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Mercury Inactivates Transcription and the Generalized Transcription Factor TFB in the Archaeon *Sulfolobus solfataricus*

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Mercury has a long history as an antimicrobial agent effective against eukaryotic and prokaryotic organisms. Despite its prolonged use, the basis for mercury toxicity in prokaryotes is not well understood. Archaea, like bacteria, are prokaryotes but they use a simplified version of the eukaryotic transcription apparatus. This study examined the mechanism of mercury toxicity to the archaeal prokaryote *Sulfolobus solfataricus*. In vivo challenge with mercuric chloride instantaneously blocked cell division, eliciting a cytostatic response at submicromolar concentrations and a cytocidal response at micromolar concentrations. The cytostatic response was accompanied by a 70% reduction in bulk RNA synthesis and elevated rates of degradation of several transcripts, including *tfb-1*, *tfb-2*, and *lacS*. Whole-cell extracts prepared from mercuric chloride-treated cells or from cell extracts treated in vitro failed to support in vitro transcription of 16S rRNAp and *lacSp* promoters. Extract-mixing experiments with treated and untreated extracts excluded the occurrence of negative-acting factors in the mercury-treated cell extracts. Addition of transcription factor B (TFB), a general transcription factor homolog of eukaryotic TFIIB, to mercury-treated cell extracts restored >50% of in vitro transcription activity. Consistent with this finding, mercuric ion treatment of TFB in vitro inactivated its ability to restore the in vitro transcription activity of TFB-immunodepleted cell extracts. These findings indicate that the toxicity of mercuric ion in *S. solfataricus* is in part the consequence of transcription inhibition due to TFB-1 inactivation.

The heavy metal mercury has been widely used as an antimicrobial agent for treatment of bacterial and fungal infections. At low levels, it is ubiquitous in the environment due to degassing of the earth's surface, while contamination by human activities, including its use in medicine and industry, have resulted in more concentrated occurrences (13). Mercury is a redox-active metal that depletes cellular antioxidants, particularly thiol-containing antioxidants, and enzymes in higher animals (15). Once absorbed into cells, both inorganic and organic types of mercury covalently bond with cysteine residues in proteins and small molecules. Although resistance to mercury has been intensively studied in bacteria, the mechanism of toxicity remains unclear. For example, RNA and protein syntheses in vivo have been reported to be inhibited by $HgCl_2$ (8), but in vitro analysis failed to detect an effect on general bacterial transcription (42). Additional support for the notion that mercury toxicity must be an important evolutionary force derives from the widespread phylogenetic and geographic distribution of mercury resistance genes. In contrast to the situation in bacteria, mercury is known to inhibit transcription in eukaryotes, reflecting action against a limited portion of the transcription apparatus. Immunofluorescence microscopy indicated that $HgCl_2$ blocked the synthesis of rRNA by RNA polymerase I (Pol I), whereas the activity of Pol II remained relatively unchanged (11). Radiolabeling experiments also showed that methylmercury treatment of isolated HeLa cell nuclei inhibited DNA and alpha-amanitin-resistant RNA synthesis catalyzed by Pol I and Pol III (16).

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Prokaryotes comprise bacteria and archaea (45, 46). While archaeal hyperthermophiles, extreme halophiles, and methanogens dominated early studies, more recently, uncultivated microbial biodiversity studies have revealed that nonextremophilic archaea are widespread in the environment (14). Archaeal metabolic genes show similarity with those of bacterial prokaryotes; however, biochemical and genomic studies indicate that they use mechanisms like those of eukaryotes for many subcellular processes, including transcription (7). The archaeal general transcription apparatus is homologous to the eukaryotic Pol II system but is simpler (25, 27, 41). Archaea contain a single RNA polymerase composed of 12 subunits orthologous to those in eukaryotes (25). Archaeal promoters have architecture similar to that of eukaryotic Pol II promoters, consisting of a TATA box located 26 nucleotides upstream from the transcription start site (36). Archaea also contain generalized transcription factors that are orthologous to the eukaryotic TATA box-binding protein (TBP) and transcription factor II B (TFB) for archaea (32, 33, 44).

There are no reports yet on the effect of mercury on archaea. Methanogenic archaea, however, are known to produce volatile methyl and hydride derivatives of metals and metalloids (30). *Sulfolobus solfataricus* is a hyperthermophile and a member of the *Crenarchaeota*, one of two major subdivisions of cultivated archaea. It is aerobic and easy to grow in the laboratory and has a sequenced genome (40). Antimicrobials effective against *S. solfataricus* are rare, which reflects the lack of suitable targets rather than the extremity of its niche. The limited examples include archaeon-specific microcins (20), actinomycin D (5), the hypusination inhibitor N1-guanyl-1,7-diaminoheptane (22), and hygromycin B (10). To clarify the biological significance of the heavy metal mercury for archaea, this

study examined the molecular mechanism of toxicity using *S. solfataricus* as a model organism.

MATERIALS AND METHODS

Archaeal cultivation. *S. solfataricus* (38) was cultivated in batch cultures, as described previously (19, 39). Cells were grown at 80°C in the medium of Allen (1) as modified by Brock et al. (9) at a pH of 3.0 in 250-ml screw-cap flasks. Fifty-milliliter volumes of medium were supplemented with 0.2% (wt/vol) sucrose as the sole carbon and energy source. The cells were aerated by vigorous shaking in orbital shakers containing glycerol to maintain the temperature. Growth was monitored at a wavelength of 540 nm using a Cary 50 Bio UV-visible spectrophotometer (Varian). When the effect of mercuric ion was investigated, cells were treated with various concentrations of $HgCl_2$ (Sigma) from a freshly made 10 mM stock for the times indicated in the figure legends.

Molecular biology methods. Genomic DNA from *S. solfataricus* was isolated as described previously (47). Recombinant molecular biology procedures, including DNA cloning, plasmid transformation, and all manipulations of RNA, were performed as described previously (39). DNA and RNA concentrations were measured using a DyNA Quant 200 fluorometer (Hoefer) and a Genesys 2 UV–visible-light spectrophotometer (Spectronics), respectively.

Bulk RNA synthesis. Measurement of bulk RNA synthesis was performed essentially as described previously (5) with the following modifications. Cultures were grown to early exponential phase, equivalent to a cell density of 6.8×10^7/ml (optical density at 540 nm [OD_{540}], 0.17) in 2× Brock salts; 250-ml volumes were transferred to capped polypropylene microcentrifuge tubes and equilibrated for 5 min at 75°C. $HgCl_2$ (0.3 μM) was added 10 min after the addition of radiolabeled uracil (5,6-^{3}H; 33.0 Ci/mmol; Perkin-Elmer) to a final concentration of 105 μCi/ml. Samples (10 μl) were collected in duplicate and transferred to tubes containing unlabeled cells (4×10^8 cells/ml) added to promote centrifugal recovery of 11.5% (wt/vol) trichloroacetic acid (TCA) precipitates. The radioactivity of pellets from TCA precipitation was measured in an LS 1701 liquid scintillation system (Beckman) using an open window.

Northern blot analysis. *S. solfataricus* total RNA was extracted as described previously (12) from wet cells obtained by filtration of early-exponential-phase cultures (OD_{540}, 0.1) at the appropriate times. Electrophoresis of RNA, RNA transfer, and generation of *lacS* antisense riboprobes were performed as described previously (19, 39). 7S RNA, *tfb-1*, and *tfb-2* antisense riboprobes were prepared as described previously (5). Northern hybridization with riboprobes was performed at 55°C with 50% (vol/vol) formamide. RNAs were detected by autoradiography on X-OMAT AR film (Kodak). Digital images were acquired using Gel Documentation System GDS7600 (UVP). Scanning densitometry of images was performed using GelBase-Pro software (UVP).

Preparation of cell extracts and in vitro transcription. All *S. solfataricus* cell extracts were prepared from cells harvested from cultures in mid-exponential phase having an OD_{540} of 0.5. Cell extracts adjusted to either pH 6.0 or 8.0 were prepared as described previously (5, 21). All reagents and plasticware used for preparation of cell extracts were made RNase free by treatment with diethylpyrocarbonate. The extraction buffer for cell extracts at pH 8.0 consisted of 50 mM Tris-Cl, pH 8.0–15 mM $MgCl_2$–1 mM EDTA, pH 8.0–1 mM dithiothreitol (DTT), while those for cell extracts at pH 6.0 consisted of 50 mM MES (morpholineethanesulfonic acid), pH 6.0–15 mM $MgCl_2$–1 mM EDTA, pH 8.0–1.0 mM DTT. Mercury-treated cell extracts were prepared by adding 1.5 μM $HgCl_2$ to cells in mid-exponential-phase growth 30 min prior to harvest. Protein concentrations were measured using a BCA Protein Assay Reagent kit (Pierce).

In vitro transcription templates consisted of linearized *S. solfataricus* promoters cloned in pUC19. The 16S rRNAp template consisted of a 177-bp PCR product corresponding to −130 to +47 relative to the transcription start site of 16S rRNA (34) obtained with primers 5′-GAATTCAAGTAACTGAAACTAG CC-3′ and 5′-CCCCTCCACGAATAAGCTTTACC-3′ and inserted at the EcoRI and HindIII sites, creating the plasmid pPB767. The *lacSp* template was prepared as described previously (5). Both templates were linearized with AflIII. In vitro transcription reactions were conducted using either control cell extract, $HgCl_2$-treated cell extract, or TFB-1-immunodepleted cell extract. The amounts of pH 8.0 and pH 6.0 cell extracts used for in vitro transcription reactions were 50 and 400 μg, respectively. The reactions were carried out at 60°C for 10 min for pH 8.0 cell extract, as described previously (21), and at 75°C for 10 min for pH 6.0 cell extract, as described previously (5). RNAs from in vitro transcription reactions were subjected to primer extension by generating cDNA using avian myeloblastosis virus reverse transcriptase (U.S. Biochemicals) or SuperScript II (Invitrogen) and [γ-^{32}P]ATP-labeled M13 primer (5′-AGCGGATAACAATTT CACACAGGAAACAGC-3′), as described previously(5). The products of reverse transcription were electrophoresed on 8% denaturing polyacrylamide gel alongside a DNA sequence ladder prepared using the primer extension primer. Plasmid DNA inserts were sequenced using a T7 Sequenase version 2.0 DNA-sequencing kit (U.S. Biochemicals). M13 primer was used to generate the sequencing ladder for mapping the start site of transcription of both 16S rRNAp and *lacSp*.

Preparation of recombinant TFB-1. The *S. solfataricus tfb-1* open reading frame (*SSO0446*) was amplified from the genome using *Taq* polymerase (Invitrogen) and oligonucleotide primers (5′-TATATATAATTCATATGTTGTATTT GTCTGAAG-3′ and 5′-GAATCCAAGCTTCTAGTTATTGAGTAGGTATT GA-3′). The PCR amplicon was digested with NdeI and HindIII and ligated into pET28b (Novagen), creating plasmid pPB929. *Escherichia coli* strain BL21 Rosetta (Novagen) was transformed with pPB929 for expression of recombinant N-terminally hexahistidine-tagged TFB-1. Cells were grown to an OD_{600} of 0.1, and expression was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), followed by 3 h of additional incubation. The cells were harvested by centrifugation, resuspended in 50 mM Tris-Cl, pH 7.8–500 mM KCl–10% glycerol–10 mM β-mercaptoethanol (buffer A) and lysed by sonication, followed by centrifugation. The soluble extract was heated at 80°C for 20 min and centrifuged at 6,000 × *g* for 20 min to remove denatured proteins. TFB-1 was purified as described previously with modifications (24). All glassware and reagents used for TFB-1 purification were made RNase free by baking them at 180°C for 4 h or by treatment with diethyl pyrocarbonate. The supernatant was applied to a 1.0-ml Ni^{2+}-nitrilotriacetic acid agarose bead column (Qiagen). The column was washed with 10 column volumes of buffer A plus 20 mM imidazole, followed by a wash with 2 column volumes of buffer A plus 50 mM imidazole. TFB-1 eluted in 2 column volumes of buffer A plus 100 mM imidazole, as indicated by analysis of fractions on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein was concentrated and buffer was exchanged using Centricon YM10 (Amicon) filters with 50 mM Tris-Cl, pH 8.0–25 mM $MgCl_2$–1 mM EDTA–1 mM DTT–20% glycerol prior to storage of aliquots at −80°C.

Preparation of TFB-immunodepleted cell extracts. Polyclonal antiserum against TFB-1 was generated in mice as described previously (6). Preimmune serum from each mouse was checked individually for the absence of any cross-reacting antibodies prior to immunization. Each mouse was injected with 1.0 μg of recombinant histidine-tagged TFB-1 following a standard immunization protocol. TFB-1-immunodepleted extracts were prepared as described previously (34) with some modifications. Briefly, 100 μl of protein A-Sepharose beads (Sigma) were washed three times with phosphate-buffered saline, and 40 μl of pooled mouse sera was added and incubated at 37°C for 1 h with gentle shaking. The beads were washed again with phosphate-buffered saline and incubated with 100 μl (1.5 mg of total protein) of pH 8.0 extract and incubated for an additional 3 h at 37°C with gentle shaking. The beads were removed by centrifugation.

Western blot analysis. Proteins were resolved on 16% polyacrylamide SDS-Tricine gels as described previously (26) using Bio-Rad protein mini-gel rigs and prestained protein standards (Invitrogen). Prior to electrophoresis, samples were adjusted to 2% (wt/vol) SDS–250 mM β-mercaptoethanol and boiled for 10 min. The protein gels were stained with Coomassie blue R250 to visualize the protein. Western blots were prepared as described previously (18). The secondary antibodies were goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (Zymed). Primary and secondary antibodies were used at 1:1,000 dilutions. Blots were developed using an enhanced-chemiluminescence detection reagent (Amersham Biosciences).

RESULTS

Growth inhibition of *S. solfataricus* by mercury. The in vivo toxicity of $HgCl_2$ in archaea has not been established; therefore, the MIC for *S. solfataricus* was determined. In order to avoid titration of $HgCl_2$ by medium components present in complex media, a defined medium was employed. Cells at early exponential phase (OD_{540}, 0.1) were treated with 0.3, 0.5, and 0.8 μM $HgCl_2$, and the cell density was monitored for an additional 45 h after $HgCl_2$ challenge. The addition of $HgCl_2$ resulted in an immediate, dose-dependent inhibition of growth (Fig. 1). Control untreated cells grew at a normal rate with a generation time of 8 to 9 h in this medium. A concentration of 0.3 μM $HgCl_2$ was found to be sublethal and cytostatic for cells, inhibiting growth for 2 h with no significant loss of cell

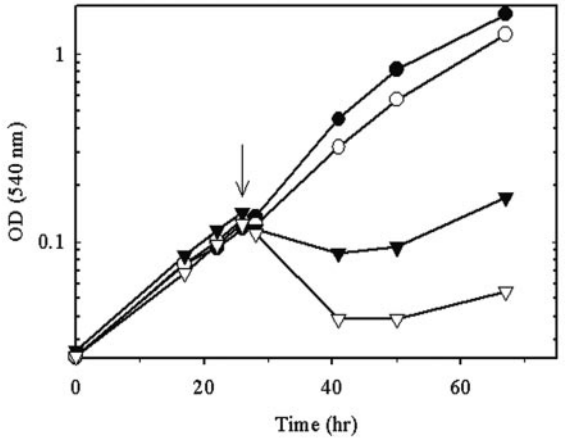

FIG. 1. Growth of *Sulfolobus* in the presence of $HgCl_2$. Cells were treated at the time indicated by the arrow with various concentrations of $HgCl_2$. Solid circles, control (no $HgCl_2$ added); open circles, 0.3 μM $HgCl_2$; solid inverted triangles, 0.5 μM $HgCl_2$; open inverted triangles, 0.8 μM $HgCl_2$.

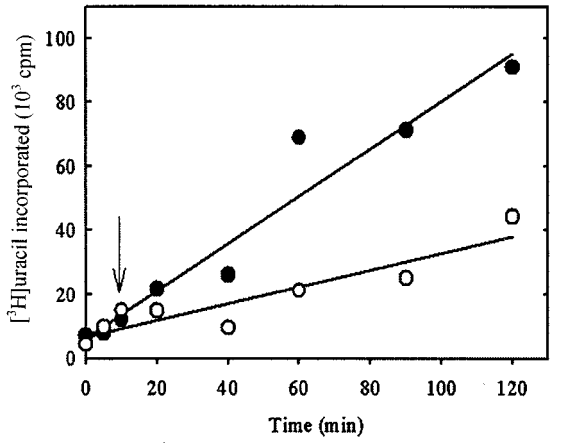

FIG. 2. Incorporation of radiolabeled uracil by intact *Sulfolobus* cells in the presence of $HgCl_2$. Shown is [^{3}H]uracil incorporation into bulk RNA following treatment with a sublethal concentration of 0.3 μM $HgCl_2$ (open circles) or with no treatment (solid circles). $HgCl_2$ was added to the cells in early exponential phase 10 min after [^{3}H]uracil was added to the cells. The values are averages of replicate samples. The arrow indicates the time of $HgCl_2$ addition.

density. Higher concentrations of 0.5 and 0.8 μM, however, were cytocidal and resulted in a growth lag lasting several hours, followed by a reduction in OD and loss of culturability. Similar results were obtained for cells in mid-exponential phase (OD_{540}, 0.5). The sublethal $HgCl_2$ concentration at this increased cell density was 1.5 μM, and it produced a growth lag lasting 4 h. When the organism dies, cell lysis ensues, reflecting energy deprivation and its acidophilic lifestyle. At higher concentrations of mercury, there is cell death and subsequent outgrowth of survivors. All the subsequent studies in this work were done at 0.3 μM $HgCl_2$.

Inhibition of bulk RNA synthesis by mercury exposure. Eukaryotic transcription is inhibited by mercury compounds. Since the archaeal transcription system shows significant homology, it was expected that transcription of *S. solfataricus* would also be a target of mercury action. Incorporation of [^{3}H]uracil into TCA-precipitable material is a general method used to determine the in vivo rate of total RNA synthesis (31) and has been used previously in *S. solfataricus* (5). To examine total RNA synthesis during $HgCl_2$ treatment, cells in early exponential phase were treated with a sublethal concentration (0.3 μM) of $HgCl_2$, and the extent of radiolabeled uracil incorporated over a 2-h period was recorded. The rate of uracil incorporation was reduced immediately following $HgCl_2$ addition and by 70% during the period examined (Fig. 2).

Inhibition of mRNA synthesis and accelerated mRNA decay. Reduced radiolabeled-uracil incorporation could result from inhibition of RNA synthesis or merely inhibition of [^{3}H]uracil uptake or its phosphorylation. To distinguish among these possibilities, Northern blot analysis was used to test whether mercury treatment depleted the abundance of specific cellular mRNAs. The rates of decay for three *S. solfataricus* transcripts were examined, including *lacS* (β-glycosidase), a gene involved in polysaccharide hydrolysis (19), and both paralogs of the archaeal basal transcription factor, *tfb-1* (35) and *tfb-2* (40). Cells in early exponential growth phase were treated with 0.3 μM $HgCl_2$, and RNA was isolated for 30 min at the appropriate times and then analyzed for mRNA content (Fig. 3). Transcript abundances were determined by normalizing band intensities determined using transmittance densitometry for each mRNA for each sample time to those of 7S RNA present in the same lane, as described previously (5). Within 30 min after $HgCl_2$ treatment, the transcript abundances of *lacS*, *tfb-1*, and *tfb-2* were reduced to 55.41, <5, and 69.85%, respectively, of pretreatment levels.

Inhibition of transcription in vitro by mercury treatment. To clarify the mechanistic basis for mercury-mediated in vivo inhibition of bulk RNA and mRNA synthesis, in vitro transcription assays were employed using two distinct approaches.

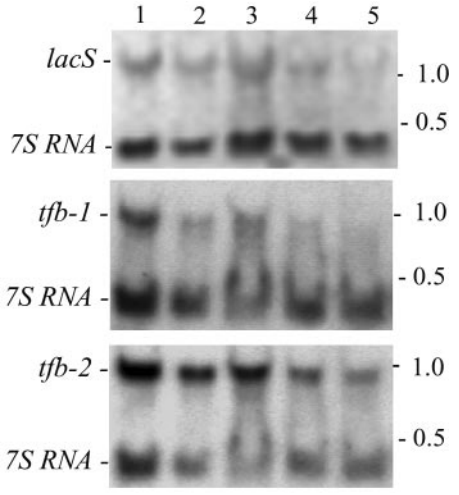

FIG. 3. Northern blot analysis of total RNA from $HgCl_2$-treated *Sulfolobus* cells. Shown are autoradiograms of blots with total cellular RNA of *S. solfataricus*. RNA was isolated from cells at an OD_{540} of 0.1. Lane 1, 5 μg of total RNA isolated prior to $HgCl_2$ addition; lanes 2 to 5, 5.0 μg of RNA loaded per lane 5, 10, 15, and 30 min, respectively, after treatment with 0.3 μM $HgCl_2$. Molecular weight markers (in thousands) are indicated on the right.

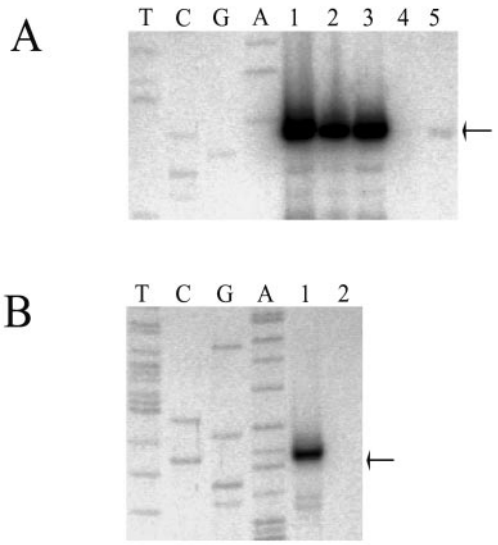

FIG. 4. In vitro transcription using $HgCl_2$-treated cell extracts. (A) Primer extension analysis of RNA from in vitro transcription of 16S rRNAp at pH 6.0. The samples loaded on the gel were from reactions using 400 or 200 μg of untreated cell extract (lanes 1 and 2), a combined reaction using 200 μg each of untreated and $HgCl_2$-treated cell extracts (lane 3), and reactions using 200 or 400 μg of $HgCl_2$ (0.3 μM)-treated cell extracts (lanes 4 and 5). (B) Primer extension analysis of RNA from in vitro transcription of *lacSp* at pH 6.0. The samples loaded on the gel were from reactions using 400 μg of untreated or $HgCl_2$ (0.3 μM)-treated cell extracts (lanes 1 and 2, respectively). Both panels include dideoxy sequencing reactions; the arrows indicate the primer extension products.

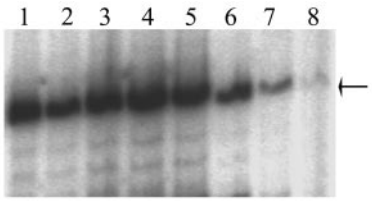

FIG. 5. In vitro transcription of 16S rRNAp with exogenous $HgCl_2$ addition. Shown is primer extension analysis of RNA from in vitro transcription of 16S rRNAp at 75°C for 10 min using 400 μg of cell extract at pH 6.0. Lanes 2 to 8, products of transcription using cell extracts to which 0.0, 0.003, 0.03, 0.3, 3.0, 30, and 200 μM $HgCl_2$, respectively, was added and incubated at 75°C for 15 min prior to use for in vitro transcription assays. Lane 1 contains products of transcription using cell extract with no pretreatment at 75°C. The products of primer extension are indicated by the arrow.

In the first approach, the ability of cell extracts prepared from $HgCl_2$-treated cells to support in vitro transcription was examined using two promoters, 16S rRNAp and *lacSp*. The second approach investigated the effect of exogenous $HgCl_2$ addition to extracts prepared from untreated cells. Earlier studies (5) had shown that improved *lacSp* transcription occurred when transcription assays were conducted at pH 6.0 and 75°C rather than at pH 8.0 and 60°C as originally described for promoters of untranslated genes (21). Consequently, *S. solfataricus* cell extracts were prepared at both pH values according to the promoter under examination. 16S rRNAp and *lacSp* were cloned into plasmid vectors and tested for the ability to direct transcription using cell extracts made from cells growing in the presence of sublethal concentrations of $HgCl_2$. In each case, the in vitro transcription product was detected by primer extension analysis using an oligonucleotide complementary to a plasmid sequence located 3′ to the promoter insert. Although *lacSp* was active at pH 6.0, 16S rRNAp was active at both pH 6.0 and 8.0.

Transcription of 16S rRNAp was strongly inhibited using $HgCl_2$-treated cell extracts at pH 6.0 compared to the level of transcription produced using control untreated cell extracts (Fig. 4A, lanes 1 and 5). Transcription of *lacSp* was also strongly inhibited using $HgCl_2$-treated cell extracts (Fig. 4B), indicating that the inhibitory effect was not promoter dependent. Mercury inactivation of transcription assay cell extracts could result from the creation of a toxic factor or merely passively inactivate an essential transcription component. To distinguish between these possibilities, control untreated cell extracts and $HgCl_2$-treated cell extracts were mixed in equal amounts and used in transcription assays. Transcription of 16S rRNAp using mixed extracts produced primer extension products in direct proportion to the amount of untreated extract added to the transcription reaction mixture (Fig. 4A, lanes 2, 3, and 4). This excluded the presence of a negatively acting factor in the mercury-treated extracts and instead indicated that $HgCl_2$ inactivated an essential transcription component. Since these extracts had no negative effect when mixed with active extracts, residual mercury was not present.

In the second approach, an untreated cell extract was preincubated at 75°C for 15 min with a wide range of amounts of $HgCl_2$ before in vitro transcription assays were conducted. Transcription of 16S rRNAp was inhibited by this treatment at 30 μM $HgCl_2$ (Fig. 5). The dose-response relationship between the degree of inhibition of transcription and the amount of $HgCl_2$ used was almost 100-fold higher than that observed for the amount of $HgCl_2$ required in vivo to produce a similar degree of inactivation of transcription. This difference is likely due to the presence of the reductant DTT, which may titrate added $HgCl_2$, in the in vitro transcription buffer. Since the addition of $HgCl_2$ to in vitro transcription reaction mixtures inactivated transcription, $HgCl_2$ appeared to interact directly with a component of the archaeal transcription apparatus. While the role of reductant on transcription is of general interest, it was not further examined, as the in vitro mercury addition experiment was conducted merely to provide additional confirmation of TFB-1 inactivation.

TFB-1 restores in vitro transcription activity. Heavy-metal targeting of eukaryotic zinc finger proteins is selective, since only Pol I- and Pol III-mediated transcription, but not Pol II-mediated transcription, was inhibited (11, 16). The *S. solfataricus* genome encodes two paralogs of eukaryotic TFIIB. One of these, TFB-1, has been studied extensively and has been shown to have a zinc finger motif at its N-terminal end (4, 33, 34). Unlike eukaryotes, archaea contain a single RNA polymerase; therefore, the inhibition of transcription by mercury in *S. solfataricus* must target some aspect of this enzyme. However, since *S. solfataricus* TFB-1 does not have the typical metal-coordinating sequence in its zinc finger motif, it was of interest to determine if $HgCl_2$ targets TFB-1. To test this possibility, recombinant hexahistidine-tagged *S. solfataricus*

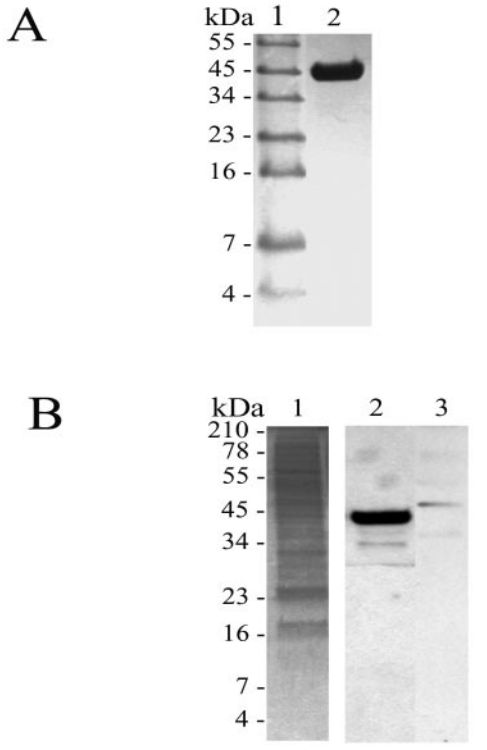

FIG. 6. Purification of recombinant TFB-1 and immunodepletion of *Sulfolobus* cell extracts. (A) Purity of recombinant N-terminal hexahistidine-tagged TFB-1 determined by Coomassie blue R250 staining of an SDS–16% Tricine PAGE gel (lane 2) and molecular mass standards (lane 1). (B) Production of TFB-1-specific polyclonal antisera. Coomassie blue R250-stained SDS–16% Tricine PAGE of 15 μg of *Sulfolobus* cell extract (lane 1) and chemiluminescent Western blot of the same material before (lane 2) and after (lane 3) TFB-1 immunodepletion.

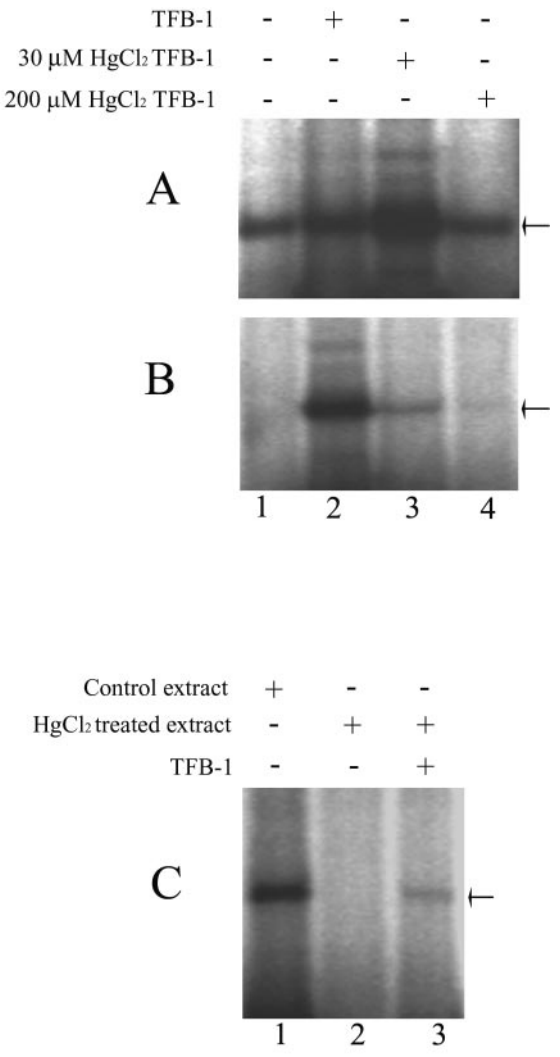

FIG. 7. TFB is the target of mercury action. Shown are primer extension analysis of RNA from in vitro transcription of 16S rRNAp at 60°C for 10 min using 50 μg of cell extract at pH 8.0 and the effect of TFB addition. (A) normal (undepleted) extracts. (B) TFB-depleted extracts. Lanes 1, no addition; lanes 2, addition of 40 nM TFB; lanes 3 and 4, addition of 40 nM TFB treated with 30 or 200 μM mercuric chloride, respectively. +, present; −, absent. (C) Lane 1, untreated cell extract; lane 2, mercuric chloride-treated extract; lane 3, addition of 40 nM TFB to $HgCl_2$-treated extract.

TFB-1 was prepared, along with specific anti-TFB-1 polyclonal antibodies and TFB-1-immunodepleted *S. solfataricus* cell extracts, to validate recombinant TFB-1 activity. The *tfb-1* open reading frame was amplified by PCR and cloned into the pET28b expression vector. The natural GTG start codon was replaced with an artificial ATG start codon to facilitate expression in *E. coli*. Bulk *E. coli* proteins were removed by heat fractionation of cell extracts prior to fractionation over Ni^{2+}-nitrilotriacetic acid agarose. The purity of recombinant TFB-1 was checked by examination of Coomassie blue-stained SDS-Tricine PAGE gels. The mass of the hexahistine-tagged recombinant protein appeared to be slightly larger than the predicted value of 35.47 kDa (Fig. 6A). Mouse polyclonal antibodies raised against this protein were found to be specific, and they could detect the protein in crude *S. solfataricus* cell extracts (Fig. 6B, lane 2). TFB-1-immunodepleted *S. solfataricus* cell extracts were prepared using the anti-TFB-1 polyclonal antibodies (Fig. 6B, lane 3).

The activity of recombinant TFB-1 was confirmed by demonstrating its ability to restore in vitro transcription of the TFB-1-immunodepleted *S. solfataricus* cell extract. TFB-1-immunodepleted cell extracts failed to support transcription of 16S rRNAp (Fig. 7B, lane 1), while addition of 40 nM TFB-1 to these extracts restored transcription activity (Fig. 7B, lane 2). The effect of $HgCl_2$ treatment of TFB-1 was then assessed by exposing the protein to either 30 or 200 μM $HgCl_2$ for 15 min at 60°C. These concentrations were similar to those required to inhibit transcription by exogenous addition of $HgCl_2$ in vitro to untreated cell extracts. TFB-1 treated with $HgCl_2$ in vitro failed to restore transcription (Fig. 7B, lanes 3 and 4). Treatment with 30 μM $HgCl_2$ did not inactivate TFB-1 completely, as some extension product was evident. The reduced in vitro potency of $HgCl_2$ may reflect the presence of reductant in the buffer for storage of purified recombinant TFB-1. TFB-1 addition to cell extracts prepared from $HgCl_2$-treated cells significantly restored transcription, reaching 62.5% ± 1% of pretreatment levels (Fig. 7C, lane 3). In contrast, extracts not

containing added TFB-1 failed to support transcription at all (Fig. 7C, lane 2). These results demonstrate that TFB-1 is a target of action of $HgCl_2$. Since restoration of transcription activity by TFB-1 addition was not complete, it remains possible that $HgCl_2$ acts on additional targets of the archaeal transcription apparatus. Since archaeal in vitro transcription has been shown to require only three proteins, viz., TBP, TFB, and RNAP, no other thiol-active protein is likely to play a role in the inhibition of transcription.

DISCUSSION

In this work, mercuric ion derived from $HgCl_2$ was found to inhibit archaeal transcription, and TFB-1 was one of the protein targets of mercuric ion action. In order to avoid detecting nonspecific toxic effects of mercury, we studied processes occurring at sublethal nanomolar concentrations of the metal. Mercury likely produces nonspecific effects at higher concentrations, in the millimolar range. Since not all zinc finger proteins are targets of mercury action and each zinc finger protein has a unique sensitivity to metal (2), it was of interest to find that *S. solfataricus* TFB-1 is a target of $HgCl_2$ action, because the protein has an atypical metal-coordinating sequence consisting of $ThrX_2CysX_{15}CysX_2Thr$. Both of the eukaryotic transcription factors TFIIIA and Sp1—assisting Pol III and Pol II, respectively, and known targets of mercury—have the canonical Cys_2His_2 zinc finger motif (37).

This study shows for the first time that the archaeal Pol II-type general transcription factor TFB can serve as a target of mercury action. This finding may have implications for the eukaryotic Pol II transcription apparatus and the historical effectiveness of mercury as a fungicide. *S. solfataricus* TFB-1 is 32% identical and 56% similar to human TFIIB (33). These homologies suggest that TFB-1 must function in a manner similar to that of eukaryotic TFIIB. TFB-1 plays a vital role in the formation of the preinitiation transcription complex. Its C-terminal domain interacts with TBP and DNA and constitutes a sequence-specific contact with the TFB-responsive element (BRE) immediately upstream of the TATA box (35) that is responsible for directing transcription in the same orientation as the eukaryotic complex (3). The N-terminal domain of TFB-1, containing a zinc ribbon (48), recruits RNA polymerase (4). Thus, inactivation of TFB-1 by $HgCl_2$ must disrupt either the formation of a ternary complex or the recruitment of RNAP or both. When TFB-1 treated with $HgCl_2$ was added to untreated cell extracts, there was no competition between this TFB-1 and the native TFB-1, suggesting that $HgCl_2$-treated TFB-1 lost its capacity to bind to DNA and or interact with TBP. This suggests that $HgCl_2$ prevents the formation of the ternary complex. One possible mode of TFB-1 inactivation by $HgCl_2$ could be replacement of zinc in its zinc ribbon motif. TFB-1 also has two cysteines in its C-terminal region that could be the targets of $HgCl_2$ action. All multisubunit RNA polymerases bind zinc (29, 43), including one of the archaeal RNA polymerase subunits (28). Since restoration of transcription was not complete, RNA polymerase could be the other target. The *S. solfataricus* genome has a second gene, called *tfb-2*, annotated as a paralog of eukaryotic TFIIB (40). The sequence of TFB-2 has a zinc finger motif of the canonical $CX_2CX_nCX_2C$ type, which makes it a possible target for $HgCl_2$. Although this gene is expressed (5), its functionality has yet to be proven.

Attempts to determine the mode of TFB inactivation by $HgCl_2$ were made using the recombinant TFB preparation. Since TFB has two cysteines in its N-terminal noncanonical zinc finger motif (responsible for recruiting RNAP) and has one cysteine in the C-terminal region (the region for binding to TBP and the promoter), it was postulated that TFB inactivation could be a result of zinc release from the zinc finger motif; however, zinc release was not observed by 4-(pyridylazo)resorcinol assay (23), a method that had been used earlier for zinc finger proteins (17). It remains possible that the lack of zinc release could be due to the fact that the cysteines in the zinc finger motif were inaccessible, perhaps reflecting unusual structural stability of this thermostable protein; however, the lack of zinc release precluded definitive conclusions regarding the site of TFB inactivation.

This work demonstrates for the first time the mechanism of toxicity of a heavy metal on a member of the archaea and that this mechanism is different from that of bacteria and similar to that of eukaryotes. Although it would be of interest to discover the toxicity target of mercury in other archaea, it is known that TFB is universally conserved, and hence, it seems likely that the mechanism of mercury toxicity would also be conserved. While it is true that the redox environments of organisms differ, it is generally agreed that the cytoplasmic chemical environments of prokaryotic cells are reduced. Although there is genomic evidence that the *Crenarchaea* (to which *Sulfolobus* belongs) are richer in disulfide bonds than the *Euryarchaea* (to which methanogens belong), their transcription factors are highly conserved. Hence, $HgCl_2$, at various concentrations depending on the redox environment, will continue to have a similar effect on these proteins. Additional studies of the generality of these findings to other archaea and of the toxicity of other heavy metals to *S. solfataricus* will clarify the significance of the results presented here.

Northern blot analysis demonstrated that transcription of *tfb-1* mRNA was inhibited by $HgCl_2$ in vivo. Inactivation and subsequent depletion of *tfb-1*, and therefore TFB-1, would preclude a mechanism in which TFB-1 negatively autoregulates its own expression. In addition, rapid inhibition of in vivo transcription by submicromolar $HgCl_2$ indicates the existence of a mercury transport system in *S. solfataricus*. Curiously, there is an apparent lack of annotated bacterial mercury transport orthologs in the *S. solfataricus* genome. This may suggest that such transport proteins are unique to this organism or perhaps to archaea in general. The finding that TFB-1 is one of the targets of mercury action shows that mercury has a direct effect on mRNA synthesis and therefore on gene regulation, a process vital for growth and differentiation.

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