Anaerobic Expression of the Ferredoxin-Encoding *FDX5* Gene of *Chlamydomonas reinhardtii* Is Regulated by the Crr1 Transcription Factor

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Aerobic organisms depend on molecular oxygen (O\textsubscript{2}) for efficient energy production as well as for several biosynthetic pathways such as heme, ubiquinone, or cholesterol synthesis. However, O\textsubscript{2} availability can be limited depending on the environment or developmental stage. Organisms react to hypoxic or anaerobic conditions by differential gene expression in order to adjust metabolic and biosynthetic pathways (26). The unicellular eukaryotic green alga *Chlamydomonas reinhardtii* has a complex anaerobic metabolism (10, 19), which is accompanied by the differential expression of many genes (25, 29). Among these genes are transcripts encoding fermentative enzymes like HydA1 (FeFe-hydrogenase) (8) and Pfl1 (pyruvate formate lyase) (10) or enzymes that need O\textsubscript{2} as a substrate for certain steps in biosynthetic pathways such as Cpx1 (oxidative coproporphyrinogen oxidase 1) (33) or Crd1 (magnesium-protoporphyrin IX monomethyl ester aerobic oxidative cyclase) (34).

While the complex anaerobic metabolism of *C. reinhardtii* and the concomitant differential expression of genes, respectively, have been analyzed for years, there is little knowledge about the messengers and factors that are involved in low-O\textsubscript{2} sensing and adjusting the transcription of certain genes.

Only one transcriptional factor is known to be responsible for the transcriptional activation of several genes under hypoxic conditions (33, 34). This factor is the Crr1 (copper response regulator 1) protein (16), which has been identified to be essential for the adaptation of *C. reinhardtii* to copper (Cu) deficiency (4).

Crr1 is a member of the *SQUAMOSA* promoter binding protein (SBP) family carrying a characteristic zinc finger domain, which is responsible for the interaction of Crr1 with the cis-acting DNA sequence GTAC (2, 16). SBP box transcription factors have been identified in many plant species, including green algae, moss, lycophyta, gymnosperms, and angiosperms (7), and have been shown to have functions in different plant organs and diverse stages of development (e.g., see references 17, 22, 38, and 40).

In *C. reinhardtii*, Crr1 is an essential transcriptional activator of several genes that are transcriptionally upregulated under conditions of Cu deficiency, such as *CYC6* (encoding cytochrome c\textsubscript{b}), *CPX1*, and *CRR1* (1, 33, 34), as well as the Cu transporter-encoding genes *CTR1*, *CTR2*, and *CTR3* (30). The 5’ upstream regions of these genes contain at least one critical GTAC core within so-called CuREs (copper response elements), which are essential for the interaction with Crr1 (30, 34). Notably, the transcripts of the Cu-regulated genes mentioned above also accumulate under hypoxic or anaerobic conditions (30, 34). It was shown previously that this transcriptional response is also Crr1 regulated and dependent on GTAC motifs (16, 34).

We reported previously that the *FDX5* gene, one of at least six ferredoxin-encoding genes in *C. reinhardtii* (21), is strongly upregulated at both the transcript and the protein levels in anaerobic algae (13). The Fdx5 protein is a chloroplast-localized plant-type 2Fe2S-ferredoxin with a redox potential similar to that of the photosynthetic ferredoxin PetF (Fdx) (13), but the metabolic function of Fdx5 is still unknown. Very recently, it was shown that the *FDX5* transcript also accumulates in Cu-deficient *C. reinhardtii* cultures (39).

In order to gain deeper insights into the transcriptional regulation of the *FDX5* gene, we made use of a luciferase-encoding gene (*RLUC*) from the soft coral *Renilla reniformis*, which has been codon adapted (*CRLUC*) and established as a reporter gene for *C. reinhardtii* (6). By analyzing algal transformants carrying the *CRLUC* gene under the control of the putative *FDX5* promoter, we could identify two critical GTAC

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motifs that are essential for the transcriptional activation of the FDX5 gene. The SBP domain of C. reinhardtii Crr1 is able to bind these GTAC cores, as shown by electrophoretic mobility shift assays (EMSSAs). Eventually, we confirmed that FDX5 is a strict Crr1 target by showing that a Crr1-deficient C. reinhardtii strain is not able to accumulate FDX5 transcripts or the Fdx5 protein.

**MATERIALS AND METHODS**

**Organisms and growth conditions.** All C. reinhardtii strains (wild-type CC-125 [mt + 137C], cell-wall-less strain CC-425, and crr1 mutant strain CC-3960 crr1-2arg7 [16]) were obtained from the Chlamydomonas Culture Collection at Duke University. If not indicated otherwise, stocks and precultures were grown in Tris-acetate-phosphate (TAP) medium (9) at a light intensity of 80 μmol of photons · s⁻¹ · m⁻² and at a temperature of 20°C.

To analyze CRLUC expression in recombinant C. reinhardtii strains carrying chimeric constructs of CRLUC under the control of various fragments of the putative FDX5 promoter, individual colonies from each transformation were grown in 2 ml TAP in 15 ml Sarstedt centrifuge tubes (Sarstedt, Nürnberg, Germany) in light (80 μmol of photons · s⁻¹ · m⁻²). After the cultures had reached a chlorophyll concentration of about 20 μg · ml⁻¹, conditions known to induce FDX5 gene expression were applied, as described below.

Sulfur (S) deprivation of strains CC-425 and CC-3960 was achieved as described previously (11). For analyzing a large number of individual C. reinhardtii transformants, experiments were conducted with 12-ml-headspace bottles (PJK GmbH, Kleinblittersdorf, Germany) in light (80 μmol of photons · s⁻¹ · m⁻²). A 5-mm glass bead was added to each vessel to improve mixing of the cells. The flasks were incubated on a reciprocal shaker and illuminated from the bottom with 80 μmol of photons · s⁻¹ · m⁻². The algal cells were used for luciferase activity assays after 72 h of S deficiency. At this time point, the cultures were hypoxic and producing hydrogen, as confirmed by gas chromatography (using a GC-2010 gas chromatograph [Shimadzu, Kyoto, Japan] equipped with a fused silica-coated Molsieve column [5Å, 10 m by 0.32 mm; Vario, Palo Alto, CA]) (data not shown).

Anaerobic adaptation by N₂ flushing was conducted as described previously (11). To anaerobically adapt many individual C. reinhardtii transformants, the protocol was modified. C. reinhardtii cultures were grown in 15-ml Sarstedt tubes, concentrated to 80 μmol of photons · s⁻¹ · m⁻². The algal cells were used for luciferase activity assays after 72 h of S deficiency. At this time point, the cultures were hypoxic and producing hydrogen, as confirmed by gas chromatography (using a GC-2010 gas chromatograph [Shimadzu, Kyoto, Japan] equipped with a fused silica-coated Molsieve column [5Å, 10 m by 0.32 mm; Vario, Palo Alto, CA]) (data not shown).

**Luciferase activity assay.** For determining luciferase activity, 200 μl of sulfur-deprived or anaerobically adapted cultures was removed with a syringe from the incubation flask by piercing through the rubber seal and transferred into the individual wells of a 96-well plate (96-well white microplate; Berthold, Bad Wildbad, Germany). Background light emission was detected for 10 s on a photon-counting microplate luminometer (Orion microplate luminometer connected to a personal computer [PC] with Simplicity software 2.1; Berthold Detection Systems, Pforzheim, Germany). Next, 0.5 μl of 2 mM coelenterazine (PKJ GmbH, Kleinblittersdorf, Germany) in methanol was added to the cell samples, and light emission was detected for 10 s again.

In the case of Cu-depleted cells, 2 ml of each culture was disrupted by freezing in liquid nitrogen, thawing, and subsequent sonication three times for 30 s (Transsonic T 460; Elma, Singen, Germany). The cell lysates were stored in liquid nitrogen until the analysis occurred. For measurements of luciferase activity, 200 μl of thawed lysates was transferred into multiwell plates as described above.

**Construction of plasmids.** In order to circumvent the necessity of cotransformation, plasmids containing both the codon-optimized luciferase gene from R. reniformis (CRLUC) (6) and the paromomycin resistance cassette aphVIII (which was under the control of the HSP70 promoter [Fig. 1c], were constructed.

CRLUC was obtained by digesting plasmid pCRLUC (6) with BamHI and Xhol and ligating the fragment into the BamHI- and Xhol-cut vector pBSI(−) (Stratagene, La Jolla, CA) to obtain plasmid pBSLUC.
Vector pSL18 (37) was used as a template to amplify the apvIII gene by PCR, including the 5' and 3' untranslated regions (UTRs) of the C. reinhardtii RBCS2 gene and the 5' region of HSP70A, and to add flanking PdiI restriction sites by using oligonucleotides TTGGCGGCGGTACCCGCTTCAAATACGC and CGCGCGGAGGTACCGCAGGTGG. The amplified fragment was inserted into pGemT-Easy (Promega, Madison, WI) to obtain plasmid pGemParo.

The apvIII cassette was cut from pGemParo by PdiI digestion and inserted into PdiI-cut pBSLuc, which resulted in plasmid pBSLucParo. Finally, the KpnI restriction site in the pBSLucParo plasmid was re-introduced, resulting in the desired promoter cassette sequence for the reporter gene analysis. The cassette was cloned into pCL20 to produce the reporter plasmid pCL20.

The individual PCR products were digested with XhoI and ApaI and inserted into pCL20 digested by the same endonucleases. Mutations within some fragments of the putative FDX5 promoter were introduced by site-directed mutagenesis using oligonucleotides carrying the desired mutation (Table 1). All constructs were sequenced at the sequencing facility at the Ruhr University of Bochum, Germany (Department of Biochemistry I, Receptor Biochemistry).

RNA analyses, RT-PCR, and 5'-RACE PCR. Total RNA was isolated according to a method described previously by Johannimeier and Howell (14). Most parts of the genomic DNA contamination were removed by LiCl precipitation. Ten nanograms of the heterologously produced Crr1 SBP domain and 60 fmol of the individual double-stranded fragments were used for each sample. After incubation, samples were separated via 0.5× TBE (Tris-borate-EDTA buffer) native PAGE and blotted onto a nylon membrane by electroblotting (400 mAh for 30 min). Fragments were visualized via chemiluminescence after immunodetection with anti-digoxigenin antibodies coupled with horseradish peroxidase.

Western blot analyses with polyclonal anti-C. reinhardtii Fdx5 antibody were done as described previously (13).

**RESULTS**

FDX5 transcription depends on two GTAC motifs in both anaerobiosis and copper depletion. FDX5 transcripts accumulate in the absence of both O2 and Cu (Fig. 1a), as was shown previously (13, 39). By applying RT-PCR analyses using mRNA samples isolated from C. reinhardtii cells under different conditions of O2 or Cu availability, no FDX5 transcript could be detected in aerobic C. reinhardtii cultures in the presence of Cu, whereas strong signals appeared in samples from algal cells that had become anaerobic due to sulfur depletion in the light (18) or N2 flushing in the dark or from Cu-depleted, aerobic cells (Fig. 1a).

To gain insights into the regulatory elements within the putative FDX5 promoter, we first carried out a 5'-RACE analysis. The FDX5 transcription start (position +1) was found 99 nucleotides upstream of the translation start codon (position +100). This result is in a 5' untranslated region (UTR) that is 14 bp longer than that indicated by gene model asg.7064.t1 in JGI 4.0.

In silico analyses of the putative FDX5 promoter region 1 to 300 bp upstream of the transcription start point (TS) revealed the presence of three GTAC motifs at positions −116, −96, and −74 relative to the TS (Fig. 1b). GTAC cores were shown previously to be essential for the activation of transcription by the Crr1 transcription factor under both Cu-deficient and hypoxic conditions (30, 33, 34). To analyze the role of these three GTAC motifs in the regulation of the FDX5 gene, chimeric constructs of the CRLUC gene (6) under the control of various fragments of the putative FDX5 promoter were generated. The FDX5 promoter fragments were chosen to contain three, two, or only one of the GTAC motifs as well as GTAC motifs in which single point mutations (GTAC→ATAC) were introduced (Fig. 1b). These constructs were used to transform C. reinhardtii wild-type strain CC-125, and the resulting transformants were exposed to S deprivation in the light. N2 bubbling in the dark, or Cu deficiency to analyze the dimensions of luciferase activity under these conditions.

Several (19 to 55) randomly chosen transformants from each library were analyzed. Smaller numbers of strains were exam-
FIG. 2. Expression of the CRLUC reporter gene controlled by various fragments of the putative FDX5 promoter in C. reinhardtii transformants under different physiological conditions. (a and c) Average luciferase activities in RLU (relative luminescence units) for S- and Cu-deprived C. reinhardtii transformants carrying the respective chimeric constructs. Bars indicate standard deviations, and n indicates the number of individual transformants analyzed for each library. (a) Luciferase activity in S-depleted cells. In the cases of CL24 and CL27, only the luminescent transformants (numbers in parentheses) were included in the calculation. (b) Transcript analysis of CRLUC via RT-PCR using mRNA of C. reinhardtii transformants that had been subjected to anaerobic conditions by N₂ flushing in the dark. The results shown are representative of three individual strains of each construct library. mRNA isolated from strain CC-125 served as an untransformed control, and mRNA from CL20 (in which CRLUC is promoterless) served as the background control. Each RNA sample was also tested for FDX5 transcript accumulation to verify the physiological status of the cells and RNA quality. (c) Luciferase activity in Cu-deficient C. reinhardtii transformants. In the case of strains carrying CL24 and CL27, the activities of four individual luminescent strains were measured. Forty individual strains of each library, CL31, CL36, and CL41, were analyzed. (Inset) Immunoblot analysis with anti-C. reinhardtii Cyc6 antibody using crude protein extracts of randomly chosen Cu-depleted transformants in order to verify the Cu-deficient status of the cells. +, sample isolated from Cu-replete CC-125 cells.

FIG. 2. Expression of the CRLUC reporter gene controlled by various fragments of the putative FDX5 promoter in C. reinhardtii transformants under different physiological conditions. (a and c) Average luciferase activities in RLU (relative luminescence units) for S- and Cu-deprived C. reinhardtii transformants carrying the respective chimeric constructs. Bars indicate standard deviations, and n indicates the number of individual transformants analyzed for each library. (a) Luciferase activity in S-depleted cells. In the cases of CL24 and CL27, only the luminescent transformants (numbers in parentheses) were included in the calculation. (b) Transcript analysis of CRLUC via RT-PCR using mRNA of C. reinhardtii transformants that had been subjected to anaerobic conditions by N₂ flushing in the dark. The results shown are representative of three individual strains of each construct library. mRNA isolated from strain CC-125 served as an untransformed control, and mRNA from CL20 (in which CRLUC is promoterless) served as the background control. Each RNA sample was also tested for FDX5 transcript accumulation to verify the physiological status of the cells and RNA quality. (c) Luciferase activity in Cu-deficient C. reinhardtii transformants. In the case of strains carrying CL24 and CL27, the activities of four individual luminescent strains were measured. Forty individual strains of each library, CL31, CL36, and CL41, were analyzed. (Inset) Immunoblot analysis with anti-C. reinhardtii Cyc6 antibody using crude protein extracts of randomly chosen Cu-depleted transformants in order to verify the Cu-deficient status of the cells. +, sample isolated from Cu-replete CC-125 cells.

The same C. reinhardtii transformants were analyzed for luciferase activity after being flushed with N₂ in the dark to confirm that the luciferase activity detected in sulfur-depleted, anaerobic cells was indeed due to the absence of O₂ rather than to the absence of sulfur. However, no luciferase activity could be detected by using the luminometer. Therefore, mRNA was isolated from 10 to 15 individual transformants of each library and used to analyze CRLUC transcription by RT-PCR (Fig. 2b). These analyses showed the presence of CRLUC transcripts in anaerobic strains carrying the constructs CL24 and CL27 but not in the other transformants (Fig. 2b) and therefore revealed the same pattern of CRLUC expression as what had been observed as luciferase activity in sulfur-depleted C. reinhardtii transformants.

Finally, the transformants were subjected to Cu deficiency. It is known that conditions of trace element deficiency are difficult to establish, since the diminutive amounts that are essential for an organism can be supplied even by diffusion out of glassware or by the entry of dust (31). We have applied a chelator-based method for inducing Cu deficiency in C. reinhardtii cells grown in low but sufficient Cu concentrations (0.3 μM). This approach was shown to allow CYC6 gene expression (5). The C. reinhardtii transformants that had been cultivated under these conditions indeed experienced a Cu deficiency, as shown by the accumulation of the Cyc6 protein in several selected strains (Fig. 2c, inset). After the copper-depleted state of the C. reinhardtii transformants had been proven, they were analyzed by the luciferase activity assay. Again, only transformants carrying the constructs CL24 and CL27 exhibited luciferase activity (Fig. 2c), while C. reinhardtii strains transformed with the constructs CL31, CL36, and CL41 did not.

To confirm that no CRLUC transcript accumulated in Cu-depleted C. reinhardtii transformants of the CL31, CL36, and CL41 libraries, RT-PCR analyses were conducted. RNA isolated from 11 individual strains of each construct library, which had been Cu deprived for 4 days, was tested for the presence of CRLUC and FDX5 transcripts. While strong signals appeared using oligonucleotides specific for FDX5 mRNA, no signals were obtained by using CRLUC-specific primers in
strains carrying the constructs CL31, CL36, and CL41 (data not shown).

The SBP domain of Crr1 binds to the GTAC motifs of the FDX5 promoter. Crr1 is a member of the SBP box family (2, 16). SBP domains harbor a zinc finger domain that is known to interact with the GTAC motif of DNA (2). It was shown previously that the SBP domain of Crr1 binds to the functionally defined (33) CuREs of CPX1 and CYC6 (16). We analyzed whether the SBP domain of Crr1 can also bind to the two proximal GTAC motifs within the FDX5 upstream region described above to be essential for FDX5 promoter activity. By using the heterologously produced Crr1 SBP domain, electrophoretic mobility shift assays were conducted with digoxigenin-labeled 36-bp fragments of the FDX5 promoter, including the two proximal GTAC motifs either intact or mutated to ACTG or TAGA (Fig. 3a). The labeled FDX5 promoter fragments were incubated in the presence or in the absence of 200 ng of a heterologously produced Crr1 SBP domain. No retardation of the fragments could be observed in the absence of the SBP domain (Fig. 3b, lanes 1 to 4). However, the incubation of a fragment of the putative FDX5 promoter including the two proximal (positions −96 and −74) GTAC motifs (construct I) (Fig. 3a) with the Crr1 SBP domain resulted in two shifted bands of the digoxigenin-labeled oligonucleotides (Fig. 3b, lane 5). The fragments carrying only one intact GTAC site (constructs II and III) (Fig. 3a) showed a single band shift, independently of which of the two GTAC motifs was present (Fig. 3b, lanes 6 and 7). The mutation of both GTAC motifs (construct IV) (Fig. 3a) prevented the retardation of the fragment (Fig. 3b, lane 8).

The specificity of the binding of the Crr1 SBP domain to the FDX5 promoter fragments was verified by incubating the SBP domain with constant amounts (60 fmol) of labeled FDX5 construct II (Fig. 3a) and increasing amounts (0 fmol to 6,000 fmol) of unlabeled fragment (construct II or IV) (Fig. 3a). The strength of the shifted band decreased with increasing amounts of unlabeled construct II but not in the presence of unlabeled construct IV, which carries both mutations (Fig. 3c).

FDX5 gene expression is absent in a Crr1-deficient C. reinhardtii strain. The fact that the expression of the FDX5 gene is induced by anaerobiosis, Cu deficiency, and the addition of Ni (13, 39; this study) and the requirement for intact GTAC sites for the activity of the FDX5 promoter, as shown above, strongly indicate that the FDX5 gene is a target of the Crr1 transcriptional activator. To confirm this hypothesis, FDX5 expression was analyzed in O2- or Cu-deficient cultures of a crr1 mutant strain (CC-3960) (16). C. reinhardtii strain CC-425 served as the control strain. Both strains were incubated in sulfur-free medium in the light, flushed with nitrogen in the dark, or transferred into TAP ENEA2 medium including TETA to establish a Cu deficiency (5). To ensure the establishment of anaerobic conditions in the first two cases, in vitro hydrogenase activity as a sensitive indicator of the absence of O2 (8) was analyzed (data not shown). The Cu deficiency of the cells was verified by Western blot analysis using a polyclonal anti-Cyc6 antibody (kindly donated by S. Merchant, UCLA, Los Angeles, CA) (data not shown).

Northern blot analyses using a probe specific for FDX5 mRNA showed a typical pattern of FDX5 transcript accumulation in C. reinhardtii strain CC-425. No FDX5 mRNA was detectable in RNA samples isolated from untreated cells (Fig. 4a), while the FDX5 transcript levels increased significantly in RNA samples isolated from CC-425 cultures subjected to sulfur deprivation for 24, 48, and 72 h; N2 flushing for 0.5, 1, and 3 h; or Cu deficiency for 45 h after the second transfer to copper-free medium (Fig. 4a). However, no FDX5 transcript could be detected in RNA samples isolated from equally treated C. reinhardtii strain CC-3960 (Fig. 4a).

In the case of sulfur-deprived algal cultures, the results obtained by RNA hybridization were confirmed at the protein level (Fig. 4b). By using polyclonal anti-Fdx5-antibodies (13), the Fdx5 protein was detected in crude protein extracts isolated from strain CC-425 subjected to sulfur deficiency for 24,
either in dark-adapted and N2-flushed cells (13, 25, 39) or in shown to be strongly upregulated under anaerobic conditions, the indicated time points of S depletion and N2 flushing and after 48 h the indicated time points of S depletion and N2 flushing and after 48 h of Cu deficiency (−Cu), respectively. Control samples (0 h in the case of −S and −O2 and + in the case of Cu) were isolated from the strains grown in TAP medium in the light. (b) Immunoblot analysis using anti-Fdx5 antibody of crude protein extracts isolated from S-deficient strains CC-425 and CC-3960 after the indicated time points of S deprivation. A protein amount equivalent to 2 μg of chlorophyll was loaded into each lane.

48, and 72 h, while no signals were visible using protein extracts from sulfur-deprived Crr1-deficient strain CC-3960 (Fig. 4b).

**DISCUSSION**

The expression of the FDX5 gene of *C. reinhardtii* has been shown to be strongly upregulated under anaerobic conditions, either in dark-adapted and N2-flushed cells (13, 25, 39) or in illuminated, sulfur-deprived cultures (13). FDX5 transcripts also accumulate significantly in Cu-limited cultures (39; this study). The transcription of several genes is activated by both O2 and Cu deprivation in *C. reinhardtii* (1, 23, 30, 34), and this dual response is mediated by one transcriptional factor, called Crr1 (16, 30, 34). Crr1 has been identified to be a SBP domain protein (16), and the recombinant Crr1 SBP domain is able to bind GTAC motifs (2, 16).

In the upstream regions of CYC6, CPX1, CRD1, and CTR1, one or more GTAC motifs have been detected, and their influence on gene expression was analyzed by using reporter gene assays. The GTAC motif was shown to be the core of copper response elements (CuREs), and in the case of the CPX1 gene, it was also proven that GTAC is a hypoxia response element (HyRE) (33, 34).

In view of the accumulation of the FDX5 transcript under both hypoxic and Cu-deficient conditions, a regulation of the FDX5 gene similar to that of the reported genes mentioned above was compelling. *In silico* analyses of the region at positions −300 to +1 relative to the transcription start point of the putative FDX5 promoter indeed showed the presence of three GTAC sites at positions −116, −96, and −74 relative to the transcriptional start point of the FDX5 gene.

Using the *Renilla* luciferase-encoding gene CRLUC as a reporter gene (6), we were able to show that a fragment of the putative FDX5 promoter involving bases −146 to +100 relative to the transcriptional start point (construct CL24) was able to activate the expression of the luciferase-encoding gene in response to anaerobiosis or to Cu deficiency. The successive truncation of this promoter region revealed the two proximal GTAC sites that are responsible for the hypoxic and Cu responses (31, 32) (Fig. 5). In both the CPX1 (33) and CTR1 (30) promoters, one single GTAC motif has been shown to be the CuRE. The CYC6 promoter contains two Cu-responsive GTAC sites, which, however, are both able to confer Cu responsiveness independently (33) (Fig. 5).

Regarding the response to hypoxia, only the GTAC sites of

![FIG. 4. Expression of the FDX5 gene in *C. reinhardtii* strain CC-425 and Crr1-deficient strain CC-3960 at the transcript level (a) and the protein level (b). (a) RNA hybridization analyses of RNA isolated from strains CC-425 and CC-3960 subjected to S deprivation (−S), N2 bubbling in the dark (−O2), or Cu deficiency (−Cu) using probes specific for FDX5 or RPL10a. RNA was isolated from the cells after the indicated time points of S depletion and N2 flushing and after 48 h of Cu deficiency (−Cu), respectively. Control samples (0 h in the case of −S and −O2 and + in the case of Cu) were isolated from the strains grown in TAP medium in the light. (b) Immunoblot analysis using anti-Fdx5 antibody of crude protein extracts isolated from S-deficient strains CC-425 and CC-3960 after the indicated time points of S deprivation. A protein amount equivalent to 2 μg of chlorophyll was loaded into each lane.

![FIG. 5. Schematic comparison of the promoter regions of several Crr1 target genes. Sequences were derived from the original research articles (CYC6 [33], CPX1 [32], CTR1 [30], and CRD1 [1]) and from the Augustus 5 gene model annotated in the *C. reinhardtii* genome sequence in JGI 4.0 for FDX5: au5.g7064.t1 (transcription start site 14 bp upstream at position chromosome_17:702568 in JGI 4.0 [identified in this work]). Positions of GTAC sites are indicated as squares and triangles and numbered relative to the transcription start site at position +1. In the case of CPX1, the transcription start point of the longest transcript described previously by Quinn et al. (32) was chosen. Black symbols indicate that a function of the respective GTAC motif as a CuRE (black squares) or HyRE (black triangles) was proven, gray symbols represent GTAC sites which were shown to have no function, and white symbols stand for GTAC sequences that have not been analyzed regarding their function. The asterisks above the CuRE symbols in the CYC6 scheme indicate that both sites are able to confer Cu responsiveness individually.]
the CPX1 promoter have been analyzed in detail. In contrast to the Cu response, a single CuRE is not sufficient for an enhanced expression of a reporter gene under hypoxic conditions. Rather, the CuRE and a second GTAC motif, referred to as the HyRE, have to be present in the promoter region to allow hypoxia-responsive expression of the reporter gene (34) (Fig. 5).

Thus, regarding the transcriptional activation of the FDX5 gene as a response to hypoxia, the FDX5 promoter is similar to the promoter of the CPX1 gene (34) (Fig. 5).

It is noteworthy that while both proximal GTAC sites of the FDX5 promoter are necessary to induce gene transcription in vivo, the SBP domain of Crr1 is able to bind to both GTAC sites of the FDX5 promoter individually, as shown by a clear band shift of all constructs of the respective region that contain at least one wild-type GTAC motif. Obviously, the fact that Crr1 is able to bind to one GTAC site via its SBP domain is not sufficient to confer transcriptional activation in vivo.

It has been suggested from a detailed mutational analysis of the CYC6 promoter that additional nucleotides in the CYC6 promoter are important for the proper expression of a reporter gene (33). Again, this indicates that the binding of the SBP domain to a GTAC core alone is not sufficient to activate transcription properly. The appearance of a double-shift band in EMSAs using the wild-type FDX5 promoter fragment, containing both GTAC sites, might hint at a double binding of the SBP domain to sequences containing two GTAC motifs (27).

Extrapolating this hypothesis to the in vivo background of living C. reinhardtii cells, this might indicate that two SBP domain proteins are necessary to activate the transcription of the FDX5 gene. This has to be analyzed in more detail in the future, also taking into account additional domains of the responsible transcriptional activator that influence the binding and/or dimerization capacity of the factor.

The fact that the Crr1 SBP domain binds to the GTAC sites of the putative FDX5 promoter is still no ultimate evidence for FDX5 being regulated by the Crr1 transcriptional activator. Several SBP domains have been shown to bind to GTAC motifs, and the SBP domain of Crr1 also binds an Arabidopsis thaliana promoter fragment, including a GTAC site, while on the other hand, a C. reinhardtii CYC6 promoter fragment can be retarded by SBP domains from Arabidopsis and Physcomitrella (2). At least seven genes that putatively encode SBP domain proteins have been detected in the C. reinhardtii genomic sequence (7). However, the hypothesis that the FDX5 gene is under the transcriptional control of the Crr1 protein was confirmed by the fact that a C. reinhardtii crr1 mutant strain does not show any detectable accumulation of FDX5 transcripts or the Fdx5 protein. Thus, FDX5 is clearly regulated by Crr1 in response to Cu and O2 limitation.

It remains a matter of debate as to why the two environmental conditions of Cu and O2 deficiencies are sensed and signaled by the same pathway. Cu is less soluble and bioavailable under anaerobic conditions, so the evolution of one signaling pathway to react to these two coupled conditions might make physiological sense (3). The proteins encoded by the known Cu- and hypoxia-responsive genes have specific functions in Cu or in O2 deficiency but not always obviously in both. While the role of the Cyce protein is evident in Cu-depleted C. reinhardtii cells, where it replaces the Cu protein plastocyanin (20), a role of Cyc6 under anaerobic conditions is not known. The same is true for copper transporters like Ctrl1 (30). Cpx1 is involved in heme biosynthesis, which might be enhanced to provide a sufficient amount of heme as the cofactor of Cyc6 (12). On the other hand, the enhanced expression of the CPX1 and CRDI genes under hypoxic conditions (23, 24, 34) might be an attempt to scavenge O2 molecules, since both encoded enzymes are O2 dependent.

Ferredoxins are small soluble electron carriers like cytochrome c, and there are manifold pathways that involve electron transfer reactions mediated by ferredoxins. Some of these are O2 dependent, such as fatty acid desaturation (36), which might provide a link to the O2 responsiveness of a ferredoxin-encoding gene.

C. reinhardtii has at least six ferredoxin-encoding genes, which are differentially expressed under various environmental conditions (13, 39). Interestingly, the putative promoter regions of PETF, FDX2, FDX4, and FDX6 each contain one GTAC motif as well. However, FDX5 is the only gene whose transcript strongly and significantly accumulates upon treatment known to elicit Crr1-dependent responses (13, 39). Obviously, the small electron carrier ferredoxin 5 has a specific role in anaerobic and/or Cu-deficient C. reinhardtii cells, which, however, remains to be elucidated.

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