinogens, but soluble nickel salts such as nickel chloride (NiCl2) exert weaker effects. The molecular mechanisms involved in the cytotoxicity and carcinogenicity of nickel compounds are not fully understood, but nickel might be associated with the intracellular production of reactive oxygen species (ROS), including superoxide, H2O2, singlet oxygen, and hydroxyl radicals (4–8). Nickel also increases lipid peroxide (LPO) levels, resulting in the generation of peroxyl radicals, lipid hydroperoxides, and alkoyl radicals (9–11). Carcinogenesis related to nickel is explained by several types of DNA damage, such as cleavage, depurination, cross-linking, and DNA base damage caused by ROS (12). Nickel inhibits processes in the DNA repair system, such as DNA ligation and DNA polymerization, that are involved in rejoining DNA breaks (6). Nickel also modifies the antioxidant system; for example, nickel chloride alters hepatic reduced glutathione levels (13) as well as renal glutathione S-transferase (GST) (14) and hepatic glutathione peroxidase (GPx) activities (4) in rodents. Nickel ions inhibit the ciliary beat of the unicellular protozoan Paramecium. Transferring Paramecium caudatum into a solution containing nickel ions causes a gradual decrease in the frequency and the amplitude of the ciliary beat without influencing the orientation of the cilia (15). The effects of nickel ions on ciliary beat also were examined in the ATP-Mg2+-reactivated cilia of detergent-extracted Paramecium models (16). Nickel ions have detrimental effects on microtubule translocation mediated by 14S dynein, which might be one of the factors directly affected during the nickel-induced paralysis of axonemal beats (17). The effects of nickel ions on the cellular functions of paramecia other than ciliary movement have not been documented in detail. Paramecium and other ciliated protozoa were used in bioassays designed to measure the cytotoxic and carcinogenic effects of soluble and particular nickel compounds (18, 19) or of waste treatment plants (20). Although the cytotoxic effects of nickel ions on Paramecium are documented, genes for which the expression levels are altered by nickel ions remain unidentified.

We performed subtractive cDNA hybridization to identify nickel-induced genes to elucidate mechanisms mediating nickel toxicity and associated detoxifying systems in Paramecium. Two genes, NCI16 and P. caudatum GST1 (PcGST1), obviously were upregulated in P. caudatum that had been treated with NiSO4 but not in control cells. NCI16 encoded a predicted four-transmembrane domain protein of unknown function, and PcGST1 encoded a GST protein that exhibits the enzymatic activities of both GST and GPx. A region of the putative PcGST1 promoter (~0.42 kb) was isolated and cloned into the pBsc-tel3 vector (21) with a
luciferase gene to generate a reporter construct. This putative PcGST1 promoter drove a significant increase in nickel-depend-ent luciferase activity, implying that PcGST1 upregulation is mediated by cis-acting sequences in the promoter region. Our nickel-inducible system also may be applicable to the efficient expression of proteins that are toxic to host cells or require temporal control.

MATERIALS AND METHODS

Strains and culture methods. The Paramecium caudatum BW6-1 strain (syngon 12, odd mating type) and the NH2 strain (syngon 3, even mating type) was transformed with the pGT1-MpLuc1H vector. Cells were cultured either in 1.25% (wt/vol) fresh lettuce juice diluted with K-DS (Dryl’s solution modified by the substitution of KH2PO4 for NaH2PO4), pH 7.0 (22), or in wheat grass powder (WGP) (23) medium. Both culture media were inoculated with Klebsiella pneumoniae 1 day before use.

cDNA subtraction of nickel-induced genes. BW6-1 cultures were grown until early log phase (approximately 300 cells/ml) in 200 ml of WGP medium in 500-ml glass flasks. Half (100 ml) of each such culture was transferred into identical 500-ml glass flasks. A stock solution of NaH2PO4 (0.1 M) was added to one culture at a final concentration of 10 μM, and the other served as a control culture without additives. Both cultures were incubated for 3 days without additional medium supply. Cells were collected onto a 5-μm membrane filter (Millipore). Total RNA and mRNA were isolated using IsoGen (Nippon Gene) followed by Oligotex-dT30 (TaKaRa Bio), and then cDNA was synthesized from 300 ng of mRNA using the Smart rapid amplification of cDNA ends (RACE) cDNA synthesis kit (TaKaRa Bio). cDNAs that were specifically expressed in nickel-treated cells were enriched using the DsDD cDNA subtraction kit (Wako Pure Chemical) according to the manufacturer’s instructions. The subtracted cDNAs were cloned in pGEM-T Easy vector (Promega) and introduced into DH5α; transformed cells were spread and grown on LB agar containing carbenicillin. Plasmid vectors (n = 39) containing a ≥0.85-kb insert identified by colony PCR were purified using Wizard plus SV minipreps (Promega) and sequenced. The nickel-induced expression of candidate cDNAs in Paramecium was assessed by agarose gel electrophoresis of the reverse transcription-PCR (RT-PCR) products.

SDS-PAGE and Western blotting. Cells were fixed in medium containing 10% trichloroacetic acid for 10 min at 4°C, pelleted, washed with distilled water, and lysed in an appropriate volume of 5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), 65 mM dithiothreitol. Cell lysates (2 to 10 μg) were resolved by electrophoresis on 13.5% SDS-PAGE gels, and proteins were stained using Coomassie brilliant blue (CBB) or the silver stain MS kit (Wako). Proteins were blotted onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h at room temperature in 1% Western blocking reagent (Roche Applied Science), incubated with anti-His6 (2) antibody (Roche Applied Science) diluted (1:2,500) in 0.5% Western blocking reagent, and then developed with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody and ECL prime (GE Healthcare). Chemiluminescent signals were captured on Hyperfilm ECL (GE Healthcare).

Phylogenetic analyses. The deduced amino acid sequences of NC16 and PcGST1 were aligned using MUSCLE with the default parameters in the MEGA5 program (24). Phylogenetic trees were generated using maximum-likelihood (ML) analysis.

qPCR. NC16 and PcGST1 mRNA expression in control and nickel-treated cells were measured using Thunderbird SYBR quantitative real-time PCR (qPCR) mix (Toyobo) and an ABI Prism 7900HT sequence detection system (Life Technologies). The primer sequences were the following for NC16 cDNA, Ni466UP1 (5’-AATTTAACCTCCTCTCGGACCTGCTTGTG-3’) and Ni466LP1 (5’-TCCAGCGCATAGTTGTTTTATTTTTTTT-3’); for PcGST1 cDNA, Ni866UP3 (5’-CATTAAACAAGATGCTGGAAGAAAGACTAT-3’) and Ni866LP3 (5’-AACGATGCTTACAGACTC-3’); for P. caudatum α-tubulin cDNA, a-Tub RT-UP1 (5’-GCAACATCAAGAAGAAGAGACCGAC3’) and a-Tub RT-LP1 (5’-ACAAGGCTCCTTGGCATGACTA-3’). The primer sequences for quantitative RT-PCR (qRT-PCR) analyses of four PcGST1 homologs are listed in Table S2 in the supplemental material. All primers were designed using Oligo 7 primer analysis software.

Expression and purification of recombinant proteins. Each coding sequence of NC16 and PcGST1 was synthesized de novo (Eurofins Genomics) to optimize codon usage for protein expression in Escherichia coli and subcloned into pET-16b and pET-20b vectors (Novagen), respectively. The pET16b-NC16 construct carried a full-length NC16 open reading frame (ORF) with an N-terminal 10× His tag; the pET20b-PcGST1 construct carried a full-length PcGST1 ORF with a C-terminal 6× His tag. The recombinant proteins were expressed in E. coli BL21(DE3) cells (Stratagene) cultured in 10 ml of YM5052 medium (25) at 30°C for 24 h with vigorous shaking. Pelleted cells expressing PcGST1–6× His-tagged protein were resuspended in 2 ml of 20 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 0.5 M NaCl, and 5 mM imidazole and sonicated on ice. PcGST1–6× His was bound to Talon Superflow (GE Healthcare) and eluted in 1 ml of 20 mM Tris-HCL, pH 8.0, 10 mM MgCl2, 0.5 M NaCl, and 500 mM imidazole (1 ml500).

GST and GPx assays. GST and GPx assays with purified PcGST1–6× His were performed using a glutathione S-transferase assay kit (Cayman Chemical) and glutathione peroxidase activity colorimetric assay kit (BioVision), respectively, according to the manufacturers’ instructions.

Measurement of H2O2. H2O2 formation was measured using an ROS-Glo H2O2 assay kit (Promega). Cells were treated with 10 μM NiSO4 or 10 μM tert-butylhydroquinone (tBHQ) for 18 h. Cell cultures (80 μl) were transferred to each well of a 96-well plate, mixed with 20 μl of H2O2 substrate, and incubated for an additional 6 h (24 h of total treatment). Resulting luminescent signals were measured using a Varioskan Flash microplate reader (Thermo Scientific).

Cloning of the PcGST1 promoter and construction of inducible expression vector. Genomic DNA (1 μg) isolated from BW6-1 cells was digested with DraI at 37°C for 16 h, extracted with phenol, and precipitated with isopropanol. Digested genomic DNA was self-ligated and served as a template to amplify the putative PcGST1 promoter region by PCR using the primers Nib66UP309, 5’-AGTCTTGAGGATTCTACCCGATTTTC-3’, and Ni66LP265, 5’-TAAATACGTCGTTATCTGATGTTCTAGGAGGTA-3’. The resultant PCR product (~1.1 kb) was cloned into pGEM-T Easy vector and sequenced. A smaller region of the putative PcGST1 promoter (~0.42 kb) was amplified from the plasmid DNA using the primers Ni66PreSelUP1, 5’-AACTGTCGAGAAGATATATATAACAACA-3’, and Ni66PreSelLEP1, 5’-GAATTCTTATATCTGATGTTCTAGGAGGTA-3’. The PCR fragment was subcloned into an SpeI-EcoRI-digested pBluesc-t3 vector (21) to generate a potentially nickel-inducible expression vector, designated pG71-MCS. Sequences encoding a secreted luciferase from the marine copepod Metridia pacifica, MpLuc1 (AB195233), were amplified from plasmid DNA harboring a cloned luciferase cDNA (26); the luciferase sequence was subcloned into EcolRI-SacI-digested pG71-MCS to generate the plasmid pG71-MpLuc1H. Plasmid DNA was isolated using a Qiagen-tip 500 (Qiagen), linearized by BamH1 digestion, purified by phenol-chloroform extraction, precipitated with isopropanol, and resuspended in sterile distilled water at a final concentration of 1 μg/μl.

Microinjection. Linearized pG71-MpLuc1H was microinjected as described previously (27). About 2.0 × 104 copies of plasmid DNA in a volume of ~10 pl were injected into the macronucleus of NH2 cells. Viable recipient cells were transferred to a glass depression slide containing fresh lettuce juice medium at 25°C. Luciferase assay. Three transformed clonal cell lines were grown in 100 ml of WGP medium. Early-log-phase cultures (100 ml of ~300 cells/ml) were equally divided into two flasks each. Thereafter, NiSO4 (0.1 M) was added to one flask at a final concentration of 10 μM, and nothing was added to the other. Cells were collected onto a 5-μm membrane filter, filtered, and resuspended in sterile distilled water at a final concentration of 1 μg/μl.
NiSO₄ was supplied once on day 3 immediately after harvest on the same day (see Fig. 7C). Pelleted cells from 1 ml of each culture were resuspended in 100 μl of 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂ and then flash frozen at −80°C for luciferase assays. Thawed cellular lysate was vortexed and separated by centrifugation at 21,600 × g for 1 min. Supernatants (10 μl) were transferred to tubes and then placed in a MiniLumat LB 9506 luminometer (Berthold). Coelenterazine (50 μl of 1 ng/μl in 20 mM Tris-HCl, pH 8.0, 50 mM MgCl₂) was added to the lysate, and then 10-s measurements were immediately started.

**RESULTS**

**Nickel cytotoxicity in *P. caudatum***. Cells were incubated with various concentrations of NiSO₄, CoCl₂, or CdCl₂ for 2 days to compare the cytotoxic effects of nickel ions on *P. caudatum* to those of other metal ions. Of the three metal ions, NiSO₄ was moderately toxic (Fig. 1), since at ≈15 μM, NiSO₄ did not significantly decrease cell viability determined as a ratio (i.e., a percentage of untreated cells). Exposure to 5 μM NiSO₄ (see Fig. S1 in the supplemental material) and 10 to 20 μM CoCl₂ (data not shown) for 2 days slightly increased cell density, whereas CdCl₂ was significantly lethal at all tested concentrations (*P* < 0.01). The 50% lethal doses (LC₅₀) for exposure to NiSO₄, CdCl₂, and CoCl₂ for 2 days were 18.6, 2.10, and 36.5 μM, respectively.

**Nickel ions induce NCI16 and PcGST1 genes in *P. caudatum***. To investigate whether nickel ions could induce the expression of specific genes in *P. caudatum*, cells were cultured in medium with or without NiSO₄. Protein expression from these cells was resolved and compared by SDS-PAGE with silver staining. The intensity of at least two protein bands substantially increased in cells incubated with 10 or 20 μM NiSO₄ (Fig. 2A, arrowheads). These two proteins were partially purified by DEAE Sepharose FF resin to determine their N-terminal amino acid sequences using conventional Edman degradation. However, no amino acid could be interpreted from chromatograms, because the peaks were quite vague (data not shown). Therefore, we used cDNA subtraction to identify genes induced by nickel ions. Thirty-nine candidate cDNAs that were ≈0.85 kb were isolated, sequenced, and evaluated by semiquantitative RT-PCR using gene-specific primers. Two genes, designated NCI16 and PcGST1, obviously were upregulated in the cells incubated with 10 μM NiSO₄ (Fig. 2B). The deduced sequences of NCI16 and PcGST1 comprised 148 (16.2 kDa; pI, 10.5) and 208 (25.0 kDa; pI, 5.12) amino acids, respectively. The PcGST1 gene sequence contained a single intron, whereas the NCI16 gene had no intronic sequences. The molecular mass of NCI16 and PcGST1 proteins was evaluated by Western blotting with 10× His-tagged NCI16 and PcGST1 proteins were evaluated by Western blotting with anti-His antibody. M, MagicMark XP protein standard (Life Technologies); LacZ, His-tagged LacZ protein as a positive control on Western blotting.

Two genes, designated NCI16 and PcGST1, obviously were upregulated in the cells incubated with 10 μM NiSO₄ (Fig. 2B). The deduced sequences of NCI16 and PcGST1 comprised 148 (16.2 kDa; pI, 10.5) and 208 (25.0 kDa; pI, 5.12) amino acids, respectively. The PcGST1 gene sequence contained a single intron, whereas the NCI16 gene had no intronic sequences. The molecular mass of NCI16 and PcGST1 proteins was evaluated by Western blotting with 10× His-tagged recombinant proteins expressed in *E. coli* and detected using an anti-His antibody. NCI16 protein was found exclusively in the phosphate-buffered saline (PBS)-insoluble fraction; it was detectable only on Western blots of cells that had been sonicated, denatured with SDS-PAGE sample buffer, and directly loaded onto SDS-PAGE gel. A smeared signal at a high-molecular-mass location (Fig. 2C) indicated that recombinant NCI16 tended to aggregate in *E. coli*. Estimating positions of nontagged protein bands by subtracting the molecular mass of the His tag and the protease recognition sequence (~2.5 kDa), it is possible that PcGST1 corresponds to the upregulated protein band at the higher molecular mass (Fig. 2A, upper arrowhead).

**Molecular phylogeny and functions of NCI16 and PcGST1**. A BLAST homology search revealed that NCI16 was most orthologous to a protein of unknown function (XP_001432069.1) in a closely related species, *Paramecium tetraurelia* (75% amino acid sequence identity) (Fig. 3A). NCI16 orthologs were found in the following eukaryotic and prokaryotic microorganisms: an amoeba (*Dictyostelium discoideum*), cnidaria (*Hydra vulgaris* and *Hydra vulgaris*).
FIG 3 Phylogeny of NCI16 and PcGST1. (A) Multiple-amino-acid sequence alignment of NCI16 and 16 NCI16 orthologs. (B) Multiple-amino-acid sequence alignment of PcGST1 and 21 PcGST1 orthologs. Outlined and shaded text represents at least 90% identical amino acid residues. The ML consensus tree obtained from bootstrap analysis with 1,000 replications of NCI16 (C) and PcGST1 (D) was based on amino acid sequence alignments shown in panels A and B. Bootstrap values of >60% are given to the left of selected nodes. Accession numbers or identifiers used in ParameciumDB are indicated with species names.
Nickel-induced Genes in *Paramecium*

*Nematostella vectensis*, a fungus-like oomycete (*Phytophthora infestans*), microalgae (*Galdieria sulphuraria* and *Coccomyxa subellipsoidea*), methylothrophs (*Methyllobacterium nodulans*, *Methylobacterium nodulans*, and *Hyphomicrobium denitrificans*), a methanotroph (*Methyloblobus morosus*), and root nodule bacteria (*Rhizobium giardinii*, *Sinorhizobium fredii*, and *Sphingobium quinquisquiliarum*) (Fig. 3A). Phylogenetic analysis of NC116 and 16 orthologous proteins showed that NC116 and other eukaryotic orthologs were paralysied, but most prokaryotic orthologs formed a monophyletic group, except for *Methyloblobus morosus*, which formed a small monophyletic clade with *G. sulphuraria* (Fig. 3C). Both S0SU1 1.11 ([http://harrier.nagahama-i-bio.ac.jp/sosui/sosui _submit.html](http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submi.html)) and TMMHMM 2.0 ([http://www.cbs.dtu.dk/services /TMHMm-2.0/](http://www.cbs.dtu.dk/services/TMHMM-2.0/)) predicted that the amino acid sequence of NC116 contains four-transmembrane segments (see Fig. S2 and Table S1 in the supplemental material).

A BLAST search of *Paramecium*DB ([http://paramecium.cgm .cnrs-gif.fr/cgi/tool/blast](http://paramecium.cgm.cnrs-gif.fr/cgi/tool/blast)) (28) using the amino acid sequence of PcGST1 revealed similarity to six *P. caudatum*, six *P. multimeric-nucleatum*, and seven *P. tetraurelia* GST-like proteins. However, none of these protein sequences were annotated (Fig. 3B). Among the six *P. caudatum* homologs found in the database, PCAUDP12020 was the most similar to PCGST1 (E value, 1e−107). We assumed that PCAUDP12020 corresponded to PcGST1 and that protein sequences differ according to strain. Aside from the *Paramecium* PcGST1 homologs, >50 similar proteins were identified (cut-off E value, >1e−18) by a BLAST search of the NCBI protein database, and most were designated GSTs (Fig. 3B). An ML analysis of five PcGST1 proteins, and 20 orthologs revealed that the *Paramecium* GST-like proteins formed a monophyletic clade (Fig. 3D) that diverged into two monophyletic clades, 1 and 2. Clade 1 contains GST-like proteins (including PcGST1) of three *Paramecium* species, whereas clade 2 did not contain any *P. caudatum* proteins. PcGST1 and a GST-like protein of *P. caudatum* (PCAUDP12019) formed a monophyletic clade (clade 3) with a well-supported bootstrap value (89%), suggesting that their amino acid sequences were relatively unique compared to those of the other *Paramecium* GST-like proteins analyzed in the present study.

**Nickel ions enhance GST and GPx activities in *P. caudatum*.**

To confirm that PcGST1 has GST activity, a 6× His-tagged PcGST1 protein was expressed in *E. coli* and purified on immobilized metal affinity chromatography resin charged with cobalt. Recombinant PcGST1-His protein eluted with a buffer containing Im500 migrated as a single band on SDS-PAGE (Fig. 4A). The specific GST activity of purified PcGST1-His toward the substrate, 1-chloro-2,4-dinitrobenzene (CDNB), was comparable to that of *Pc*GST1 and that protein sequences differ according to strain. Nickel ions increase GST and GPx activities in *PcGST1* homologs (Fig. 3B and D) to the overall GST and/or GPx activities measured in nickel-treated *P. caudatum* lysate, we examined their relative expression levels by qRT-PCR analyses with gene-specific primers. PcGST1 was upregulated >3,000-fold in cells incubated with 10 μM NiSO4 compared to untreated control cells on days 3 and 6, whereas the four other *PcGST1* homologs were induced only <150-fold (Fig. 4E). One homolog (PCAUDP15663) was not significantly amplified and was not further analyzed. Thus, it is likely that PcGST1 significantly contributes to changes in GST and/or GPx activity in cellular extract, although the enzymatic nature of the other five homologs remains undetermined. We cloned qRT-PCR products containing partial sequences of PCGST1 homologs and sequenced them to confirm the specificity of the PCR amplification.

**Metal ion specificity, dose dependency, and time course of NC116 and PcGST1 induction evaluated by quantitative RT-PCR.** Incubation with either NiSO4 or NiCl2 at 10 μM for 3 days increased the levels of NC116 and PcGST1 mRNAs >40-fold and >6,000-fold, respectively, compared to untreated controls (Fig. 5A and B). Both genes also were significantly upregulated 118- and 225-fold, respectively, by CoCl2 at 10 μM. The concentration-related effects of NiSO4 on the induction of NC116 and *PcGST1* genes differed notably from those of CoCl2 (Fig. 5C and D). The mRNA levels of NC116 and *PcGST1* were not altered at NiSO4 concentrations ranging from 0 to 5 μM, whereas CoCl2 exerted dose-dependent effects on both genes. Both NC116 and *PcGST1* mRNAs were rapidly induced 10.5- and 267-fold, respectively, at 6 h after NiSO4 exposure and continued to increase up to days 6 and 3, respectively (Fig. 5F and E).

**NC116 and PcGST1 expression was enhanced by H2O2 and antioxidant tBHQ, respectively.** We investigated whether exposure to nickel causes intracellular ROS accumulation in *Paramecium*. Exposure to 10 μM NiSO4 caused a notable increase in the H2O2 concentration in *P. caudatum* (Fig. 6A). Treatment of cells with 1 mM H2O2 for 24 h significantly enhanced NC116 expression ~700-fold but increased *PcGST1* by only ~15-fold (Fig. 6B). The antioxidant tBHQ is capable of inducing phase II detoxification enzymes, including GSTs. When cells were incubated with 10 μM tBHQ for 24 h, *PcGST1* expression was prominently induced (~780-fold) (Fig. 6B), but tBHQ induced minimal NC116 expression (~2-fold) (Fig. 6B) and no intracellular H2O2 (Fig. 6A).

**Putative PcGST1 promoter can induce luciferase reporter gene upon nickel exposure.** Lastly, we examined whether or not nickel-responsive expression of the *PcGST1* gene is regulated by elements in a putative *PcGST1* gene promoter region. Genomic sequence data for *P. caudatum* were not yet listed in the *Paramecium*DB at the time this experiment started. Therefore, we isolated *PcGST* genomic DNA with its 5′ and 3′ flanking regions by inverse PCR. We amplified a DNA product containing a 5′ flanking region (~0.42 kb) immediately upstream of the *PcGST1* coding sequence. This putative *PcGST1* gene promoter contained two putative TATA boxes at −31 and −44 bp relative to the transcription start site for *PcGST1* (Fig. 7A), but neither canonical GC boxes nor sequences similar to the consensus metal-responsive or antioxidant-responsive element were found in the mammalian metallothionein (29) or GST (30) gene promoter, respectively.

We performed a reporter gene assay to determine whether or not this putative *PcGST1* gene promoter was sufficient to induce nickel-responsive transcription. We generated the expression vector pGT1-MpLuc1H that encoded the marine planktonic luciferase, MpLuc1 (26), as a reporter gene immediately downstream of the putative ~0.42-kb *PcGST1* promoter fragment (Fig. 7B).
FIG 4 GST and GPx activities of PcGST1. (A) Recombinant PcGST1 His-tagged protein was expressed in *E. coli*, purified by cobalt affinity chromatography, resolved by SDS-PAGE, and stained with CBB. M, protein molecular mass marker. Crude, soluble fraction of *E. coli* crude extract. FT, flowthrough fraction. Im20, Im50, and Im500, fractions eluted with buffers containing 20, 50, and 500 mM imidazole, respectively. (B) Specific GST and GPx activities of purified recombinant PcGST1. Values for the positive control indicate specific activities of equine liver GST and bovine erythrocyte GPx that are components of GST and GPx assay kits, respectively. Values for the empty vector indicate specific GST and GPx activities of crude extract from *E. coli* transformed with empty expression vector as a negative control. Specific GST (C) and GPx (D) activities of *P. caudatum* whole-cell lysate after treatment with 10 μM NiSO₄ for 3 or 6 days. Bars show means ± SD from 5 different cultures (*, P < 0.01 by Student’s t test). (E) Relative mRNA levels of PcGST1 and 4 other homologs normalized to α-tubulin expression upon nickel exposure. Cells were incubated with 10 μM NiSO₄ for 3 or 6 days. Bars represent mean fold changes ± SD (n = 3 per group). The level of mRNA in untreated control cells is defined as 1.0.
Three clonal transformant lines were established by microinjecting pGT1-MpLuc1 into the macronucleus of *P. caudatum*. The relative abundance of the transduced luciferase gene in the genomic DNA isolated from these three lines was determined by qPCR. When the amount of the luciferase gene in clone 1 was defined as 1.0, those of clones 2 and 3 were 0.57 and 0.14, respectively. Each clonal line was grown in medium with or without 10 μM NiSO₄ (Fig. 7C). Luciferase activity was considerably enhanced by NiSO₄ treatment for 3 days in clones 1 and 2 (211- and 167-fold, respectively, compared to the untreated control) and moderately enhanced in clone 3 (6.4-fold) (Fig. 7D). Luciferase activity did not seem to change in a transformant line transduced with a constitutive reporter expression vector, pTT3-MpLuc1H, that harbors an α-tubulin promoter sequence driving MpLuc1 gene transcription (Fig. 7D). Based on qRT-PCR analyses, the induction of MpLuc1 mRNA by NiSO₄ began on day 1 and ended precipitously on day 3 in all inducible clone 3 lines (Fig. 7E).

**DISCUSSION**

The present study aimed to identify genes that are significantly induced by nickel ions. Using subtractive cDNA hybridization, we identified *NCI16* and *PcGST1* as genes that were upregulated in cDNA prepared from cells exposed to 10 μM NiSO₄, a concentration at which cellular viability did not significantly decrease (Fig. 1). Recombinant 6× His-tagged NCI16 protein expressed in *E. coli* was largely insoluble, and that expressed in mammalian cultured cells could not be detected on immunoblots (data not shown). Thus, we could not use recombinant NCI16 protein for functional analysis. The hydrophobic nature of the predicted multiple membrane-spanning domains (see Fig. S2 in the supplemental material) might have rendered recombinant NCI16 insoluble. We are still in the process of expressing and purifying recombinant NCI16 to reveal its functions and roles in cellular responses to nickel and/or ROS. Recombinant PcGST1 protein was pro-
Nickel treatment caused a notable increase in the \( \text{H}_2\text{O}_2 \) concentration in *P. caudatum* (Fig. 6A), indicating the intracellular formation of various ROS, including superoxide, which is converted to \( \text{H}_2\text{O}_2 \) by superoxide dismutase, hydroxyl radicals, and LPO. Incubating cells with \( \text{H}_2\text{O}_2 \) significantly enhanced *NCI16* expression ~700-fold (Fig. 6B). Taken together, these findings suggest that *NCI16* gene induction was caused by \( \text{H}_2\text{O}_2 \) and/or superoxide produced by nickel ions. Interestingly, the antioxidant tBHQ notably induced PcGST1 but not *NCI16* (Fig. 6B). In mammals, tBHQ might have both chemoprotective and carcinogenic effects (32). The chemoprotective mechanism of tBHQ might involve the induction of phase II detoxification enzymes, such as GSTs, UDP-glucurononyltransferases, and NAD(P)H:quinone oxidoreductase. Our results indicated that *P. caudatum* and mammals share a mechanism by which tBHQ enhances transcription of GST and other phase II genes. To date, various mechanisms have been proposed for tBHQ-mediated phase II enzyme induction in mammals, for example, via ROS-mediated dissociation of Nrf2-Keap1, Nrf2 stabilization, mitogen-activated protein kinase pathway activation, phosphatidylinositol 3-kinase/Akt activation, or a combination of these (32). We found that incubating cells with tBHQ did not lead to intracellular \( \text{H}_2\text{O}_2 \) accumulation (Fig. 6A). Thus, we postulated that the PcGST1 gene was induced by oxidative intermediates derived from tBHQ, such as phenoxyl free radicals (33), rather than ROS generated during tBHQ metabolism. This hypothesis also was supported by the finding that \( \text{H}_2\text{O}_2 \) treatment only weakly induced PcGST1 (Fig. 6B). However, the mechanisms by which PcGST1 is induced by nickel exposure remain to be elucidated.

The 5′-flanking region of the PcGST1 gene isolated from *P. caudatum* genomic DNA was capable of inducing MpLuc1 luciferase transcription in transformed *Paramecium* organisms incubated with NiSO4. This finding indicated that the ~0.42 kb of the PcGST1 promoter region contains a putative nickel-responsive element, although little is known about any gene regulatory element in paramecia. The kinetics of MpLuc1 mRNA expression and luciferase activity induced by nickel in transformant cells apparently differed (Fig. 7D and E). This delayed induction of luciferase activity relative to that of mRNA expression might be due to protein synthesis and proper MpLuc1 folding. The kinetics of MpLuc1 mRNA expression driven by the PcGST1 promoter (Fig. 7E) and endogenous PcGST1 expression (Fig. 5F) also differed; the former sharply declined on day 3 after nickel exposure. Differences in the expression mechanism of each gene product might explain this discrepancy. First, the reporter construct might not have the cis- and/or trans-regulatory element required to maintain long-term MpLuc1 mRNA expression. These elements might be located at a distant region extending upstream or downstream of the PcGST1 gene. Second, the reporter construct might lack epigenetic regulation, such as histone modifications, for long-term mRNA expression. Furthermore, the mRNA sequences of MpLuc1 and PcGST1 fundamentally differ, which might result in different mRNA stability and expression profiles.

Importantly, we have shown that luciferase activity as a reporter could serve for gene promoter activity in paramecia. MpLuc1, like commercially available *Gaussia* and *Metridia longa* luciferases, is a secreted luciferase isolated from marine plankton. The secretion of planktonic luciferases into the extracellular milieu might require the initial direction of these proteins to the endoplasmic reticulum by signal sequences at their N termini. Here, we found that MpLuc1 was not secreted into the culture media, whereas an intracellular fraction exhibited substantial luciferase activity (data not shown). This finding indicated that the signal sequence encoded by MpLuc1 does not function in signal-dependent secretion in *Paramecium*. Thus, the reporter system...
using MpLuc1 can serve as a highly sensitive and efficient nonsecreted luciferase assay in *Paramecium*. We plan to utilize this reporter system to further elucidate the mechanisms of *Pc* GST1 and NC16 gene regulation in nickel-treated paramecia.

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**FIG 7** Analysis of *PcGST1* gene promoter. (A) Putative promoter sequence of the *PcGST1* gene. Canonical TATA binding boxes are outlined. The bent arrow indicates the putative transcription initiation point. (B) Map of expression vector pGT1-MpLuc1H, carrying the planktonic luciferase gene (MpLuc1) downstream of the SpeI-EcoRI fragment of the *PcGST1* gene promoter. Telomere sequences were designed to stabilize a linearized vector in the macronucleus of *P. caudatum*. (C) Schema of pGT1-MpLuc1H transformant clones incubated with (Ni⁺) or without (Ni⁻) 10 μM NiSO₄. Control (Ni⁻) cells were cultured in regular medium at the same time as Ni⁺ cells, prep., preparation. (D) Relative luciferase activities were measured in pGT1-MpLuc1H transformant clones at 1, 3, and 6 days after treatment with or without 10 μM NiSO₄. Values represent mean fold induction determined from triplicate measurements. The activity of untreated cells is defined as 1.0. (E) Induction kinetics of MpLuc1 mRNA in 3 transformant clones after treatment with (Ni⁺) or without (Ni⁻) 10 μM NiSO₄. Values obtained for MpLuc1 mRNA were normalized to those for *P. caudatum* α-tubulin mRNA.

**FIG 7A** A schematic of the *PcGST1* gene promoter region showing the putative promoter sequence. The bent arrow indicates the putative transcription initiation point.

**FIG 7B** Diagram of the expression vector pGT1-MpLuc1H, which contains the *PcGST1* gene promoter region downstream of the MpLuc1 luciferase gene.

**FIG 7C** Time-course analysis of luciferase activity in *Paramecium* cells treated with NiSO₄.

**FIG 7D** Graph showing the fold induction of luciferase activity in *Paramecium* cells over different induction periods.

**FIG 7E** Graph depicting the induction kinetics of MpLuc1 mRNA in *Paramecium* cells treated with NiSO₄.
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