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AnCF, the CCAAT Binding Complex of *Aspergillus nidulans*, Is Essential for the Formation of a DNase I-Hypersensitive Site in the 5′ Region of the *amdS* Gene

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The CCAAT sequence in the *amdS* promoter of *Aspergillus nidulans* is recognized by AnCF, a complex consisting of the three evolutionary conserved subunits HapB, HapC, and HapE. In this study we have investigated the effect of AnCF on the chromatin structure of the *amdS* gene. The AnCF complex and the CCAAT sequence were found to be necessary for the formation of a nucleosome-free, DNase I-hypersensitive region in the 5′ region of the *amdS* gene. Deletion of the *hapE* gene results in loss of the DNase I-hypersensitive site, and the positioning of nucleosomes over the transcriptional start point is lost. Likewise, a point mutation in the CCAAT motif, as well as a 530-bp deletion which removes the CCAAT box, results in the loss of the DNase I-hypersensitive region. The DNase I-hypersensitive region and the nucleosome positioning can be restored by insertion of a 35-bp oligonucleotide carrying the CCAAT motif. A DNase I-hypersensitive region has been found in the CCAAT-containing *fmdS* gene and was also *hapE* dependent. These data indicate a critical role for the AnCF complex in establishing an open chromatin structure in *A. nidulans*.

Most of the genomic DNA in a eucaryotic cell is packed into nucleosomes representing a potentially repressive chromatin structure. In such a chromatin environment the regulatory regions of genes must be accessible to specific transcription factors and the components of the general transcriptional machinery. Such sites, often characterized by their increased sensitivity to nucleases, have been identified in many genes and appear to be generated by the displacement or disruption of nucleosomes within the promoter region (9, 34). DNase I-hypersensitive sites often coincide with the binding sites for transcription factors (1, 51). Hypersensitive sites can be generated by a variety of different mechanisms. In some cases replication is required in order to disrupt a nucleosome, e.g., to activate promoters silenced by their proximity to telomeric sequences (4). Replication-independent pathways have been described for a number of inducible promoters (59, 61). The precise positioning of nucleosomes, which leave some transcription factor binding sites exposed, could create a hypersensitive site (2, 17, 73). Increased DNase I susceptibility could also result from the binding of a transcription factor which locally distorts the DNA within or adjacent to this site (62).

Recently, a variety of complexes have been identified that assist transcription factors to reconfigure chromatin. An ATP-dependent multiprotein complex, the SWI-SNF complex, capable of altering the chromatin structure and facilitating binding of TFIIA-TBP and activators to nucleosome templates is required for activation of certain genes (10, 72). Another class of transcriptional coactivators are the histone acetyltransferases (HAT), whose enzymatic activity may contribute to chromatin disruption and transcription. For example, the yeast ADA complex contains GCN5, a subunit with intrinsic HAT activity, that is necessary for transcriptional activation, and this complex is known both to modify histones locally in the vicinity of the regulated promoter and to facilitate chromatin disruption (11, 69, 70). Additional coactivators in yeasts and higher eukaryotes, including TATA-binding protein (TBP)-associated factor TAFII 250, p300/CBP and P/CAF, have been identified to be HATs (50, 56, 77).

The sequence CCAAT is found in the 5′ region of approximately 30% of eukaryotic genes (46). In *Saccharomyces cerevisiae* a heteromeric complex of proteins encoded by *HAP2*, *HAP3*, *HAP4*, and *HAP5* genes binds to sequences containing a core CCAAT element upstream of genes involved in respiration (18, 24, 48). In vertebrates the NF-Y complex containing NF-YA, NF-YB, and NF-YC homologs of Hap2p, Hap3p, and Hap5p, respectively, has been found to bind to CCAAT containing sequences (39, 54). NF-YA and NF-YC contain a histone fold motif, a structural feature of histones suggesting that NF-Y might be involved in the organization of the chromatin structure (40).

The *amdS* gene of *Aspergillus nidulans* encodes an acetamidase required for the utilization of acetamide (28). The 5′ region contains a CCAAT sequence that is required for setting the basal level of expression (43). The AnCF complex, comprising the HapB, -C, and -E homologs of the Hap2p, Hap3p, and Hap5p subunits, respectively, has been shown to bind to the CCAAT sequence (57, 63, 67). Disruption of each of the *hapB*, *hapC*, and *hapE* genes has been shown to affect *amdS* expression to the same extent as mutations of the CCAAT sequence (57, 63). So far evidence for a *HAP4* homologue is lacking.

We have investigated the role of AnCF in influencing the chromatin structure and found that AnCF is necessary for the establishment of a nucleosome-free, DNase I-hypersensitive site in the 5′ region of the *amdS* gene. Deletion or point mutation of the CCAAT box greatly decreases gene expression and loss of DNase I hypersensitivity, as well as the loss of positioned nucleosomes upstream of the core promoter and over the transcriptional unit. Disruption of the *hapB*, *hapC*, and *hapE* genes also results in loss of the DNase I-hypersensitive site. In addition, CCAAT sequences in the 5′ region of another gene in *A. nidulans* have been found to result in AnCF-dependent DNase I hypersensitivity. We have also

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found that AnCF function is likely to be dependent on other promoter elements since insertion of a functional CCAAT-containing motif outside a promoter context did not result in DNase I hypersensitivity. Our data implicate AnCF as a critical determinant of DNase I-hypersensitive regions in *A. nidulans*, a function that may be central to its role as a transcription activator.

While this manuscript was in preparation, it has been shown that a CCAAT sequence bound by NF-Y is essential for the formation of a DNase I-hypersensitive site and defines the acetylation responsiveness of the *Xenopus laevis hsp70* promoter via interaction with p300/CBP in vivo (38). Further, the association of the acetyltransferase P/CAF with the mammalian CCAAT binding factor NF-Y in vitro has been reported (13, 31). Therefore, AnCF involvement in chromatin organization may occur via acetylation of components of the chromatin.

MATERIALS AND METHODS

Medium. The minimal medium used was that of Cove (12), with 10 mM ammonium tartrate as the sole nitrogen source.

Construction of strains containing *amdS* 5′ mutations. The construction of plasmids pLIT1, pLIT23, pLIT1011, and pLIT14 have been described (43). These plasmids were used to generate the strains MH 3408, MH5103, MH5095, and MH5788 by two-step gene replacement at the *amdS* locus (14). Point mutations of the CCAAT sequence of the *amdS* 5′ region were introduced by site-directed mutagenesis by the method of Kunkel (33) with an oligonucleotide of sequence 5′-TAGCTGGAGATCTGCTGGCT-3′. The resulting plasmid containing an *amdS-lacZ* fusion (pMH3352) was used to generate strain MH5733 by gene replacement at the *amdS* locus. The PCR method of Higuchi (26) was used to introduce mutations into the putative TATA box by using the oligonucleotide pair 5′-GGCATGAGAGCTCTGTAGGC-3′ and 5′-GCCTACAGAGCTCTCT CATGCC-3′. The resulting mutated fragment was cloned upstream of the *amdS-lacZ* fusion in pMH3779 containing a mutated *argB* gene, and strain MH8709 was made by insertion of a single copy of the plasmid at the *argB* locus by the method of Punt et al. (58). Strain MH8907 was generated by insertion of a single copy of plasmid pMH3776 containing an *amdS-lacZ* fusion and the mutated *argB* gene at the *argB* locus by the method of Punt et al. (58).

Chromatin analysis. Micrococcal nuclease and DNase I-based mapping of chromatin organisation was carried out as described by Gonzales and Scazzocchio (22). Strains grown for 16 h in 0.1% glucose plus 10 mM ammonium tartrate were transferred for 2 h either to repressing conditions (1% glucose) or derepressing conditions (glucose free). The mycelia were harvested by filtration through a nylon mesh, pressed dry with a paper towel, and frozen in liquid nitrogen. Then, 200-mg portions of mycelia were ground under liquid nitrogen and resuspended in 1 ml of nuclease digestion buffer (250 mM sucrose, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 15 mM Tris-Cl [pH 7.5]). Digestion mixtures containing 200 μl of crude nuclei were incubated with micrococcal nuclease (50 to 150 U/ml) or DNase I (5 to 15 U/ml) at 30°C for 5 min. The reaction was terminated with 1% sodium dodecyl sulfate–12.5 mM EDTA (final concentration). DNA was purified by two rounds of phenol-chloroform extraction and ethanol precipitation, and RNA was removed by treatment with 50 μg of RNase A at 37°C for 30 min.

Indirect-end-labeling analysis. Indirect end labeling was carried out as described by Wu (75). After secondary digestion with the appropriate restriction enzyme, the samples were electrophoresed in 1.7% agarose gels in 1× TAE, transferred onto Hybond N^+ nylon membrane (Amersham), and hybridized according to standard protocols. Labeling of specific probes was done by random oligonucleotide priming.

RESULTS

The promoter region of *amdS* is organized in strictly positioned nucleosomes and a DNase I-hypersensitive region. We investigated the chromatin organization of the *amdS* gene by using micrococcal nuclease (MNase) and DNase I. Strain MH1 (wild type) was grown in 0.1% glucose for 15 h, harvested, and transferred to either repressing conditions (1% glucose) or derepressing conditions (C-free) for 2 h. Nuclease-treated DNA was then analyzed by the indirect end-labeling technique. The DNase I experiments clearly showed a strong DNase I-hypersensitive site in the promoter region, while the transcriptional unit was resistant to DNase I treatment (Fig. 1A). Regions of increased DNase I sensitivity have been proposed to be free of nucleosomes or altered in DNA-histone interaction, leading to an open chromatin structure more accessible to transcription factors (9, 34). The hypersensitive site in the *amdS* promoter corresponded to positions −250 to −70 (±20 bp) relative to the translational start point and overlapped the binding sites for known transcription factors in the *amdS* promoter (29). This DNase I-hypersensitive site is interrupted by a region of lower sensitivity at approximately position −180, which coincides with the binding site for AnCF. The faint bands over the transcriptional unit indicate the presence of nucleosomes.

To address more directly whether the *amdS* promoter is free of positioned nucleosomes, we repeated the indirect end-labeling experiments with MNase. Under repressing conditions MNase generated a ladder of nucleosome-specific bands, exhibiting a repeat length of 170 bp (Fig. 1B) over the transcriptional unit (nucleosomes +1 to +4). However, the promoter region was cleaved in a pattern similar to that of naked control DNA, indicating the absence of nucleosomes. The size of the nucleosome-free region determined by use of MNase corresponded to the DNase I-hypersensitive site. Note that the sequence-specific bands in the chromatin samples were hypersensitive compared to the naked control DNA, suggesting that DNA in this region is more sensitive to MNase in a chromatin environment (Fig. 1B and Fig. 4). This result supported the DNase I experiments in indicating the absence of positioned nucleosomes over the *amdS* promoter. Another nucleosome is positioned upstream of this nuclease-hypersensitive site (nucleosome −1). After derepression, nucleosomal bands became more diffuse and additional, sequence-specific cutting sites appeared over the transcriptional unit in the chromatin samples, indicating that the strict positioning of the nucleosomes over the transcriptional unit was lost. Nucleosomes further upstream were unaffected by this rearrangement (data not shown).

These data showed that the promoter region of *amdS* is preset in a nucleosome-free region, whereas an array of nucleosomes is positioned over the coding region and upstream of the promoter. After relief of carbon catabolite repression the chromatin structure in the *amdS* gene undergoes rearrangement.

DNase I hypersensitivity over the promoter region is dependent on the CCAAT sequence and functional AnCF. The CCAAT site in the promoter region of the *amdS* gene is located 180 bp upstream of the translational startpoint and shows significant homology to the HAP2/3/4/5 consensus binding site (46). Gel mobility shift assays have shown that AnCF binds to this sequence in vitro and is required for setting the level of the *amdS* expression (43, 57, 63).

We examined the effect of mutations in the CCAAT sequence on the activity and chromatin architecture of the *amdS* promoter. Expression of the *amdS* promoter was assessed by using an *amdS-lacZ* fusion reporter replaced at the *amdS* locus (14, 33). A 530-bp deletion (−117 to −647 relative to the translational startpoint) in MH5103 resulted in greatly reduced expression of an *amdS-lacZ* reporter (Fig. 2). Insertion of a 35-bp sequence representing the region −185 to −151, including the *amdS* CCAAT sequence, in strain MH5095 restored expression (Fig. 2). We mutated this element by site-directed mutagenesis and subsequent gene replacement (32). In strain MH5733 the alteration of two bases of the CCAAT sequence resulted in a reduction in *amdS-lacZ* expression by an order of magnitude (Fig. 2). The level of expression was similar to the previously described affect of disruption of the *hapB*, *hapC*, and *hapE* genes (57, 63). These data confirm previous results

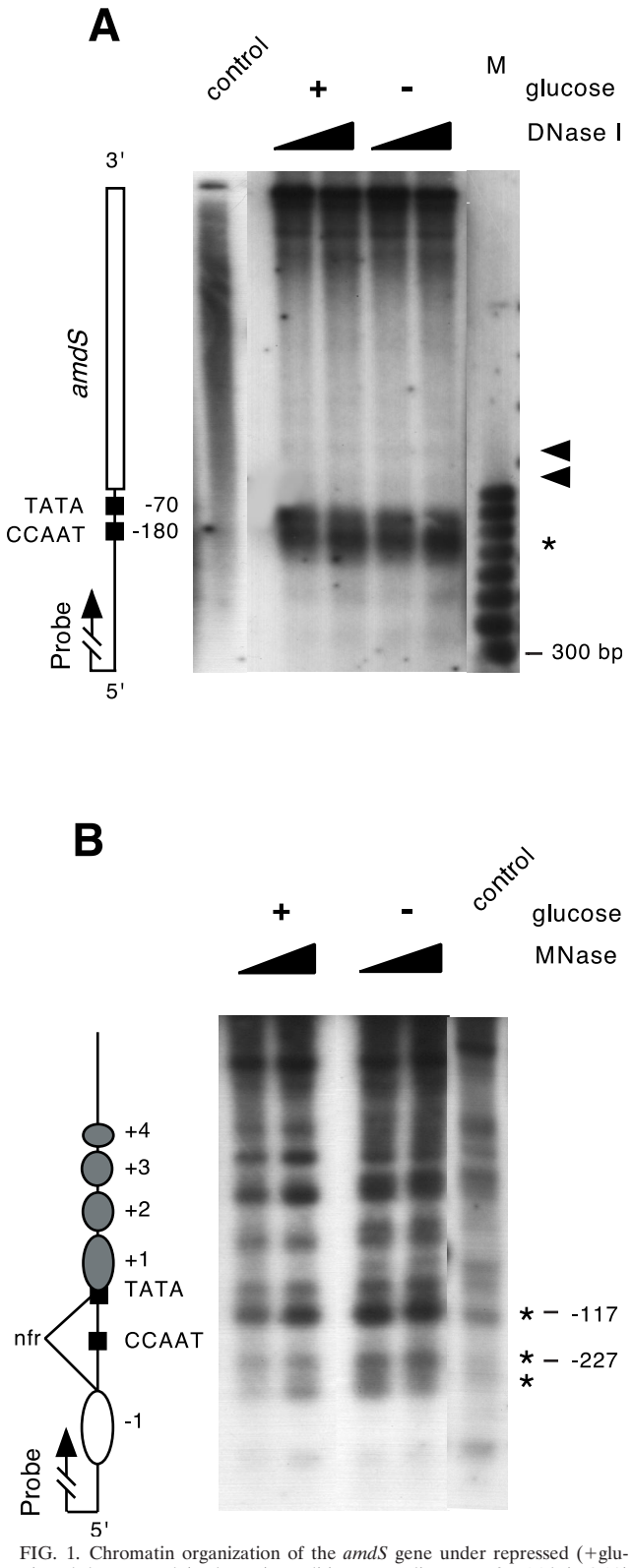


FIG. 1. Chromatin organization of the *amdS* gene under repressed (+glucose) and derepressed (−glucose) conditions. Mycelia grown for 16 h in 0.1% glucose plus 10 mM ammonium tartrate were transferred for 2 h either to repressing conditions (1% glucose) or derepressing conditions (carbon free). (A) Crude nuclei were treated for 5 min with 5 and 15 U of DNase I per ml at 30°C. DNA was digested with *Spe*I and subjected to indirect end labeling with a *Spe*I (−1008)/*Xba*I (−650) fragment of the *amdS* promoter region as a probe. The control is naked genomic DNA treated with DNase I and processed similarly to chromatin samples. The arrowheads indicate internucleosomal cutting of DNase I. Lane M contains a 100-bp ladder (Bio-Rad). The vertical map at the left indicates the relative positions of TATA and CCAAT sequences and the *amdS* coding region. (B) Crude nuclei were treated for 5 min with 50 and 150 U of MNase per ml. DNA was digested with *Sac*I and subjected to indirect end labeling with a *Sac*I (−751)/*Sac*II (−227) fragment of the *amdS* promoter region as a probe. The control is naked genomic DNA treated with MNase and is processed similarly to chromatin samples. Asterisks indicate nuclease-hypersensitive sites. The positions of nucleosomes are pictured at the right as ellipses and are numbered divergently from the nucleosome-free region (nfr). The closed ellipses denote nucleosomes remodeled upon derepression. The vertical map at the left indicates the relative positions of TATA and CCAAT sequences and the *amdS* coding region. Numbers on the right refer to the position of restriction sites in the *amdS* promoter that were determined by double digests of genomic DNA with *Sac*I and *Sma*I (−117) and *Sac* and *Sac*II (−227).

(43) showing that the CCAAT motif is required for high levels of *amdS* expression.

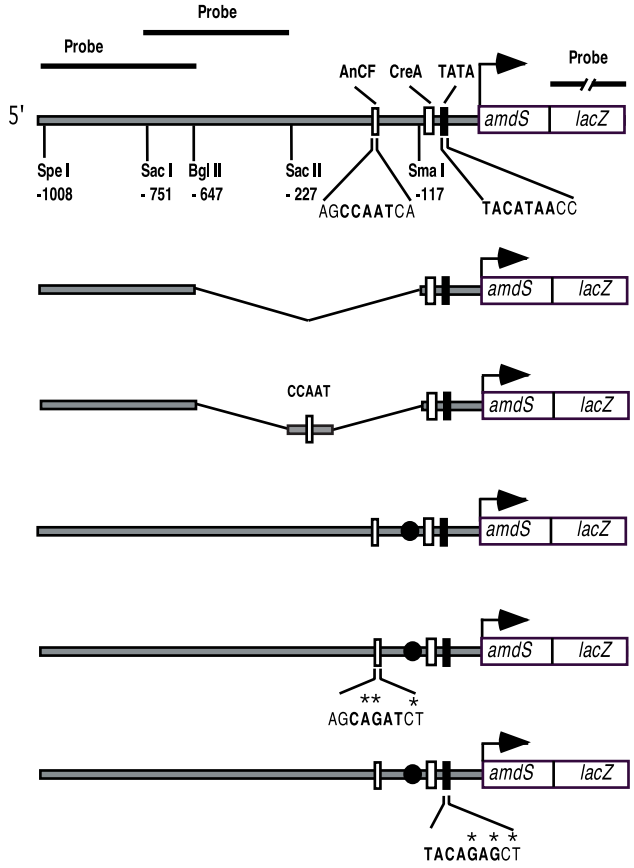
We examined the effect of mutations in the CCAAT motif on the chromatin architecture of the *amdS* promoter. Point mutations in the CCAAT sequence completely eliminated the DNase I hypersensitivity in the promoter region (Fig. 3A), indicating a dramatic change in the chromatin structure.

To determine whether the observed effect results from the binding of the AnCF complex, we analyzed the chromatin structure of *amdS* in Δ*hapB*, Δ*hapC*, and Δ*hapE* deletion strains where AnCF is not functional (57, 63). Strains MH9207 (Δ*hapB*), MH8194 (Δ*hapC*), and MH9206 (Δ*hapE*) showed no DNase I hypersensitivity in the *amdS* promoter (Fig. 3B). These results revealed a crucial role for AnCF in the formation of a defined chromatin structure in the *amdS* promoter.

To test whether the positioning of the nucleosomes is affected by these mutations, strain MH5733 and MH9206 were analyzed by MNase treatment under repressing conditions (Fig. 4). The nucleosomal organization in the MH5733 strain carrying the mutated CCAAT sequence was changed compared with the wild-type situation. The strict positioning of the nucleosomes over the transcriptional unit was lost. The appearance of sequence-specific cutting sites in addition to weaker nucleosomal bands indicated a changed organization of the nucleosomes. The same result was obtained with the *hapE* deletion strain (MH9206). We conclude that AnCF not only is responsible for the formation of a DNase I-hypersensitive site in the *amdS* 5′ region but also influences the positioning of the adjacent nucleosomes.

To determine whether the CCAAT sequence alone is sufficient to generate DNase I hypersensitivity in the *amdS* promoter, we carried out DNase I analysis with strains MH5103 and MH5095 (Fig. 3C). The DNase I-hypersensitive site was absent in strain MH5103 which contains the deletion from −117 to −650 but was restored in strain MH5095 in which the deleted region is replaced by a 35-bp oligonucleotide containing the *amdS* CCAAT sequence. However, the architecture of this DNase I-hypersensitive site was clearly different from that in the wild type. Whereas the wild-type promoter showed a clear double band (Fig. 1A), the DNase I-sensitive site in MH5095 was a more diffuse region lacking the intervening protected area.

In addition to the DNase I-hypersensitive site recreated after insertion of the oligonucleotide containing the CCAAT sequence, two positioned nucleosomes were observed downstream of the DNase I-sensitive site (indicated by the arrows in Fig. 3C). This finding strongly supports the idea that the CCAAT-mediated formation of the DNase I-sensitive site directly or indirectly determines the position of the adjacent nucleosomes, possibly by defining the position of the first nucleosome. Thus, AnCF plays a crucial role in the organization



Strain	β-Galactosidase Activity	
	1 % glucose	0.1 % glucose
MH3408	1.8 (0.7)	31.9 (5.8)
MH5103	0.2 (0.0)	2..8 (0.7)
MH5095	0.6 (0.1)	31.5 (5.2)
MH5788	0.5 (0.1)	16.4 (2.0)
MH5733	0.2 (0.0)	0.9 (0.2)
MH8709	0.3 (0.1)	3.5 (0.9)

FIG. 2. Effect of promoter mutation on *amdS-lacZ* expression. The arrow indicates the translational startpoint. The relevant restriction sites and *cis*-acting sites are shown. The sequences for the CCAAT and TATA box are given in detail. Mutated nucleotides within these sequences are indicated by asterisks. β-Galactosidase activity is shown with standard errors in brackets. Repressed conditions were growth for 16 h in minimal medium with 1% glucose plus 10 mM ammonium tartrate; derepressed conditions were growth for 19 h in minimal medium with 0.1% glucose plus 10 mM ammonium tartrate. The *amdS-lacZ* construct in strain MH8709 is integrated into the *argB* locus. A corresponding control *amdS-lacZ* reporter strain (MH8907) showed comparable values to MH5788. The closed circle indicates a 10-bp insertion (CGGGATCCCG [43]). Probes used in indirect-end-labeling experiments are shown.

of the regulatory chromatin structure of the *amdS* gene. The experiments with strains MH5103 and MH5095 also showed that the regulatory elements for AreA mediated nitrogen derepression, FacB mediated acetate induction, and AmdR mediated omega amino acid induction, which are deleted in these strains, were not necessary for the formation of the DNase I-hypersensitive site.

We further tested the ability of the CCAAT sequence to generate a DNase I-hypersensitive site outside a promoter. For this purpose the 35-bp oligonucleotide containing the *amdS* CCAAT sequence was inserted 788 bp 3′ to the stop codon of the *argB* gene, and this construct was transformed into an *argB* mutant strain. DNase I experiments were carried out on five independent *argB*$^+$ transformants, but none showed a nuclease-sensitive site in the 3′ region of the *argB* gene (data not shown). Therefore, additional elements may be necessary for the CCAAT-mediated formation of the DNase I-sensitive site, and these elements must be present in the truncated *amdS* promoter of MH5095.

Presetting the chromatin structure of the *amdS* promoter is not dependent on the TATA box or a CreA binding site. The truncated *amdS* promoter in MH5095 still carries a potential TATA box. Mutation of this sequence in MH8709 resulted in greatly reduced *amdS-lacZ* expression compared to the control strain MH5788 (Fig. 2), indicating a functional role for this sequence. As the yeast homologue of AnCF, the HAP2/3/4/5 complex interacts with the ADA2/ADA3/GCN5 complex, and ADA2 is associated with TBP, we supposed that TBP could be the component acting together with AnCF (5, 20, 47, 64). However, the DNase I-hypersensitive site was still present in the mutated promoter (Fig. 3D).

Two sequences present between positions −90 and −107 have been found to be binding sites for CreA, a C2H2 finger protein responsible for carbon catabolite repression, as well as for binding by the AmdA and AmdX C2H2 finger activating proteins (3, 41, 53). Deletion of these sequences (in plasmid p23 [41]), followed by gene replacement at the *amdS* locus, resulted in strain MH6900. This deletion was not found to affect the formation of a DNase I-sensitive site (Fig. 3E).

The promoter regions of *fmdS* are organized in DNase I-sensitive sites which are dependent on AnCF. We investigated whether other genes containing CCAAT sequences showed a similar chromatin organization in their promoter regions. The promoter region of *fmdS*, a gene encoding a formamidase,

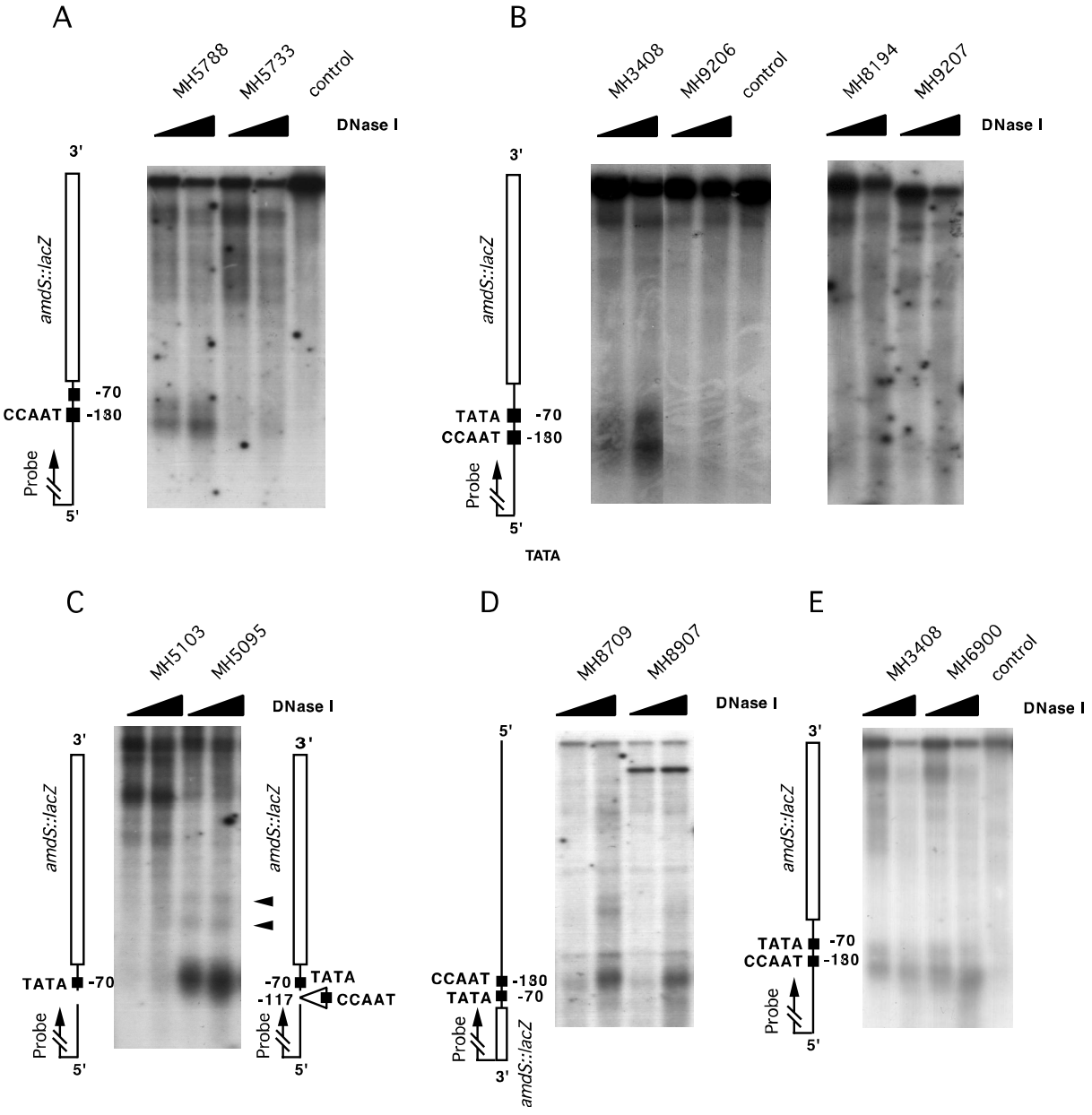


FIG. 3. Effect of mutations on the DNase I sensitivity of the *amdS* promoter. Mycelia were grown for 16 h on 1% glucose plus 10 mM ammonium tartrate. DNA was digested with *Sac*I, and a *Sac*I (−751)/*Sac*II (−227) fragment of the *amdS* promoter region was used as a probe except as noted otherwise. (A) Strains MH5788 and MH5733 carry a 10-bp oligonucleotide containing a *Bam*HI site inserted into the *Sma*I (−117) site in the *amdS* promoter. In strain MH5733 the CCAAT motif is mutated to CAGAT (see Fig. 2). (B) Effect of the *hapBCE* gene deletions on the DNase I sensitivity of the *amdS* promoter. In MH9207 *hapB* is deleted, in MH8194 *hapC* is deleted, and in MH9206 *hapE* is deleted. MH3408 is wild type at all *hap* loci. All strains carry an *amdS*::*lacZ* fusion. (C) MH5103 carries a 530-bp *Bgl*II (−647)/*Sma*I (−117) promoter deletion which removes most of the regulatory sites including the CCAAT sequence. In strain MH5095 a 35-bp oligonucleotide carrying the CCAAT motif is inserted into the deletion site (see Fig. 2). DNA was digested with *Spe*I, and a *Spe*I (−1008)/*Xba*I (−650) fragment of the *amdS* promoter region was used as a probe. The arrowheads indicate internucleosomal cutting of DNase I. (D) In MH8709 the TATA box is mutated as indicated in Fig. 2. Both strains carry an *amdS*::*lacZ* fusion gene integrated into the *argB* locus. DNA was digested with *Cla*I and a *Hin*dIII (+113)/*Cla*I (+907) fragment of the *lacZ* coding region was used as a probe. (E) Strain MH6900 carries a 29-bp deletion which removes the two CreA-binding sites.

contains a CCAAT sequence (position −108) which exhibits significant homology to the CCAAT element consensus sequence (46) and binds AnCF (19). We investigated the promoter regions of this gene by using DNase I and indirect end labeling. A DNase I-sensitive site was found which coincides with the CCAAT motif (Fig. 5). The *hapE* deletion present in strain MH 9206 abolished the formation of the DNase I-hypersensitive site in the promoter region of *fmdS*. These data indicate that the mechanism by which AnCF presets the chromatin structure in a promoter region may be the same in the two genes investigated in this work.

DISCUSSION

In the present study we found that the formation of a DNase I-hypersensitive site in the *amdS* promoter region is strictly dependent on the presence of a functional CCAAT box and the AnCF complex. Further, the positioning of the adjacent

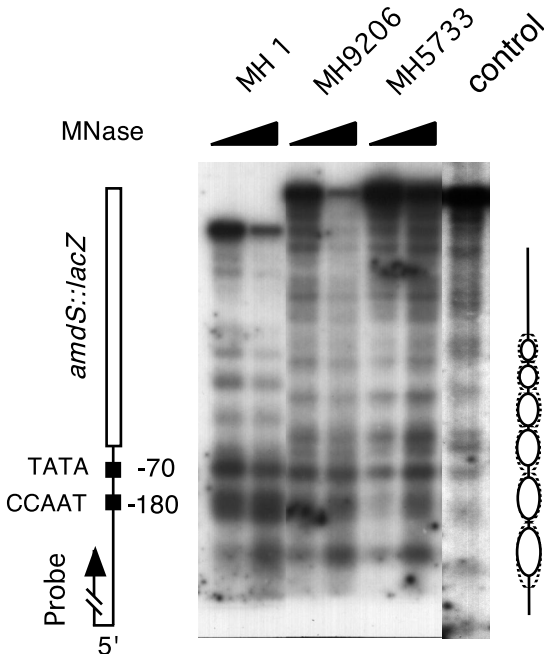


FIG. 4. Chromatin organization of the *amdS*::*lacZ* fusion gene in a CCAAT and *hapE* mutant strain. MH1 corresponds to the wild-type strain. In strain MH9206, *hapE* is deleted. In strain MH5733, the CCAAT motif is mutated to CAGAT (Fig. 2). Mycelia were grown for 16 h on 1% glucose plus 10 mM ammonium tartrate. Chromatin analysis was performed as indicated in the legend of Fig. 1B. Nucleosomes are pictured at the right as ellipses. The dashed ellipses indicate a less strict positioning of nucleosomes. The vertical map at the left indicates the relative positions of TATA box, CCAAT sequence, and *amdS*::*lacZ* coding region.

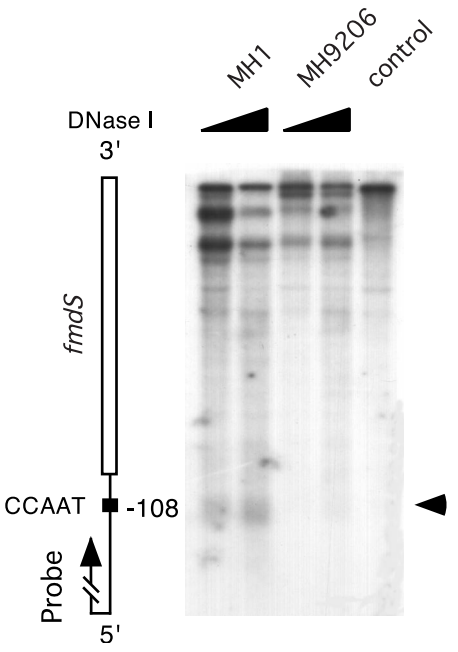


FIG. 5. DNase I analysis of the *fmdS* gene in a wild-type strain (MH1) and a *hapE* deletion strain (MH9602). Mycelia were grown for 16 h on 1% glucose plus 10 mM ammonium tartrate. Chromatin analysis was performed as indicated in the legend to Fig. 1A except that DNA was digested with *Xho*I, and a *Xho*I (−599)/*Pst*I (−90) fragment of the *fmdS* promoter region was used as probe. The vertical map on the left indicates the relative position of the CCAAT sequence in the *fmdS* promoter. The arrow indicates the DNase I-hypersensitive site.

nucleosomes is dependent on the presence of the DNase I-hypersensitive site. Our results indicate that AnCF is crucial for presetting the *amdS* promoter in an open chromatin structure.

The *amdS* locus is organized in a well-defined chromatin structure in the repressed state, with an array of positioned nucleosomes over parts of the promoter and the coding region. In both the repressed and derepressed states a constitutive DNase I-hypersensitive site exists from positions −250 to −70 relative to the translational startpoint. Adjacent to the DNase I-hypersensitive site an ordered nucleosome array covering the coding region and sequences upstream of the promoter exists under repressing conditions. Upon derepression, the discrete MNase bands become diffuse, indicating that the exact positioning of the nucleosomes is lost and that they can slide along the DNA, a change also observed in other genes and characteristic of the active state (15, 36, 68, 76).

The constitutive DNase I-hypersensitive site observed in the promoter region covers all previously identified *cis*-acting sequences (for a review, see reference 29), suggesting that the transcription factors required for regulation of the *amdS* gene may have permanent access to their binding sites. This stretch of DNA (ca. 180 bp) is nucleosome-free under the growth conditions tested. Thus, the transcription factors acting at the *amdS* promoter may be able to bind to their sites without first disrupting nucleosomes. This differs from the remodelling of the *PHO5* promoter by Pho4p, in which transcription factor binding and nucleosome disruption seem to be linked (2, 66).

The DNase I-hypersensitive site is interrupted by a region of decreased nuclease sensitivity, a structural feature observed in DNase I-sensitive regions of several other promoters (6, 23). This protection may be caused by the binding of protein(s) to the hypersensitive region that protects DNA from DNase I. This idea is supported by the fact that some MNase cleavage sites within the promoter show hypersensitivity, indicating a structural change causing increased accessibility of the DNA in the chromatin samples compared with naked DNA. It is known that bending of DNA by DNA binding proteins can cause an increased accessibility to nucleases (25), and such a bending of DNA has been shown for the CCAAT binding complex PENR1 (which is probably identical to AnCF) (44) and NF-Y (60). Alternatively, the protection in the DNase I-hypersensitive region may reflect a conformational change of the DNA structure that prevents DNase I from cutting in both strands.

The TATA box of *amdS* is located at the border of the first downstream nucleosome (nucleosome +1) and the DNase I-hypersensitive site. A similar situation is found in the yeast *HSP82* and the *Drosphila hsp26* genes (23, 42). This is in contrast to a number of other genes where the incorporation of the TATA box into a nucleosome severely inhibits the binding of TBP (21, 30) and greatly reduces transcription initiation in vitro (32, 35, 74) and in vivo (37, 65).

Deletion or mutation of the CCAAT motif, the DNA-binding motif for AnCF in the *amdS* promoter, or deletion of the *hapB*, *hapC*, and *hapE* genes, which results in a nonfunctional AnCF complex, reduces *amdS* expression by an order of magnitude (Fig. 2) (43, 57, 63). Moreover, such mutations result in a distinct rearrangement of the chromatin structure of *amdS*. The DNase I-hypersensitive site in the promoter region and the positioning of the nucleosomes is lost. Importantly, this effect on chromatin structure is not a consequence of transcriptional inactivation, since point mutations in the TATA box which greatly reduce *amdS* expression (Fig. 2) retain the DNase I-hypersensitive site in the promoter region (Fig. 3D). The importance of the CCAAT box is further demonstrated by

the restoration of both the DNase I sensitivity and *amdS* expression when a CCAAT containing sequence is inserted into a truncated *amdS* promoter (Fig. 3C and Fig. 2).

However, the CCAAT sequence itself is not sufficient to initiate an open chromatin structure since the CCAAT-containing oligonucleotide cloned 3′ to the *argB* gene failed to create such a nuclease-sensitive site. Thus, the remaining part of the promoter must carry an additional feature necessary to generate the DNase I-hypersensitive site which acts in concert with AnCF. Two known *cis*-acting sites in the truncated promoter are the binding site for the CreA carbon catabolite repressor with homology to Mig1p (16) and the TATA box. The lack of effect of the TATA box mutation on DNase I sensitivity (Fig. 3D) is consistent with data from the yeast *HSP82* gene, where it has been shown that TBP has no influence on the organization of the DNase I-hypersensitive region (23), although in vitro data suggest that TBP may prevent the assembly of nucleosomes in a core promoter region (7, 49, 74). A strain carrying a deletion of the CreA-binding sites showed a chromatin structure corresponding to the carbon-derepressed phenotype (data not shown) but was unaffected in DNase I hypersensitivity (Fig. 3E).

Of particular interest is the fact that the insertion of the CCAAT sequence into a truncated *amdS* promoter not only restores the DNase I-hypersensitive site but also reassembles the downstream region into positioned nucleosomes (Fig. 3C). Together with the observation that the strict positioning of the nucleosomes over the coding region is lost in the CCAAT sequence mutant, this suggests that the AnCF-mediated assembly of the DNase I-hypersensitive site directly or indirectly determines the position of the adjacent nucleosomes, possibly by defining the position of the first nucleosome. Widlund et al. (71) found that particular sequences, e.g., A runs, extended repeats of CA, or tetramers of TATAA, are responsible for the positioning of nucleosomes. However, the *amdS* promoter does not contain such strong nucleosome-positioning motifs, indicating that the underlying DNA sequence is not involved in the positioning of nucleosomes. Mutation of the major HSF binding site in the yeast *HSP82* promoter leads to the displacement of the DNase I-hypersensitive site by two strictly positioned nucleosomes (23). In contrast, a loss of nucleosomal positioning results from mutation of the *amdS* CCAAT or from inactivation of AnCF.

The *amdS* gene is subjected to multiple regulatory controls (29). Mutations affecting the CCAAT sequence do not eliminate responses to *amdA*-, *areA*-, *facB*-, or *creA*-mediated regulatory circuits, although overall levels of expression are greatly reduced (27, 63). Deletion of the *areA* gene, a major determinant of *amdS* expression under carbon sufficient but nitrogen limiting conditions, does not affect the DNase I-hypersensitive site (55). This indicates that the products of these genes can bind in a nucleosomal environment. A similar effect is seen in the *niiA-niaD* promoter, where *areA*-dependent chromatin remodelling still occurs in a mutant where only AreA binding sites outside the nucleosome-free region are intact (45). However, it has been shown that regulation by AmdR which binds to a sequence partially overlapping the CCAAT sequence is abolished in mutants lacking AnCF. For this transcription factor, therefore, it is probable that the AnCF-mediated chromatin structure is necessary for binding to DNA (63).

An AnCF-dependent DNase I-hypersensitive site corresponding to a CCAAT sequence has been found in the promoter region of the *fmdS* gene (Fig. 5). This indicates that the mechanism by which AnCF is acting at the *amdS* promoter could also apply to other promoters in *A. nidulans*.

Our results are consistent with the finding that Y boxes, bound by the AnCF homologue NF-Y, serve a similar role in the *X. laevis hsp70* promoter (34, 38). The biochemical mechanism which finally prevents a tight DNA-histone interaction in a DNase I-hypersensitive site remains unclear. The NF-Y subunits B and C (corresponding to HapB and HapC in AnCF) carry a histone fold motif, showing similarity to histone H2B and H2A (40). It has been shown that NF-Y can bind to a Y box even in the presence of reconstituted nucleosomes (52). The action of acetyltransferases may play a role in the local disruption of nucleosomes since an association of GATA-1 and NF-Y with acetyltransferases p300/CBP has been shown (8, 38). However, Trichostatin A, an inhibitor of deacetylases which clearly activates the p300-triggered transcription of the *X. laevis hsp70* gene, has no influence on the formation of a DNase I-sensitive site in this promoter (38).

Our data support the idea that CCAAT sequences could play a conserved role in the generation of an open chromatin structure necessary for full transcriptional activation in eukaryotic promoters.

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