Deciphering the Combinatorial DNA-binding Code of the CCAAT-binding Complex and the Iron-regulatory Basic Region Leucine Zipper (bZIP) Transcription Factor HapX*  

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Background: HapX and the CCAAT-binding complex (CBC) are the master regulators of fungal iron homeostasis. 

Results: HapX DNA binding requires previous CBC-DNA complex formation and a 3′-flanking GAT motif with variable nucleotide spacing.

Conclusion: Combinatorial CBC-HapX DNA recognition allows discrimination of CBC and CBC-HapX targets. 

Significance: Conservation of functional domains in HapX orthologs suggests a similar DNA-binding code in most fungi.

The heterotrimeric CCAAT-binding complex (CBC) is evolutionarily conserved in eukaryotic organisms, including fungi, plants, and mammals. The CBC consists of three subunits, which are named in the filamentous fungus Aspergillus nidulans: HapB, HapC, and HapE. HapX, a fourth CBC subunit, was identified exclusively in fungi, except for Saccharomyces cerevisiae and the closely related Saccharomycotina species. The CBC-HapX complex acts as the master regulator of iron homeostasis. HapX binds to the class of basic region leucine zipper transcription factors. We demonstrated that the CBC and HapX bind cooperatively to bipartite DNA motifs with a general HapX/CBC/DNA 2:1:1 stoichiometry in a class of genes that are repressed by HapX-CBC in A. nidulans during iron limitation. This combinatorial binding mode requires protein-protein interaction between the N-terminal domain of HapE and the N-terminal CBC binding domain of HapX as well as sequence-specific DNA binding of both the CBC and HapX. Initial binding of the CBC to CCAAT boxes is mandatory for DNA recognition of HapX. HapX specifically targets the minimal motif 5′-GAT-3′, which is located at a distance of 11–12 bp downstream of the respective CCAAT box. Single nucleotide substitutions at the 5′- and 3′-end of the GAT motif as well as different spacing between the CBC and HapX DNA-binding sites revealed a remarkable promiscuous DNA-recognition mode of HapX. This flexible DNA-binding code may have evolved as a mechanism for fine-tuning the transcriptional activity of CBC-HapX at distinct target promoters.

The basic region leucine zipper (bZIP)2 transcription factor HapX acts as the master regulator of iron homeostasis in fungal species. HapX transmits the information on the cellular iron status to the site of DNA transcription within the nucleus to adapt iron uptake, consumption, and storage (1–4).

In the filamentous fungi Aspergillus nidulans (an important model eukaryote) and Aspergillus fumigatus (the most common air-borne fungal pathogen of humans), HapX coordinates the adaptation to iron starvation by repression of iron-consuming metabolic pathways such as heme biosynthesis, respiration, tricarboxylic acid cycle, amino acid, and ribosome biosynthesis (1, 2). Importantly, HapX repressor activity depends on protein-protein interaction with the heterotrimeric CBC, which is structurally a sequence-specific histone and is highly conserved in all eukaryotes (termed Hap complex in Saccharomyces cerevisiae and NF-Y in humans) (1). Additionally, for iron acquisition, HapX activates a subset of genes that are involved in the biosynthesis of fungus-specific ferric iron chelators (termed siderophores) and reductive iron assimilation (2, 5). Whether a physical interaction of HapX and the CBC is required for these activities as well has to be elucidated by further studies.

During adaptation to iron sufficiency, the Cys2-Cys2-type GATA zinc finger transcription factor SreA acts as the second player within the fine-tuned iron homeostatic regulatory network (6). SreA represses high affinity iron uptake, including reductive iron assimilation and siderophore biosynthesis, to

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‡ The abbreviations used are: bZIP, basic region leucine zipper; CBC, CCAAT-binding complex; MEME, Multiple Em for Motif Elicitation; SPR, surface plasmon resonance; RU, resonance unit.

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avoid iron overload. SreA and HapX are interconnected in a negative feedback loop, i.e. SreA represses expression of hapX during iron sufficiency and HapX represses sreA during iron starvation (7). Additionally, both SreA and HapX appear to be regulated post-translationally by iron, blocking HapX function and activating SreA function (8).

Despite the SreA-mediated transcriptional repression of hapX during iron sufficiency, a very recent study surprisingly revealed that HapX is not only crucial for adaptation to iron starvation but also for coping with iron toxicity via activation of cccA, which encodes a vacuolar iron importer (8, 9). Strikingly, cccA is regulated post-translationally by iron, blocking HapX function during iron sufficiency, a very recent study surprisingly revealed that hapX and the CBC with CCAAT box containing promoter regions from the HapX-CBC target genes cycA, sreA, acoA, and lysF of A. nidulans. Dnase I footprinting analyses, multicomponent electrophoretic mobility shift assays (EMSAs), and surface plasmon resonance (SPR) biosensor analysis using recombinant HapX-CBC revealed a remarkable promiscuous binding site recognition of these promoters. Nevertheless, we could identify the minimal HapX nucleotide recognition sequence motif 5′-GAT-3′ that is located at a distance of 11–12 bp downstream of the respective CCAAT box.

**EXPERIMENTAL PROCEDURES**

**Bacterial Expression and Purification of the CBC and HapX for in Vitro DNase I Footprinting and Electrophoretic Mobility Shift Assays**—Recombinant HapB, HapC, and HapE were prepared as GST-HapB, GST-HapC, and MalE-HapE fusion proteins, as described previously (17). A CDNA fragment encoding A. nidulans hapX (1–200) (covering the CBC binding domain, basic region, and coiled-coil domain) with Myc and His tags was amplified by PCR with hapX-s(Ndel) and hapX200Myc-nol primers and subcloned into the pET-29a(+) vector (Novagen). The resultant plasmid was used for transformation of Escherichia coli BL21(DE3). Recombinant HapX(1–200) was purified with a nickel-nitritoltriacetic acid-agarose resin (QiaGen). Reconstitution of the CBC from its subunits solubilized in a buffer containing 6 M guanidine hydrochloride was carried out as described (17).

**In Vitro Dnase I Footprinting**—DNase I footprinting was carried out as described previously (18). Briefly, A. nidulans cycA and lysF promoter fragments were amplified by PCR with the cycA550as/cycA745s and lysF200s/lysFus as primer pairs, followed by digestion with BamHI and HindIII or BamHI only. DNA fragments were labeled with [α-32P]dCTP by T7 DNA polymerase and purified with ProbeQuant™ G-50 micro-columns (GE Healthcare). The labeled DNA fragments (2 × 10^4 cpm) were incubated with reconstituted CBC (10 pmol) in the presence or absence of HapX (50 pmol) for 15 min at 37 °C in binding buffer consisting of 60 mM KCl, 25 mM HEPES/KOH, pH 7.9, 5 mM MgCl2, 1 mM EDTA, 0.1 mM PMSF, 1 μg/ml antipain, leupeptin, chymostatin, and pepstatin, respectively, and 10% (v/v) glycerol, and then digested at 25 °C with DNase I for 1 min. The reaction was stopped by addition of 100 μl of DNase I stop solution (1.5 mM ammonium acetate, 60 mM EDTA, 10 ng/μl of calf thymus DNA), followed by ethanol precipitation. The samples were analyzed by electrophoresis on a DNA sequencing gel, as described previously (18).

**Electrophoretic Mobility Shift Assays (EMSA)**—EMSA were carried out as described previously with minor modifications (18). Reconstituted CBC (10 pmol) and HapX (50 pmol) were incubated with 32P-labeled DNA fragments (10^3 cpm) in the same binding buffer that was used for DNase I footprinting. DNA probes were prepared by PCR with oligonucleotides listed.

**CBC-HapX DNA-binding Code**

Although cooperative binding of HapX and the CBC to a bipartite cccA promoter element has been explored in A. fumigatus, a comprehensive picture of CBC-HapX target sites within the 5′-upstream regions of HapX-regulated genes is still lacking. In an attempt to analyze whether this kind of motif is commonly recognized, we analyzed the interaction of HapX and the CBC with CCAAT box containing promoter regions from the HapX-CBC target genes cycA, sreA, acoA, and lysF of A. nidulans. Dnase I footprinting analyses, multicomponent electrophoretic mobility shift assays (EMSAs), and surface plasmon resonance (SPR) biosensor analysis using recombinant HapX-CBC revealed a remarkable promiscuous binding site recognition of these promoters. Nevertheless, we could identify the minimal HapX nucleotide recognition sequence motif 5′-GAT-3′ that is located at a distance of 11–12 bp downstream of the respective CCAAT box.

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TABLE 1

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<th>Strain designation in text</th>
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<td>15</td>
</tr>
<tr>
<td>ΔhapX</td>
<td>BPUΔX1</td>
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<td>15</td>
</tr>
<tr>
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<td>WXCZW1</td>
<td>pyrYG89; biA1; wA3; argB2; pyroA4; pAR5 (argB); pycyAp-latM-lacZ (pyroA); PyroA&lt;sup&gt;+&lt;/sup&gt;; PyroA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>TGAFlm/WT</td>
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<td>This study</td>
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</table>

in Table 2. The DNA fragments were labeled with [α-<sup>32</sup>P]dCTP by T7 DNA polymerase as described for the DNase I footprinting analysis.

**Bacterial Expression and Purification of the CBC and HapX for SPR Analysis**—All three *A. nidulans* CBC-forming subunits HapB, HapC, and HapE were overproduced in *E. coli* Rosetta 2 (DE3) and purified as described previously (1). A cDNA fragment encoding *A. nidulans* HapX(1–198) (covering the CBC binding domain, basic region, and coiled-coil domain) with an N-terminus extension, including a cleavage site for tobacco etch virus protease, was amplified and subcloned into the pMAL-c2X (New England Biolabs) vector. The resulting plasmid was transformed into *E. coli* Rosetta 2 (DE3) cells for overnight induction. Crude bacterial lysates were purified by detergent-Sepharose affinity chromatography (GE Healthcare). The maltose-binding protein HapX(1–198) fusion was cleaved with tobacco etch virus protease and further purified sequentially using CelluloseSulfate (Millipore) affinity chromatography, (NH₄)₂SO₄ precipitation (50% w/v), and Superdex 200 prep grade (GE Healthcare) size exclusion chromatography. The absolute molecular mass of HapX(1–198) was determined by dynamic light scattering experiments on a miniDawn TREOS monitor in series with an OPTILab T-rEX differential refractometer (Wyatt). HapX(1–198) was characterized on a Superdex 200 Increase 10/300 GL column (GE Healthcare), and molar mass was calculated using ASTRA 6 software (Wyatt).

**Surface Plasmon Resonance Measurements**—Real-time analyses were performed on a BLAcore 2000 System (GE Healthcare) at 25 °C. DNA duplexes were produced by annealing complementary 50-bp oligonucleotides using a 5-fold molar excess of the nonbiotinylated oligonucleotide. The dsDNA was injected on flow cells of a streptavidin (Sigma)-coated CM3 sensor chip at a flow rate of 10 μl/min until 50 RU of DNA had been bound. The CBC was reconstituted from lyophilized Hap subunits by mixing equimolar amounts of HapB, HapC, and HapE as 0.1 mM solutions of each subunit in water. Samples for SPR analysis were generated by 500-fold dilution of this stock solution in running buffer (10 mM phosphate, pH 7.4, containing 2.7 mM KCl, 137 mM NaCl, 1 mM DTT, and 0.005% (v/v) surfactant P20) followed by serial 2-fold dilution.

CBC samples were injected in running buffer at a flow rate of 30 μl/min. Injection of HapX(1–198) containing 25 μg/ml poly(dl-dc) and 25 μg/ml poly(dA-dT) (Sigma) on preformed binary CBC-DNA complexes within the equilibrium phase was performed by using the coinjection command. Each injection was performed at least two times. The chip surface was regenerated with 10 mM Tris/HCl, pH 7.5, containing 0.5 mM NaCl, 1 mM EDTA, and 0.005% (w/v) SDS for 1 min. Refractive index errors due to bulk solvent effects were corrected with responses from DNA-free flow cell 1 as well as subtracting blank injections. Kinetic raw data were processed and globally fitted with the Kinetic module of Mathematical Software using a 1:1 interaction model including a mass transport term.

*A. nidulans Strains, Oligonucleotides, Growth Conditions, and Enzyme Assays*—*A. nidulans* strains and oligonucleotides used in this study are listed in Tables 1 and 2, respectively. *A. nidulans* strains were grown for 24 h at 37 °C in Aspergillus minimal medium (19), containing 1% (w/v) glucose as carbon source, 20 mM glutamine as nitrogen source, 30 μM FeSO₄ as iron source, and the appropriate supplements to complement strain-specific auxotrophies. For iron-depleted conditions, iron was omitted. β-Galactosidase activity in mycelial extracts was measured as described (20). One unit of β-galactosidase activity was defined as the amount of the enzyme that hydrolyzes 1 nmol of o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol and D-galactose per min.

**Reporter Gene and Strain Construction**—A promoter region of the *cycA* gene was amplified by PCR with chromosomal DNA and primers cycA-615s and cycA-615as. The amplified fragment was inserted at the cloning site of pGEM-T easy vector (Promega) to construct pT-cycA. With the plasmid pT-cycA as a template and primers cycA-5as (BamHI) and cycA-5as (PstI), the second PCR was carried out, and the amplified fragment was digested with BamHI and PstI and subcloned into pUC119 to construct pycyAp-607<sup>−</sup>~<sup>+</sup>5(PstI-BamHI). To construct the mutated *cycA* promoters, pycyAp-607<sup>+</sup>~<sup>+</sup>5(PstI-BamHI) was used for site-directed mutagenesis by applying the QuikChange site-directed mutagenesis kit (Agilent Technologies). Two sets of primers (cycA-665s/tg/cycA-615as-629at and cycA-635Ms/cycA-612Ms) were used to construct pycyAp-607<sup>−</sup>~<sup>+</sup>5(PstI-BamHI)attM<sub>cycA</sub> and pycyAp-607<sup>+</sup>~<sup>+</sup>5(PstI-BamHI)attM<sub>cycA</sub>, respectively. The BamHI-PstI DNA fragments containing the wild-type and mutated promoters and the BamHI-HindIII fragment of pAN923<sup>−</sup>42B (21) carrying the lacZ gene were ligated to the PstI-HindIII fragment of pPY-7 carrying the pyroA marker gene (22) to construct plasmids pycyAp-wt-lacZ, pycyAp-latM-lacZ, and pycyAp-attG-lacZ, respectively. The plasmids were digested by SacI and ligated in the pyroA marker gene and used to transform the *A. nidulans* wild-type strain BPU1 as well as the hapX deletion strain BPUDX1 (15). The location and copy number of the integrated plasmids in the transformants were examined by Southern blot analysis. Strains carrying single genomic insertions at the pyroA locus were selected (Table 1).
RESULTS

Both the CBC and HapX Recognize a Highly Conserved Bipartite cycA Promoter Motif—Previously, we have explored the structural basis for CCAAT box recognition by the functionally active core CBC from A. nidulans on a 23-bp DNA duplex containing the CCAAT box at position 611 derived from the natural promoter sequence of the cytochrome c encoding cycA gene (14). The CBC binds asymmetrically to the CCAAT box by minor groove sensing and widening. The HapE N-terminal helix (N9251 N) that has been shown to associate with HapX adopts an orientation directed to the 3' end of the covered and bent CCAAT sequence. This structural arrangement prompted us to hypothesize that HapX might recognize a DNA motif downstream of the CBC-bound CCAAT box.

To identify evolutionarily conserved regulatory motifs in the cycA promoter, the 1-kb 5'-upstream region of cycA homologs from 19 Aspergillus species were subject to MEME analysis (23). The identified sites and their positions in the promoters of the different species are shown in Table 3. The highest scoring sequence (e-value of 6.2 × 10^-139), present in all 19 species, was a bipartite motif separated by a 9-bp spacer region with low conservation (Fig. 1A). The 5'-conserved submotif perfectly matches the CBC consensus DNA-binding motif CCAAT(C/T)(A/G) (14). The 3'-conserved submotif 5'GATGATTCA-3' does not match the recently identified nonpalindromic HapX-binding site 5'ATTGTCAGC-3' present in the promoter of the A. fumigatus cccA gene but contains the pseudo-palindromic sequence TGATTCA that is reminiscent of the AP-1 (Jun-Fos) and Gcn4 bZIP transcription factor-binding site TGACTCA (24–26).

To analyze whether HapX specifically binds to the region containing the 3'-submotif consensus sequence, we carried out DNase I footprinting analysis of the CBC alone and in combination with HapX (Fig. 1B). The CBC protects a region of 26 and 24 bp on the coding and noncoding strands of the cycA promoter fragment, respectively. This protection pattern is in perfect agreement with the CBC-DNA crystal structure. The CBC partially protects the 5'-GATGATTCA-3' motif, and interestingly, co-incubation of the DNA probe with both the CBC and HapX extends the protected region by additional 9 bp and led to complete protection of the 5'-GATGATTCA-3' motif. Therefore, we con-
clude that HapX and the CBC simultaneously recognize the conserved regulatory motif.

Only the Left Half-site of the TGATTCA Motif in the cycA Promoter Is Necessary for HapX Binding—To examine which part of the conserved 5'-GATGATTCA-3' motif is involved in HapX binding, multicomponent EMSAs were performed with mutant cycA promoter fragments carrying mutations within the putative HapX-binding site. Binding of the CBC to the wild-type promoter fragment shows a distinct band shift, whereas CBC-HapX binding induced a supershift of the primary CBC-DNA complex into a new discrete CBC-DNA-HapX complex (Fig. 2). Mutations within the right TCA-3' half-site (m1) as well as of the preceding 5'-AGG to CAT (m6) had none or only minor impact on the HapX-dependent supershift. By contrast, mutations in the left 5'-TGAT (m4 and m5) led to a complete loss of HapX binding. This demonstrates that only the left half-site of the TGATTCA motif in the cycA promoter is necessary for high affinity HapX binding.

CBC and HapX Cooperatively Bind the Conserved Bipartite cycA Promoter Motif—To confirm these results and to define whether CBC binding is a prerequisite for HapX binding, we also tested the same mutations used in EMSAs by protein-DNA real time SPR biosensor interaction analysis. Therefore, we overexpressed and purified the A. nidulans CBC (comprising the full-length domains of subunits HapB, HapC, and HapE) as well as a peptide corresponding to residues 1–198 of A. nidulans HapX, which includes the CBC binding, basic region, and coiled-coil domains (Fig. 3A). Light scattering analysis of purified HapX(1–198) revealed a molmass of 45.63 kDa, demonstrating that this domain is dimeric in solution (theoretical mass of 43.87 kDa), as expected for a bZIP protein (Fig. 3B).

Kinetic SPR binding responses of the CBC to the wild-type 50-bp cycA promoter duplex fitted with a dissociation constant (KD) of 0.42 nM (Fig. 4A, panel 1). Interestingly, no binding was detectable during concentration-dependent injection of HapX alone (Fig. 4A, panel 2, red lines). However, by co-injection of HapX on CBC-bound DNA, binding of HapX was clearly measurable (Fig. 4A, panel 2, blue lines) and fitted with a KD of 2.54 nM after CBC response subtraction (Fig. 4A, panel 3), indicating that the CBC together with HapX cooperatively bind the conserved bipartite cycA promoter motif. The same set of SPR experiments was performed on the mutated cycA promoter duplexes (Fig. 4, B–G). In perfect agreement with the supershift EMSA data, HapX affinity to CBC-DNA harboring mutations within the left half-site of the TGATTCA sequence decreased 58-fold (Fig. 4E) and 32-fold, respectively (Fig. 4F).

In Vitro Binding Pattern of CBC-HapX Is Reflected by cycA Promoter Activity in Vivo—Mutations of the cycA promoter CCAAT box at position −611 completely abolished CBC interaction in vitro (14), and as described here, mutations of the 5'-TGAT HapX-specific submotif strongly attenuate HapX affinity. Therefore, we were interested whether the in vitro binding affinities of the mapped binding sites reflect cycA promoter activity in vivo. To address this question, we generated cycA-lacZ translational fusion reporter gene constructs. One construct harbored the wild-type cycA promoter region fused with the E. coli lacZ gene. For the second construct, the 5'-TGAT HapX site was mutated to TGCA (matching mutation m4 in Figs. 2 and 4), whereas the CCAAT box was mutated.

### TABLE 3

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to CGTAA for the third construct. All three constructs were introduced into both an A. nidulans wild-type and hapX deletion strain, and transformants with a single integration of plasmids at the pyroA genomic locus were selected for analysis of β-galactosidase activity under iron-starvation and iron-sufficient growth conditions.

Like the native cycA transcript (1), the cycA-lacZ reporter gene was highly expressed during iron-replete conditions in both the wild-type and ΔhapX strain. Consistent with the CBC-HapX repressor function, expression of the wild-type like cycA-lacZ reporter was reduced to a level of 50% in the wild-type strain but not in the ΔhapX strain during iron starvation (Fig. 5).

Mutation of cycA promoter CCAAT box at position −611 rendered cycA-lacZ expression in the wild-type strain insensitive to iron availability, confirming the involvement of the CBC in the iron regulation of cycA expression. Notably, this mutation strongly decreased cycA-lacZ expression in both the wild-type and ΔhapX strains, indicating that this CCAAT box is also required for full activation of cycA promoter activity. Previously, we showed that inactivation of the CBC by deletion of hapC led to a strong derepression of cycA at the transcript level during both iron deficiency and sufficiency (1). Consequently, cycA expression is inversely affected by global CBC inactivation compared with mutation of only one of its binding sites. These data are in agreement with HapX- and iron-independent functions of the CBC and most likely exerted via cycA promoter organization (14). Mutation of the 5′-TGAT HapX left half-site conferred only partially derepressed cycA-lacZ reporter activity during iron limitation to a level of about 80% compared with that measured under iron-replete conditions. These results might be explained by a residual HapX repressor activity in vivo, solely based on protein-protein interaction with the CBC, as well as higher HapX levels during iron deficiency. By contrast, activity of the same cycA-lacZ reporter mutant was not affected in a ΔhapC strain consistent with the lack of the regulator HapX. Taken together, the CBC-HapX-binding sites predicted from in silico and in vitro analyses are indeed required for proper iron-dependent regulation of cycA promoter activity in vivo.

Mapping of Further HapX DNA-binding Sites in CBC Regulated Promoters—Because we identified a conserved bipartite CBC/HapX-binding site in the cycA promoter, we expected
CBC-HapX DNA-binding Code

1. CBC (1.56-100 nM) + HapX (3.13-100 nM)

- cycAp-611 (-) ATCTGGGAAATTGAACCATGAGGATGATTGCCAGCATGTGAGCACT
- TAGACCCATTAACTGGTATGTCCTCATTACTAAGTGCGTGCAGGTC

2. 6.25 nM CBC

- m1 AGGATGATTcC TCCTACTAAGg

- m2 AGGATGATTtA TCCTACTAAaT

- m3 AGGATGATcCA TCCTACTAgGT

- m4 AGGATGcaTCA TCCTACgtAGT

- m5 AGGAatATTCA TCCTtaTAAGT

- m6 cATGATTCa gtaTACTAAGT

K_D = 0.42 nM

K_D = 2.54 nM

K_D = 3.65 nM

K_D = 5.60 nM

K_D = 28.4 nM

K_D = 147.2 nM

K_D = 81.7 nM

K_D = 19.3 nM
that similar motifs were present in the promoter regions of further HapX-CBC targets, i.e. genes, which are down-regulated during iron starvation in *A. nidulans*. MEME analyses of the 2-kb 5'-upstream region of *sreA* (27) and 1-kb 5'-upstream regions of *acoA* and *lysF* (encoding ac oxidase and homoaconitase) from 19 different *Aspergillus* species did not identify evolutionarily conserved bipartite CCAAT-containing motifs in *A. nidulans*. As all of the *A. nidulans* sreA, *acoA*, and *lysF* promoters contain at least one CCAAT box (1, 28), we inspected the sequences at the 3'-flanking region of these CCAAT boxes for the presence of 5'-TGAT 5'-TGAT half-sites that might represent putative HapX-binding motifs. We were actually able to identify one 5'-TGAT half-site in each of the promoter regions with 10-16 bp spacing sequence relative to the upstream located CCAAT box (Fig. 6).

To analyze whether the identified 5'-TGAT half-sites were cooperatively recognized by HapX-CBC, we determined CBC-DNA affinity, as well as binding of HapX to CBC-bound DNA by real time SPR biosensor interaction analysis. Kinetic CBC binding responses to 50-bp cycA, sreA, *acoA*, and *lysF* promoter duplexes fitted with *K*_D^o^ values ranging from 0.42 to 3.9 nM (Fig. 6, panel 1). The measured saturating CBC responses (R^max^ values) on the cycA, sreA, and *acoA* 50-bp DNA duplexes reached values that corresponded to a 1:1 stoichiometry of the formed CBC-DNA binary complex, as expected (Fig. 6, A–C, and Table 4). However, CBC interaction analysis on the *lysF* duplex fitted with poor quality and revealed an R^max^ value that indicates binding of about 1.8 CBCs per duplex (Fig. 6D and Table 4).

Next, we performed co-injection analyses of HapX on the preformed binary CBC-DNA complexes (Fig. 6, panel 2). After CBC response subtraction, HapX binding responses fitted with apparent dissociation constants varying from 4.4 to 26 nM (Fig. 6, panel 3, and Table 5). These high affinity values and the lack of HapX binding in the absence of the CBC led us conclude that HapX and the CBC cooperatively bind the CCAAT box and the identified 5'-TGAT half-sites in all of the analyzed promoter regions. Comparison of the calculated and measured HapX R^max^ responses on preformed CBC-DNA revealed molar HapX/CBC ratios from 2.7 to 3.7. These values exceeded the expected 2:1 ratio, probably due to a decreased CBC dissociation rate in the presence of HapX, but nevertheless indicated binding of a HapX homodimer on all duplexes tested. Intriguingly, on the *lysF* promoter duplex, the observed HapX/CBC molar ratio upon co-injection was not elevated despite pre-binding of 1.8 CBCs, suggesting preferential HapX binding to one of these CBCs. Taken together, our data are consistent with a general 2:1:1 stoichiometry of HapX-CBC-DNA ternary complex and in agreement with our previous results concerning HapX-CBC binding on a bipartite *A. fumigatus cccA* promoter element (8).

**CBC-HapX Binding on the lysF Promoter Is Dictated by the Spacing of Their Target Motifs**—To examine the unexpected 1.8:1 stoichiometry of CBC DNA binding on the *lysF* promoter fragment in more detail, we performed DNase I footprinting analysis of the CBC alone and in combination with HapX. Surprisingly, the CBC did not protect the perfect CCAAT box at position −181 but specifically at a region upstream of this motif (Fig. 7A). A closer inspection of this sequence revealed the presence of a 5'-CGAATCA motif on the complementary strand at position −154, which matches the CBC consensus DNA-binding motif CCAAT(C/T)(A/G) with a C to G exchange at position 2 (14). Furthermore, a GGAT motif is found on the same strand with a 10-bp spacing sequence downstream of the 5'-located CGAAT box. Co-incubation of the DNA probe with both the CBC and HapX extended the protected region at the 3'-flanking end of the CGAAT box on the noncoding strand, suggesting that this sequence motif acts as the primary CBC target in the presence of HapX (Fig. 7A).

To confirm this hypothesis, we carried out multicomponent EMSAs using ³²P-labeled wild-type DNA as well as mutant *lysF* promoter fragments carrying mutations within one or both putative CBC-binding sites. CBC binding of the wild-type promoter fragment caused DNA shifts consistent with CBC-DNA complexes containing either one or two bound CBCs, which is in perfect agreement with the apparent 1.8:1 stoichiometry observed by SPR biosensor interaction analysis. Strikingly, CBC-HapX DNA binding led to the loss of DNA complexes containing two CBCs and induced a supershift of only the 1:1
CBC-DNA complex into a new CBC-DNA-HapX complex (Fig. 7B), suggesting that one CBC-binding site is protected upon HapX binding. Substitution of the G at position 2 of the imperfect CGAAT box to C (m1) had no effect on the band shift pattern, indicating the functionality of this motif. By contrast, mutation of two nucleotides within the CGAAT box (m2) completely abolished HapX binding but allowed the formation of DNA complexes only containing one CBC on the CCAAT box at position −181. Mutations within this CCAAT box (m3) led to the loss of DNA complexes containing two CBCs, but it

**FIGURE 6.** Real time SPR characterization of in vitro formation of the CBC-DNA-HapX ternary complex on conserved motifs present in *A. nidulans* promoters of *cycA*, *sreA*, *acoA*, and *lysF*. Nucleotides marked in blue represent the putative HapX 5′-TGAT-3′ half-sites as identified in the cycA promoter by EMSA and SPR analysis. Data are presented as described in Fig. 4 legend.
allowed for CBC-DNA-HapX complex formation, demonstrating that only the CGAAT box at position −154 is recognized in the presence of HapX. Mutagenesis of both CBC-binding sites (m4) abolished CBC as well as HapX-CBC binding, which is consistent with the notion that HapX binding requires previous CBC-DNA complex formation.

Next, we addressed the question whether the GGAT motif was important for HapX-CBC recognition. Full mutation of the

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### TABLE 4

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### TABLE 5

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**FIGURE 7.** CBC and HapX specifically recognize CGAAT and GAT motifs in the lysF promoter. A. DNase I footprinting patterns of the CBC and CBC-HapX on the A. nidulans lysF promoter. As indicated on top, the reconstituted CBC alone or in combination with HapX was incubated with an end-labeled DNA fragment of the lysF promoter. Protected sequences are shown on the right side of the footprint. Nucleotides colored in red indicate the CGAAT and CCAAT boxes at positions −154 and −181. The putative HapX-binding site GAT is marked in blue. Numbers represent the nucleotide positions relative to the start of the open reading frame. B and C. Multicomponent EMSAs of the CBC and CBC-HapX with natural and mutant lysF promoter probes. Partial sequences of 69-bp DNA probes used in EMSAs are shown on top of the autoradiograms. The CGAAT and CCAAT boxes and the HapX-binding motif 5′-GGAT-3′ are highlighted in red and blue, respectively. Substituted nucleotides relative to the wild-type sequence are underlined and shown in lowercase.
GAT sequence at position −170 (m4) led to the complete loss of HapX binding in multicomponent EMSAs, whereas single nucleotide substitutions of the 5′-flanking G by C or T (m2 and m3) had only a minor impact on CBC-DNA-HapX complex formation (Fig. 7C). However, single nucleotide exchanges of the 3′-flanking T by C or G (m5 and m6) strongly weakened HapX binding. Therefore, we conclude that cooperative CBC-HapX binding on the 3′-flanking T by C or G (m5 and m6) strongly weakened HapX binding. Consequently, the N-terminal domain of the CBC subunit HapE that is required for interaction with the N terminus of HapX is likewise present only in HapX-employing fungal species. The CBC is a global regulatory complex involved in control of a wide range of processes (12, 31), whereas the CBC-HapX complex appears to be involved exclusively in iron regulation.

After identification of the physical interaction between the CBC and HapX, starting in A. nidulans (1), the mechanism for discrimination between general CBC and specific CBC-HapX target genes remained an open question. In agreement with a mechanism that involves interaction of HapX not only with the CBC but also with DNA, mutation of the basic DNA binding motif present at positions −154 and −170, respectively.

**DISCUSSION**

In most fungal species, with the exception of S. cerevisiae and the closely related Saccharomyces species such as Candida glabrata, adaptation to iron availability is coordinated at the transcriptional level by the CBC-HapX transcription factor complex (7). Because of its crucial role in adaptation to iron starvation, HapX was found to be essential for fungal pathogenicity in animal and plant hosts, e.g. in A. fumigatus, Candida albicans, and Cryptococcus neoformans in murine models as well as Fusarium oxysporum on tomato plants (2–4, 29, 30). In A. fumigatus, HapX was recently identified to be additionally involved in adaptation to iron excess. This is mechanistically mediated by cysteine-rich protein domains, which are conserved in most HapX orthologs indicating conservation of this function in other fungal species (8). The heterotrimeric CBC is conserved in all eukaryotes, although HapX is confined to fungal species, making HapX a fungus-specific virulence determinant.

The cooperative action of multiple transcriptional regulatory proteins that bind to promoter DNA and specifically activate or
repress downstream target genes is a general feature of eukaryotic transcription (33). bZIPS are very ancient transcription factors, and it is estimated that they underwent extended gene duplications during the ~950 million years of evolution after the divergence of metazoa and fungi, which allowed them to evolve new DNA binding specificities as well as complex dimerization networks (34). Combinatorial gene regulation involving bZIP transcription factors seems to be a common principle and has been analyzed at the atomic level in a few cases, e.g. for AP-1 (Jun-Fos) and NFAT1 (35) or CAAT/enhancer-binding protein β and c-Myb (36).

Here, we aimed for the first time to analyze the combinatorial DNA-binding mode of the CBC and the bZIP factor HapX in A. nidulans focusing on target genes that are repressed during iron starvation. By using several independent methods, we demonstrated that the CBC and HapX cooperatively bind to bipartite DNA motifs in the A. nidulans cyaA, sreA, acoA, and lysF promoter regions (Fig. 9). This synergetic binding mode not only requires protein-protein interaction between the N-terminal domain of HapE and the N-terminal CBC binding domain of HapX (1) but also sequence-specific DNA binding when part of the HapX-CBC-DNA ternary complex. The observed general 2:1 stoichiometry of these complexes raises the important question of whether both or only one of the HapX CBC binding domains are bound by the single HapX binding domain of the CBC subunit HapE. Furthermore, our closer investigation of the CBC-HapX DNA-binding code revealed that HapX binding requires the previous CBC-DNA complex formation and additionally the presence of a 5′-GAT-3′ motif with a spacing of 11 or 12 bp relative to the 5′-flanking CCAAT box.

Based on its invariant amino acid residues within the basic DNA-binding region (NXNLYAQXXFR), HapX belongs to the fungus-specific Pap1/Yap1 subfamily of bZIP transcription factors (37) that are known to recognize overlapping or adjacent TTAC half-sites (38). However, the minimal HapX recognition site 5′-GAT-3′ identified by our study shows limited similarity to half-sites of the TGACTXA motif that is recognized by members of the AP-1 (Jun-Fos)/Gcn4 subfamily (26). This unexpected DNA-binding specificity tolerated single nucleotide substitutions at the 5′- and 3′-end of the GAT motif, and the variable spacing between the CBC and HapX DNA-binding sites revealed a remarkable promiscuous DNA recognition mode of HapX. A closer inspection of the recently identified HapX-binding motif 5′-ATTGTCAGC-3′, present downstream of a CCAAT box in the promoter of the A. fumigatus cccA gene (8), revealed a 5′-TGAC-3′ AP-1-like half-site on the reverse complement strand with a 16-bp spacing relative to the 5′-flanking CCAAT box. Despite the lack of experimental proof for functionality of the 5′-TGAC-3′ motif in the A. fumigatus cccA promoter, opposite strand orientation of the CBC and HapX submotifs might offer an additional level of variability for target promoter recognition. The well known conformational flexibility of bZIP transcription factors in general, and most likely also in the case of HapX, provides an explanation for the observed DNA recognition promiscuity (39).

In summary, our studies strongly suggest that DNA binding by HapX is involved not only in control of genes that are activated during iron excess, as shown recently in A. fumigatus (8), but also in regulation of genes that are repressed during iron starvation as shown here in A. nidulans. The conservation of the N-terminal CBC interaction domain, the basic DNA-binding domain, and the coiled-coil dimerization domain in most HapX orthologs (8) suggest a similar DNA binding mode in most fungi. A possible exception is Schizosaccharomyces pombe Php4, which, similar to other HapX orthologs, contains an N-terminal CBC interaction domain as well as a coiled-coil domain but lacks a basic DNA binding domain (40), suggesting that DNA binding might be dispensable in this system.

Acknowledgment—Sylke Fricke (Leibniz Institute for Natural Product Research and Infection Biology) is gratefully acknowledged for excellent technical assistance.

REFERENCES


FIGURE 9. Alignment of experimentally mapped bipartite CBC-HapX DNA-binding motifs in the A. nidulans cyaA, sreA, acoA, and lysF promoter regions. The alignment was used to generate the WebLogo consensus motif for CBC-HapX.
CBC-HapX DNA-binding Code


Deciphering the Combinatorial DNA-binding Code of the CCAAT-binding Complex and the Iron-regulatory Basic Region Leucine Zipper (bZIP) Transcription Factor HapX

Peter Hortschansky, Eriko Ando, Katja Tuppatsch, Hisashi Arikawa, Tetsuo Kobayashi, Masashi Kato, Hubertus Haas and Axel A. Brakhage

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