Transcription Factor–Dependent Chromatin Remodeling at Heat Shock and Copper-Responsive Promoters in Chlamydomonas reinhardtii

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How transcription factors affect chromatin structure to regulate gene expression in response to changes in environmental conditions is poorly understood in the green lineage. To shed light on this issue, we used chromatin immunoprecipitation and formaldehyde-assisted isolation of regulatory elements to investigate the chromatin structure at target genes of HSF1 and CRR1, key transcriptional regulators of the heat shock and copper starvation responses, respectively, in the unicellular green alga Chlamydomonas reinhardtii. Generally, we detected lower nucleosome occupancy, higher levels of histone H3/4 acetylation, and lower levels of histone H3 Lys 4 (H3K4) monomethylation at promoter regions of active genes compared with inactive promoters and transcribed and intergenic regions. Specifically, we find that activated HSF1 and CRR1 transcription factors mediate the acetylation of histones H3/4, nucleosome eviction, remodeling of the H3K4 mono- and dimethylation marks, and transcription initiation/elongation. By this, HSF1 and CRR1 quite individually remodel and activate target promoters that may be inactive and embedded into closed chromatin (HSP22F/CYC6) or weakly active and embedded into partially open (CPX1) or completely open chromatin (HSP70A/CRD1). We also observed HSF1-independent histone H3/4 deacetylation at the RBCS2 promoter after heat shock, suggesting interplay of specific and presumably more generally acting factors to adapt gene expression to the new requirements of a changing environment.

INTRODUCTION

Living organisms may acclimate to abiotic stress by the up- and downregulation of specific sets of genes. Since chromatin remodeling plays an important role in the regulation of gene expression in all eukaryotes examined to date (Kouzarides, 2007; Li et al., 2007), we are interested in how chromatin remodeling affects gene expression in plant systems as a consequence of changes in environmental conditions. We studied this issue in the unicellular green alga Chlamydomonas reinhardtii. Chlamydomonas has the advantage that changes in environmental conditions may be homogeneous and instantaneous applied to all cells in a cell culture. Moreover, in contrast with land plants, Chlamydomonas cells are not differentiated into different cell types or organized into different tissues. The stress responses on which our studies focus, those permitting acclimation to heat shock and copper deficiency, are well characterized in Chlamydomonas and therefore well suited for investigations into transcriptional regulation at the chromatin level (Merchant et al., 2006; Schulz-Raffelt et al., 2007).

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The heat shock response is regulated by evolutionarily conserved heat shock transcription factors (HSFs), which are activated by hyperphosphorylation and bind as trimers to cis-regulatory motifs known as heat shock elements (HSEs) (Sorger and Pelham, 1988; Sorger and Nelson, 1989). HSEs contain at least three 5′-nGAAn-3′ repeats in alternating orientations and are present in the promoters of heat shock genes in a diverse set of organisms (Pelham, 1982). As deletion of HSEs from the Chlamydomonas HSP70A promoter entirely abolishes its heat shock inducibility, HSEs are also clearly indispensable for the regulation of the heat shock response in Chlamydomonas (Lodha et al., 2008). Chlamydomonas contains a single canonical HSF (HSF1), which possesses all features typical for plant (class A) HSFs and represents a key regulator of the stress response in this alga (Schulz-Raffelt et al., 2007). Like in the yeast Saccharomyces cerevisiae (but in contrast with the situation for other organisms), Chlamydomonas HSF1 forms trimers constitutively and becomes activated by hyperphosphorylation. The two heat shock genes investigated in this work are HSP70A and HSP22F. HSP70A encodes a cytosolic chaperone, which is constitutively expressed and further induced after heat shock (Müller et al., 1992). HSP22F encodes a small heat shock protein that is most likely targeted to the chloroplast (Schroda and Vallon, 2008) and only expressed under stress conditions like heat shock (this work). The copper response regulator (CRR1) is the key regulator of copper homeostasis in Chlamydomonas as it mediates activation and repression of target genes of the copper response pathway. CRR1 contains a plant-specific DNA binding domain named SBP that recognizes defined copper response elements (CuREs) with a 5′-GTAC-3′ core sequence. CRR1 binding to the CuREs within the CYC6, CPX1, and CRD1 promoters leads to
transcriptional activation of these genes (Quinn and Merchant, 1995; Kropat et al., 2005; Sommer et al., 2010). The CYC6 gene encodes cytochrome c6 (Merchant and Bogorad, 1986), which substitutes for the copper-containing plastocyanin in photosynthetic electron transport under copper-deficiency conditions (Wood, 1978; Merchant and Bogorad, 1987). The CPX1 gene encodes coprogen oxidase (Quinn et al., 1999), and the copper response defect1 (CRD1) gene encodes a plastid-localized putative diiron protein that is required for the synthesis of protochlorophyllide (Moseley et al., 2000; Tottey et al., 2003). Hence, the CPX1 and CRD1 gene products both are involved in tetrapyrrole biosynthesis.

Chromatin structure is dictated in large part by posttranslational modifications of the unstructured N termini of histones (Luger, 2003; Kouzarides, 2007). Of the many known histone modifications, several are especially intensely studied because they are consistently associated with increased or reduced levels of transcription. These include acetylation of histone H3 at Lys-9 and -14, of histone H4 at Lys-5/8/12/16 and methylation of histone H3 at Lys-4 (Li et al., 2007).

Histone Lys acetylation is mediated by histone acetyltransferases, which in turn are recruited by transcription factors that bind to cis-regulatory elements on the underlying DNA (de la Cruz et al., 2005). Histone acetylation again may be recognized by proteins containing bromodomains (Owen et al., 2000) or tandem PHD fingers (Zeng et al., 2010) that may themselves be histone acetyltransferases, factors with ATP-dependent chromatin remodeling activity like SNF2 or Brahma, or components of chromatin remodeling complexes like CHRAC, SAGA, or RSC (Aalfs and Kingston, 2000).

Methyl marks are deposited by methyl-transferases that may, for example, be recruited by the Ser5-phosphorylated RNA polymerase II to target methylation of nucleosomes at the 5’ ends of active genes (Krogan et al., 2003; Ng et al., 2003). Lys methylation is recognized by proteins containing chromodomains, WD40 repeats, or PHD fingers via aromatic cages, which allow discriminating between mono-, di-, and trimethylated lysines (Couture et al., 2006; Li et al., 2006; Ruthenburg et al., 2006). ING2 (inhibitor of growth) is an example of a protein that harbors a PHD finger that recognizes trimethylated Lys-4 at histone H3, which is typically present at promoters of highly transcribed genes (Peña et al., 2006). ING2 in turn may recruit the mSin3a-histone deacetylase complex to repress active genes in response to DNA damage (Shi et al., 2006).

With the goal of determining the relationship between chromatin state and transcriptional activation of heat shock and copper-regulated genes in Chlamydomonas, we monitored transcription factor binding, nucleosome occupancy, and levels of histone H3/4 acetylation and histone H3 Lys 4 (H3K4) mono- and dimethylation at heat shock and copper-regulated genes using chromatin immunoprecipitation (ChIP), while in parallel monitoring changes in RNA abundance by quantitative real-time RT-PCR (qRT-PCR). The gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase 2 (RBCS2) was included as control. We also used formaldehyde-assisted isolation of regulatory elements (FAIRE) (Giresi et al., 2007) to obtain additional information concerning the chromatin state under different environmental conditions. Combined, these approaches provided us with insights into the underlying mechanisms of chromatin remodeling preceding transcriptional activation in Chlamydomonas.

RESULTS

HSF1 Is Required for Target Gene Activation by Heat Shock

To study the role of the HSF1 transcription factor in regulating chromatin structure at its target genes, we needed hsf1 mutant strains. As a stable hsf1 knockout mutant is not available, we generated strains that are downregulated for HSF1 using RNA interference (RNAi) and artificial microRNA (amiRNA) approaches as described previously (Schulz-Raffelt et al., 2007; Schmollinger et al., 2010). HSF1-RNAi and HSF1-amiRNA strains were both selected on the basis of thermosensitivity and therefore contained similarly low levels of residual HSF1 protein (Figure 1A; see Supplemental Figure 1A online). As expected, the downregulation of HSF1 strongly impaired but did not entirely abolish the transcription of HSF1 target genes under heat stress: compared with control strains, heat shock-induced transcript accumulation for HSP70A, HSF1, and HSP22F in these lines was reduced on average from ~6.5-fold to ~2.5-fold, ~16-fold to ~2.7-fold, and ~840-fold to ~6.6-fold, respectively (Figure 1B; see Supplemental Figure 1B online). Heat shock had no effect on the accumulation of RBCS2 and CYC6 transcripts in control and HSF1-underexpressing lines (Figure 1B).

HSF1 Binds to the Control Regions of the HSP70A and HSP22F Promoters

To characterize the interaction of HSF1 with its predicted target promoters, we performed ChIP assays on control and HSF1-RNAi/amiRNA lines with an affinity-purified polyclonal antiserum against HSF1 (Schulz-Raffelt et al., 2007; see Supplemental Figures 2C and 2D online). In control strains, under nonstress conditions, ChIP with HSF1 antibodies enriched the HSP70A, HSP22F, RBCS2, and CYC6 promoters, respectively, compared with control strains. Heat shock led to ~1.6 times less HSP70A, ~1.4 times less HSP22F, ~3.4-fold less HSP70A and ~4.1-fold less HSP22F promoter fragments, respectively, compared with the control strain, while no change was observed in the amount of precipitated HSP22F promoter fragments. Under heat shock conditions, ~3-fold less HSP70A promoter fragments was precipitated from HSF1-RNAi/amiRNA strains compared with the control strain and enrichment
of HSP22F promoter fragments was completely abolished. These data suggest that HSF1 constitutively binds to the HSP70A promoter, but not the HSP22F promoter, and that binding at both promoters increases 4-fold during heat stress. Moreover, HSF1 appears to have a higher affinity for the HSP70A promoter than for the HSP22F promoter, as judged from the binding of residual HSF1 to HSP70A but not to HSP22F in HSF1-RNAi/amiRNA strains.

HSF1 Appears to Be Responsible for Nucleosome Remodeling

To analyze whether HSF1 affects nucleosome occupancy at its target promoters, we performed ChIP with antibodies against the C-terminus of core histone H3, which is known not to be modified. For a better comparability of biological replicates, we normalized values resulting from qPCR quantification of precipitated DNA fragments relative to those obtained for amplification of 10% input DNA and to the values obtained for the CYC6 promoter. The CYC6 promoter is inactive in the presence of copper (Quinn and Merchant, 1995), which explains why heat shock had no effect on mRNA expression, nucleosome occupancy, or histone modifications at CYC6 (Figure 1B; see Supplemental Figures 4B and 5A online).

In nonstressed control cells, nucleosome occupancy at the heat shock gene promoters was 30 to 50% lower than at the CYC6 promoter. Heat shock led to a further reduction of nucleosome occupancy by ~1.6-fold at the HSP22F promoter and by ~10-fold at the HSP70A promoter (Figure 4A; see Supplemental Figure 4A online). The latter result was consistent with the observation that much lower levels of HSP70A promoter fragments were precipitated with antibodies against modified histones from heat shock samples compared with nonstressed controls (see below). In the HSF1-RNAi/amiRNA strains, we observed the same ~10-fold reduction of nucleosome occupancy at the HSP70A promoter after heat shock as seen in the control strain, whereas the reduction of nucleosome occupancy at the HSP22F promoter was less pronounced. This suggested

Figure 1. Analysis of Protein and Transcript Levels in HSF1-Underexpressing Strains Prior to ChIP Analysis.

(A) HSF1 abundance is reduced in HSF1-amiRNA and -RNAi strains. Control, HSF1-amiRNA, and HSF1-RNAi lines were kept under nonstress conditions (CL) or subjected to heat shock (HS) for 30 min. Whole-cell proteins were extracted, and proteins corresponding to 2 μg chlorophyll were separated by SDS-PAGE and analyzed by immunoblotting using antisera against HSF1 and CF1β (as loading control).

(B) Accumulation of selected transcripts in control and HSF1-RNAi/amiRNA cells. RNA was extracted from nonstressed cells and cells subjected to a 30-min heat shock for analysis by qRT-PCR using the comparative CT method with CBLP2 as control gene. Primer efficiencies and qRT-PCR end products (amplicons) for all target transcripts are presented in Supplemental Figure 3 online. Shown are fold changes in transcript accumulation between stressed versus nonstressed conditions in control (black) and HSF1-amiRNA/amiRNA lines (white), respectively. Three technical replicates each from HSF1-RNAi line #10 (triangles) and HSF1-amiRNA line #5 (diamonds) were performed.

Figure 2. Regions Amplified from Chromatin Immunoprecipitates by qPCR.

Shown are the six genes investigated in this study. Promoter regions are indicated by gray boxes, transcriptional start sites (TS) by arrows, translated regions by black boxes, untranslated regions by white boxes, and introns by thin lines. The HSP70A promoter has two transcriptional start sites designated TSA1 and TSA2 (von Gromoff et al., 2006). Vertical black lines designate putative HSEs in the HSP70A and HSP22F promoter regions and putative C-terminal proteins at the CYC6, CPX1, and CRD1 promoters. Gray bars designate the regions amplified by qPCR (if not indicated otherwise, region I was used by default for HSP22F and CYC6).
that HSF1 was to some extent responsible for reducing nucleosome occupancy at the HSP22F promoter. This may be true also for the HSP70A promoter but might be concealed by its higher affinity for HSF1, leading to binding of the residual HSF1 present in HSF1-RNAi/amiRNA strains (Figure 3).

Interestingly, nucleosome occupancy at the RBCS2 promoter increased by ~20% after heat shock (Figure 4A). As this effect was observed equally in control and HSF1-RNAi/amiRNA strains, it appears to be independent of HSF1.

**HSF1 Promotes Increased Levels of Histone H3/H4 Acetylation at Heat Shock Gene Promoters**

The levels of histone H3 and H4 acetylation and the methylation state of Lys-4 (K4) at histone H3 are known to be crucial marks for the regulation of euchromatic genes. Hence, we wanted to determine whether HSF1 binding to the heat shock gene promoters influences levels of H3/4 acetylation and H3K4 mono- and dimethylation of local nucleosomes. We first performed ChIP analyses using antibodies against di-acetylated histone H3 and tetra-acetylated histone H4. We chose to generally express histone modifications (e.g., Figure 4B) relative to the abundance of nucleosomes at the DNA fragment investigated (Figure 4A) to account for variations in nucleosome occupancy.

Strikingly, in control cells, histone H3 was acetylated at ~14-fold higher levels at the HSP70A promoter than at the CYC6 promoter (Figure 4B). Although the low nucleosome occupancy of the HSP70A promoter during heat shock makes correct quantification of histone modifications difficult, H3 acetylation levels appeared to be equally high under nonstress and stress conditions. By contrast, histone H3 acetylation levels at the HSP70A promoter were ~35 to 50% lower in HSF1-RNAi/amiRNA strains under nonstress and stress conditions, thus pointing to a role of HSF1 in promoting histone H3 acetylation. In contrast with the HSP70A promoter, histone H3 acetylation levels at the HSP22F promoter under nonstress conditions were only slightly elevated when compared with the CYC6 promoter. Levels of H3 acetylation at the HSP22F promoter were the same in control cells as in the HSF1-RNAi/amiRNA strains under nonstress conditions but increased more than 3-fold in the control strain following heat shock, while they did not increase at all in the HSF1-RNAi/amiRNA strains. Again, these results point to a role of HSF1 in promoting H3 acetylation at heat shock gene promoters. Histone H3 acetylation levels at the RBCS2 promoter were ~4-fold higher than at the CYC6 promoter and tended to decline during heat shock. As the effect was similar in control and HSF1-RNAi/amiRNA strains, it appears to be HSF1 independent.

ChIP analyses using antibodies against acetylated H4 yielded similar results. In control cells under nonstress conditions, histone H4 at the HSP70A promoter was acetylated at ~10-fold higher levels than histone H4 at the CYC6 promoter (Figure 4C). Although difficult to assess accurately, levels of H4 acetylation of the few nucleosomes remaining on HSP70A promoter fragments tended to increase even more during heat shock. This tendency was not observed in HSF1-RNAi/amiRNA strains, thus suggesting a role of HSF1 also in promoting H4 acetylation. In control cells, histone H4 acetylation levels at the HSP22F promoter were ~2- and ~16-fold higher than at the CYC6 promoter during nonstress and heat shock conditions, respectively (Figure 4C). By contrast, in HSF1-RNAi/amiRNA strains under nonstress conditions, H4 acetylation levels at the HSP22F promoter were as low as at the CYC6 promoter, and during stress, they were only ~7-fold higher, supporting the conclusion that HSF1 is required for H4 acetylation at the heat shock gene promoters. Under nonstress conditions, histone H4 acetylation levels at the RBCS2 promoter were even ~6-fold higher than at the CYC6 promoter, but during heat shock dropped to the same low levels as at the CYC6 promoter. Since this effect was observed in both control and HSF1-RNAi/amiRNA strains, it appears to be HSF1 independent.

Under nonstress conditions, histone H3K4 dimethylation levels at the CYC6 and HSP22F promoters were comparable, whereas they were ~3- and ~8-fold lower at the HSP70A and RBCS2 promoters than at CYC6, respectively (Figure 4D). Heat shock appeared to result in an ~2.7-fold increase in H3K4 dimethylation levels at the HSP70A promoter; however, given the low nucleosome occupancy at the HSP70A promoter during heat shock, this observation may not be meaningful. In control cells, H3K4 dimethylation levels were comparable at promoters CYC6, HSP22F, and RBCS2 under nonstress and heat shock conditions (Figure 4E). By contrast, H3K4 dimethylation was ~6-fold lower at the HSP70A promoter than at CYC6 under nonstress conditions but seemed to increase ~4-fold during heat shock. As for dimethylation of H3K4, this result might not be
Figure 4. Analysis of Nucleosome Occupancy and Histone Modifications at Heat Shock–Responsive and Control Promoters.

(A) Nucleosome occupancy declines at heat shock gene promoters after heat shock. ChIP was done as described in Figure 3 but using antibodies against the unmodified C terminus of histone H3 to determine nucleosome occupancy at the indicated promoters in control (black bars) and HSF1-underexpressing strains (gray bars).

(B) HSF1 promotes acetylation of histone H3 at the HSP70A and HSP22F promoters. ChIP was done using antibodies against acetylated Lys-9 and -14 of histone H3.

(C) HSF1 promotes acetylation of histone H4 at the HSP22F promoter. ChIP was done using antibodies against acetylated Lys-5, -8, -12, and -16 of histone H4.

(D) HSF1 has no effect on histone H3 dimethylation at the heat shock gene promoters. ChIP was done using antibodies against dimethylated Lys-4 at histone 3 (H3K4).

(E) HSF1 might reduce histone H3 monomethylation at the HSP22F promoter. ChIP was done using antibodies against monomethylated Lys-4 at histone 3 (H3K4).

qPCR data from the experiments in (B) to (E) are given relative to the nucleosome occupancy at the respective promoter region (data from [A]). Error bars indicate standard errors of the mean of two biological replicates, with each analyzed in triplicate. Asterisks indicate the significance of change at the respective promoter compared with control cells under nonstress conditions (t test, P value ≤ 0.05).
meaningful because few nucleosomes remain on HSP70A promoter fragments under heat shock conditions. While the same patterns of H3K4 dimethylation were observed in control and HSF1-RNAi/amiRNA strains and therefore appeared not to depend on HSF1, levels of H3K4 monomethylation were higher at the HSP22F promoter under nonstress and stress conditions in the hsf1 mutant compared with control cells. This suggests that HSF1 might be responsible for the reduced levels of monomethylation at the HSP22F promoter.

HSF1 Binding at the HSP22F Promoter Precedes Histone Acetylation/Eviction and Transcriptional Activation

To gain mechanistic insights into how transcriptional activation by HSF1 is mediated in Chlamydomonas, it is necessary to resolve when exactly after onset of heat stress the processes of transcription factor binding, histone modification, histone eviction, and transcription take place. Under nonstress conditions, the HSP22F gene is not transcribed, HSF1 does not bind to the promoter, nucleosome occupancy is relatively high, and histones H3 and H4 contain low levels of acetylation (Figures 3 and 4; see Supplemental Figure 2D online). Hence, the HSP22F gene appears to be an ideal target to study the sequence of events leading to transcriptional activation by heat stress. To this end, we performed a time-course analysis of HSF1 binding, histone occupancy, and histone H3/4 acetylation at the HSP22F promoter and of HSP22F mRNA accumulation within the first 10 min after exposing control cells to heat stress. This analysis revealed that occupation of the HSP22F promoter by HSF1 is detectable already 30 s after the onset of heat shock, which correlates with an ~2-fold increase in levels of histone H4 acetylation (Figure 5). Within 60 s after onset of heat stress, HSF1 has already reached ~30% of its maximal occupancy at the HSP22F promoter and acetylation levels of histones H3 and H4 have increased ~2- and ~5.6-fold, respectively, which coincides with a reduction of histone occupancy by ~35%. Note that almost identical results were obtained when a region located at the HSP22F transcriptional start site rather than at the HSEs was amplified from chromatin precipitates (region II of HSP22F in Figure 2; see Supplemental Figure 6A online). As a strong increase in HSP22F transcript levels was detected only 2 min after onset of heat stress, nucleosome remodeling at the HSP22F promoter is more likely a prerequisite for rather than a consequence of transcription.

Nucleosome Remodeling at Promoters of Copper-Responsive Genes Depends on CRR1

To elucidate whether our results from HSF1-mediated chromatin remodeling at heat shock promoters can more generally be applied to other Chlamydomonas promoters responsive to changes in environmental conditions, we extended our studies to the copper response. In contrast with the situation for HSF1, a mutant harboring a stable knockout of the gene encoding the key regulator of copper homeostasis, CRR1, is available (Eriksson et al., 2004). As expected, the induction of CRR1 target genes CYC6, CPX1, and CRD1 after copper depletion was entirely abolished in the crr1 knockout mutant (Figure 6), hence corroborating the results reported previously by Kropat et al. (2005). The variation in CRR1 target gene expression levels in control (CRR1+) cells under copper-depleted conditions is due to slight variations in residual copper ion concentrations in the cell cultures.

As we were not able to immunoprecipitate native or green fluorescent protein–tagged CRR1, we could not directly test for preloading of copper-responsive promoters by CRR1. Hence, we had to limit our analysis to the investigation of nucleosome occupancy and histone modifications in control and crr1 mutant strains under copper-replete and copper deprivation conditions. This time, we normalized qPCR quantification values relative to those obtained for the RBCS2 promoter, whose associated expression levels, nucleosome occupancy, and histone modifications remained unaffected by copper starvation (see Supplemental Figure 5B online). In control and crr1 mutant cells, nucleosome occupancy under copper-replete conditions was similar between the CYC6 and RBCS2 promoters, whereas it was 40 to 60% lower at the CPX1 and CRD1 promoters (Figure 7A). In control cells, copper depletion led to a 1.3- to 2-fold reduction of nucleosome occupancy at the CYC6, CPX1, and CRD1 promoters, whereas no such effect was observed in crr1 mutant cells. These results suggest that, similar to what was observed for HSF1 at the HSP22F promoter, CRR1 appeared to be responsible for reducing nucleosome occupancy at copper-responsive promoters.

CRR1 Promotes Higher Levels of Histone H3/H4 Acetylation and Lower Levels of H3K4 Mono- and Dimethylation at the CYC6 and CPX1 Promoters

We next asked whether CRR1, like HSF1, promotes histone acetylation at its target promoters. As shown in Figure 7B, this is indeed the case for some targets: in control cells, after copper depletion, H3 acetylation increased at the CYC6 and CPX1 promoters by factors of ~4 and ~1.6, respectively, whereas this effect was not observed in the crr1 mutant. No changes in histone H3 acetylation levels were observed at the CRD1 promoter. Interestingly, under copper-replete conditions, H3 acetylation levels were ~2-fold lower at the CYC6 promoter than at the RBCS2 promoter, whereas they were ~1.5- and 2.5-fold higher at the CPX1 and the CRD1 promoters, respectively, than at RBCS2.

A similar picture was obtained for H4 acetylation. Here, under copper-replete conditions, acetylation levels at the CYC6 and CPX1 promoters were ~6- and ~3-fold lower, respectively, than at the RBCS2 promoter. After copper depletion, however, H4 acetylation at CYC6 and CPX1 increased more than 8-fold relative to copper-replete conditions (Figure 7C). This effect was not observed in the crr1 mutant, indicating that H4 acetylation of nucleosomes at the CYC6 and CPX1 gene promoters is mediated by the CRR1 transcription factor. Histone H4 acetylation at the CRD1 promoter was independent of both copper availability and CRR1: in all strains and under all conditions tested, it was ~2.5-fold higher than at the RBCS2 promoter.

To get an estimate on how much the region that was chosen within the target promoter for amplification from chromatin immunoprecipitates influenced the results, we analyzed nucleosome occupancy and H3/4 acetylation at a different region of the CYC6 promoter in control and crr1 mutant cells under
copper-replete conditions and after copper depletion (region II of the CYC6 promoter in Figure 2). While histone occupancy was ~1.7-fold higher within the CYC6 5' untranslated region (region I) than at the relevant copper-responsive elements (region II), CRR1-dependent reduction in nucleosome occupancy and relative increases in H3/4 acetylation were the same at both regions (see Supplemental Figure 6B online). Combined with the results obtained for the two different regions analyzed within the HSP22F promoter (Figure 2), these data indicate that the chromatin state at the actual promoter regions appears to spread into the flanking regions.

Under copper-replete conditions, the CYC6 and CPX1 promoters are associated with 7- to 8-fold higher levels of histone H3K4 dimethylation than the RBCS2 promoter, and the CRD1 promoter possesses ~2.5-fold higher levels than RBCS2 (Figure 7D). At the CYC6 and CPX1 promoters, H3K4 dimethylation levels decreased ~2-fold in response to copper depletion. This effect was not as pronounced in the crr1 mutant, suggesting a role for the CRR1 transcription factor in mediating remodeling of the dimethylation mark. H3K4 dimethylation levels at the CRD1 promoter were the same regardless of copper or CRR1 availability.

Under copper-replete conditions, levels of histone H3K4 monomethylation were ~2.5-fold higher at the CYC6 promoter than at the RBCS2 promoter but ~2-fold lower at the CPX1 and CRD1 promoters than at RBCS2 (Figure 7E). Interestingly, in

Figure 5. Analysis of the Sequence of Events at the HSP22F Promoter within the First 10 min after Onset of Heat Stress.

(A) HSF1 binding precedes chromatin remodeling and transcription. Control cells were subjected to heat stress, and samples for RNA extraction and ChIP were taken immediately prior to the temperature shift and at the indicated time points after shift from 25 to 40°C. HSP22F mRNA levels were quantified by qRT-PCR as described in Figure 1B. Shown are fold changes in transcript accumulation relative to the nonstressed state. Values derive from two biological replicates, with each analyzed in triplicate. ChIP was done as described in Figure 3, again amplifying region I of the HSP22F promoter (Figure 2). The enrichment relative to 10% input DNA was calculated and normalized to the values obtained for the CYC6 promoter. Error bars indicate standard errors of two biological replicates, each analyzed in triplicate.

(B) Graphical overview of the sequence of events at the HSP22F promoter after onset of heat stress. The data from (A) are given as percentage of the respective maximal values.
response to copper depletion, H3K4 monomethylation levels decreased by a factor of \( \sim 10 \) at the CYC6 promoter and by a factor of \( \sim 2 \) at the CPX1 and CRD1 promoters. These effects were largely abolished in the \( \text{crr1} \) mutant, suggesting that CRR1 also plays a role in remodeling of the H3K4 monomethylation mark.

FAIRE Analysis Indicates That Transcription Factor–Mediated Chromatin Remodeling Occurs at Target Promoters

Our ChIP results suggest that the HSF1 and CRR1 transcription factors under inducing conditions mediate chromatin remodeling toward an open chromatin structure at the heat shock and copper-responsive promoters, respectively. To test this conclusion using a second assay, we employed the FAIRE technique. FAIRE is a non-antibody-based method that involves formaldehyde cross-linking of DNA-protein complexes and that enriches for DNA fragments that correspond to regions of open chromatin structure (Giresi et al., 2007).

As shown in Figure 8A, \( \sim 2.5 \) times more HSP70A than HSP22F promoter fragments were enriched by FAIRE (relative to input DNA) in nonstressed cells, indicating that the HSP70A promoter is constitutively in a more open conformation than the HSP22F promoter. Furthermore, enrichment of HSP70A and HSP22F promoter fragments was \( \sim 1.5- \) and \( \sim 2 \)-fold greater, respectively, for control cells subjected to heat shock relative to nonstressed cells, while there was no enrichment for HSP22F promoter fragments in heat-shocked HSF1-amirNA cells, relative to nonstressed cells. Interestingly, in control and \( \text{crr1} \) mutant cells grown under copper-replete conditions, roughly the same quantity of CYC6, CPX1, and CRD1 promoter fragments were enriched by FAIRE (Figure 8B), and these amounts were comparable to those obtained for the inactive HSP22F promoter (Figure 8A).

Copper depletion led to a 1.6- to 2-fold increase in FAIRE-enriched fragments of the CYC6, CPX1, and CRD1 promoters, but there was no enrichment of these promoter fragments in \( \text{crr1} \) mutant cells (Figure 8B). When compared with ChIP, FAIRE indicated a more pronounced opening of chromatin structure at the CRD1 promoter in copper-depleted control cells (cf. Figures 7A and 8B). This might be explained by the comparably low sensitivity of the CRD1 gene to copper depletion (Figure 6), which might prevent a clearer detection of changes in nucleosome occupancy. Overall, the data obtained from these FAIRE experiments corroborate the ChIP results reported above, namely, that HSF1 and CRR1 transcription factors mediate chromatin remodeling at their target promoters.

DISCUSSION

We employed the ChIP and FAIRE techniques to study how transcription factors affect chromatin structure to regulate the expression of target genes in response to changes in environmental conditions in Chlamydomonas. We focused our analysis on five genes of the heat shock and copper response pathways that in Chlamydomonas are regulated by the HSF1 and CRR1 transcription factors, respectively. Our results, summarized in Figure 10, reveal that both transcription factors regulate the expression of these genes via conserved mechanisms involving histone acetylation, histone methylation, nucleosome eviction, and polymerase loading/activation. However, at each target promoter, these means are employed quite individually to establish a characteristic chromatin state, presumably to allow for a fine-tuning of gene expression that meets the requirements of the respective environmental condition.

Preloading of Transcription Factors

ChIP assays using antibodies against HSF1 revealed that HSF1 constitutively binds the HSP70A promoter and that the association increased \( \sim 4 \)-fold after heat shock (Figures 3 and 10; see Supplemental Figure 2D online). This finding is in line with previous findings showing that constitutive hypersensitive sites exist at the HSE1/TATA box and HSE4 within the HSP70A
Figure 7. Analysis of Nucleosome Occupancy and Histone Modifications at Copper-Responsive and Control Promoters.

(A) Nucleosome occupancy declines at copper-responsive gene promoters under copper depletion. ChIP was done on control (black bars) and crr1 knockout cells (gray bars) grown under copper-replete or copper deprivation conditions. From DNA fragments precipitated with antibodies against the unmodified C terminus of histone H3, the promoter regions shown in Figure 2 were amplified by qPCR. The enrichment relative to 10% input DNA was calculated and normalized to the values obtained for the RBCS2 promoter. Error bars indicate standard errors of two biological replicates, each analyzed in triplicate.

(B) CRR1 promotes histone H3 acetylation at the CYC6 and CPX1 promoters after copper depletion. ChIP was done using antibodies against acetylated Lys-9 and -14 of histone H3.

(C) CRR1 promotes histone H4 acetylation at the CYC6 and CPX1 promoters after copper depletion. ChIP was done using antibodies against acetylated Lys-5, -8, -12, and -16 of histone H4.

(D) CRR1 promotes reduction of H3K4 dimethylation at promoters CYC6 and CPX1 after copper depletion. ChIP was done using antibodies against dimethylation of Lys-4 at histone H3 (H3K4).

(E) CRR1 promotes reduction of H3K4 monomethylation at promoters CYC6 and CPX1 after copper depletion. ChIP was done using antibodies against monomethylation of Lys-4 at histone H3 (H3K4).

qPCR data from the experiments in (B) to (E) are given relative to the nucleosome occupancy at the respective promoter region (data from [A]). Asterisks indicate the significance of change at the respective promoter compared with control cells under copper replete conditions (t test, P value ≤ 0.01).
promoter (Lodha and Schroda, 2005). Constitutive binding to heat shock gene promoters was reported for yeast HSF1, but not for human, fly, or plant HSFs (Sorger et al., 1987; Zhang et al., 2003; Erkina and Erkine, 2006; Kodama et al., 2007). This discrepancy is presumably related to the fact that like yeast HSF1, Chlamydomonas HSF1 is constitutively trimeric, but HSF1 trimerization is induced only under stress conditions in higher plants, flies, and humans (Sorger and Nelson, 1989; Rabindran et al., 1993; Lee et al., 1995; Schulz-Raffelt et al., 2007). While HSF1 preloading was evident at the HSP70A promoter, little or none was observed at the HSP22F promoter (Figures 3, 5, and 10; see Supplemental Figure 2D online). Similar observations were made in yeast, where preloading was observed at the HSP82 and SSA4 promoters but not at the HSP12 promoter (Erkina and Erkine, 2006; Erkina et al., 2010). Apparently, it may be a widespread phenomenon that HSFs occupy small heat shock gene promoters only after stress (Erkina and Erkine, 2006; Kodama et al., 2007), and this generalization correlates well with the expression of many sHSP genes only under stress conditions (Haslbeck, 2002).

In the absence of a functional antibody against CRR1, we could not perform ChIP experiments to analyze whether CRR1 binds to its target promoters also in the presence of copper. However, as ChIP and FAIRE analyses revealed no striking differences between the chromatin states of noninduced control and crr1 mutant strains (Figures 7 and 8), it appears more likely that CRR1 is not preloaded to its target promoters in the presence of copper.

Constitutive versus Inducible Histone Modification

We observed constitutively high levels of histone H3 and H4 acetylation at the HSP70A and CRD1 promoters (Figures 4B, 4C, 7B, 7C, and 10). In HSF1-underexpressing strains, constitutive H3 acetylation at the HSP70A promoter was lower and therefore appears to be mediated by preloaded HSF1. On the contrary, as H3/4 acetylation levels at the CRD1 promoter were constitutively high in control and crr1 mutant cells, they must be mediated by an activator distinct from CRR1. In contrast with the HSP70A and CRD1 promoters, the HSP22F, CYC6, and CPX1 promoters had low levels of H3 and particularly H4 acetylation under noninducing conditions. However, levels of acetylation increased under heat shock (HSP22F) or copper deprivation (CYC6 and CPX1) conditions. Constitutive H3/4 acetylation at these promoters appears to be mediated by HSF1 and CRR1, as it was reduced or abolished in the respective mutant strains (Figures 4B, 4C, 7B, 7C, and 10; see Supplemental Figure 6B online). A direct role for HSF1 in mediating histone acetylation at target promoters has also been demonstrated in yeast by the use of strains carrying a mutated HSE (Zhao et al., 2005) or expressing an HSF1 variant without transactivation domain (Erkina and Erkine, 2006). Good candidates for coactivators of Chlamydomonas HSF1 with histone acetylase activity are homologs of the yeast NuA4 and SAGA complexes that, following heat shock, have been shown to rapidly enrich at HSF1-dependent heat shock gene promoters (Reid et al., 2000; Robert et al., 2004; Kremer and Gross, 2009). In contrast with Chlamydomonas, it is not clear whether preloaded HSF1 also drives constitutive histone acetylation in yeast.
Figure 9. Gene-Wide Overview of the Relative Abundance of Histone Occupancy and Modifications.

ChIP was done on control cells grown under the following conditions: nonstress in copper-replete medium (CL or Cu+), 30 min heat shock (HS), and medium depleted from copper (Cu−). ChIP was done using antibodies against the unmodified C terminus of histone H3 (A), acetylated Lys-9 and -14 of histone H3 (B), acetylated Lys-5, -8, -12, and -16 of histone H4 (C), dimethylation of Lys-4 at histone H3 (H3K4me2) (D), and monomethylation of Lys-4 at histone H3 (H3K4me1) (E). Fragments corresponding to transcribed regions shown in Figure 2 and intergenic regions separating genes au5. g14265_t1/P23 (IGR-1) and RBCS2/au5.g9204_t1 (IGR-2) were amplified by qPCR. The enrichment relative to 10% input DNA was calculated and normalized to the values obtained for the CYCS promoter (heat stress experiments) or the RBCS2 promoter (copper depletion experiments). The data on the promoter regions correspond to that shown in Figures 4 and 7. In case of ChIP analysis with antibodies against modified histones, an additional
A remarkable property of the HSP70A promoter is that in a transgene setting it strongly increases the likelihood that a promoter fused downstream becomes active (Schroda et al., 2000, 2002). This effect is dependent on the presence of HSEs within the HSP70A promoter, suggesting that it is mediated by HSFs (Lodha et al., 2008). As the constitutively high acetylation levels at the HSP70A promoter depend on HSF1, it is tempting to speculate that in the transgene setting the activation of downstream promoters is mediated by histone acetyltransferase activities recruited by HSF1. In turn, the constitutively high acetylation levels at the CRD1 promoter suggest that like the HSP70A promoter it may also be capable of activating neighboring transgenic promoters.

Levels of H3K4 mono- and dimethylation declined in a CRR1-dependent manner at the CYC6, CPX1, and CRD1 promoters (Figures 7D, 7E, and 10). Hence, CRR1 appears to recruit histone demethylase activities to its target promoters. Alternatively, CRR1 may recruit histone methyltransferase activities that convert mono- and dimethylated H3 to the trimethylated state. As H3K4 monomethylation in Chlamydomonas was shown to be linked to inactive chromatin (van Dijk et al., 2005) and H3K4 trimethylation is widely accepted as a typical mark of active euchromatin (Lachner and Jenuwein, 2002; Santos-Rosa et al., 2002), we favor the latter scenario. In contrast with the copper-responsive promoters, induction of the HSP22F promoter was not accompanied with a decline of H3K4 mono- and dimethylation levels (because of the low nucleosome occupancy at the HSP70A promoter after heat shock, we cannot draw any conclusions on the methylation state of nucleosomes at that promoter) (Figures 4D, 4E, and 10). In contrast with growth under copper-deficient conditions, cells experienced heat shock only for 30 min. Hence, it is possible that remodeling of the H3K4 methylation state proceeds more slowly than that of the H3/4 acetylation state. In consequence, the acetylation state of a nucleosome appears to be directly connected with promoter activation, while the H3K4 methylation state, as suggested previously (Ng et al., 2003), may serve a memory function to mark promoters that have been active for a certain time. Accordingly, yeast mutants defective in the Set1 and Set2 methyltransferases, catalyzing methylation of histones H3K4 and H3K36, respectively, were hardly impaired in the transcriptional output of the HSP82 gene (Kremer and Gross, 2009).

Nucleosome Displacement by Activated HSF1 and CRR1 Transcription Factors

Both ChIP and FAIRE analyses revealed that during copper starvation and heat shock, histone occupancy at the copper-responsive promoters and at the HSP22F promoter declined in a CRR1- and HSF1-dependent manner, respectively (Figures 4A, 7A, 8, and 10; see Supplemental Figure 6 online). As there is a good correlation between high levels of histone H3/4 acetylation and histone loss at these promoters, it appears that binding of activated CRR1 or HSF1 mediates histone acetylation, thus facilitating histone eviction. This conclusion is supported by a time-course experiment, where HSF1 binding, nucleosome occupancy, H3/4 acetylation, and transcription from the HSP22F promoter were monitored within the first 10 min after exposure of cells to heat stress (Figure 5; see Supplemental Figure 6A online). The results indicate the following sequence of events: within the first 30 s after onset of heat stress, HSF1 is activated, binds to the HSP22F promoter, and already mediates acetylation of histone H4. Within 60 s after temperature shift, acetylation of H3 and H4 strongly increases, which coincides with nucleosome eviction. Higher levels of HSP22F transcripts are detected only 2 min after onset of heat stress, thus indicating that chromatin remodeling at the HSP22F promoter most likely is a prerequisite and not a consequence of transcription. Our data agree very well with observations made at the yeast HSP82 promoter: there, nucleosome occupancy declines drastically within the first 60 s after onset of heat stress and is preceded by a burst of acetylation of histones H2A, H3, and H4 (Zhao et al., 2005). A correlation between histone acetylation and nucleosome loss was demonstrated previously also at the yeast PHO5, SSA4, and HSP12 promoters (Reinke and Hörm, 2003; Erkina and Erkine, 2006), at the viral HTLV-1 promoter in human cells (Sharma and Nyborg, 2008), or at the Arabidopsis thaliana HSP18.2 promoter (Kodama et al., 2007).

However, we observed that high levels of histone acetylation do not necessarily always correlate with low histone occupancy. For example, H3/4 acetylation levels under nonstress conditions were higher at the RBCS2 promoter than at the HSP22F promoter, but nucleosome occupancy at HSP22F was lower than at RBCS2 (Figures 4A to 4C). Moreover, levels of H3 acetylation at the HSP70A promoter were lower in HSF1-underexpressing strains than in the control strain, but these lower acetylation levels were not accompanied by reduced nucleosome occupancy.

Interestingly, the most dramatic nucleosome loss among the promoters studied here, as detected by ChIP and FAIRE, was observed at the HSP70A promoter during heat shock (Figures 4A, 8A, and 10). This loss appeared to be HSF1 independent but also may have been mediated by residual HSF1 in HSF1-underexpressing cells (Figure 3). The evidence for strong chromatin remodeling at the HSP70A promoter during heat shock, which was obtained by both ChIP and FAIRE experiments, corroborates previous results obtained from micrococcal nuclease digestion studies (Lodha and Schroda, 2005). In that set of experiments, the HSP70A promoter under nonstress conditions was found to be embedded into a nucleosome array, which was strongly perturbed by heat shock.
Chlamydomonas Promoter Categories Based on Their Chromatin State

Based on their activation by chromatin remodeling, we may place the six promoters analyzed here into four categories (Figure 10): the 
HSP22F and CYC6 promoters belong to the first category. Under nonstress or copper-replete conditions, these promoters are inactive. The inactive state is mediated by a closed chromatin structure as judged from high nucleosome occupancy, low levels of histone H3/4 acetylation, and high levels of histone H3K4 mono- and dimethylation. HSF1 and CRR1 transcription factors that are activated by heat stress and copper deprivation, respectively, mediate an opening of the chromatin structure at the promoters that is characterized by reduced nucleosome occupancy, high levels of histone H3/4 acetylation, and, presumably only after prolonged activation, reduced levels of histone H3K4 mono- and dimethylation. Moreover, activated HSF1 and CRR1 transcription factors mediate transcription initiation/elongation and, thus, high level transcription of the HSP22F and CYC6 genes.

The HSP70A and CRD1 promoters belong to the second category. They are constitutively in an open chromatin state, as judged from low nucleosome occupancy, high levels of histone H3/4 acetylation, and low levels of histone H3/K4 mono- and dimethylation. Although the chromatin state of the noninduced HSP70A and CRD1 promoters resembles that of the induced CYC6 promoter, the HSP70A and CRD1 genes under nonstress and copper-replete conditions are only weakly expressed (von Gromoff et al., 1989; Moseley et al., 2000). Apparently, high-level expression under heat stress and copper deprivation conditions requires that the activated HSF1 and CRR1 transcription factors enhance transcription initiation/elongation. At least activated CRR1 also mediates further reduction of nucleosome occupancy. In case of the HSP70A promoter, the open chromatin state and basal expression is mediated to a large part by preloaded HSF1, whereas at the CRD1 promoter it is mediated by an unknown activator.

The CPX1 promoter belongs to a third category that represents an intermediate between the HSP22F/CYC6 and HSP70A/CRD1 promoter categories in that it has a partially open chromatin structure under noninducing conditions. The latter is characterized by intermediate levels of nucleosome occupancy, high levels of histone H3 acetylation and H3K4 dimethylation, but low levels of histone H4 acetylation and H3K4 monomethylation. Similar to the fully opened HSP70A and CRD1 promoters, the partially opened chromatin state at the CPX1 promoter allows for low level expression of the CPX1 gene (Quinn et al., 1999). Thus, HSP70A, CRD1, and CPX1 promoters are poised for full transcriptional activation. The fully opened chromatin state at the CPX1 promoter, mediated by activated CRR1 under copper-deprived conditions and leading to high level expression of the CPX1 gene, resembles exactly that observed at the CYC6 promoter.

A fourth category is represented by the RBCS2 promoter, which is constitutively active and drives constitutive high-level expression of the RBCS2 gene (Goldschmidt-Clermont and Rahire, 1986). A constitutively active chromatin state at the RBCS2 promoter is suggested by high levels of histone H3/4 acetylation and low levels of H3K4 dimethylation. However, the RBCS2 promoter also exhibits high levels of nucleosome

![Figure 10](image-url)
occupancy and H3K4 monomethylation, which rather are characteristic for inactive chromatin. Heat shock leads to a closed chromatin structure, as levels of histone H3/4 acetylation strongly decrease and nucleosome occupancy increases. These results suggested reduced activity of the RBCS2 promoter under heat stress conditions, which indeed was observed previously for RBCS2 promoter driven transgenes that are much more weakly expressed than the endogenous RBCS2 gene (Schroda et al., 2002). Expression levels also of other Chlamydomonas genes were observed to decline during heat stress (Dorn et al., 2010). This response might be part of a global, heat shock--induced loss of histone acetylation, which was first observed long ago in Drosophila melanogaster (Arrigo, 1983). In contrast with what was reported from a recent study in mammalian cell cultures (Fritah et al., 2009), this effect appears not to depend on HSF1 in Chlamydomonas.

In summary, Chlamydomonas adjusts gene expression levels in response to changes in environmental conditions by specific transcription factors, such as HSF1 and CRR1 that individually remodel chromatin structure at their target genes, but also by yet unknown factors that appear to generally remodel the chromatin state of many promoters. The most important mark indicative of open chromatin and transcriptionally active promoters appears to be histone acetylation: basal activity of promoters was observed only when at least histone H3 carried high acetylation levels and strong activity was observed only when both histones H3 and H4 were acetylated at high levels. Moreover, histone acetylation preceded nucleosome eviction. By contrast, levels of nucleosome occupancy, H3K4 monomethylation, or H3K4 dimethylation appeared not to have a crucial influence on promoter activity.

The Gene-Wide Distribution of Histone Marks in Chlamydomonas versus Yeast

Nucleosome occupancy and histone modifications were determined at a genome-wide scale in yeast by ChIP-on-chip assays (Bernstein et al., 2004; Lee et al., 2004, 2007; Pokholok et al., 2005). When compared with these yeast studies, the glimpse we obtained here by examining selected regions in the Chlamydomonas genome suggests similar, but also distinct, features. Similar to yeast, nucleosome occupancy in Chlamydomonas in general was low at active promoters and high in transcribed regions (Figures 9 and 10). Moreover, histone H3/4 acetylation was high at promoters of active genes and low at inactive promoters and transcribed and intergenic regions. Furthermore, histone H3/4 acetylation was generally low at active promoters and high toward the 3′ end of transcribed regions. Finally, H3K4 dimethylation appeared to be higher at 5′ regions of inactive/weakly transcribed genes compared with actively transcribed genes, which correlated with the notion derived from studies on metazoans that H3K4 dimethylation may mark regions of poised, inactive genes (Schneider et al., 2004; Bernstein et al., 2005; Sims and Reinberg, 2006).

In contrast with what has been observed for yeast, where nucleosome occupancy is low at intergenic regions, we observed high nucleosome occupancy at two intergenic regions (Figure 9). Also, in human cells, nucleosome occupancy appears to be more or less evenly distributed, but contrary to what has been observed for yeast and now Chlamydomonas, histones are not particularly depleted at promoter regions (Bernstein et al., 2005). Hence, there appear to be organism-specific differences in histone occupancy and modifications, which can only be elucidated in depth by ChIP-on-chip or ChIP-seq approaches. As the chromatin structure of Chlamydomonas appears to be of particularly repressive nature in that nucleosomes exhibit overall low levels of acetylation and high levels of H3K4 monomethylation (Waterborg et al., 1995; van Dijk et al., 2005), it will be of special interest to investigate chromatin structure at a genome-wide level in Chlamydomonas.

METHODS

Strains and Cultivation Conditions

To generate strains for investigating the heat shock response, Chlamydomonas reinhardtii strain cw15–325 (cw d,m t +, kindly provided by R. Matagne, University of Liège, Belgium) was transformed with pCB412 (containing only the wild-type ARG7 gene; control strain), pMS418 (containing ARG7 and an HSF1-RNAi construct), and pMS540 (containing ARG7 and an HSF1-amRNA construct) as described previously (Schulz-Raffelt et al., 2007; Schmollinger et al., 2010). Arg prototrophic transformants were screened for thermosensitivity by exposing cells on agar plates three times within 48 h to a 1-h heat shock by floating plates in a water bath prewarmed to 40°C. To generate strains for investigating the copper response, strain CC3980 (crr1-2, arg7-7; kindly provided by S. Merchant, UCLA, CA) was transformed with plasmid pARG7.8 (Debuchy et al., 1989) or cotransformed with pARG7.8 and pCRR1F1B6 (CRR1 control strain) as described previously (Kroppat et al., 2005). Strains were grown mixotrophically to a density of 7 × 106 cells/mL in Tris-acetate-phosphate medium (Harris, 2008) on a rotary shaker at 24°C and ~30 μE m−2 s−1. For heat shock experiments, cells were pelleted by a 4-min centrifugation at 24°C and 270 g, resuspended in Tris-acetate-phosphate medium prewarmed to 40°C, and incubated under agitation in a water bath at 40°C and ~30 μE m−2 s−1 for 30 min. Prior to harvest, ice was added to the cells. Copper depletion experiments were performed as described previously (Quinn and Merchant, 1998).

Protein Extraction, Immunodetection, RNA Extraction, and qRT-PCR

Protein extraction and immunoblot analyses were done as described previously (Liu et al., 2005). RNA was isolated from ~106 cells with the TRIzol reagent (Invitrogen) using the manufacturer’s protocol except for the last steps: before RNA precipitation, two additional chloroform/isoamyl alcohol (24:1) extractions were performed. A DNase digest was done using RNase-free Turbo DNase (Ambion). The quality of the RNA preparations was estimated by agarose gel electrophoresis, and RNA concentration and purity were determined spectrophotometrically (NanoDrop-1000). cDNA synthesis was performed using the MULV reverse transcriptase (Promega), deoxynucleotide triphosphate, and oligo-d(T)18 primers. Primers for qRT-PCRs were selected based on ≥90% primer efficiency, a single melt curve, a single band on a 1.5% agarose gel, and on the correct sequence of the amplicon. They are listed in Supplemental Table 1 online. qRT-PCR was performed using the StepOnePlus RT-PCR system (Applied Biosystems) and the Maxima SYBR Green kit from Fermentas. Each reaction contained the vendor’s master mix, 200 nM of each primer, and cDNA corresponding to 10 ng input RNA in the reverse transcriptase reaction. The reaction conditions were as follows: 95°C for 10 min, followed by cycles of 95°C for 15 s and 65°C for 60 s, up to a total of 40 cycles. Primer efficiencies and amplicon sizes for all eight targets are listed in Supplemental Figure 3B online. Controls without template or reverse transcriptase were always included.
ChIP
A total of 10^9 cells that were grown under nonstress conditions and heat shocked for 30 min or grown under copper-replete and copper deprivation conditions were harvested by a 2-min centrifugation at 4°C and 3220g. To cross-link protein–DNA interactions, cells were resuspended in 10 mL freshly prepared cross-linking buffer (20 mM HEPES-KOH, pH 7.6, 80 mM KCl, and 0.35% formaldehyde) and incubated for 10 min at 24°C. Cross-linking was quenched by the addition of Gly at a final concentration of 125 mM and further incubation for 5 min at 24°C. Cells were collected by a 2-min centrifugation at 4°C and 3220g, washed twice with 1 mL 20 mM HEPES-KOH, pH 7.6, and 80 mM KCl, and lysed by the addition of 400 μL lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 0.25× protease inhibitor cocktail [Roche]). Cells were sonicated on ice using a BANDELIN Sonopuls HD2070 sonicator with sonication tip MS 73 (55% output control and 60% duty cycle) to gain an average DNA fragment size of ~200 bp. Sonication efficiency was verified for each sample by agarose gel electrophoresis. ChIP was performed with aliquots corresponding to ~2 × 10^7 cells that were diluted 1/10 with ChIP buffer (1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris-HCl, pH 8) and supplemented with BSA and sonicated λ-DNA at final concentrations of 100 and 1 μg/mL, respectively. Antibodies specific for the following epitopes were used: histone H3 (5 μL; Abcam ab1791); diacetyl H3K9 and H3K14 (10 μL; Upstate 06-599); tetra-acetyl H4K8, H4K12, and H4K16 (10 μL; Upstate 06-866); monomethylated H3K4 (5 μL; Abcam ab8895); dimethylated H3K4 (10 μL; Upstate 07-030); HSF1 (40 μL; affinity purified from rabbit antiserum; Schulz-Raffelt et al., 2007); vesicle-inducing protein in plastids 2 (VIPP2) (40 μL; affinity purified from rabbit antiserum, used as mock control). Affinity purification was done as described previously (Willmund and Schrodka, 2005). Antibody-protein/DNA complexes were allowed to form during a 2-h incubation at 4°C, were complexed with 6 mg preswollen protein A Sepharose beads containing 150 mM NaCl, once with washing buffer 1 containing 500 mM NaCl, once with washing buffer 2 (250 mM LiCl, 1% Nonidet P-40, 1% Na-deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8), and twice with TE (1 mM EDTA and 10 mM Tris-HCl, pH 8). Protein-DNA complexes were eluted by incubating twice for 15 min at 65°C in elution buffer (1% SDS and 0.1 M NaHCO₃), and cross-links were reverted by an overnight incubation at 65°C after addition of NaCl to a final concentration of 0.5 M. Proteins were digested by incubating for 1 h at 55°C after the addition of proteinase K (3.5 μg/mL), EDTA (8 mM), and Tris-HCl, pH 8.0 (32 mM). DNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1), once with chloroform/isoamyl alcohol (24:1), and precipitated by incubation with 2 volumes of ethanol after addition of 0.3 M Na-acetate, pH 5.2, and 10 μL glycogen for 3 h at ~20°C. Precipitated DNA was collected by a 20-min centrifugation at 4°C and 16,000g, washed with 70% ethanol, air-dried, and resuspended in TE for ChIP (see above).

FAIRE
A total of 10^9 cells that were grown under nonstress conditions and heat shocked for 30 min or grown under copper-replete and copper deprivation conditions were harvested by a 2-min centrifugation at 4°C and 3220g. Cross-linking of DNA–protein interactions was performed exactly as described above for the ChIP Protocol. Cells were sonicated on ice using a BANDELIN Sonopuls HD2070 sonicator with sonication tip MS 73 (55% output control and 60% duty cycle) to give an average DNA fragment size of ~200 bp. Sonication efficiency was verified for each sample by agarose gel electrophoresis. FAIRE was performed with aliquots corresponding to ~2 × 10^7 cells. DNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1) and precipitated by incubation with 2 volumes of ethanol after addition of 0.3 M Na-acetate, pH 5.2, for 4 h at ~20°C. Precipitated DNA was collected by a 20-min centrifugation at 4°C and 16,000g, washed with 70% ethanol, air-dried, and resuspended in TE, pH 8. Resuspended DNA was incubated at 65°C for 10 min; 1/40th of 10% of the precipitated DNA was used for qPCR using the same primer pairs as for ChIP (see above).

Accession Numbers
Accession numbers for all genes investigated in this study are given in Supplemental Tables 1 and 2 online.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Analysis of HSF1 Target Gene Expression in Different HSF1-RNAi and HSF1-amiRNA Strains.

Supplemental Figure 2. Test of the Specificity of Affinity-Purified HSF1 Antibodies.

Supplemental Figure 3. Experimental Parameters Underlying Transcript Quantification by qRT-PCR.

Supplemental Figure 4. PCR End Products Amplified on Selected Chromatin Precipitates.

Supplemental Figure 5. Nucleosome Occupancy and Histone Modifications at Promoters CYC6 and RBCSV remain Unaltered after Heat Shock and Copper Depletion, Respectively.

Supplemental Figure 6. Different Amplicons within the HSP22F and CYC6 Promoters Confirm Results.

Supplemental Table 1. Primers Used for qRT-PCR.

Supplemental Table 2. Primers Used for qPCR on Chromatin Precipitates.

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AUTHOR CONTRIBUTIONS
M.S. and D.S. conceived the project and designed the experiments. D.S. performed and analyzed all qRT-PCR, ChIP, and FAIRE experiments. D.S. and S.S. performed the immunoblotting experiments and the statistical analyses. F.S. contributed to setting up copper starvation conditions were harvested by a 2-min centrifugation at 4°C and 3220g.
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Transcription Factor–Dependent Chromatin Remodeling at Heat Shock and Copper-Responsive Promoters in *Chlamydomonas reinhardtii*

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