A regulator of nutritional copper signaling in *Chlamydomonas* is an SBP domain protein that recognizes the GTAC core of copper response element

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The *CRR1* (Copper Response Regulator) locus, required for both activating and repressing target genes of a copper- and hypoxia-sensing pathway in *Chlamydomonas*, encodes a 1,232-residue candidate transcription factor with a plant-specific DNA-binding domain named SBP, ankyrin repeats, and a C-terminal Cys-rich region, with similarity to a *Drosophila* metallothionein. The recombinant SBP domain of Crr1 shows zinc-dependent binding to functionally defined copper-response elements associated with the *CYC6* and *CPX1* promoters that contain a critical GTAC core sequence. Competition experiments indicate equivalent selectivity for copper-response elements from either promoter and 10-fold greater selectivity for the wild-type sequence vs. a sequence carrying a single mutation in the GTAC core. The SBP domain of *Chlamydomonas* Crr1 binds also to a related GTAC-containing sequence in the *Arabidopsis AP1* promoter that is the binding site of a defining member of the SBP family of DNA-binding proteins. *Chlamydomonas* Crr1 is most similar to a subset of the *Arabidopsis* SBP domain proteins, which include SPL1, SPL7, and SPL12. The abundance of the *CRR1* mRNA is only marginally copper-responsive, and although two mRNAs that differ with respect to splicing of the first intron are detected, there is no indication that the splicing event is regulated by metal nutrition or hypoxia. It is likely that the dramatic copper-responsive action of Crr1 occurs at the level of the polypeptide.

acclimation | copper homeostasis | hypoxia | metal | transcription factor

The transition metals like Cu, Mn, Fe, and Zn are essential for life because of their role in the catalysis of biochemical reactions, especially redox reactions or reactions involving oxygen. The acquisition of these metals is a nutritional problem for many forms of extant life because bioavailability has changed during the course of evolution, owing to the build up of oxygen in the atmosphere and the movement of life from aquatic to terrestrial habitats. At the same time, many of the essential metal nutrients are toxic to cellular constituents because of their propensity for catalyzing oxidative chemistry in the presence of oxygen. Nutritional supply can vary from deficiency to plentitude to excess, and the variation occurs in the background of deficiency or superabundance of other essential nutrients. Most organisms, therefore, have evolved tightly regulated and highly metal-selective homeostatic mechanisms for acquiring, distributing and storing metal nutrients.

Copper is one of the toxic, but nutritionally essential, metals for aerobic organisms and its metabolism is, accordingly, tightly controlled (1, 2). In *Saccharomyces cerevisiae*, two related transcription factors, Mac1p and Ace1p, regulate copper homeostasis by activating either assimilatory molecules (reductases and transporters) under nutritional deficiency or copper-sequestering molecules and cellular stress responses under copper excess, respectively (3–7). In other fungi, copper homeostasis is controlled in a similar manner by related transcription factors, Amt1, Cuf1, and GRISEA (2). In each case, copper binds to the transcription factor at a cysteine-rich domain and determines its activity. For Ace1 and Amt1, copper binding promotes DNA binding, whereas, for Mac1, copper binding inhibits DNA binding and transcription activation via intramolecular communication between the copper-binding and DNA-binding domains. In *Drosophila*, copper homeostasis is determined through transcriptional activation of *CTR1B* encoding one of three Ctr-type copper transporters in the deficiency situation vs. activation of four metallothionein-encoding *MTN* genes in the copper overload situation (8, 9). The transcription factor, dMTF-1, which is a homolog of mammalian MTF-1 that is involved in zinc homeostasis and activation of metallothionein-encoding *MT* genes, binds metal response elements associated with target genes. DNA binding is promoted by zinc ions: Thus, the response of the Drosophila *MTN* genes to high copper (or cadmium or H_2O_2) occurs by an elegant mechanism involving release of zinc from metallothionein in the presence of inducers and, accordingly, an increase in intracellular zinc availability for binding to MTF-1 (10). Zn binding also affects the subcellular localization of MTF-1. The mechanism by which dMTF-1 activates transcription of *CTR1B* in response to low copper is not known, but it is likely that it would involve the metal-binding sites on dMTF-1.

Copper homeostasis in plants has been studied in the *Chlamydomonas* model (11). Among several responses to copper-deficiency in *Chlamydomonas*, the *CYC6* gene is activated to provide a copper-independent substitute for plastocyanin in the photosynthetic electron transfer chain (12). Transcriptional activation occurs through copper-response elements (CuREs) that contain a critical GTAC core sequence where each nucleotide in the core is critical for *in vivo* CuRE activity (13). This sequence is different from the fungal CuREs bound by the Ace1/Mac1 family of transcription factors and from the metal response elements bound by MTFs, suggesting a different DNA-binding domain in the *Chlamydomonas* copper signaling pathway. Furthermore, the metal selectivity of the *Chlamydomonas* response is unique: Hg(II), but not Ag(I), ions mimic the effect of copper ions in turning off the expression of target genes (14), whereas Ni(II) or Co(II) ions can recapitulate the copper-deficiency response, even in the presence of repressing amounts of copper

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Abbreviation: CuRE, copper-response element.

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(15), suggesting that the *Chlamydomonas* factor has a different metal sensing/signaling mechanism.

Recently, we identified two *Chlamydomonas* mutants that define a regulatory locus, *CRR1*, for nutritional copper signaling (16). The *crr1* strains are unable to grow in copper-deficient medium because they cannot activate any of the known copper-deficiency target genes, e.g., *CYC6*, *CPX1*, and *CRD1/CHL27A* (17, 18). The mutants are also unable to repress the *CTH1/CHL27B* gene (19). In this work, we cloned *CRR1* by complementation and show that it encodes a putative transcription factor that recognizes the GTAC core of the CuRE. Crr1 displays two candidate metal-sensing domains, the Zn-binding SBP domain, and a C-terminal cysteine-rich domain.

Materials and Methods

Strains and Culture Conditions. Wild-type strain CC425, mutant strains *crr1-1arg7cw15* and CC3960 *crr1-2arg7*, and rescued *crr1* strains were cultured in copper-supplemented or copper-deficient TAP medium as described in ref. 20. The *crr1-1arg7cw15crd1-5* strain was derived from previously described mutant CC3952 by crosses (16, 18, 19). For analysis of nickel-induced gene expression, cultures were supplemented with $NiCl_2$ from a 100 mM stock solution. For hypoxic conditions, strains were cultured as described in refs. 21 and 22.

Cloning of *CRR1*. A 6-kb BamHI fragment, corresponding to the minimal complementing fragment (see Fig. 7, which is published as supporting information on the PNAS web site), was cloned into pBluescript II SK− (resulting in plasmid pCRR1F1B6) and sequenced by Qiagen Genomics (Bothell, WA). The corresponding sequence was submitted to GenBank under accession no. AY484394, and the plasmid was submitted to the *Chlamydomonas* Culture Collection. Nucleic acid analyses and assembly of the Crr1 cDNA is detailed in *Supporting Materials*, which is published as supporting information on the PNAS web site.

Fluorescence Analysis. Room temperature chlorophyll fluorescence induction kinetics were measured by using an open FluorCam detector (Photon Systems Instruments, Brno, Czech Republic). Fluorescence emission was recorded from colonies of cells grown on either +Cu or −Cu TAP plates after dark adaptation periods of at least 5 min by using an actinic light intensity of ≈60 μmol·m^{-2}·sec^{-1} for 2–3 sec.

His-6-Tagged SBP Domain. Details of the expression, purification, and assay of the recombinant Crr1-SBP domain are described in *Supporting Materials*.

Electrophoretic Mobility Shift Assay. For assessing DNA binding, 100 ng (final concentration 500 nM) of the expressed SBP domain was incubated with 4 pmol labeled fragment (4.9×10^5 to 9.4×10^5 cpm/pmol), and the samples were processed as described in ref. 23. After electrophoresis for 1 h, the gel was exposed to a PhosphorImager screen for 1 h.

Results

Complementation of *crr1*. We used an indexed cosmid library carrying *Chlamydomonas* genomic DNA to complement the *crr1* strains (see *Supporting Materials* for details). DNA from three microtiter plates, 9, 33, and 81, supported the restoration of Crr1 function in one or two Arg+ colonies per Petri dish in both sets of transformations of *crr1* strains. Five transformants were tested for cosegregation of growth on −Cu medium and Arg prototrophy by backcross to *crr1arg7* or (from plate 81) were confirmed to be photosynthetically competent in −Cu medium on the basis of fluorescence rise and decay kinetics, which suggests restoration of *CYC6* expression (e.g., Fig. 1*B*). Representative transformants (12 corresponding to plate 9 and 6 to plate 33) were

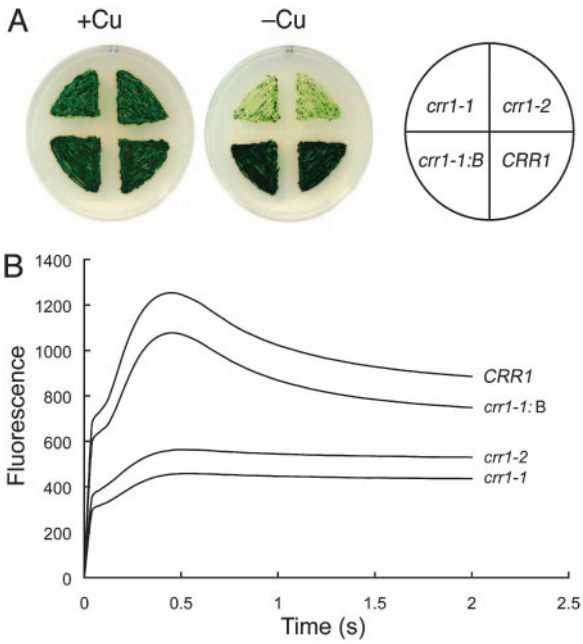

Fig. 1. Rescue of *crr1* growth by the cloned gene. (*A*) Growth of strains on TAP medium with (+) or without (−) 6 μM supplemental copper. The strains [wild-type (CC125), *crr1-1*, *crr1-2*, and a complemented *crr1-1* strain (*crr1-1*:B)] were grown for 1 week at 20°C under 100 to 125 μmol photons·m^{-2}·s^{-1}. (*B*) Fluorescence rise and decay kinetics from strains grown without supplemental copper.

further analyzed for copper-responsive *CYC6* and *CPX1* expression. The pattern of expression of these copper-deficiency response marker genes confirmed that each transformant was likely to be a genuine rescued strain. The complementing cosmids from microtiter plates 9 and 33 were specifically localized by testing DNA preparations from columns and rows of each plate for their ability to rescue strain *crr1arg7cw15*. Approximately 20 candidate *CRR1* colonies were obtained for each positive column or row, and, of these, 4 representatives were tested from each set specifically for copper-responsive *CYC6* and *CPX1* expression. The complementing cosmids 9F1, 9A3, and 33E8 were isolated and shown to rescue the *crr1* phenotype.

Restriction mapping indicated that the inserts in cosmids 9F1 and 9A3 overlap ≈90% with each other and share a common 13-kb section of DNA with 33E8, suggesting that the complementing cosmids represented a single region of the genome. The minimal complementing fragment was localized to a 6-kb BamHI fragment (Fig. 1*A*; also see Fig. 3) and was confirmed to rescue both growth and photosynthetic competence of strain *crr1–2arg7* (data not shown). Transformants derived from rescue of *crr1-1* and *crr1-2* by the 6-kb BamHI fragment showed normal copper-responsive expression of each of the marker genes *CYC6*, *CPX1*, and *CRD1* and the 3-kb form of the *CTH1* mRNA (Fig. 2*A*).

Previously, we had shown that several target genes of the copper response pathway were up-regulated by growth in low O_2 or by provision of high concentrations of nonessential transition metals like cobalt or nickel ions (13, 15, 21). The hypoxia- or nickel-responsive expression of these genes required the CuREs and was absent in the *crr1* mutant, suggesting that the same signal transduction components were responsible for the response to low O_2 and nickel/cobalt ions. Therefore, we tested and confirmed restoration of nickel- and hypoxia-responsive expression of target genes in representative rescued colonies (Fig. 2 *B* and *C*, respectively).

Sequence analysis (see below and *Supporting Materials*) indi-

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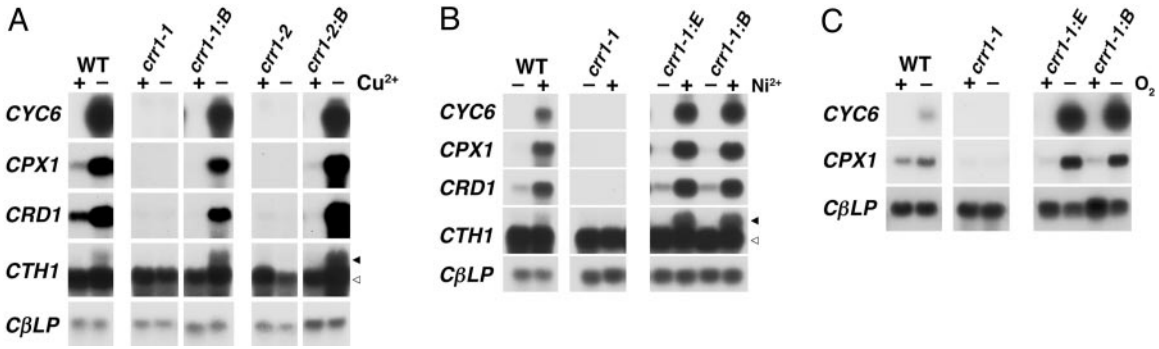

Fig. 2. Rescue of all *crr1* expression phenotypes by the cloned gene. Total RNA was prepared from the indicated allelic mutant strains *crr1-1* and *crr1-2*, a wild-type strain (CC425), or strains rescued with either the 9,201-bp EcoRI fragment (:E) or the 6,096-bp BamHI fragment (:B), and analyzed for the expression of target genes *CYC6*, *CPX1*, *CRD1*/*CHL27A*, or *CTH1*/*CHL27B* and a loading control *CBLP* by hybridization (specific activity of probes were in the range 2.4 to 11×10^8 cpm/μg DNA). *CTH1* produces two transcripts, the 2-kb form (white arrow) in +Cu cells encodes the protein, whereas the 3-kb form (black arrow) occurs only in −Cu cells dependent on Crr1 function. (*A*) The strains were grown in copper-supplemented (+) or copper-deficient (−) TAP medium. (*B*) The strains were grown in +Cu TAP medium. $NiCl_2$ was added (+) or not (−) to a final concentration of 25 μM, and RNA was isolated 5 h later. (*C*) The strains were grown at room temperature on a shaker with aeration (100% air) (+O_2) or were transferred for 24 h to hypoxic conditions (96%N_2/2% air/2% CO_2) (−O_2) before RNA isolation.

cated that the BamHI fragment did not include the entire 3′ UTR of the *CRR1* gene, and a 9-kb EcoRI fragment, which included the entire gene (Fig. 3), was shown to rescue the *crr1* phenotype. Nevertheless, the expression of target genes was indistinguishable from wild-type strains in transformants carrying the 3′ truncated (:B) vs. intact (:E) *CRR1* gene (e.g., Fig. 2 *B* and *C*).

Crr1 Sequence Analysis. Southern hybridization analyses and sequencing of the cloned complementing DNA and cDNAs derived from the locus revealed that the *CRR1* locus represents a single gene with three small exons that produced a 5.8-kb mRNA encoding a 1,232 residue polypeptide (Fig. 3). A significant portion of the polypeptide consists of low complexity regions

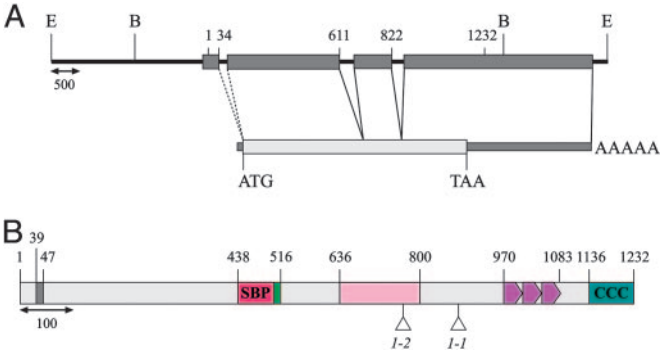

Fig. 3. Structure of the *CRR1* locus. (*A*) Structure of the *CRR1* gene. The EcoRI (E) and BamHI (B) sites are indicated. The double-headed arrow indicates the length scale (500 bp). The gray rectangles indicate exons, and the thin lines intervening indicate introns. The numbers mark the corresponding amino acids encoded by the exons. The dashed line indicates an ''inefficient'' splicing event (see text). The diagram of the mRNA is shown below with the ORF indicated from ATG to a TAA codon. (*B*) Diagram of the protein. The numbers mark the amino acyl residues in the protein. The red box marks the DNA-binding SBP domain, and the pale pink box indicates an extended region of conservation consisting of a conserved sequence $WL(X)_3P(X)_3E(X)_2IRPGC$ found in a subset of SBP domain proteins in *Arabidopsis* and rice. A putative nuclear localization signal is indicated in green within the SBP domain. Ankyrin repeats are shown as purple arrows, and a C-terminal cysteine-rich sequence is in turquoise. A gray rectangle at the N terminus of the protein represents the core of a region that shows sequence characteristics of an ''AHA motif'' found in a subset of SBP domain proteins. In strains *crr1-1* and *crr1-2*, there is a single nucleotide deletion at codon 895 or 780, respectively, resulting in a frame shift and, presumably, premature termination of translation.

(shown in light gray), but we noted three regions that spoke to function: An SBP domain was identified by both BLAST analysis (7×10^{-17} on August 2002) and via a search at the Pfam site (1.3×10^{-35} on February 2004) as was a repeat of three ankyrin motifs, and visual inspection of the sequence revealed five sets of Cys-containing motifs in the C-terminal region of the protein, CxxCxC at 942, 1146, and 1185, and CxxC at 1164 and 1220, plus a number of His, Met, and additional Cys residues.

The ≈78-residue SBP domain (see Fig. 8, which is published as supporting information on the PNAS web site) is named for a novel Zn-dependent DNA-binding domain, which was discovered originally in two nuclear proteins SBP1 and SBP2 from *Antirrhinum majus* that bound the promoter of a floral meristem identity gene called *SQUAMOSA* (24, 25). The SBP domain occurs in a number (16 in *Arabidopsis*, >12 in *Chlamydomonas*, and >6 in rice) of otherwise unrelated proteins in the plant kingdom that function in fundamental developmental processes, especially associated with reproduction (26–29). The consensus DNA sequence bound by the SBP domain contains a GTAC core (23, 29), and this point was immediately relevant in terms of Crr1 function because the *Chlamydomonas* CuRE had been identified as having a critical GTAC core sequence by extensive mutagenesis of two such elements associated with *CYC6* and one element associated with *CPX1* (13). The SBP domain also contains a nuclear localization signal, which has been shown to target a reporter construct to the nucleus (23), and a recent study showed that a GFP fusion with *Arabidopsis* SPL14 was targeted to the nucleus (30). The presence of the SBP domain in Crr1, therefore, is consistent with its function as a transcription factor.

Southern analysis of the *crr1-1* and *crr1-2* strains did not reveal any major alteration of the *CRR1* locus in mutants relative to the wild-type and the locus, corresponding to position −1092 to +4839 of the BamHI fragment, was amplified from each strain and analyzed by sequencing (see *Methods*). The *crr1-2* and *crr1-1* strains were both found to carry single nucleotide deletions at positions 2834 and 3343, respectively, leading to frameshifts between the SBP domain and the ankyrin repeats (at codons 780 and 895) and, hence, premature termination of translation (Fig. 3).

Expression of *CRR1*. RNA blot hybridization indicated that the locus produces a low abundance, ≈6 kb mRNA (Fig. 4). In the wild-type strains 2137, CC125, and CC425, the abundance of the RNA is always slightly (0.7× to 0.8×) lower in RNA prepared from −Cu vs. +Cu cells, as confirmed by real-time PCR measurement (Table 1), but it is unlikely that this differ-

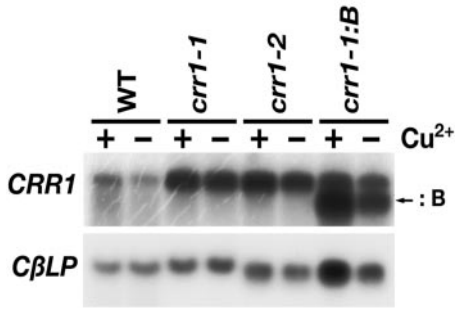

Fig. 4. Expression of *CRR1*. RNA blot hybridization. RNA was isolated from copper-supplemented (+) or copper-deficient (−) wild-type (CC425), *crr1-1*, *crr1-2*, or *crr1-1* complemented with the 6-kb BamHI genomic DNA fragment (*crr1-1*:B) strains and analyzed for *CRR1* expression by using fragment A (see Fig. 7) as a probe for hybridization. *CβLP* was used as a loading control. The size of the *CRR1* mRNA was estimated by comparison to size markers (0.24–9.5 kb RNA ladder, Invitrogen). The difference in mRNA abundance between mutant and wild-type strains evident in this figure was not observed in six other experiments (e.g., Table 1).

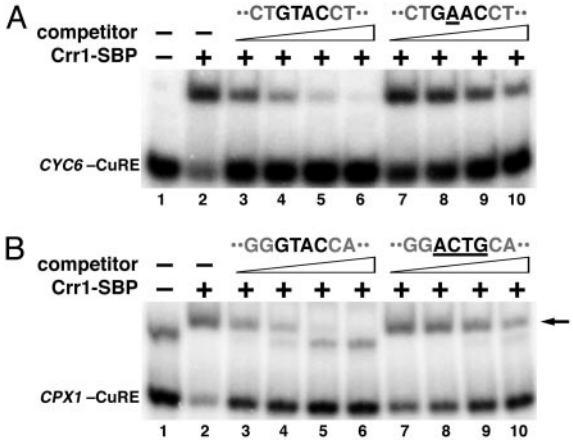

Fig. 5. The SBP domain of Crr1 binds to the CuRE. (*A*) Recombinant His-6-Crr1-SBP was incubated with radiolabeled DNA corresponding to a CuRE (position −134 to −110) from the *CYC6* promoter (lanes 2–10) in the presence of increasing concentration (5×, 10×, 50×, and 100× molar ratio) of unlabeled wild-type binding site (lanes 3–6) or a binding site carrying a T to A change in the GTAC core (lanes 7–10). (*B*) Radiolabeled DNA representing the CuRE from the *CPX1* promoter (position −49 to −26 relative to the 5′ end of the longest *CPX1* mRNA) (lanes 2–10) was incubated with increasing concentrations (5×, 10×, 50×, and 100× molar ratio) of unlabeled wild-type binding site (lanes 3–6) or a mutated binding site where the nucleotides of the GTAC core were rearranged to ACTG (lanes 7–10). The specific band corresponding to the DNA–protein complex is marked with an arrow. The other band is probably a primer-trimer.

ence is significant in terms of the dramatic (up to 10^3-fold for *CYC6*) metal-responsive regulation of the Crr1 target genes. The abundance of the *CRR1* mRNA is not changed much in the *crr1* mutants relative to wild-type (e.g., if it were degraded by nonsense mediated decay) (Table 1), and this observation is consistent with the position of the frame-shift mutations in the third and fourth exons of the gene. The strains (:B transformants) carrying the 3′ truncated *CRR1* gene produce an appropriately truncated mRNA of 4.8 kb, whose pattern of expression resembles that of the full-length endogenous gene (Fig. 4).

RT-PCR and 5′ RACE analysis of *CRR1* mRNAs indicated the presence of two populations of mRNAs in both the total RNA preparation and also the polyadenylylated RNA preparation, and these mRNAs represented molecules in which the first intron was either spliced or not (diagrammed in Fig. 3; see also Fig. 9, which is published as supporting information on the PNAS web site, for data). The abundance of the unspliced mRNA was equivalent to or greater than the abundance of the spliced mRNA. The unspliced mRNA encodes an in-frame termination codon within the first intron, which could lead to nonsense mediated decay of the mRNA or, if translation were reinitiated at the next methionine, to a slightly shorter protein lacking the first 50 amino acids, including the AHA motif. To assess whether alternative splicing of the first intron might be biologically significant, we tested the ratio of spliced to unspliced *CRR1* mRNAs under conditions where Crr1 target genes are regulated, including copper-, zinc-, or iron-deficiency vs. sufficiency, in normoxic vs. hypoxic, or with nickel supplementation (see Fig. 9). The ratio of the spliced to the unspliced form was not affected under any condition, and we conclude that the first intron is spliced slowly relative to other steps of mRNA processing. Slow splicing events have been observed previously for *Chlamydomonas* RNAs (31).

Table 1. Abundance of *CRR1* mRNA in *crr1* strains relative to the wild type

	Experiment 1		Experiment 2		Experiment 3	
Strains	+Cu	−Cu	+Cu	−Cu	+Cu	−Cu
WT	1.00	0.83	1.00	0.70	1.00	0.80
crr1-1	0.39	0.23	0.50	0.41	0.58	0.86
crr1-2	0.55	0.41	0.46	0.21	0.37	0.53

Accumulation of *CRR1* mRNA was assessed by real-time PCR by using the comparative C_T method (for details, see Table 2, which is published as supporting information on the PNAS web site). For each experiment, *CRR1* mRNA abundance is expressed relative to the amount in copper-replete wild-type cells.

Crr1-SBP Domain Binds the CuREs. Genetic analysis had suggested that the *CRR1* locus functions through the CuREs of the target genes of the nutritional copper-signaling pathway (unpublished results and ref. 16). To assess whether Crr1 interacts biochemically with the CuREs, we tested the putative DNA-binding SBP domain of the protein in an electrophoretic mobility shift assay with labeled CuREs from the *CYC6* and *CPX1* genes (Fig. 5). The His-6-tagged SBP domain of Crr1 could bind each of the two CuREs from the *CYC6* gene (Fig. 5*A* for the distal CuRE and data not shown for the proximal CuRE) and the CuRE from the *CPX1* gene. DNA binding was shown to be specific for the wild-type vs. the mutated element by competition with unlabeled DNA: a 10-fold higher concentration of unlabeled mutant oligonucleotide was required compared to the wild-type sequence to achieve the same degree of competition (Fig. 5). An irrelevant nucleotide sequence from the *Arabidopsis CAULIFLOWER* promoter was also not an effective competitor in the gel-shift assay (data not shown).

In previous work, we had indicated that the GTAC core of the CuRE was absolutely essential for copper-responsive gene expression *in vivo* because alteration of any nucleotide in the core to any other nucleotide completely abolished gene expression (13). This finding was recapitulated in the *in vitro* assay, where the single T to A substitution in the GTAC core (Fig. 5*A*) was as effective as the scrambling of all four nucleotides (Fig. 5*B* for *CPX1* CuRE and data not shown for scrambled *CYC6* CuRE). The Crr1 SBP domain is also able to bind the putative binding site of the *Arabidopsis* SBP domain in the *AP1* promoter, and the SBP domain from *Arabidopsis* SPL1 is able to bind the CuRE (23), which confirms that the GTAC core is a key recognition element for the SBP domain.

Consistent with the structural model, which indicated the presence of two bound zinc ions in the SBP domain, preincu-

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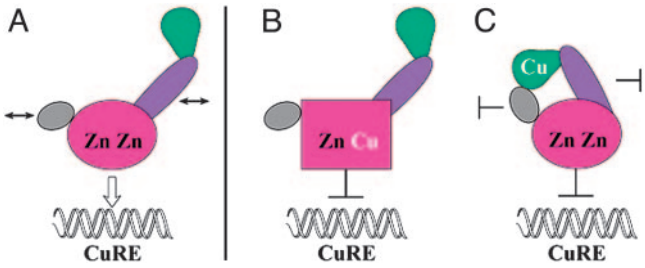

Fig. 6. Alternative models for copper-responsive modification of Crr1 function. (*A*) In copper-deficient cells, Crr1 binds the CuRE through the SBP and activates transcription by interaction with the transcription apparatus (double-headed arrows). In copper-replete cells, the structure of Crr1 is modified, either by binding of copper to the SBP domain (*B*) or to the C-terminal Cys-rich region (*C*), such that the protein no longer binds the CuRE or is no longer able to activate transcription.

bation or renaturation of the protein in the presence of EDTA inhibited the DNA binding activity of the recombinant protein, whereas renaturation in the presence of zinc could restore activity to protein that had been treated previously with EDTA (Fig. 10, which is published as supporting information on the PNAS web site).

Discussion

Crr1 is required for up-regulation of *CYC6*, *CPX1*, *CRD1*, and *CTR* genes and also for down-regulation of the *CTH1* locus. Both types of responses rely on transcriptional activation through CuREs associated with the target genes. For up-regulation, Crr1-dependent activation produces templates for synthesis of the respective polypeptides, whereas for down-regulation of Cth1, Crr1-dependent promoter activity interferes with the production of mRNA templates (19). Crr1 is, therefore, a key regulator of copper homeostasis in *Chlamydomonas*, and it works by transcriptional activation. Three biochemical activities are required for transcriptional activation of the copper-deficiency response: recognition of the CuRE on target genes, copper sensing, and transcriptional activation. These activities may reside on a single molecule or on different molecules that interact with each other. Genetic analysis has revealed only a single regulatory locus for nutritional copper signaling and, therefore, we consider and discuss a model where Crr1 carries all three activities (Fig. 6).

CuRE and the GTAC Core. Sequence and functional analysis of Crr1 confirms the presence of a DNA-binding domain with selectivity for the CuRE (Figs. 3 and 5), and, therefore, we conclude that Crr1 houses at least one of the three required properties of a regulator of nutritional copper signaling. Mutational analysis of the promoters on the *CYC6* and *CPX1* genes had indicated the importance *in vivo* of a GTAC core in the CuRE, where every position in the core was critical for CuRE activity (13). The DNA-binding activity of the Crr1-SBP domain on the GTAC core of the CuRE now provides the biochemical basis for this *in vivo* sequence requirement.

The prototypical SBP domain was discovered on the basis of its ability to bind the *SQUAMOSA* promoter in *Antirrhinum majus* (24). Subsequently, a sequence comparison of the *SQUA* promoter, the promoter of the homologous *AP1* gene in *Arabidopsis* and the promoters of other MADS-box genes that are expressed specifically during floral development, suggested a consensus SBP domain-binding site T-CGTACAA− (29). However, specific *in vivo* targets of each SBP domain protein are not known, and, hence, an *in vivo* mutational analysis of the binding site was not possible. The discovery of an SBP domain protein as a regulator of target genes with well defined cis-regulatory sequences (13) and the recovery of the GTAC motif in a target site selection study (23) now allows us to authenticate the DNA-binding site of the SBP domain. We conclude that all positions in the GTAC core (but not the flanking nucleotides) are critical. Nevertheless, other nucleotides must contribute to selectivity because the core by itself is insufficient for *in vivo* CuRE or HyRE (hypoxia responsive element) activity (13, 21).

Copper Sensing. A provocative aspect of the Crr1 sequence was the presence of two candidate metal-binding domains: the SBP domain and the C-terminal Cys-rich sequence, which opens the door to the possibility that the copper sensing activity also resides on Crr1. The SBP domain consensus sequence includes 10 highly conserved His and Cys residues that are potential metal-binding ligands (see Fig. 8). Eight of these residues were shown to bind two Zn ions in an NMR study of two bacterially expressed SBP domains from *Arabidopsis*, and mutagenesis confirmed the importance of the zinc-liganding cysteine residues for the DNA-binding activity of the SBP domain (23, 25). The two remaining His residues could potentially be involved in metal-sensing. The C-terminal Cys-rich sequence (16 Cys and 3 His in 120 C-terminal residues) is unique to Crr1, and its similarity to *Drosophila* metallothionein 2 (see Fig. 11, which is published as supporting information on the PNAS web site) suggests that it may participate in some aspect of metal-sensing. We are considering two, not necessarily mutually exclusive, models for metal sensing based on models developed in other laboratories (2).

In the copper-deficient cell, the zinc-binding site of the SBP is occupied and the protein is located in the nucleus, where it interacts through the ankyrin repeats directly or indirectly with a component of the transcription apparatus, and activates the target genes (Fig. 6*A*). In one model, the structure of the SBP domain is modified, owing to direct interaction of the conserved His residues in SBP with copper and resulting in rearrangement of the zinc-binding site (Fig. 6*B*), and in another model from intramolecular communication with the copper-bound form of the C-terminal Cys-rich region (Fig. 6*C*). The structural change could block DNA binding and/or interaction with coactivators through activation domains, or it could prevent nuclear localization, in either case preventing activation of target genes. The displacement of zinc by copper is precedented by mammalian metallothioneins, which release zinc in the presence of copper, and the intramolecular communication between a DNA-binding domain and a regulatory metal site is precedented by Mac1 (10, 32). An intramolecular interaction between a metal-binding Cys cluster and a transcriptional activation domain is also proposed for MTF-1 (33).

It is also possible that there are multiple layers of metal-responsive regulation of Crr1 function, and metal binding to the C terminus may modify protein localization or degradation. Unlike other essential nutrients, the transition metals are toxic in excess, and, therefore, it is not uncommon for nature to call on multiple layers of regulation, including transcription factor trafficking and degradation, to maintain homeostasis (2).

Nickel and O_2 Sensing. Crr1 function is modified not only by copper, but also by nickel and cobalt ions and oxygen supply. Nickel and cobalt ions function antagonistically to copper so that the target genes are turned on by nickel ions, even in fully copper-replete cells (15). Nickel and cobalt are not essential elements for *Chlamydomonas* and the response, therefore, is clearly artificial, but it provides a useful pharmacological tool to understand the mechanism of copper sensing by Crr1. Comparison of the nickel- and cobalt-induced responses of the target genes suggested that these ions were interfering with copper sensing because the pattern of expression of the target genes resembled closely the pattern noted in the copper-deficiency

state. The most likely scenario is that nickel or cobalt bind at the copper-sensing site with an affinity or an off-rate constant that precludes displacement by copper, and, hence, Crr1 would be locked in a state where it is constitutively active. This idea is precedented by structural studies of the prokaryotic ArsR-SmtB family of metalloregulators, which indicate that the same site can accommodate different metal ions with variation in coordination geometry (34, 35). In this context, the conserved but unliganded histidines in the zinc-bound form are of interest.

The hypoxic activation of the target genes is clearly separate from nutritional copper signaling, and it represents a physiologically relevant mechanism. For instance, the *CPX1* gene responds much more strongly to hypoxia than does the *CYC6* gene, whereas the opposite is true in copper deficiency, and although Crr1 and a CuRE are required for the hypoxic response, a separate HyRE is also required (21). Therefore, we concluded that the hypoxia signaling is not a simple recapitulation of the copper-deficiency response. We proposed instead that hypoxia signaling represents a physiological intersection with the nutritional copper network. One possibility is that hypoxic growth conditions affects aspects of metal metabolism that indirectly impact Crr1 function. Another attractive possibility is that hypoxia alters the redox state of the C-terminal Cys residues leading to a modification of Crr1 function.

The distinction between the hypoxia- and copper-deficiency responses is attributed to the presence of a HyRE on the *CPX1* gene but not the *CYC6* gene. The HyRE also contains a GTAC core, but it cannot function as a CuRE (13). Perhaps the HyRE represents a binding site for another SBP-domain transcription factor, indicating that other domains in the transcription factor contribute to binding site selectivity *in vivo*.

SPB-Domain Proteins in Plants. The SPB domain is a plant-specific DNA-binding domain occurring in 16 proteins in the *Arabidopsis* genome. A subset of the *Arabidopsis* SPLs contain motifs found in Crr1: an AHA motif (found in SPL1, 7, 12, 14, and 16) that is important for activity in other transcription activators (36) at the N terminus, a region of homology linked to the SBP domain that we refer to as an "extended" SBP domain (in SPL1, 3, 7, and 12), and three ankyrin repeats (in SPL1, 12, 14, and 16). It is possible that some of these Crr1-type SPLs are regulated by trace metal nutrients like copper. A recent study suggested a role for a copper transporter of the COPT family for pollen sac development in *Arabidopsis*, but the target copper protein is unknown (37). It may be relevant to consider metal-containing transcription factors as targets for micronutrient dependent phenomena in plant development.

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