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 belongs 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 HapX 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) 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, the latter mode of gene activation not only requires CBC-HapX protein–protein interaction but also cooperative CBC-HapX binding of an evolutionarily conserved bipartite DNA motif within the cccA promoter. This entirely new iron regulatory mechanism depends on evolutionarily conserved protein domains within HapX that are exclusively essential for adaptation to either limitation or excess of iron. In conclusion, HapX is a Janus-type transcription factor acting as both an activator and repressor depending on the ambient iron availability.

In A. nidulans, the CBC consists of the subunits HapB, HapC, and HapE (10) that together bind CCAAT boxes in the limited number of gene promoter sequences identified so far (11, 12). Genome-wide identification in human cells resulted in 5000–15,000 NF-Y-binding sites depending on the cell type, but only 25% of these NF-Y sites mapped to promoter regions (13). It has long been an open question of how the CBC employs the HapC/HapE histone fold motifs to gain sequence specificity for the CCAAT pentanucleotide. The recently reported crystal structure of the A. nidulans CBC-DNA complex revealed a novel mode of sequence-specific DNA binding and provided deep insights into transcription initiation, which constitutes a fundamental biological process (14). HapC and HapE induce nucleosome-like DNA bending by interacting with the sugar-phosphate backbone, whereas HapB tightly anchors the CBC to the CCAAT box by minor groove sensing and widening. The CBC-DNA structure visualized for the first time the position of the HapE N-terminal helix (αN) within the CBC and relative to the DNA backbone. This domain was shown to be important for protein-protein interaction with HapX (1).

Originally, a yeast two-hybrid screen using HapB as bait identified HapX as an additional subunit of the CBC in A. nidulans (15). HapX displays no similarity to S. cerevisiae Hap4p, except for an N-terminal 17-amino acid motif, which has been shown to be essential for interaction of Hap4p with the S. cerevisiae Hap2p-Hap3p-Hap5p complex (16).

HapX orthologs in ascomycetes contain both the “b(ZIP)” basic region and “coiled-coil” subdomains, which together mediate DNA binding of bZIP-type transcription factors, and an N-terminal CBC binding domain that is essential for HapX function due to its requirement for interaction with the CBC subunit HapE (1). In addition, the HapX-type transcription factors contain up to four conserved cysteine-rich regions, each of which contains four cysteine residues with different specific architectures. Meanwhile, it became clear that these cysteine-rich regions are dispensable for the HapX functions in adaptation to iron starvation but are required for HapX activity during iron detoxification (8).

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. DNsA 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(NdeI) and hapX200Myc-as(NotI) 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-nitrilotriacetic 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/lysF-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 ProbeQuantTM 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 aprotinin, 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 (EMSAs)—**EMSAs 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...
in Table 2. The DNA fragments were labeled with [α-^{32}P]dCTP by T7 DNA polymerase as described for the DNase I footprinting analysis. Biological 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 His six tag was cleaved with tobacco etch virus protease and further purified sequentially using CelluloseSulfate (Millipore) affinity chromatography (GE Healthcare). The maltose-binding protein HapX(1–198) fusion was cleaved with tobacco etch virus protease and further purified using CelluBlue (New England Biolabs) vector. The resulting plasmid was transformed into *E. coli* Rosetta 2 (DE3) cells for overnight autoinduction. Crude bacterial lysates were purified by dye-affinity chromatography, (NH₄)₂SO₄ precipitation (50% w/v), and Superose (GE Healthcare), and molar mass was calculated using miniDawn TREOS monitor in series with an OPTILab T-rEX autoinduction. 

**Table 1**

**Fungal strains used in this study**

| Strain designation in text | Strain Genotype Ref. |
|---------------------------|----------------------|
| Wild type                 | BPU1                 |
| ΔhapX                     | BYP3ΔX1              |
| cycAp/ΔhapX               | BYP3ΔX1              |
| TGAAtm/Δ hapX             | BYP3ΔX1              |
| CCAAtm/Δ hapX             | BYP3ΔX1              |

| Strain designation in text | Strain Genotype Ref. |
|---------------------------|----------------------|
| Wild type                 | BPUG1               |
| ΔhapX                     | BYP3ΔX1              |
| cycAp/ΔhapX               | BYP3ΔX1              |
| TGAAtm/Δ hapX             | BYP3ΔX1              |
| CCAAtm/Δ hapX             | BYP3ΔX1              |

**Table 2**

**CBC-HapX DNA-binding Code**

**Table 1**

**Fungal strains used in this study**

| Strain designation in text | Strain Genotype Ref. |
|---------------------------|----------------------|
| Wild type                 | BPUG1               |
| ΔhapX                     | BYP3ΔX1              |
| cycAp/ΔhapX               | BYP3ΔX1              |
| TGAAtm/Δ hapX             | BYP3ΔX1              |
| CCAAtm/Δ hapX             | BYP3ΔX1              |

| Strain designation in text | Strain Genotype Ref. |
|---------------------------|----------------------|
| Wild type                 | BPUG1               |
| ΔhapX                     | BYP3ΔX1              |
| cycAp/ΔhapX               | BYP3ΔX1              |
| TGAAtm/Δ hapX             | BYP3ΔX1              |
| CCAAtm/Δ hapX             | BYP3ΔX1              |

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| TGAAtm/Δ hapX             | BYP3ΔX1              |
| CCAAtm/Δ hapX             | BYP3ΔX1              |

| Strain designation in text | Strain Genotype Ref. |
|---------------------------|----------------------|
| Wild type                 | BPUG1               |
| ΔhapX                     | BYP3ΔX1              |
| cycAp/ΔhapX               | BYP3ΔX1              |
| TGAAtm/Δ hapX             | BYP3ΔX1              |
| CCAAtm/Δ hapX             | BYP3ΔX1              |
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 (αN) 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(CT/T)(A/G) (14). The 3'-conserved submotif 5'-GATATTCA-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'-GATATTCA-3' motif, and interestingly, co-incubation of the DNA probe with both the CBC and HapX extended the protected region by an additional 9 bp and led to complete protection of the 5'-GAATTCAAGCTTGAGGTTGACAC-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 (627 to 630) half-site (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 molar mass 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 ($K_D$) 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 $K_D$ 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'-GATGATTCA-3' motif 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'-GATGATTCA sequence was mutated to TGCA (matching mutation m4 in Figs. 2 and 4), whereas the CCAAT box was mutated.
FIGURE 1. HapX binds to an evolutionarily conserved motif identified in promoters of cycA homologs. A, an evolutionarily conserved, bipartite motif in promoters of Aspergillus cycA homologs identified by MEME analysis. The 23 underlined nucleotides are covered upon CBC binding according to the CBC-DNA binary complex crystal structure (14). B, DNase I footprinting patterns of the CBC and CBC-HapX on the A. nidulans cycA promoter. As indicated on top, the reconstituted CBC alone or in combination with HapX was incubated with end-labeled DNA fragments of the cycA promoter. Protected sequences on either strand are shown on the right side of the footprints. Nucleotides colored in red and blue indicate the CCAAT box at position −611 and the conserved 3′-submotif, respectively. Numbers represent the nucleotide positions relative to the start of the open reading frame.

FIGURE 2. Multicomponent EMSAs of the CBC and CBC-HapX with natural and mutant cycA promoter probes carrying mutations outside and within the putative pseudo-palindromic HapX-binding motif 5′-GAT-GATTTCA-3′. Partial sequences of 129-bp DNA probes used in EMSAs are shown on top of the autoradiogram. The CCAAT box and the left half-site of the TGATTTCA motif are highlighted in red and blue, respectively. Substituted nucleotides relative to the wild-type sequence are underlined and shown in lowercase.

FIGURE 3. Characterization of purified recombinant proteins used in this study. A, SDS-PAGE analysis of the CBC and HapX(1–198). B, analysis of the solution oligomeric state of HapX(1–198) by size exclusion chromatography and multiangle static light scattering. The light scattering signal (LS) is shown overlaid with the calculated molar mass (Mw) across the elution profile monitored by the absorbance at 280 nm (UV) and changes of the refractive index (dRI).

to CGTAA for the third construct. All three constructs were introduced into both an A. nidulans wild-type and hapC 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 ΔhapC 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 ΔhapX 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)  
\[
\begin{align*}
\text{cycAp-611} (-) & \quad \text{ATCGGGATTTGCACAGGTTAGAGTTGCACACTGTCGTCAGA} \\
& \quad \text{TAGACCTTAACTGGTTAGTCCTCAATCTACTAATGCTG} \\
\text{50 bp} & \\
\end{align*}
\]

2 + HapX (3.13-100 nM)  

3 HapX (3.13-100 nM) - CBC response

\[ K_D = 0.42 \text{ nM} \]

\[ K_D = 2.54 \text{ nM} \]

\[ K_D = 3.65 \text{ nM} \]

\[ K_D = 5.60 \text{ nM} \]

\[ K_D = 28.4 \text{ nM} \]

\[ K_D = 147.2 \text{ nM} \]

\[ K_D = 81.7 \text{ nM} \]

\[ K_D = 19.3 \text{ nM} \]
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 aconitate 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 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 a 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 *Kd* values ranging from 0.42 to 3.9 nM (Fig. 6, panel 1). The measured saturating CBC responses (*Rmax* 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 *Rmax* 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 *Rmax* 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 stoichiometry of HapX-CBC-DNA ternary complexes and in agreement with our previous results concerning HapX-CBC binding on a bipartite *A. fumigatus ccaA* 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′-CGAAT box 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-HapX DNA-binding Code**

![Figure 5](image-url)

**FIGURE 5.** Regulatory function of the CBC and HapX-binding motifs on the expression of translational *cycA-locZ* reporter gene fusions. Iron-dependent expression of cycA-locZ gene fusions carrying mutations within the CBC or HapX-binding sites was determined as β-galactosidase-specific activity from soluble cell extracts of wild-type and ΔhapX mycelia grown under iron-replete (+Fe) and iron-depleted (−Fe) conditions. The host strains, BPU1 (wt) and BPUΔX1 ΔhapX, were used as negative controls. Data represent the mean ± S.D. of three independent experiments.

![Figure 4](image-url)

**FIGURE 4.** SPR co-injection analysis of HapX binding to CBC-bound cycAp DNA duplexes carrying mutations within the conserved 3′-submotif. SPR analyses included binding of the CBC to DNA (panel 1), HapX alone to DNA (panel 2, red lines), and HapX to preformed CBC-DNA complexes (panel 2, blue lines). Binding responses of CBC-DNA-HapX ternary complex formation (panel 2, blue lines) were obtained by concentration-dependent co-injection of HapX on preformed binary CBC-DNA complexes after 150 s within the steady-state phase. Concentrations of 3.13, 6.25, 12.5, 25, 50, and 100 nM HapX were used, respectively. Sensorgrams in panel 3 depict the association/dissociation responses of HapX on preformed CBC-DNA and were generated by CBC response (co-injection of buffer instead of HapX) subtraction from HapX co-injection responses. Binding responses of the indicated CBC or HapX concentrations (black lines) are shown overlaid with the best fit derived from a 1:1 interaction model, including a mass transport term (red lines). Dissociation constants (*Kd*) are plotted inside the graphs. Substituted nucleotides relative to the wild-type sequence are indicated as in Fig. 2.
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 −11002.

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.

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 −11002. Mutations within this CCAAT box (m3) led to the loss of DNA complexes containing two CBCs, but it
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

| DNA duplex   | Molar mass duplex | DNA bound | \( R_{\text{max}} \) calculated \({}^a\) | \( R_{\text{max}} \) measured | Molar ratio CBC-DNA | \( K_D \) (nM) |
|--------------|-------------------|-----------|---------------------------------|----------------------------|-------------------|----------------|
| cycA-p-611(−) | 31,208            | 52.9      | 162.3                           | 146.8 ± 0.1                | 0.904             | 0.425 ± 0.003 |
| srcA-p-1235(+) | 31,211            | 50.9      | 156.1                           | 143.4 ± 0.1                | 0.919             | 0.729 ± 0.003 |
| acoA-p-568(−) | 31,201            | 49.6      | 152.2                           | 153.8 ± 0.2                | 1.011             | 0.960 ± 0.003 |
| lysP-p-181(+) | 31,211            | 51.8      | 158.9                           | 282 ± 2\(^b\)              | 1.775             | 3.9 ± 0.2\(^b\) |

\(^a\) \( R_{\text{max}} \) calculated = 95,735 (molar mass CBC)/molar mass DNA × DNA bound.

\(^b\) Data were determined by steady-state analysis.

**TABLE 5**

Dissociation constants and stoichiometry of HapX-CBC-DNA ternary complex formation

| DNA duplex   | CBC bound | \( R_{\text{max}} \) HapX-CBC\(^a\) (1:1) calculated | \( R_{\text{max}} \) HapX-CBC measured | \( K_D \) (nM) HapX-CBC-DNA | Molar ratio HapX-CBC |
|--------------|-----------|---------------------------------|----------------------------|----------------|----------------|
| cycA-p-611(−) | 145.9     | 33.4                            | 102.8 ± 0.1                | 4.36 ± 0.03     | 3.1            |
| srcA-p-1235(+) | 134.1     | 30.7                            | 103.6 ± 0.7                | 25.9 ± 0.4      | 3.4            |
| acoA-p-568(−) | 124.5     | 28.5                            | 105.6 ± 0.2                | 6.27 ± 0.07     | 3.7            |
| lysP-p-181(+) | 183.6     | 42.1                            | 113.0 ± 0.3                | 6.06 ± 0.07     | 2.7            |

\(^a\) \( R_{\text{max}} \) HapX-CBC (1:1) calculated = 21,936 (molar mass HapX monomer)/95,735 (molar mass CBC) × CBC bound.

**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′-GGATT-3′ are highlighted in red and blue, respectively. Substituted nucleotides relative to the wild-type sequence are underlined and shown in lowercase.
GAT subsequence 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 11-bp spacing between the CGAAT box and the 3′-flanking GAT 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. Conversely, 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 domain of the *C. albicans* HapX ortholog Hap43 led to reduced growth during iron starvation conditions, whereas mutation of the Hap43 N-terminal domain that is required for interaction with the CB5-Hap43 led to reduced growth during iron starvation conditions, whereas mutation of the Hap43 N-terminal domain that is required for interaction with the *C. albicans* HapX ortholog Hap43 led to reduced growth during iron starvation conditions, whereas mutation of the Hap43 N-terminal domain that is required for interaction with the Hap5 completely blocked growth during iron starvation (32). These data indicate that the Hap43 interaction with the CBC might be more important than interaction with DNA, at least in *C. albicans*. However, a chromatin immunoprecipitation/MEME-guided search for Hap43 target genes and a possible CBC-HapX-specific promoter element only yielded the CBC consensus sequence CCAAT (29). Recently, *A. fumigatus* HapX was found to interact physically not only with the CBC but also with DNA, i.e. the CBC-HapX complex cooperatively recognizes an evolutionarily conserved bipartite DNA motif within the cccA promoter to activate vacuolar detoxification during iron excess conditions (8).
HapX-binding motif 5
the variable spacing between the CBC and HapX DNA-binding to half-sites of the cases, involving bZIP transcription factors seems to be a common dimerization networks (34). Combinatorial gene regulation reverse complement strand with a 16-bp spacing relative to the cccA stream of a CCAAT box in the promoter of the fungus-specific Pap1/Yap1 subfamily of bZIP 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 cycA, 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 2:1:1 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 (NXVAXQXXR), 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 TGACTCA motif that is recognized by members of the AP-1 (Jun-Fos)/Gcm4 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’-ATTGTGACAGC-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.

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