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Analysis on the Transcriptional Regulation Mechanisms Controlled by the CCAAT-box Binding Complex as a Key Factor in Filamentous Fungi

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Research aims

The CCAAT box is one of the most common *cis*-elements present in the promoter regions of many eukaryotic genes. A statistical analysis of over 500 promoters revealed that the CCAAT sequence is one of the widespread elements that is present in approximately 30% of the eukaryotic promoters.[1,2)]

The CCAAT sequence in filamentous fungi has been shown to modulate the expression of many genes.[3–5)] CCAAT-binding proteins, which are homologs of the yeast Hap complex, have also been characterized.[3–5)] The *hapC* gene, which is a homolog of the *hap3* gene of *Saccharomyces cerevisiae*, has been isolated from *Aspergillus nidulans* by M. Hynes and colleagues. We analyzed this factor in cooperation with Hyne's group. Disruption of the *hapC* gene resulted in the loss of binding between the CCAAT-binding protein and the CCAAT sequence, indicating that HapC is a component of the CCAAT-binding protein complex.[5–7)] We further confirmed the result by a supershift assay using anti-HapC antibodies.[6)] Furthermore, a DNA-binding protein complex has been successfully reconstituted using recombinant HapC together with the recombinant *S. cerevisiae* Hap2p and Hap5p subunits.[6)] The *hapB* and *hapE* genes, which are orthologs of the *hap2* and *hap5* genes of *S. cerevisiae*, have also been cloned from *A. nidulans*, and a DNA-binding complex has been reconstituted with recombinant HapC, HapE, and HapB.[6)] All these data indicate that the CCAAT-binding protein complex of *A. nidulans* is a counterpart of the *S. cerevisiae* Hap complex. Therefore, we designated this factor as the *A. nidulans* Hap complex.

A yeast-two-hybrid screen suggested physical interaction of *A. nidulans* HapB with a protein of an as yet unknown function, termed HapX.[8)] HapX displays no similarity to *S. cerevisiae* Hap4p, besides 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. It has recently been shown that various iron-dependent pathways are repressed under iron-deficient conditions via the interaction of HapX with the HapB/C/E complex.[9)] HapX harbors a basic domain from the bZip motif except for a coiled-coil domain instead of leucine-zipper; this suggests that HapX possesses a DNA-binding ability that is different from that of *S. cerevisiae* Hap4p. In this study, therefore, we analyzed the interaction of the HapX-HapB/C/E complex with the promoter of the cytochrome *c* gene (*cycA*) of *A. nidulans*, which is one of the HapX-dependent genes. Electrophoretic mobility shift assays (EMSAs) and DNA footprint analyses using recombinant HapX-HapB/C/E and various DNA probes revealed that both the CCAAT-box and an adjacent sequence of the *cycA* promoter are required for the recognition by the HapX-HapB/C/E complex. The DNA-binding ability of HapX presumably allows independent regulation by HapB/C/E and HapX-HapB/C/E.

Methods

The strains used in this study included *A. nidulans* BPU (genotype: *biA1*, *pyrG89*, *wA3*, *pyroA4*) as a wild-type strain and *A. nidulans hapX*Δ (genotype: *biA1*, *pyrG89*, *wA3*, *hapX*Δ, *pyroA4*), for the β-galactosidase assay. pPY-cycAp-lacZ, a reporter plasmid used for the analysis of the *cycA* promoter, was constructed as follows: a promoter region of the *cycA* gene was amplified with chromosomal DNA from the *A. nidulans* wild-type strain; the promoter fragment was fused to the *lacZ* gene and inserted to the integration vector pPY-7.

For the DNA binding experiments, a truncated HapX (1–200) was prepared and used. The recombinant protein was produced in *Escherichia coli* and purified with a Ni-NTA agarose column. The subunit proteins of the HapB/C/E complex were also prepared as recombinant proteins, and reconstituted to form the Hap core complex. Electrophoretic gel mobility shift assay (EMSA) and DNase I footprinting experiments were carried out as described previously.[6)]

Results

HapX interacts with a target promoter in the presence of the HapB/C/E complex: EMSAs were carried out with the HapB/C/E complex and HapX in order to examine the binding specificity for HapX-dependent (cytochrome *c* gene: *cycA*) and independent (amylase gene: *taaG2*) promoters, respectively (Fig. 1). HapX can bind to the *cycA*

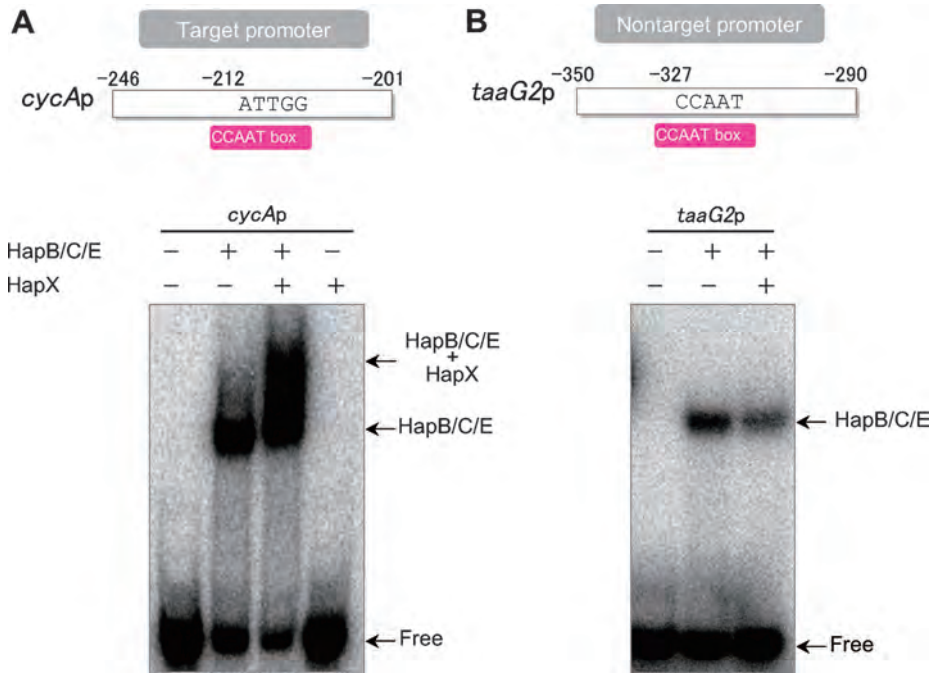

Fig. 1. EMSAs using the HapB/C/E complex and HapX. HapX interacts with a target promoter in the presence of the HapB/C/E complex.

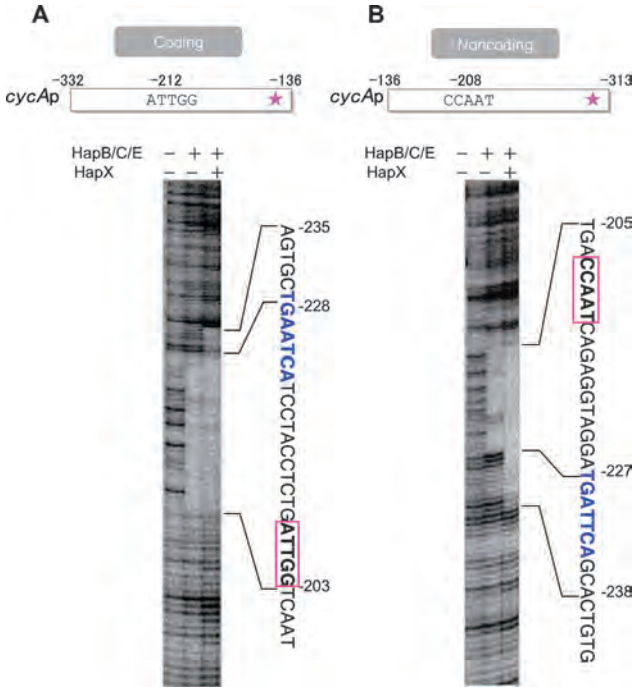

Fig. 2. Footprint analysis of the *cycA* promoter with HapX and the HapB/C/E complex. (A) Coding strand (B) Noncoding strand.

promoter in the presence of the HapB/C/E complex, while HapX cannot bind to the *taaG2* promoter despite the presence of the HapB/C/E complex, suggesting that the recruitment of HapX to the HapB/C/E complex is promoter sequence-dependent.

Footprint analysis of the *cycA* promoter with HapB/C/E/X complex: Footprint analyses of the *cycA* promoter with HapX and the HapB/C/E complex were performed (Fig. 2). When HapX was added to the HapB/C/E complex, the protected region was extended, suggesting that HapX may have a DNA binding activity. This is consistent with the fact that HapX possesses a basic domain of the bZip-like motif. It is reported that a bZip transcription factor, AP-1, recognizes TGAGTCA sequence. In our study, we found a similar sequence, TGATTCA, in the protected region.

The ATCA sequence in the *cycA* promoter is necessary for the recognition of HapX: To examine whether the TGAATCA (−633 to −627) sequence is involved in the HapX binding, EMSAs were performed with mutant *cycA* promoters (Fig. 3). When mutations were introduced in the ATCA (−630 to −627) sequence, the intensities of the upper shift band were significantly decreased. The mutation in the A (−631) residue also slightly decreased the binding affinity of HapX.

The HapX-binding sequence is conserved among the *cycA* promoters of *Aspergillus* species (data not shown). The ATCA sequence and its distance from the CCAAT box are strictly conserved among the *cycA* promoters of *Aspergillus* species, as far as we examined. We also found a downstream conserved sequence. Although its function remains unknown at present, we speculate that it could be acting as the binding site for a positive regulator of the *cycA* gene.

The ATCA sequence is required for the repression of the *cycA* promoter under the iron-depleted conditions: To examine the in *vivo* function of the ATCA sequence of the *cycA* promoter, we constructed some *lacZ* fusion genes under control of the wild-type and mutant *cycA* promoters (Fig. 4). Mutations were introduced into the ATCA (−630) and CCAAT (−611) sequences, respectively. The β-gal activities of the extracts from mycelia were measured. In the wild-type background (*hapX*$^{+}$), the β-gal activity under iron-deficient conditions (−Fe) was decreased by approximately 50% compared with that under the iron-replete conditions (+Fe). On the other hand, *lacZ*-fusion genes with mutations within the ATCA or CCAAT sequence exhibited derepression under the iron-deficient conditions. Furthermore, in the *hapX*Δ background, even the wild-type pro-

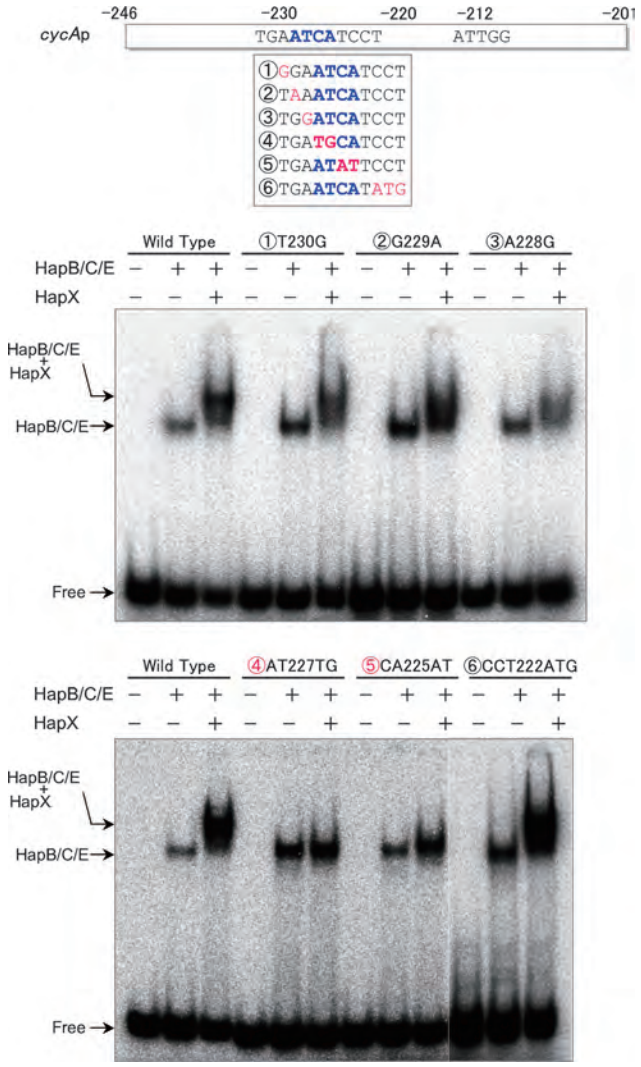

Fig. 3. The ATCA sequence in the *cycA* promoter is necessary for the recognition of HapX.

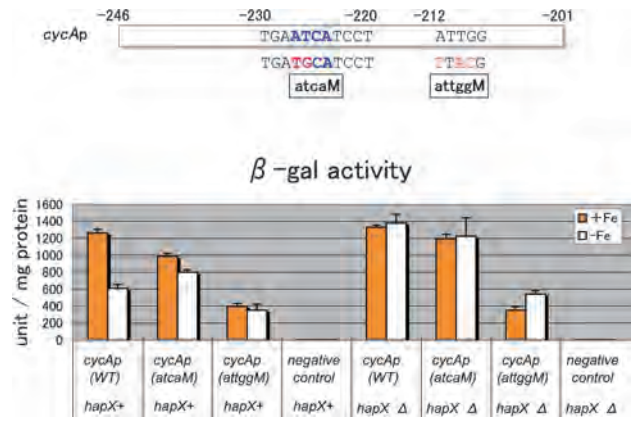

Fig. 4. Analysis of the *lacZ* fusion genes under the control of the wild-type and mutant *cycA* promoters. The ATCA sequence is required for the repression of the *cycA* promoter under the iron-deficient conditions.

moter exhibited derepression under the iron-deficient conditions. Taken together, our data led us to conclude that HapX represses iron-dependent genes by interacting with the ATCA sequence and the Hap complex.

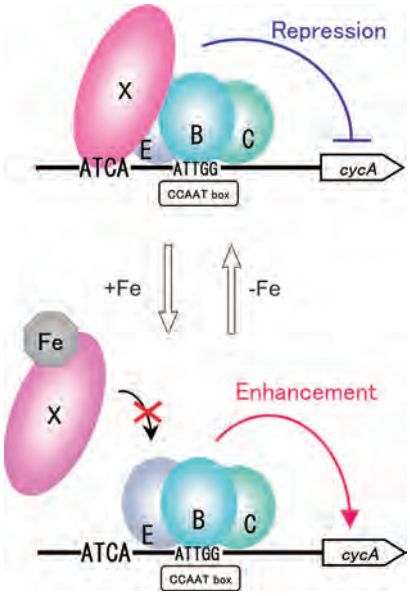

Fig. 5. A model of the transcriptional regulation by HapX and HapB/C/E complex.

Conclusion

In this study, the following new findings were revealed. (i) HapX interacts with the target promoter in the presence of the HapB/C/E complex. (ii) The ATCA sequence in the *cycA* promoter is necessary for the recognition of HapX. (iii) The HapX-binding sequence is conserved among the *cycA* promoters of *Aspergillus* species. (iv) The ATCA sequence is required for the repression of the *cycA* promoter under the iron-deficient conditions. Recently, we have shown that various iron-dependent pathways are repressed under iron-deficient conditions by interaction of HapX with the HapB/C/E complex. A tentative model (Fig. 5) for transcriptional regulation by HapX and the HapB/C/E complex is depicted, on the basis of the results presented here and some speculations. Under the iron-deficient conditions, HapX recognizes the HapB/C/E complex and the ATCA sequence in the *cycA* promoter region. HapX is thought to interact with the DNA sequence through the bZip-like domain. Under the ion-replete conditions, binding of the iron ion to the C-terminal domain (cys-rich domain) would alter the conformation of HapX, resulting in its decreased interaction with the HapB/C/E complex. This permits derepression of HapX-dependent genes.

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