

CopY Is a Copper-inducible Repressor of the *Enterococcus hirae* Copper ATPases*

(Received for publication, July 23, 1996, and in revised form, December 12, 1996)

Daniel Strausak and Marc Solioz‡

From the Department of Clinical Pharmacology, University of Berne, 3010 Berne, Switzerland

The *cop* operon of *Enterococcus hirae* effects copper homeostasis in this organism. It encodes a repressor, CopY, an activator, CopZ, and two P-type copper ATPases, CopA and CopB. Expression of all four genes is regulated by the ambient copper. In this regulation, CopY apparently acts as a copper-inducible repressor. By DNase I footprinting, it was shown that purified CopY protected two discrete sites in the region encompassing nucleotides –71 to –11 relative to the translational start site and containing hyphenated inverted repeats. Transcription is initiated between these repeats at nucleotide –42, in a domain that remained accessible to DNase I in the DNA-repressor complex. Chemical cross-linking revealed that CopY exists as a dimer in solution. In DNA band-shift assays, it was apparent that the CopY-DNA interaction occurred in two discrete steps. Half-maximal binding of repressor to the two operator sites was observed at 2×10^{-9} M and 5×10^{-9} M CopY, respectively. Copper ions released CopY from the promoter/operator with an apparent half-binding constant for Cu(I) of 20 μ M. The site-directed mutations A–61T and A–30T essentially abolished the binding of CopY to the respective binding sites, and the double mutation A–61T/A–30T inactivated both binding sites. Thus, CopY is a copper-inducible repressor of the *cop* operon of *E. hirae*, exhibiting highly specific DNA-protein interactions with two sites on the *cop* promoter/operator and playing a key role in copper homeostasis in *E. hirae*.

Copper is an essential trace element, acting as a cofactor for enzymes as diverse as cytochrome *c* oxidases, lysyl oxidases, or tyrosinases. But copper can also cause serious cell damage through radical formation. Therefore, careful regulation of the intracellular copper concentration is required. Until a few years ago, copper homeostasis was believed to be effected by metallothioneins, small, cysteine-rich proteins that complex and are regulated by heavy metal ions (1). More recently, it was discovered that copper homeostasis in the Gram-positive bacterium *Enterococcus hirae* involves two P-type copper ATPases, CopA and CopB, of 727 and 745 amino acids, respectively (2, 3). The CopA ATPase was shown to be required for copper accumulation under conditions of copper limitation, and CopB is needed to extrude copper from the cells when ambient copper reaches toxic levels (4, 5).

The two copper ATPase genes, *copA* and *copB*, are arranged in the *cop* operon, together with two regulatory genes, *copY* and *copZ*, in the order *copY*, *copZ*, *copA*, *copB*. These four genes appear to make up the copper homeostatic system of *E. hirae*. *copY* and *copZ* encode hydrophilic proteins of 145 and 69 amino acids, respectively. Expression of the ATPases was shown to be regulated by the ambient copper concentration in a biphasic fashion: expression was minimal at 10 μ M extracellular copper, and increased as well as decreased copper concentrations led to induction of the *cop* operon (4). Disruption of the *copY* gene caused massive overexpression of CopA and CopB ATPase, whereas null mutation of *copZ* suppressed expression to very low levels. Based on these findings, a model was proposed in which CopY acts as a repressor and CopZ as an activator of the *cop* operon (6). In partial support of this model, we here show the interaction of CopY with the operator/promoter region of the *cop* operon.

The transcriptional start site of the *cop* operon was mapped by primer extension, and the site of interaction of CopY with the operator was delineated by DNase I footprinting. Through site-directed mutagenesis, nucleotide residues that are critically involved in the CopY-operator interaction were identified. It was also shown that CopY is a homodimer that interacts with the operator in two steps in a copper-sensitive fashion.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—*E. hirae* (ATCC 9790, formerly called *Streptococcus faecalis* or *S. faecium*) was obtained from the American Type Culture Collection. *Escherichia coli* strains XLmutS and XL1-Blue were obtained from Stratagene, Inc. The expression vector pQE12 was purchased from Qiagen. The construction of pOAI has been described previously (6).

Materials—Pico green was from Molecular Probes, Inc. The Chameleon mutagenesis kit (Stratagene, Inc.) was used for site-directed mutagenesis. [γ - 32 P]ATP (3000 Ci/mmol) was obtained from Amersham Corp., phenylmethylsulfonyl fluoride from Merck, and growth media additives from BBL. Purification columns, column materials, and the *Mlu*I→*Sca*I switch-toggle primer were bought from Pharmacia Biotech Inc. Moloney murine leukemia virus reverse transcriptase was from Anawa Trading, Inc. (Wangen, Switzerland). Dynazyme DNA polymerase was provided by Finnzymes (Espoo, Finland), and oligonucleotide primers were synthesized by Microsynth, Inc. (Windisch, Switzerland). All other molecular biology reagents were obtained from Boehringer Mannheim. DNase I and other chemicals were from Sigma and were of the highest grade available.

Primer Extension—Total cellular RNA of wild-type *E. hirae* was prepared either from uninduced cells or from cells that were induced for 1 h with 1 mM CuSO₄. RNA was isolated as described previously (7). The primer 5'-CCAATCCATCGAATCAGCTAATATC-3' was labeled with polynucleotide kinase and [γ - 32 P]ATP to a specific activity of 0.1 μ Ci/nmol. 20 μ g of RNA in 30 μ l of hybridization solution (40 mM PIPES,¹ pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide) were heated for 5 min to 85 °C and annealed overnight at 30 °C with 0.5 pmol of the labeled primer. The nucleic acids were then ethanol precipitated, washed, and dissolved in 100 μ l of 10 mM dithiothreitol, 0.5 mM dNTPs,

* This work was supported by Grant 3200-046804 from the Swiss National Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Clinical Pharmacology, University of Berne, Murtenstrasse 35, 3010 Berne, Switzerland. Tel.: 41-31-632-3268; Fax: 41-31-632-4997; E-mail: solioz@ikp.unibe.ch.

¹ The abbreviations used are: PIPES, 1,4-piperazinediethanesulfonic acid; PCR, polymerase chain reaction; bp, base pairs.

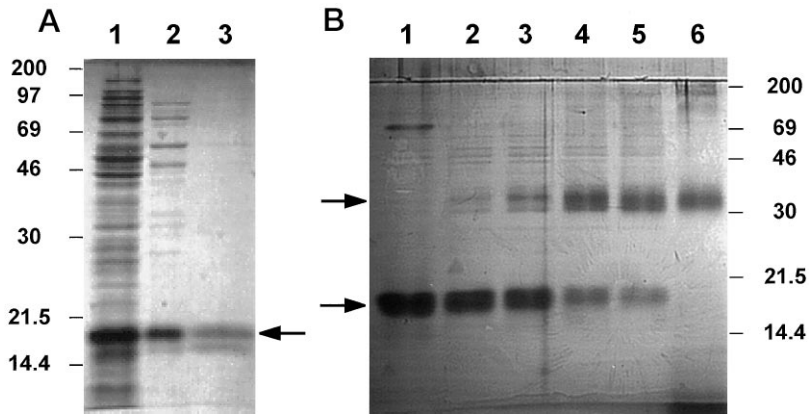


FIG. 1. A, Purification of CopY. Lane 1, 50 μ g of cell extract; lane 2, 10 μ g of Q-Sepharose-purified material; lane 3, 10 μ g of protein after gel filtration. Samples were run on a 12% SDS-polyacrylamide gel, and proteins were visualized by staining with Coomassie blue. The arrow indicates the band corresponding to CopY. B, cross-linking analysis of CopY. 100 pmol of CopY were cross-linked with the following amounts of glutaraldehyde: lane 1, 0%; lane 2, 0.001%; lane 3, 0.002%; lane 4, 0.005%; lane 5, 0.01%; lane 6, 0.1%. The proteins were resolved on a 15% SDS-polyacrylamide gel and visualized by silver staining. The arrows indicate the monomeric and dimeric forms of CopY. The vertical scales in A and B correspond to the migration of marker proteins of the indicated molecular masses in kDa.

40 units of RNase inhibitor, and reverse transcribed with 100 units of Moloney murine leukemia virus reverse transcriptase in the buffer supplied by the manufacturer for 90 min at 42 °C. Following ethanol precipitation, the products were analyzed by electrophoresis on 6% denaturing polyacrylamide sequencing gels.

CopY Overexpression—A PCR product of the *copY* gene was generated with the two primers 5'-CAAGAATGATCAAAGAATTT-3' and 5'-CCATTTTGGAAATTCGTTGCC-3' and Dynazyme DNA polymerase. Following cutting with *Bam*HI and *Hind*III, the PCR product was cloned into pQE12 and digested with the same enzymes. The resultant clone encoded a CopY protein with a five-amino acid N-terminal extension originating from the expression vector. These extra residues were deleted by site-directed mutagenesis. The final clone, pWY145, was verified by DNA sequencing.

CopY Purification—For overexpression, 500-ml cultures of XL1-Blue cells transformed with pWY145 were grown aerobically at 30 °C to an A_{550} of 0.5. Following induction with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 2 h, the cells were harvested by centrifugation for 10 min at 5000 $\times g$. The cell pellet was washed once with 200 ml of TGD buffer (50 mM Tris- SO_4 , pH 7.7, 5% (v/v) glycerol, 2 mM dithiothreitol) and resuspended in 100 ml of TGD buffer. The cells were lysed by incubation with 25 μ g of lysozyme and 1 mM phenylmethylsulfonyl fluoride for 10 min at room temperature and broken open by one passage through a French pressure cell at 40 MPa. The cell debris was collected by centrifugation for 1 h at 90,000 $\times g$ and the supernatant passed through a Q-Sepharose column. CopY was eluted with a 0–100 mM K_2SO_4 gradient in TGD buffer. Final purification was achieved by gel filtration on a Superose-12 column in TGD buffer plus 150 mM K_2SO_4 . Purified CopY was stored in 2- μ g aliquots at -70 °C. The concentration of CopY was determined by amino acid analysis.

Cross-linking Experiments—100 pmol of purified CopY were cross-linked at room temperature with the glutaraldehyde concentrations indicated under "Results" in a total volume of 40 μ l of 10 mM Na-HEPES, pH 8.0, 5 mM magnesium acetate, 5 mM dithiothreitol, 50 mM sodium acetate, 12% (v/v) glycerol, for 10 min at room temperature, followed by quenching with 5 μ l of 1 M ethanolamine. The products were resolved on a 15% SDS-polyacrylamide gel and visualized by silver staining.

Site-directed Mutagenesis—Mutation A-61T was generated in pOA1 (6) with the Chameleon mutagenesis kit using the mutagenic primer 5'-CGATTCAGTTGTAATCTAT-3' and a commercial *Mlu*I-*Sca*I switch-toggle primer. The resultant plasmid pOM214 was used to generate the double mutant A-61T/A-30T by PCR amplification with the primers 5'-CGAATCTTCCAAGTATCG-3' and 5'-GTTTTTTCACCTC-CATCGATTACATTTGAAAACCTTAAC-3'. Mutant A-30T was generated similarly, but using wild-type DNA as a template. Mutants T-23A and A-61T/T-23A were generated by PCR amplification of pOA1 and pOM214, respectively, with the primers 5'-GTTTTTTCACCTC-CATCGATTACATTTGAAAACCTTAAC-3' and 5'-CGAATCTTCCAAGTATCG-3'.

DNA Band-shift Assays—DNA band-shift assays were performed essentially as described (8). 2–3 fmol (6–10 nCi) of labeled wild-type *cop* promoter/operator DNA were incubated with a 10–300-fold molar excess of purified CopY for 30 min at room temperature in a total volume of 20 μ l of the buffer used for DNase I footprinting. The reactions were

analyzed on 6% polyacrylamide gels in 6.8 mM Tris acetate, pH 8.0, 3.3 mM sodium acetate, 2.5% (v/v) glycerol. Gels were allowed to polymerize for at least 12 h prior to use and were run at 5 W for 90 min. Promoter/operator fragments with the A-61T mutation were isolated by PCR amplification of the mutated plasmid with the two primers 5'-CCAATC-CATCGAATCAGCTAATATC-3' and 5'-CGAATCTTCCAAGTATCG-3'. The promoter/operator fragments with the mutations A-30T and T-23A and the double mutations A-30T/A-61T and T-23A/A-61T were similarly generated by PCR amplification of mutated plasmid DNA with the primers used for mutagenesis.

DNase I Footprinting—Footprinting assays were performed in 200 μ l of 20 mM Tris acetate, pH 8.0, 5 mM dithiothreitol, 5 mM magnesium acetate, 50 mM sodium acetate, 12% glycerol, 1 mM $\text{Ca}(\text{OH})_2$, 2 μ g of poly(dI-dC), and 5 μ g of bovine serum albumin. Assays contained 4–6 fmol of *cop* promoter/operator fragment, that was labeled with ^{32}P at one end with polynucleotide kinase as described (8) to a specific activity of 3 $\mu\text{Ci}/\text{pmol}$. The amounts of purified CopY specified under "Results" were added and the reaction incubated for 30 min at room temperature. The DNA was then digested with 0.012 unit of DNase I at 30 °C for 2 min. Digestion was terminated by adding 700 μ l of 92% ethanol, 0.75 M ammonium acetate, 7 $\mu\text{g}/\text{ml}$ tRNA. The products were precipitated at -70 °C for 15 min and centrifuged for 10 min at 10,000 $\times g$. The pelleted material was washed once with 70% ethanol and resolved by electrophoresis on an 8% polyacrylamide sequencing gel together with a Sanger sequencing reaction of the same fragment with the primers 5'-CGAATCTTCCAAGTATCG-3' for the sense strand and 5'-CCAATCCATCGAATCAGCTAATATC-3' for the antisense strand.

Miscellaneous Methods—Standard molecular biology methods were used according to published procedures (8). DNA was sequenced according to the method of Sanger *et al.* (9). The concentrations of the DNA fragments used for band-shift assays were determined by fluorescent measurements with pico green as described by the manufacturer.

RESULTS

To characterize the function of the CopY repressor and to study its interaction with the DNA target, CopY was overexpressed in *E. coli* and purified. When CopY was overexpressed as detailed under "Experimental Procedures," approximately 60% of the protein was trapped in inclusion bodies. However, CopY in the supernatant still made up 10% of the total protein in this fraction. CopY could be purified to approximately 90% purity by successive chromatography on Q-Sepharose and gel filtration. Fig. 1A shows the products of the different purification steps. Below the band of apparent molecular mass 17 kDa, corresponding to CopY, there was always a minor band of 16 kDa in the preparation. This contaminant increased with increasing purification and could be a degradation product of CopY. However, we could not observe variation in the results obtained with preparations containing differing amounts of the 16-kDa band.

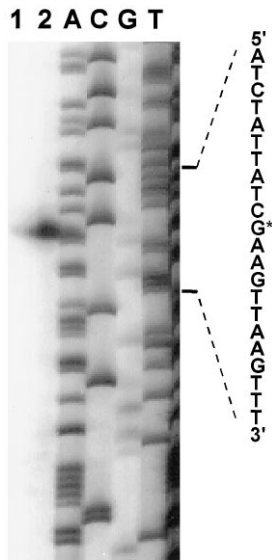


FIG. 2. **Primer extension.** 20 μ g of total RNA from uninduced (lane 1) or copper-induced (lane 2) cells were reverse transcribed with a specific 32 P-labeled primer. A, C, G, T, Sanger sequencing reactions with the same primer. The reaction products were resolved on a DNA sequencing gel, and radioactivity was visualized by phosphorimaging. The relevant DNA sequence is given on the right, and the transcriptional start site is indicated by an asterisk.

We assessed whether CopY was monomeric or multimeric in solution by cross-linking experiments. Fig. 1B shows that increasing concentrations of glutaraldehyde resulted in the formation of CopY homodimers with an apparent molecular mass of 34 kDa. No significant amounts of higher molecular mass species were generated even at very high concentrations of cross-linker, indicating that CopY homodimers are the principal species of CopY in solution. Inclusion of a 4-fold excess of lysozyme in the experiment did not result in significant cross-linking of this control protein to itself or to CopY (not shown). In analytical gel filtration, CopY migrated like a globular protein of apparent molecular mass 53 kDa, suggesting that the native CopY dimers are not globular but form an extended structure, as would be expected.²

Since CopY acts as a repressor of the *cop* operon, it was expected to interact with a DNA target at or near the site of transcription initiation. Primer extension analysis was thus carried out to determine the transcription initiation site of the *cop* operon. Total RNA from copper-induced and uninduced wild-type *E. hirae* cells was reverse transcribed with a primer as specified under "Experimental Procedures," and the resultant DNA fragments were resolved on a sequencing gel (Fig. 2). A single product was generated from induced but not from uninduced cells. This is in line with the previous observation that in copper-induced cells expression of the *cop* operon was increased 12-fold compared with uninduced cells (6). Co-electrophoresis of the primer extension products with DNA sequencing reactions conducted with the same primer allowed assignment of the transcriptional start site to guanosine at position -42 with respect to the start of translation. This assignment was further verified by running gels of mixtures of sequencing reactions and primer-extension product (not shown).

DNA binding properties of the CopY repressor were studied by band-shift assays. When a radiolabeled 316-bp DNA fragment encompassing the transcription start site was incubated with purified CopY, DNA-protein complexes could be observed

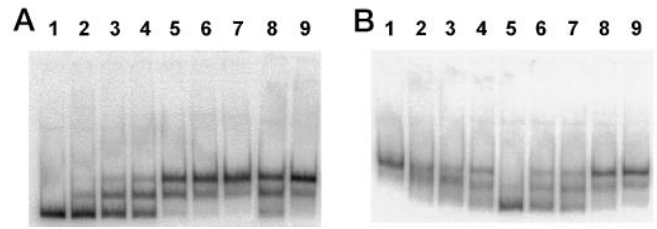


FIG. 3. **Interaction of CopY with wild-type *cop* promoter/operator.** A, wild-type *cop* promoter/operator DNA. A 32 P-labeled 316-bp *cop* promoter/operator fragment at a concentration of 0.15 nM was reacted with the following: lanes 1-7, 0, 1, 2, 3, 6, 9, and 15 nM CopY, respectively; lane 8, 15 nM CopY plus 1.5 nM cold *cop* promoter/operator DNA; lane 9, 15 nM CopY plus 1.5 nM cold β -lactamase promoter DNA from *E. coli*. Samples were electrophoresed on a nondenaturing 6% polyacrylamide gel and visualized by phosphorimaging. Other details of the method are given under "Experimental Procedures." B, effects of heavy metal ions on CopY-*cop* operator interaction. A 32 P-labeled 316-bp *cop* promoter/operator fragment at a concentration of 0.15 nM was incubated with 15 nM CopY and the following heavy metal ion concentrations: lanes 1-5, 0, 1, 5, 10, and 50 μ M Cu^+ , respectively; lane 6, 50 μ M Ag^+ ; lane 7, 50 μ M Cd^{2+} ; lane 8, 50 μ M Ni^{2+} ; lane 9, 50 μ M Zn^{2+} . The reactions were performed as described under "Experimental Procedures."

(Fig. 3). Increasing concentrations of CopY changed the apparent mobility of the DNA fragment in two steps: to an intermediate retarded form I, and to a more retarded form II, indicating two binding events. Addition of a 10-fold molar excess of unlabeled *cop* promoter/operator DNA to the binding mixture competed with the DNA-protein complex formation, whereas added promoter DNA from the β -lactamase gene of *E. coli* did not act as a competitor. Similarly, the promoter of the NaH antiporter gene of *E. hirae* (10) did not compete with the binding (data not shown), indicating the specificity of the DNA-CopY interaction. By quantitative evaluation of the band-shift data, the half-association concentrations of CopY under these conditions were estimated to be 2 nM for retarded form I and 5 nM for retarded form II.

In vivo, the expression of the *E. hirae cop* operon is induced by Cu^{2+} , Cu^+ , Ag^+ or Cd^{2+} but not by Ni^{2+} or Zn^{2+} (3, 6). It was thus of interest to test whether these heavy metal ions affected the *in vitro* interaction of CopY with the *cop* operator. Fig. 3B shows the effects of different heavy metal ions on the CopY-*cop* operator interaction as measured by band-shift assays. As little as 1 μ M added copper in the binding reaction released some CopY from the *cop* operator. At 50 μ M copper, formation of a CopY-DNA complex was completely inhibited. An approximately 50% displacement of CopY from the operator was observed with 20 μ M Cu^+ , 50 μ M Ag^+ , or 50 μ M Cd^{2+} . The same concentrations of Ni^{2+} and Zn^{2+} ions released approximately 5% of the bound repressor from the DNA. These results demonstrate that Cu^+ , Ag^+ , and Cd^{2+} are efficient inducers of the CopY repressor. This is in line with the induction properties of the *cop* operon observed *in vivo*. It is of course not possible to quantitatively compare *in vitro* band-shift experiments with whole cell studies, since it is not known how externally added metal ions change the cytoplasmic concentration in living cells. Also, the redox equilibrium between Cu(I) and Cu(II) makes it very difficult to determine the oxidation state of copper *in vivo*.

By site-directed mutagenesis of the *cop* promoter/operator DNA, specific DNA-CopY interactions were identified. Inversion of the adenosine at position -61 to a thymidine (A-61T) altered the binding of CopY to the operator. Fig. 4A shows that even a 200-fold molar excess of repressor to A-61T *cop* promoter/operator resulted in the generation of only one retarded form. The electrophoretic mobility of this complex was identical to the form I intermediate observed with wild-type promoter

² D. Strausak and M. Solioz, unpublished data.

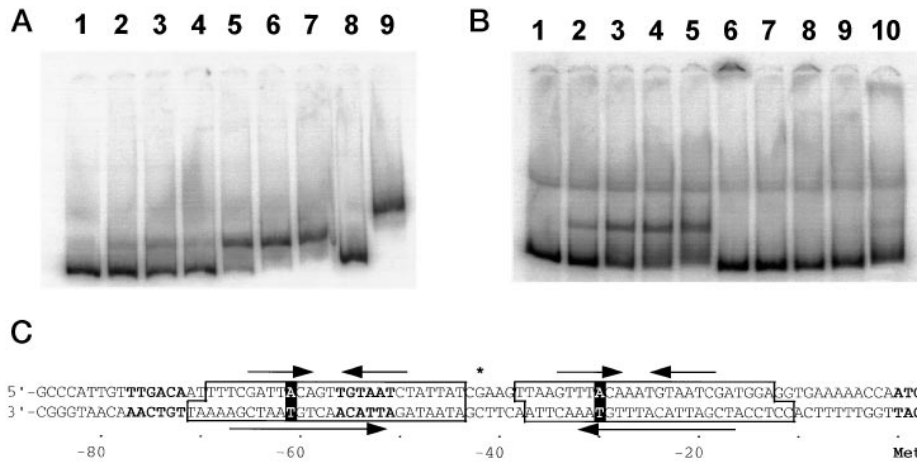


FIG. 4. Interaction of CopY with mutant *cop* promoter/operator DNA. A, A-61T mutant operator DNA. 0.145 nM 316-bp A-61T *cop* promoter/operator fragment was incubated with the following: lanes 1-7, 0, 1, 2, 3, 6, 12, and 30 nM CopY, respectively; lane 8, 0.15 nM wild-type *cop* promoter/operator without CopY; lane 9, same as in lane 8 plus 30 nM CopY. B, A-30T and A-61T/A-30T mutant promoter/operator DNA. Lanes 1-5, 0.16 nM 188-bp A-30T *cop* promoter/operator fragment was incubated with 0, 5, 10, 20, and 60 nM CopY, respectively. Lanes 6-10, 0.16 nM 188-bp A-61T/A-30T *cop* promoter/operator fragment was incubated with 0, 5, 10, 20, and 60 nM CopY, respectively. Other details of the experiment were as described in the legend to Fig. 3. C, the *cop* promoter/operator region of *E. hirae*. The nucleotide numbering is relative to the start of translation. Indicated in bold type are the conserved transcriptional signals (-35 and -10 regions) as well as the first methionine codon of the *copY* gene. The arrows delineate the inverted repeats, and the asterisk marks the start of transcription. Regions protected from DNase I by CopY are boxed. The residues leading to inactivation of the binding site when mutated are represented in reverse type.

DNA. This suggests that adenosine -61 is an essential base for the interaction of CopY with one site on the operator. The mutation A-30T revealed another essential residue, inactivating the second site of interaction of CopY with the operator (Fig. 4B), whereas the mutation T-23A in the proximal half of the inverted repeat did not have an effect on the repressor-DNA interaction (not shown). In the double mutant A-30T/A-61T, the repressor-DNA interaction was practically abolished. Clearly, single bases in the *cop* promoter/operator play key roles in the interaction with the CopY repressor. Half-maximal binding of repressor to the single remaining high affinity binding sites in the mutants was estimated to occur at 7 nM CopY for the site proximal to the translation start and at 12 nM CopY for the distal binding site. These values are 2-3-fold higher than those observed for the wild-type operators, which could indicate some cooperativity between the binding sites. Since it is unlikely that the relative binding affinities of the two sites are reversed in the mutants, this would suggest that the proximal site exhibits the higher affinity for CopY and interacts first with the repressor.

With DNase I footprinting experiments, the sites on the *cop* promoter/operator DNA that are occupied by the bound CopY repressor were mapped. Two regions were protected from DNase I digestion by CopY. They were slightly asymmetric on the two DNA strands and encompassed nucleotides -13 to -38 and -44 to -69 on the sense strand (Fig. 5A) and nucleotides -11 to -37 and -44 to -71 on the complementary strand (Fig. 5C). Between these protected sites, there is a region not protected from DNase I digestion, namely from -39 to -43 on the sense strand and from -38 to -43 on the antisense strand. The start of transcription, mapped to nucleotide -42, is located in this DNase I-sensitive domain (see Fig. 4C for a summary).

Band-shift experiments with mutant promoter/operator DNA had suggested that CopY binding by one site was impaired in some of the mutants. DNase I protection experiments with the mutated *cop* promoter/operator fragment A-61T showed indeed that only one site could bind repressor under the conditions used. The distal half of the inverted repeat that contained the A-61T mutation remained sensitive to DNase I digestion (Fig. 5B). Similarly, the A-30T mutated operator/promoter DNA was protected from DNase I by CopY at the

distal, nonmutated site but was protected only by high concentrations of CopY at the mutated site (Fig. 5D). Finally, the double mutant A-30T/A-61T remained essentially unprotected to DNase I digestion by CopY at both binding sites. Only high concentrations of CopY somewhat protected the proximal region from DNase I digestion (Fig. 5E). These observations are in agreement with the gel retardation experiments and show that mutations A-61T and A-30T both strongly interfere with the binding of the CopY repressor to its DNA target. Clearly, the nucleotides at these positions must fulfill crucial roles in the DNA-protein interaction.

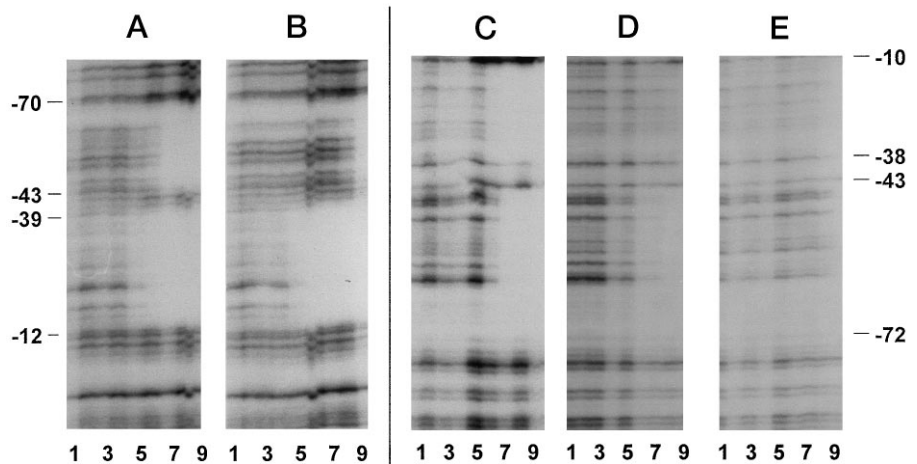
Taken together these results show that the *cop* promoter/operator possesses two independent binding sites for the CopY repressor. These sites exhibit somewhat different binding affinities for the repressor and appear well separated, since there are some DNase I-sensitive bases between the sites, including the site of transcriptional initiation.

DISCUSSION

We have characterized the interaction of the *E. hirae* copper-responsive repressor, CopY, with the *cop* operator *in vitro*. Three main features of this regulation system were revealed: (i) the CopY repressor interacts with the *cop* operator/promoter at two discrete sites at positions flanking the transcriptional start site; (ii) the repressor-DNA interaction is copper-sensitive; and (iii) single base changes can lead to half-of-the-site reactivity. Cross-linking and gel filtration experiments suggest that CopY is present as a homodimer in solution. Thus, it appears likely that the CopY homodimers interact with each of the two binding sites identified in the operator/promoter region.

The N terminus of the CopY repressor exhibits 27-32% sequence identity to the β -lactamase repressors MecI of *Staphylococcus epidermidis* (11), PenI of *Bacillus licheniformis* (12), and BlaI of *Staphylococcus aureus* (13). These repressors possess two structurally and functionally different domains (14), similar to the well characterized proteins of the large λ Cro/LysR family of repressors (15). The general concept of the function of these proteins is that the N-terminal part of the protein forms a helix-turn-helix DNA binding motif, whereas the C-terminal part interacts with the effector. This concept is supported by x-ray crystallographic studies of the bacteriophage λ Cro and

FIG. 5. DNase I footprinting of the CopY-*cop* promoter/operator complex. 0.02 nM promoter/operator DNA, 32 P-labeled at the 3'-end, was incubated with (lanes 1-9) 0, 0.1, 0.2, 0.3, 0.6, 0.9, 1.5, 4.5, and 10 nM CopY, respectively, and subjected to DNase I digestion as described under "Experimental Procedures." The reaction products were resolved on 8% denaturing polyacrylamide gels and visualized by phosphorimaging. A, sense strand of wild type. B, sense strand of mutant A-61T. C, antisense strand of wild type. D, antisense strand of mutant A-30T. E, antisense strand of double mutant A-61T/A-30T. The numbers at the left and right indicate nucleotide positions relative to the translational start site.



434 repressors (16, 17), which revealed that the Q28-Q29 pair in the helix-turn-helix motif of these proteins tightly interacts with the base pairs of an ACA triplet of the operator DNA (18) and that the mutation Q28A abolishes the interaction of the repressor with wild-type operator DNA (19). An analogous Q30-Q31 pair is present in CopY, and the *cop* promoter/operator contains four ACA triplets that would allow a similar interaction. We have shown that mutation of two of these ACA triplets to TCA almost completely abolish CopY-DNA interaction at the corresponding sites. These findings are essentially complementary to those obtained with the 434 repressors and suggest that the CopY-operator interaction is very similar in nature.

CopY concentrations required for half-maximal interaction with the two sites on the wild-type operator were determined *in vitro* as 2×10^{-9} M and 5×10^{-9} M, and thus are in the range of 10^{-9} to 10^{-10} M observed for other repressor-operator interactions (20, 21). Although most repressors are homodimeric proteins, only a few of them interact with two discrete binding sites on the operator, such as NodD, IlvY, or CadC (22, 23). In fact, the CadC regulatory system resembles the *E. hirae* CopY system in other ways. It also controls expression of heavy metal ATPases: the *cad* operons of cadmium-resistant bacteria encode a regulatory protein, CadC, and a cadmium efflux ATPase, CadA. The expression of CadA is strongly induced by Cd^{2+} but also by Pb^{2+} , Bi^{3+} , and some other heavy metal ions (24). By gel retardation assays and DNase I footprinting, it was recently shown that CadC binds to an inverted repeat in the promoter/operator region of the *cad* operon and is released by Cd^{2+} , Pb^{2+} , and, more efficiently, Bi^{3+} and Hg^{2+} (25). Similar to the CopY-DNA interaction, the binding of CadC to DNA took place in two discrete steps. However, CadC does not exhibit significant sequence identity to CopY but is related to ArsR from *E. coli*, which regulates resistance to arsenic ions (26), and to SmtB, which controls cyanobacterial metallothionein synthesis (27). So these proteins apparently represent another class of metalloregulators.

The promoter of the *cop* operon features the consensus sequences TGTAAT and TTGACA at the -10 and -35 positions relative to the transcriptional start site. These sequences are found in many bacterial promoters (28). The positioning of the two CopY binding sites relative to these transcriptional signals lies within the range of consensus positions for repressors and outside the range for activators (29). The biological significance of two CopY binding sites with similar affinities remains unclear at this time.

The control of the *cop* operon of *E. hirae* apparently also

requires the function of CopZ (6). CopZ is a protein of 69 amino acids containing the presumed copper-binding motif GMX-CXXC. In *copZ*-disrupted strains, the transcription of the *cop* operon was repressed and *copZ* function could be complemented in *trans* (6). A model for the observed biphasic regulation was proposed in which derepression of the *cop* operon can be achieved in two ways: by copper releasing CopY from the operator under conditions of excessive copper, or through activation of transcription by CopZ under copper-limiting conditions. Although our data support the activation of the *cop* operon by copper-induced release of the repressor, we could not obtain evidence of an involvement of CopZ *in vitro*, and the elucidation of the role of this protein requires further study.

Acknowledgments—We thank Thomas Weber for expert technical assistance, Hans-Ulrich Möscher for advice on DNase I footprinting, and Linda Thöni and Bernhard Erni for helpful suggestions.

REFERENCES

- Kägi, J. H., and Schaffer, A. (1988) *Biochemistry* **27**, 8509–8515
- Odermatt, A., Suter, H., Krapf, R., and Solioz, M. (1992) *Ann. N. Y. Acad. Sci.* **671**, 484–486
- Odermatt, A., Suter, H., Krapf, R., and Solioz, M. (1993) *J. Biol. Chem.* **268**, 12775–12779
- Odermatt, A., Krapf, R., and Solioz, M. (1994) *Biochem. Biophys. Res. Commun.* **202**, 44–48
- Solioz, M., and Odermatt, A. (1995) *J. Biol. Chem.* **270**, 9217–9221
- Odermatt, A., and Solioz, M. (1995) *J. Biol. Chem.* **270**, 4349–4354
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Ausubel, R. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., and Struhl, K. (1995) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. New York
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
- Waser, M., Hess-Bienz, D., Davies, K., and Solioz, M. (1992) *J. Biol. Chem.* **267**, 5396–5400
- Yoon, K. P., Misra, T. K., and Silver, S. (1991) *J. Bacteriol.* **173**, 7643–7649
- Endo, G., and Silver, S. (1995) *J. Bacteriol.* **177**, 4437–4441
- Wu, J., and Rosen, B. P. (1993) *Mol. Microbiol.* **8**, 615–623
- Morby, A. P., Turner, J. S., Huckle, J. W., and Robinson, N. J. (1993) *Nucleic Acids Res.* **21**, 921–925
- Suzuki, E., Kuwahara Arai, K., Richardson, J. F., and Hiramatsu, K. (1993) *Antimicrob. Agents Chemother.* **37**, 1219–1226
- Himeno, T., Imanaka, T., and Aiba, S. (1986) *J. Bacteriol.* **168**, 1128–1132
- Rowland, S. J., and Dyke, K. G. (1990) *Mol. Microbiol.* **4**, 961–975
- Wittman, V., and Wong, H. C. (1988) *J. Bacteriol.* **170**, 3206–3212
- Schell, M. A. (1993) *Annu. Rev. Microbiol.* **47**, 597–626
- Pabo, C. O., and Lewis, M. (1984) *Annu. Rev. Biochem.* **53**, 596–601
- Anderson, J. E., Ptashne, M., and Harrison, S. C. (1987) *Nature* **326**, 846–852
- Koudelka, G. B., Harrison, S. C., and Ptashne, M. (1987) *Nature* **326**, 886–888
- Wharton, R. P., and Ptashne, M. (1987) *Nature* **326**, 888–891
- Comess, K. M., Shewchuk, L. M., Ivanetich, K., and Walsh, C. T. (1994) *Biochemistry* **33**, 4175–4186
- Summers, A. O. (1992) *J. Bacteriol.* **174**, 3097–3101
- Fisher, R. F., and Long, S. R. (1989) *J. Bacteriol.* **171**, 5492–5502
- Wek, R. C., and Hatfield, G. W. (1988) *J. Mol. Biol.* **203**, 643–663
- Lisser, S., and Margalit, H. (1993) *Nucleic Acids Res.* **21**, 1507–1516
- Collado-Vides, J., Magasanik, B., and Gralla, J. D. (1991) *Microbiol. Rev.* **55**, 371–394