The *Escherichia coli* copper-responsive *copA* promoter is activated by gold*.

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Running title: Gold activation of a copper-responsive promoter.

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The *copA* gene of *E. coli* encodes a copper transporter and its promoter is normally regulated by Cu(I) ions and CueR, a MerR-like transcriptional activator. We show that CueR can also be activated by gold salts and that Cys112 and Cys120 are involved in recognition of gold, silver and copper salts. Gold activation is unaffected by copper chelating agents but is affected by general metal chelators. This is the first example of specific regulation of transcription by gold and we briefly speculate that the biological effects of gold antiarthritic drugs may be through their effects on copper management in eukaryotic systems.
There is no known natural biological function of gold or its salts, but it has biological effects. Solutions of gold chloride are toxic to bacteria (1), disimilatory Fe(III)-reducing bacteria and archaea are capable of reducing Au(III) to Au(0), precipitating the metal (2) and may be responsible in part for the formation of some gold deposits (1). Medical uses of gold have been limited largely to prosthetics and antiarthritic Au(I)-thiolate drugs, although the basis of antiarthritic action has been unclear (3). In contrast, the biological requirements for copper are quite well understood. Mammalian systems require copper for many enzymes (4). Recently, new elements of copper homeostasis in bacteria have been identified, including transporters, multicopper oxidases, and two-component and MerR-like regulators (5-9).

The *Escherichia coli* CopA protein is a Cu(I)/Ag(I)-translocating P-type ATPase involved in copper export and resistance (5). It shows similarity to copper pumps from several prokaryotic and eukaryotic sources, including 31% and 29% identity to the human Wilson’s and Menkes’ proteins, respectively. The transcription of *copA* is regulated solely by CueR (GeneBank AF318185), a copper- and silver-responsive (7,8,10) member of the MerR family of transcriptional activators (Figure 1). MerR acts as a dimer on the promoter of bacterial mercury resistance (*mer*) genes recruiting RNA polymerase to the promoter in the process (11) and it is activated by Hg(II) binding to 3 cysteine residues in a trigonal planar fashion (12). CueR activates expression of the *copA* promoter (P*copA*) upon addition of copper or silver, but not nickel, lead, cadmium, mercury or zinc salts (8). Here we show that CueR activates the P*copA* promoter in the presence of gold salts and that cysteine residues, equivalent to those in MerR, are required for activation by copper, silver and gold.
EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids - All experiments, other than the construction of mutants and some β-galactosidase assays, were carried out in E. coli strain TG1 (K-12, lac-pro supE thi hsdD5 [F’ traD36 proA<sup>B</sup> lacIq lacZ M15]). E.coli WK6 Δ(lac-proAB)galEstrA [F’ lacIq lacZΔM15 proAB<sup>B</sup>] and its mutS215:Tn10 variant were used for gapped duplex mutagenesis (13). E. coli CSH26Δ(lac-proAB)thi ara ΔrecA (14) was used in some β-galactosidase assays to show that the observed effects were not strain specific. The following plasmids have been described elsewhere: pMU2385 (15), pSU18 (16), pMUP<sub>copA</sub> (8), pSU<sub>cueR</sub> (8), pMUP<sub>merT</sub> (17) and pSU<sub>merR</sub> (17). Bacterial cells were grown at 37°C in Luria-Bertani (LB) medium or M9 medium supplemented with casamino acids and thiamine (18). For metal induction assays, sodium chloride was omitted to avoid AgCl precipitation (called SLB and SM9 media). Antibiotics were used as described earlier (17).

DNA manipulations - Standard DNA manipulations were carried out as described elsewhere (18). Oligonucleotides were synthesized by Alta Bioscience Ltd, University of Birmingham, by Gibco-BRL, UK, or by MWG Biotech, Germany. Cloned PCR products were sequenced using an PE Applied Biosystems Big Dye<sup>TM</sup> sequencing kit according to the manufacturers' instructions and analysed on an ABI 3700 sequencing apparatus.

β-galactosidase assays - E. coli cells transformed with plasmid pMUP<sub>copA</sub> (8) or pMUP<sub>merT</sub> (17) were assayed for β-galactosidase activity (14) as described previously (17). E.coli TG1 [cueR::kan] cells containing pMUP<sub>copA</sub> (8) were transformed with pSU<sub>cueR</sub>, or other pSU18 vectors containing mutant cueR, and induced overnight with 1mM isopropyl-β-D-thiogalactoside (IPTG). Cultures for metal induction experiments were
grown overnight in the required medium with antibiotic selection to maintain resident plasmids. The overnight culture was diluted 100-fold into fresh medium without antibiotics and grown to mid-exponential phase. Metal induction was performed for 2 h at 37°C. Assays were performed in triplicate and repeated several times.

The direct effect of gold salts on $\beta$-galactosidase was tested by a standard enzyme assay using 96 units of $\beta$-galactosidase in 1 ml Z-buffer (14) in the presence of 0, 50 or 150 $\mu$M AuCl$_3$. Reactions were started with 0.1ml 4 mg ml$^{-1}$ o-nitrophenyl-$\beta$-D-galactoside and stopped with 0.5 ml 1M Na$_2$CO$_3$. Z-buffer is reducing, containing 50mM 2-mercaptoethanol. No inhibition by gold was seen.

**Mutagenesis of cueR** - The cueR mutants C112S; C120S; C129S,C130S and H131N,H132N were generated by the gapped duplex method (13) using 24 to 30-mer oligonucleotides containing the 1 or 2 mismatched bases. Site-directed mutagenesis by PCR overlap extension (19), was used in later stages for creating H76N, H94N using primer pairs containing complementary mutating nucleotides, and flanking forward and reverse primers containing EcoRI and BamHI sites, on pSU18cueR as template. Truncated cueR mutants were generated by PCR using a downstream primer containing a stop codon and a BamHI site, with a flanking upstream primer containing an EcoRI site. PCR products were digested with EcoRI and BamHI and cloned into EcoRI-BamHI-cut pSU18, creating pSUcueR-mutant. All mutated DNA fragments were fully sequenced to confirm the required mutation and screen against undesired mutations.

**Generation and assay of Cu(I)** - Solutions of cuprous ions, Cu(I) were made freshly on a daily basis as described elsewhere (20).
RESULTS

*Gold specifically induces activation of the PcopA promoter* - The dynamic response of the copA promoter was tested at a range of copper, silver and gold ion concentrations in minimal medium (Figure 2A) and maximal responses were observed with additions of 100µM CuSO₄, 2.5µM AgNO₃ or 25µM AuCl₃ for mid-exponential phase cells. Gold toxicity caused a reduction in cell growth at higher metal concentrations. Similar relative concentrations for maximal induction were observed at different growth stages, although the absolute concentrations differed, presumably due to the production of metal-chelating extracellular material. AuCl₃ does not inhibit β-galactosidase in vitro (data not shown), so reduction in induction at high gold concentrations is not due to direct effects on the reporter enzyme activity. The values of half maximal induction, calculated from the induction profiles were: for copper ~21µM, for gold ~15.2µM and for silver ~1.9µM; these values include differences in access of the metal to the regulator (sequestration, uptake and reduction) as well as the induction *per se*. The values for silver and copper agree with those determined previously (8). The increases in induction with increasing metal ion concentrations are different for the three metals.

Induction by gold of PcopA was abolished in a disruptant strain, *E. coli* TG1[cueR::kanR] (8). As with previous results for copper and silver (8), metal induction of PcopA could be recovered by providing the cueR gene *in trans* (Figure 2B). Therefore, CueR activates transcription of PcopA in response to gold ions. To check that the response of CueR to gold was specific, a second member of this class of metal regulators, MerR and its promoter PmerTPAD, was tested in both *E. coli* TG1 and in *E. coli* CSH26ΔrecA (17). Induction of the mercury resistance promoter, PmerTPAD, was observed only when the merR gene was provided *in trans* and induced with 1µM HgCl₂, but was not observed after
induction with copper, silver and gold or when merR was not present, even though cueR was present on the chromosome (data not shown).

The growth response of *E. coli* W3110 ΔcopA (5) to increasing concentrations of AuCl₃ in LB medium was indistinguishable from that of the parental W3110 strain (data not shown), indicating that the CopA transporter does not confer gold resistance.

*Cysteine residues 112 and 120 are required for activation by copper, silver and gold* - The CueR protein contains several cysteine and histidine residues which are candidates for recognition and specific binding of activating metals. Cysteine residues at positions 112, 120, 129, 130 were mutated to serine and histidines at positions 76, 94, 131 and 132 were altered to asparagine. These variants of the cueR gene were expressed in pSU18 vector (16) in *E. coli* TG1[cueR::kanR](pMUPcopA). The response of the complemented *E. coli* TG1[cueR::kanR] strain to metals was more variable between experiments than when the wild type *E. coli* TG1 was used, possibly due to the variable copy number of pSU18::cueR plasmids. However, qualitatively clear results were obtained, showing that mutations C112S or C120S eliminated the response to copper, silver and gold salts (Figure 3) as did all double and truncated mutants containing one of these mutations (data not shown). Furthermore, none of these mutants responded to Pb, Hg, Ni, Cd or Co. A deletion from position 120 failed to respond to metals, while a deletion from position 128 had an attenuated response. The cysteines at positions C112 and C120 are analogous to C117 and C126 of Tn501 and Tn21 MerR, which are important for binding Hg(II) and activation of the transcription of the *mer* operon (11). Alanine A78 was replaced by cysteine to mimic the equivalent essential cysteine C82 in MerR (Figure 1), but the mutant protein did not activate transcription in response to Hg(II) in vivo (data not shown).
Double mutations H131N,H132N and, separately, C129S,C130S were generated to investigate the importance of these distal, potentially metal-binding, amino acids and were tested in the induction assay (Figure 3). The mutations H131N,H132N gave no significant change in the response to copper but gave an altered response to silver (decreased) and gold (increased) compared to the wild type. There was a slight increase of the background response without metal. The mutant C129S,C130S also showed relatively small changes in the response to copper and silver but there was a slight decrease in the response to gold. For neither mutant was the specificity of reaction with other metals altered. The retention of a response to copper, silver and gold salts, albeit altered in magnitude, by these double mutations and by the seven amino acid C-terminal deletion from G128 indicates that the full CCHH motif at positions 129-132 is not responsible for metal recognition by CueR, as we had originally hypothesised. A decrease of 30-40% but not abolition of the response to copper was found with the separate H76N and H94N mutations (data not shown) indicating that these two histidines are not essential to metal recognition.

The effect of chelators indicates that the metal responses are specific - The effect of different chelating agents on the induction of \( P_{\text{copA}} \) was tested (Figure 4). Bathocuproine disulfonate (21), cuprizone (22) and neocuproine (21,22) were used as copper chelators; EDTA was used as a non-specific metal chelator. When \( P_{\text{copA}} \) was induced in the presence of 100\( \mu \)M bathocuproine, activation by Cu(I) was reduced by 84-86% and the activation by Cu(II) decreased by 35-69%; activation by silver was reduced by 70% and activation by gold was increased by 9-34%. Activation of the mercury-resistance promoter, \( P_{\text{merTPAD}} \), in the presence of \( \text{merR} \) and 1\( \mu \)M Hg(II) showed no significant difference in the presence and absence of bathocuproine. With 100\( \mu \)M cuprizone, activation by Cu(I) was reduced 33-65% and by Cu(II) 16-46%, suggesting that cuprizone may not be specific for Cu(II), as advertised. Activation by silver increased by 29% and activation by gold increased by 42-
45%, both of which may be due to the removal of relatively more poorly-inducing copper ions by the chelator. No difference was observed for the activation of \( P_{merTPAD} \) by Hg(II).

Activation of \( P_{copA} \) was observed in the presence of the membrane-permeable copper chelator neocuproine even in the absence of metal, resulting in 8.8 fold base level increase (Figure 4). The increased induction by different metals was calculated by subtracting from each sample the background level in the control with chelator but without metal and comparing with the equivalent samples without chelator (with the 'no chelator, no metal' control subtracted), thus enabling direct comparison of the results. The values were as follows: 3 to 4-fold increase in activation by Cu(I), 1.8 to 3-fold increase in activation by Cu(II), no change in activation by silver or with gold. Again there was no change in the response of \( P_{merT} \) and \( merR \). These results suggest that neocuproine specifically carries copper into the cell. The background level may have increased due trace amounts of copper present in SM9 medium.

Gold (as extracellular Au(III)) is not chelated by copper-specific chelators and activates the \( copA \) promoter in their presence to approximately the same extent as in the presence or absence of these chelators. Au(I) salts are too insoluble to use directly in chelation experiments. EDTA (200\( \mu \)M) decreased the activation of the \( copA \) promoter to background levels by all metals (Figure 4). None of the copper chelators had any effect on the induction of the \( mer \) promoter by MerR and mercury, which suggests that all of the effects observed for copper, silver and gold are metal specific and not caused by the chelating agents.
DISCUSSION

Au(I) is a soft metal ion with a covalent radius of 134 pm and and electronegativity of 1.42 (Allred-Rochow), identical to those properties of silver, but different to those of copper - 117 pm and 1.75, respectively (23). The effective nuclear charge, the charge due to the protons of the nucleus less a screening factor due to the outer electrons of the atom, for gold, copper and silver is identical at 4.20 (Slater). No other element has the same effective nuclear charge.

The activation of PcopA by CueR and gold salts was unexpected from a biological point of view. The physical chemistry of gold ions suggests that they are mimicking Cu(I) and Ag(I) in the metal binding site of CueR, leading to activation of PcopA. Au(I) is a common valence for this element and may be produced in the reducing environment of the cytoplasm, or in the periplasm by a reductase such as NDH2 (24,25). Although Au(I) is practically insoluble in water, which has prevented us from simply confirming our in vivo data with in vitro experiments, the local production of Au(I) salts is the most chemically convincing explanation for the mimicry of Cu(I) by gold salts, given the identical effective nuclear charge among the three elements. The exact levels of metal required for half-maximal induction of the promoter are extracellular values and differential uptake and reduction may account for the differences between the metal ions; Ag(I) alone would not be involved in redox reactions and has the lowest concentration for half maximal induction. Carefully-designed experimental conditions for in vitro study of the effect of the metals on CueR will need to be performed to confirm the valence state of gold required for activation of CueR.
The lack of effect of a $\Delta$copA mutation on the gold sensitivity of growth of *E. coli* cells may be due to gold salts not being recognised by the CopA transporter, indicating that there are different mechanisms for proteins discriminating between Cu and Au salts, or may be due to change to the valence state of gold as it is exported to the outside (oxidizing) side of the cytoplasmic membrane, or transport may not confer resistance to the metal. Such an effect has been shown for the CopB copper exporter of *Enterococcus hirae* which transports Cu(I) and Ag(I), but confers resistance only to Cu(I) and not to Ag(I) (26).

The details of recognition of Cu(I) Ag(I) and Au(I) by the CueR regulator remain to be fully dissected. The observation that cysteine residues 112 and 120 are required for activation by all three metals indicates that the metals bind to the same site on the regulator, and the equivalence of these amino acids to Cys117 and Cys126 in MerR (Figure 1) suggest that the metal binding site is in the same relative position on both regulators. Thus, it is not surprising that replacing alanine 78 with cysteine to mimic the equivalent essential cysteine in MerR (Figure 1) has no effect on CueR response to Hg(II), as the spacing between Cys112 and 120 in CueR is different to that between Cys 117 and Cys 126 in MerR, and more subtle positioning and charge effects will undoubtedly be involved in metal recognition. The recently-described copper-dependent regulator, HmrR, from rhizobia (27) contains cysteines equivalent to Cys112 and Cys120, but lack a sequence equivalent to the CCHH motif at positions 129-132, providing indirect confirmation of our analysis. The detail of metal binding will require structural information on the CueR protein. The existing structures of MerR family regulators are for proteins responding to effectors other than metals (28,29) and cysteines 112 and 120 lie outside the regions of similarity between CueR and these regulators.
The molecular homeostatic mechanisms for copper in humans continue to be identified. The toxicity of copper is due in part to its redox chemistry. Cuprous ions can be oxidised by peroxide to generate hydroxyl ions and radicals, which can attack and damage phospholipids in biological membranes and inactivate membrane bound enzymes (30). This type of damage is characteristic in people with copper toxicosis (Wilson’s disease) due to a defect in the WND copper transport protein (31). Rheumatoid arthritis (RA) is a disease characterised by migration of phagocytes and other leukocytes into synovial and periarticular tissue and damage of these tissues by activated oxygen species from triggered phagocytes (32). Increased copper levels were found in the blood plasma of rheumatoid arthritis patients (33), copper toxicity and arthritis were linked in a patient with Wilson’s disease (34) and a copper supplemented diet was shown to ameliorate Mycobacterium butyricum-induced arthritis in rats (35).

Our speculation is that by mimicking copper, gold may act as an effective activator of the expression of copper transport proteins and alter the copper homeostasis within the arthritic tissues, by removing copper from locations where it might generate reactive oxygen species.

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**Figure Legends**

**Figure 1** Alignment of CueR and other metal-responsive members of the MerR family. The consensus amino acids indicate where three of the four regulators have an identical amino acid at that position. The sites of directed mutations tested for their effect on the response to Cu, Ag and Au salts are marked with arrows above the sequences; Cys112 and Cys120 are numbered.

**Figure 2** (A) The response of the *copA* promoter to copper, silver and gold salts. β-galactosidase activity was measured in wild-type *E. coli* TG1 (pMUP*copA*) in SM9 medium with added CuSO$_4$ (filled bars), AgNO$_3$ (open bars) or AuCl$_3$ (hatched bars); the micromolar metal concentrations are shown on the abscissa.

(B) The response of the *copA* promoter to gold salts is dependent on CueR. β-galactosidase activity was measured in response to 50µM copper and 12.5, 25 and 50µM gold salts in (i) wild-type *E. coli* TG1(pMUP*copA*), (ii) the cueR deletion strain *E. coli* TG1[cueR::kanR](pMUP*copA*), and (iii) the same deletion strain containing the plasmid pSU$cueR$. Metal concentrations (µM) are given on the abscissa. Higher gold concentrations were tolerated in (B) compared with (A), probably because of sequestration of gold at higher cell concentrations in (B).

**Figure 3** Effect of cysteine and histidine mutations on Cu, Ag and Au recognition. β-galactosidase activity was measured in SM9 medium following induction with 50µM CuSO$_4$, 2.5µM AgNO$_3$ or 35µM AuCl$_3$. pSU18 bearing wild type or mutant cueR was provided *in trans* in *E. coli* TG1[cueR::kanR](pMUP*copA*).
**Figure 4** Response of the *copA* promoter to Cu(I), Cu(II), Ag(I) and Au(III) in the presence of chelators. β-galactosidase activity in *E. coli* TG1 (pMUP*copA*) was measured in Chelex-treated SM9 medium following induction with 50µM Cu(I) as CuCl, 50µM Cu(II) as CuSO$_4$, 2.5µM AgCl or 37µM AuCl$_3$ in the presence of 100µM concentrations of bathocuproine (b), cuprizone (c), neocuproine (n), or EDTA(e). The results were normalized relative to the value determined for Cu(II) in the absence of chelating agents, given an arbitrary value of 100%.
Figure 1

CueR ~~~~~~~~~ M NISDVAKITG LTSKAILRFYE EKGLVTTPMR SENGRTYRTQ
PbrR ~~~~~~~~ MNI QIGELAKRTA CPVVTFIRFYE QEGLLPPLPR SRGNFRLYGE
ZntR ~~~~~~~~~~ MNY RIGELAKMAE VTPDTIRYYE KQQMEHEVR TEGGFLRTYE
MerR ~~~MENNLENL TIGVFAKAAG VNVTIRFYQ RKGLLLEPDQ PYGRSIRRGE
Consensus ~~~~~~~~~ -IG--AK--- ----TIRFYE --GL---P-R --G--R-Y-E

CueR QHLNELTLLR QARQVGFNLE ESGEVNLFN DPQRHSADV K R. R TLEKVAE
PbrR EHVERLQFIR HCRSLDMPLS DVRTLSSYRK RPD.OQGEV NMLLDEHIRQ
ZntR SDLQRLKFIR HARQLGFSLE SIRELLSIRI DPEHHTCQES KGIVQERLQE
MerR ADVTRVRFVK SAQRLGFSLD EIAELLRL . DGTHCEEA SSLAEHKLKD
Consensus ~~~~RL-F-R ~~~~L-F-L~ ~~~~ELL~~ ~~~~C-E~ ~~~~~~~~~

N C
↑ ↑
CueR IERHIEELQS MRDQLLALAN ACPG..DDS ADCPIIENLS GCCHHRAG~ ~~~~~
PbrR VESRIGALLE LKHLVELRE ACSG.ARPA QSCGILQGLS DCVCDEGTT AHPSD
ZntR VEARIAELQS MQRSLQRLND ACCGTASS HYCSILEAE QGASGVKSGC ~~~~~
MerR VREKMDLAR MEAVLSELVC ACHARRGN. VSCPLAISLQ GGASLAGSAM P~~~
Consensus VE--I--L-- M---L---L-- AC---------- --C-II--L-- ~~~~~~~~ ~~~

Truncated at C120
Truncated at G128
Figure 2(A)

β-galactosidase (units)

wt Cu12.5 Cu25 Cu50 Cu75 Cu100 Ag1.5 Ag2 Ag1.5 Ag2 Ag2.5 Au0.9 Au1.7 Au3.1 Au6.3 Au12.5 Au18.5 Au25 Au67.5 Au50 Au65 Au60 Au70
Figure 3

% wild type

Wild type  H131NH132N  C129SC130S  C112S  C120S
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