

A quantity control mechanism regulating levels of the HapE subunit of the Hap complex in *Aspergillus nidulans*: no accumulation of HapE in *hapC* deletion mutants

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Abstract The *Aspergillus nidulans* CCAAT-binding complex (Hap complex) consists of at least three subunits, HapB, HapC and HapE. To investigate the quantity control mechanisms of the subunits during assembly of the Hap complex, reconstitution studies with the recombinant subunits and extracts prepared from the respective *hap* subunit deletion mutants were carried out. Furthermore, Western blot analysis of the Hap subunits and Northern blot analysis of the *hap* genes with the respective deletion mutants were also performed. From all the results together, it was suggested that the number of the HapC molecule could adjust that of the HapE molecule by forming stable heterodimers prior to assembly of the Hap complex. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: CCAAT-binding complex; Hap complex; Subunit assembly; *Aspergillus nidulans*

1. Introduction

The CCAAT sequence is one of the most common *cis*-elements present in the promoter regions in eukaryotes. In a statistical analysis of over 500 promoters, the CCAAT sequence is present in approximately 30% of eukaryotic promoters [1]. The CCAAT sequence is also found in many genes of filamentous fungi and has been shown to modulate the expression of various genes such as the *amdS* gene encoding the *Aspergillus nidulans* acetamidase, the *taaG2* gene encoding *Aspergillus oryzae* Taka-amylase A, and the genes encoding the *A. nidulans* penicillin biosynthetic enzymes. Proteins binding to these sequences have been detected independently by several groups: AnCF for *amdS* [2], AnCP for *taa* [3,4], PENR1 for the penicillin biosynthesis genes [5] and AAB for *am* [6]. A similar complex designated AoCP from *A. oryzae* has been characterized by us [7,8].

The *hapC* gene, with significant similarity to the *hap3* gene of *Saccharomyces cerevisiae*, has been isolated from *A. nidulans* [9]. Deletion of the *hapC* gene resulted in loss of binding of AnCP, AnCF and PENR1 to CCAAT sequences of *taa*, *amdS* and the penicillin biosynthesis genes, respectively, indi-

cating that all these complexes contain HapC as a component [9–11]. This was further confirmed using anti-HapC antibody [10,11]. All these data clearly show that AnCP/AnCF/PENR1 could be an *A. nidulans* counterpart of the *S. cerevisiae* Hap complex. In addition to the *hapC* gene, two genes, *hapB* and *hapE*, encoding polypeptides with a central core bearing high similarity to yeast Hap2p and Hap5p, respectively, have been isolated from *A. nidulans*. [12].

A. nidulans deletion strains of the respective *hap* genes have been constructed [9,12]. Deletion of any of the *hap* genes results in abolition of transcriptional enhancement of *taa*, *amdS* and the penicillin biosynthesis genes in vivo and loss of the binding activity to the CCAAT-containing DNA probe [7,9,11]. CCAAT-binding activity was successfully reconstituted from the recombinant HapB, HapC and HapE, indicating that these three subunits are absolutely required for formation of the Hap complex [8,12]. In this study, the quantity control mechanisms of the Hap subunits during assembly were investigated by (i) reconstitution analysis using the recombinant Hap subunits and the extracts prepared from the respective *hap* deletion mutants, (ii) Western blot analysis of the Hap subunits in the deletion mutants, and (iii) Northern blot analysis of the levels of the *hap* subunit transcripts.

2. Materials and methods

2.1. Strains and media

A. nidulans UR7 (pyrG89; pyroA4) [7], UR1 (pyrG89, yA1; hapCΔ; pyroA4) [7], MH9099 (yA1, pabaA1; amdS368 argB2; amdA7; hapB::argB) [12] and MH9092 (yA1, pabaA1; amdS368 argB2; amdA7; hapE::argB) [12] were used. *A. nidulans* strains were grown at 37°C in DP medium supplemented with 2% starch or glucose [3]. *Escherichia coli* JM83 was used for DNA manipulation and expression of recombinant proteins. Strains of plasmid-carrying *E. coli* were grown at 37°C in LB medium containing 50 µg/ml ampicillin.

2.2. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was carried out with DNA fragments labeled at the 3' end with T7 polymerase and [α -³²P]-dCTP as described previously [4]. An EcoRI–HindIII fragment isolated from pUC-poly20 [10] which carried five copies of the CCAAT sequence was used as a probe DNA. The same fragment from pUC-poly20M [10] which carried the mutated CCAAT sequences was also used as a control probe.

2.3. Preparation of anti-HapE antiserum

Plasmid pMal-HapE used for production of the MalE-HapE fusion protein was constructed as follows. Oligonucleotides, ES (5'-GAA-GAAGCACTTTGCTGCAGATGGAGCAAG-3'; corresponding to

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Abbreviations: EMSA, electrophoretic mobility shift assay

positions –20 to +10 of the *hapE* cDNA where the translation start site is designated +1) and EAS (5'-CCCCTGCAGGTCGAAACAAAGAAACCGCCCGG-3'; corresponding to positions 823–851), were used as primers to introduce the *Pst*I sites (underlined) and amplify a 1.2-kb *hapE* cDNA fragment with pHapE-7 [12] carrying the entire *hapE* cDNA as a template. The resultant fragment was digested with *Pst*I and inserted at the *Pst*I site of pMal-c2 to construct pMal-HapE. The nucleotide sequence of the amplified fragment was confirmed by DNA sequencing. From *E. coli* JM83 harboring pMal-HapE, MalE-HapE was prepared as described for the MalE-HapC fusion protein [10]. Purified MalE-HapE was used for immunization of a rabbit (Sawady Technology, Japan).

2.4. Other methods

Proteins were extracted with 6 M guanidine hydrochloride from mycelia and renatured by dialysis as described previously [13]. GST-HapC and GST-HapB were prepared as described previously [8,10]. Nucleotide sequences were determined with a DNA sequencer (Li-cor 4000L). Western blot analysis was carried out as described previously [10]. The methods of preparation of total RNAs from *A. nidulans* and Northern blotting were principally the same as described previously [7].

3. Results and discussion

Deletions of any of the *hapB*, *hapC* and *hapE* genes result in loss of CCAAT-binding activity in EMSAs [12]. CCAAT-binding activity was restored by addition of the recombinant HapB and HapE subunits to the extracts prepared from the *hapB* and *hapE* deletion strains (abbreviated throughout this study as $\Delta hapB$ and $\Delta hapE$), respectively (Fig. 1). On the other hand, the recombinant HapC subunit was unable to reconstitute CCAAT-binding activity with the $\Delta hapC$ extract. The plausible explanation for these results is that the $\Delta hapC$ strain may lack the other Hap subunit as well as HapC subunit. To address this possibility, the recombinant Hap subunits were added to the $\Delta hapC$ extract (Fig. 2). The addition of each subunit alone to the $\Delta hapC$ extract was unable to reconstitute CCAAT-binding activity, whereas the simultane-

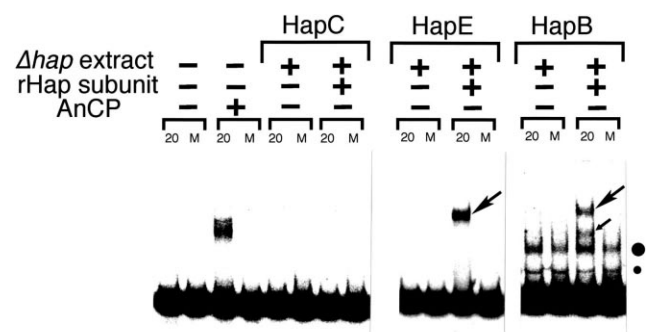


Fig. 1. Reconstitution of the CCAAT-binding complex with the extracts from the *hap* deletion strains and the corresponding recombinant subunits. EMSAs were carried out with 20 μ g of the cell-free extract from the respective *A. nidulans* Δhap strains (Δhap extracts) and 1 μ g of the respective recombinant subunits (rHap subunits) as indicated. The *Eco*RI–*Hind*III fragments of pUC-poly20 (20) and pUC-poly20M (M) were used as probe DNAs. The cell-free extract from the wild-type strain (AnCP) was used as a positive control. Fusion proteins, GST-HapC, MalE-HapE and GST-HapC, were used as recombinant subunits. The bands corresponding to the DNA-bound reconstituted Hap complexes are indicated by the larger arrows. A band indicated by the smaller arrow could correspond to a complex containing a truncated recombinant HapB, because of the instability of GST-HapB during preparation [8]. The bands indicated by the large and small dots were non-specific, since these bands were found even when the control probe (M) was used.

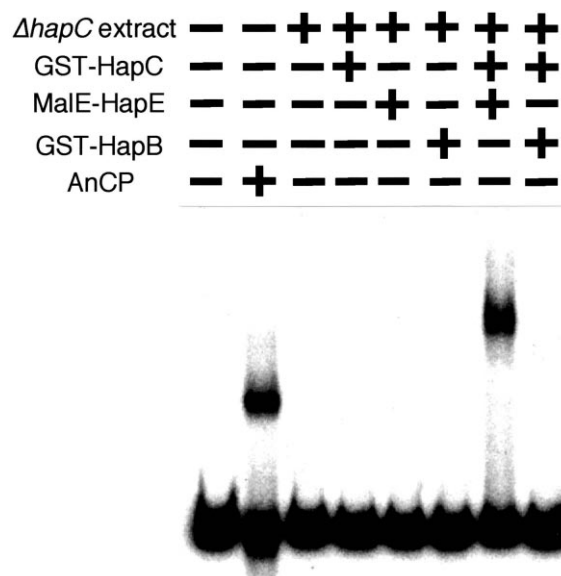


Fig. 2. Reconstitution with the $\Delta hapC$ extract and combinations of the recombinant Hap subunits. EMSAs were carried out using the same fragment of pUC-poly20 as a probe DNA as described in the legend to Fig. 1.

ous addition of both HapC and HapE subunits restored the activity. Taken together, these data indicate that the $\Delta hapC$ strain lacked both HapC and HapE subunits and contained only the HapB subunit.

To directly determine the Hap subunit components in the $\Delta hapC$ strain, the $\Delta hapC$ extract as well as the $\Delta hapB$ and $\Delta hapE$ extracts were subjected to immunological analysis (Fig. 3). The $\Delta hapC$ extract cross-reacted only with anti-HapB antibody, while the $\Delta hapB$ and $\Delta hapE$ extracts con-

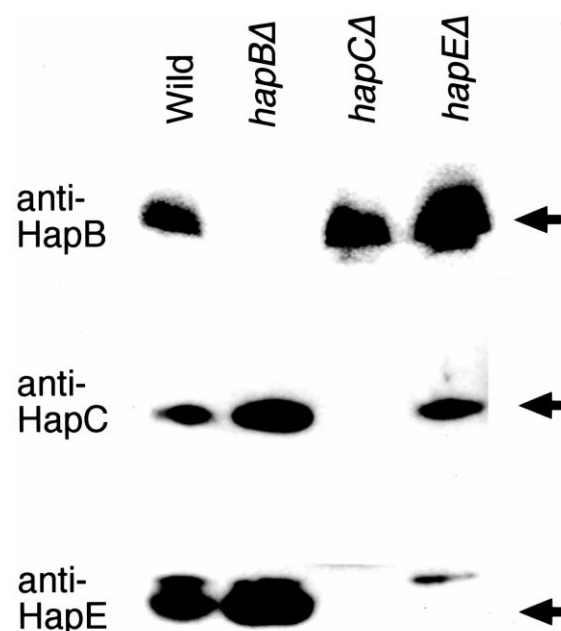


Fig. 3. Western blot analysis of the *hap* deletion strains. The same cell-free extracts used for reconstitution experiments were subjected to the Western blot analysis with anti-HapB, anti-HapE and anti-HapC antisera. The position of each Hap subunit is indicated by the arrow.

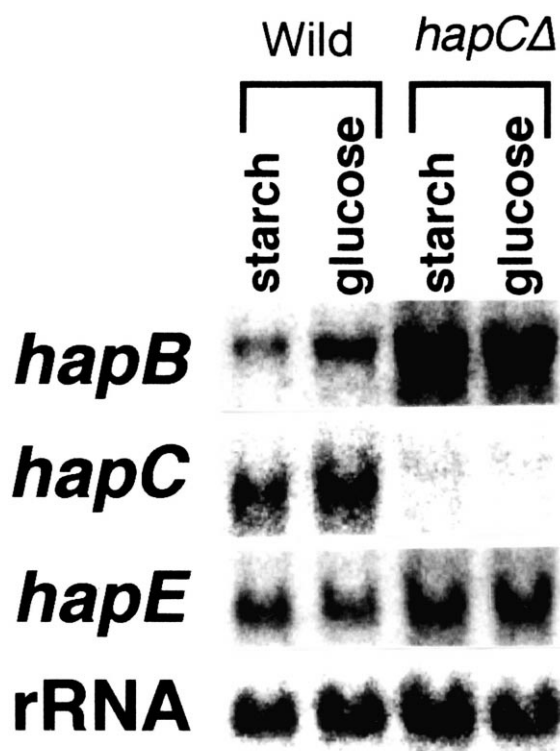


Fig. 4. Northern blot analysis of the Hap subunit genes. Total RNAs were prepared from the wild-type and $\Delta hapC$ strains grown in DP medium containing 2% starch or glucose at 37°C for 16 h. A 4-kb *Bam*HI fragment from pBShapB4.0, a 0.8-kb *Sal*I–*Ap*I fragment from pHapC-5, a 1.0-kb *Pst*I fragment from pHapE-9 and a 0.3-kb *Kpn*I–*Sac*I fragment from pBS-rRNA were used as probes. Although two transcripts of the *hapC* gene were described in the previous report [9], only one transcript was observed under the conditions used here.

tained two subunits cross-reactive with antisera to both the HapC and HapE subunits and antisera to both the HapC and HapB subunits, respectively. All these data described so far suggest two major possibilities: (1) expression of the HapE subunit might be under the control of the HapC subunit, and (2) the HapE subunit might be unstable in the absence of the HapC subunit, since the HapE subunit was detected along with the HapC subunit in the $\Delta hapB$ extract.

To examine the first possibility, the transcript level of each *hap* subunit gene was determined in both wild-type and the $\Delta hapC$ strains (Fig. 4). Although the *hapC* gene was not transcribed in the $\Delta hapC$ strain, the *hapE* gene was transcribed at the same or a somewhat higher level as that in the wild-type strain. Consistent with the previous results [4], the transcript level of each *hap* subunit gene remained the same regardless of the carbon source, starch or glucose. All these results clearly show that the HapC subunit does not modulate expression of the *hapE* gene. To directly confirm stabilization of HapE by HapC, we monitored degradation of the recombinant HapE in the absence ($\Delta hapC$ extract) and presence ($\Delta hapE$ extract) of HapC by Western blotting. No conclusive results were obtained in spite of several trials under various conditions (data not shown).

In higher eukaryotes, it is known that NF-YB (HapC homologue) and NF-YC (HapE homologue) tightly interact with each other through the histone-fold motifs within both subunits and form a heterodimer which is a prerequisite for

NF-YA (HapB homologue) binding [14–16]. Furthermore, introduction of the *A. oryzae hapC* gene into the *A. nidulans* $\Delta hapC$ strain restored CCAAT-binding activity [7]. Taken together, we favor the interpretation that the HapC subunit stabilized the HapE subunit by interaction through the histone-fold motif localized in each subunit, although other possibilities, such as HapC-dependent translation of *hapE* RNA, could not be completely excluded.

It is notable that the transcript level of the *hapB* gene in the $\Delta hapC$ strain was significantly higher than that in the wild-type. The increase in the transcript could be caused by de-repression of the *hapB* gene in the $\Delta hapC$ strain, since the *hapB* gene has been shown to be negatively regulated by the Hap complex [17].

All the results described so far suggest that the number of the HapC molecule could adjust that of the HapE molecule by forming stable heterodimers prior to assembly of the Hap complex. Although many CCAAT complexes have been characterized from various eukaryotes, little is known about stability of their subunits. The findings reported here suggest that similar quantity control mechanisms of the subunits may exist in other eukaryotes.

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