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The GATA factor AreA is essential for chromatin remodelling in a eukaryotic bidirectional promoter

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The linked *niiA* and *niaD* genes of *Aspergillus nidulans* are transcribed divergently. The expression of these genes is subject to a dual control system. They are induced by nitrate and repressed by ammonium. AreA mediates derepression in the absence of ammonium and NirA supposedly mediates nitrate induction. Out of 10 GATA sites, a central cluster (sites 5–8) is responsible for ~80% of the transcriptional activity of the promoter on both genes. We show occupancy *in vivo* of site 5 by the AreA protein, even under conditions of repression. Sites 5–8 are situated in a pre-set nucleosome-free region. Under conditions of expression, a drastic nucleosomal rearrangement takes place and the positioning of at least five nucleosomes flanking the central region is lost. Remodelling is strictly dependent on the presence of an active *areA* gene product, and independent from the NirA-specific and essential transcription factor. Thus, nucleosome remodelling is independent from the transcriptional activation of the *niiA–niaD* promoter. The results presented cast doubts on the role of NirA as the unique transducer of the nitrate induction signal. We demonstrate, for the first time *in vivo*, that a GATA factor is involved directly in chromatin remodelling.
Keywords: ascomycetes/*Aspergillus nidulans*/chromatin/GATA factors/nitrate assimilation

Introduction

Chromatin remodelling has for a long time been associated with activation of eukaryotic gene expression. The 'repressed state' of chromatin is generally equated with strict nucleosome positioning. The onset of transcription is accompanied by the loosening of nucleosome positioning. A vexing problem is whether chromatin rearrangement is a pre-requisite for eukaryotic gene transcription or whether the rearrangement is a somewhat trivial consequence of transcription itself (Felsenfeld, 1992; Wolffe, 1994; Steger and Workman, 1996; Gaudreau *et al.*, 1997; Stafford and Morse, 1997). The association of transcription and chromatin rearrangement has been demonstrated many times. In very few instances, however, it has been shown rigorously that the rearrangement is not a result of transcriptional activation (Hirschhorn *et al.*, 1992; Axelrod *et al.*, 1993; Verdin *et al.*, 1993; Verdone *et al.*, 1997; Svaren and Hörz, 1997). The method of choice to distinguish these possibilities has been to prevent transcription by, for example, deleting essential promoter elements, and then to show that the induction of chromatin remodelling is unimpaired (Hirschhorn *et al.*, 1992; Axelrod *et al.*, 1993; Fascher *et al.*, 1993). In particular, it has been shown that the Pho4p, Adr1p and Gal4p factors of *Saccharomyces cerevisiae* are able to modify chromatin structure even in the absence of transcription (Axelrod *et al.*, 1993; Svaren and Hörz, 1997; Verdone *et al.*, 1997). For the 'pre-set' promoters of higher eukaryotes, it is clear that a nucleosome-free region is essential for transcriptional activation to occur (Wallrath *et al.*, 1994; Lu *et al.*, 1995; O'Brien *et al.*, 1995; Shopland *et al.*, 1995).

GATA factors are a class of eukaryotic transcriptional activators or repressors which are found in plants, fungi and metazoans. They are characterized by a highly conserved DNA-binding motif comprising a Cys(4) zinc finger followed by a basic domain (Omichinski *et al.*, 1993). In metazoans, they are involved in differentiation of a number of cell lines ranging from the erythroid line of vertebrates to the endoderm line of *Caenorhabditis elegans* (Pevny *et al.*, 1991; Tsai *et al.*, 1994; Pandolfi *et al.*, 1995; Zhu *et al.*, 1997). In fungi, well studied GATA factors are involved in processes as diverse as regulation of nitrogen metabolism (Fu and Marzluf, 1990a; Kudla *et al.*, 1990), repression of siderophore biosynthesis (An *et al.*, 1997) and transcriptional activation of light-induced processes (Ballario *et al.*, 1996; Linden and Macino, 1997).

In higher eukaryotes, it has been shown that GATA-1-binding motifs, together with binding sites for other erythroid-specific factors, are required for the formation of DNase I-hypersensitive sites in the β-globin locus control region (LCR) (Barton *et al.*, 1993; Stamatoyannopoulos *et al.*, 1995; Boyes and Felsenfeld, 1996).

The best-studied fungal GATA factors are the AreA protein of *Aspergillus nidulans* and, to a lesser extent, its homologue NIT2 in *Neurospora crassa*. These proteins are necessary for the transcription of almost every gene involved in the utilization of nitrogen sources (Arst and Cove, 1973). Ammonium and glutamine repress the expression of all these genes. Thorough physiological, molecular and structural work on AreA has been carried out (Platt *et al.*, 1996; Ravagnani *et al.*, 1997; Starich *et al.*, 1998a,b). The mechanism by which ammonia and

glutamine inhibit the activation of gene expression by AreA and NIT2 has also been elucidated. In brief, in the presence of free glutamine in the cell, a second protein, NMR, would form a complex with AreA, thus preventing its binding to DNA (Xiao *et al.*, 1995; Platt *et al.*, 1996; Andrianopoulos *et al.*, 1998).

There are a multiplicity of GATA factors in a given cell, involved in different metabolic or developmental pathways. These factors, at least qualitatively, recognize identical DNA sequences. It remains an open question how the specificity of co-existing GATA factors is determined. One possibility (which is not exclusive of others) is that specificity is achieved through interactions with other specific factors. This may well be the case in the β-globin promoter (Merika and Orkin, 1995). AreA, NIT2 and presumably their homologues described in other fungi (Marzluf, 1997) operate in almost every case by coupling to pathway-specific transcription factors of the zinc binuclear cluster class. Substantial evidence for direct or indirect interactions with AreA exists for NirA (Rand and Arst, 1978; Tollervey and Arst, 1981). NirA is the specific activator of transcription of the genes coding for the enzymes and the permease of the nitrate assimilation pathway. This protein binds as a dimer to a CTCCGHGG asymmetric consensus sequence (Fu *et al.*, 1995; Punt *et al.*, 1995; Strauss *et al.*, 1998). Significant expression of the genes coding for nitrate and nitrite reductase is only achieved by inducing with nitrate in the absence of repressing nitrogen sources (ammonium or glutamine). This is a general feature of nitrate utilization in fungi (Crawford and Arst, 1993; Marzluf, 1997). Both NirA and AreA (in *A.nidulans*) or NIT4 and NIT2 (in *N.crassa*) are necessary for induced, derepressed expression to occur. It has been accepted as obvious that AreA (and NIT2) mediates nitrogen derepression, and NirA (and NIT4) mediates specific induction by nitrate. In *N.crassa*, where the genes coding for nitrate and nitrite reductase are not linked, preliminary results suggest that the presence of nuclease-hypersensitive sites in the promoter of the gene coding for nitrate reductase is dependent on the activity of both NIT4 and NIT2 (Brito *et al.*, 1993).

The genes coding for nitrate and nitrite reductase of *A.nidulans* (*niaD* and *niiA*) are transcribed divergently. Their transcription is regulated by elements included in a 1267 bp intergenic region. Four NirA-binding sites and 10 AreA-binding sites are included in this region (Fu and Marzluf, 1990b; Punt *et al.*, 1995). A schematic representation of this region is given in Figure 1. It has been shown previously that NirA sites 2 and 3 act bidirectionally on both *niiA* and *niaD* transcription, that site 1 is only involved in the expression of *niiA* and site 4 contributes mainly to the expression of *niaD* (Punt *et al.*, 1995). Preliminary evidence indicated that the AreA (HGATAR) sites located centrally, in the proximity of NirA site 2, were sufficient to ensure AreA-mediated stimulation of transcription (Punt *et al.*, 1995).

Here we establish the role of the four centrally located GATA sites (5, 6, 7 and 8) and we show that they are in a nucleosome-free region flanked by six positioned nucleosomes. We further show that a drastic chromatin rearrangement occurs under conditions of expression of the gene cluster, that this rearrangement is strictly dependent on a functional AreA protein and virtually independent of NirA, and that it occurs equally in the absence of transcription.

Results

Expression of the niiA and niaD genes

The expression of the *niaD* and *niiA* genes depends on two signals and these are operationally independent. These signals are induction by nitrate and repression by ammonium. Expression of both genes is obtained in the absence of ammonium and in the presence of nitrate. Thus four different conditions can be defined. (i) Non-induced–derepressed: these correspond to transfer of pre-cultured mycelia (see Materials and methods) to a neutral nitrogen source, urea; neither inducing nor repressing metabolites are present. (ii) Induced–derepressed: these are the conditions of significant expression; the mycelia are transferred to medium containing nitrate. (iii) Induced–repressed: the mycelia are transferred to medium containing both nitrate and ammonium. (iv) Non-induced–repressed: the mycelia are transferred to medium containing ammonium. Figure 2 shows the levels of expression of the *niiA* and *niaD* genes under the four different conditions described above. Within the limits of sensitivity of a Northern blot, expression of both genes occurs only in conditions of simultaneous induction and derepression. This figure also shows that transcription of both genes is absolutely dependent on the NirA and AreA proteins.

Physiological role of the central cluster of AreA (GATA)-binding sites

The *niiA–niaD* intergenic region contains 10 GATA sites which were revealed by gel shift and DNase I protection experiments carried out with a β-galactosidase fusion protein carrying the NIT2 DNA-binding domain (Fu and Marzluf, 1990b). NIT2 differs from the AreA-binding domain by only one conservative substitution (Fu and Marzluf, 1990a). We have confirmed these results by carrying out gel shift experiments with both a glutathione *S*-transferase (GST)–AreA(663–809) fusion protein carrying the DNA-binding domain and the untagged AreA(663–809) peptide. The four central binding sites (sites 5, 6, 7 and 8, see below) were confirmed by DNase I footprinting (results not shown).

Preliminary results of Punt *et al.* (1995) suggested that the central cluster of GATA sites (5, 6, 7 and 8) neighbouring the crucial bidirectionally acting NirA-binding site 2 are important in setting the derepressed transcription rate of both *niiA* and *niaD*. We have investigated the role of the four central GATA sites by fusing the *niiA–niaD* intergenic region to two reporter genes transcribed in opposite directions, as described previously. This is shown schematically in Figure 1. All constructions were inserted in one copy in exactly the same place in the genome (Punt *et al.*, 1995). We have mutagenized sites 5 and 6 individually, and all four sites in a number of combinations, including simultaneous mutation of all four central sites. All mutations consisted of single base G→T replacements of the first guanine in the HGATAR consensus-binding site. The results are shown in Table I. It is shown that the site affording the most important contribution to AreA-mediated activation of the bidirectional promoter is site 5. The elimination of all four central sites lowers

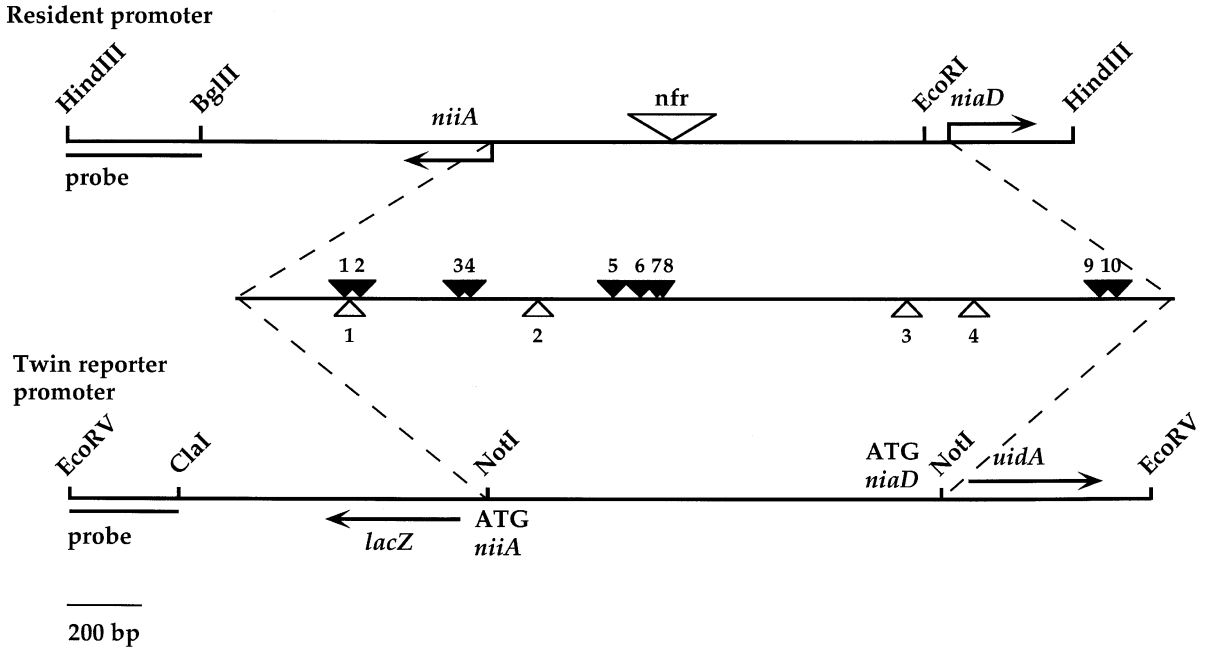

Fig. 1. Schematic structure of the *niiA–niaD* bidirectional promoter. On the top line, the intergenic region between the *niiA* and *niaD* gene is represented. The wide triangle above this line indicates the DNase I- and MNase-sensitive region present in this promoter (nfr, nucleosome-free region). The bottom line represents a scheme of the construction where the 1267 bp region between the ATGs of *niiA* and *niaD* is inserted between the two reporter genes *lacZ* and *uidA*. The middle line shows the position of NirA- (open triangles, under the line) and AreA (black triangles, above the line)-binding sites. Numbering of the sites is as in Punt *et al.* (1995), which also includes the complete sequence of the *niiA–niaD* intergenic region. The *Hin*dIII–*Bgl*II and *Eco*RV–*Cla*I probes shown are those used to reveal, by the indirect end-labelling technique (see Materials and methods), the nucleosome structure of the resident promoter (Figures 4–8) and of the transgene promoter, respectively (Figure 9). The complete sequence of the *Hin*dIII fragment shown is reported by Johnstone *et al.* (1990).

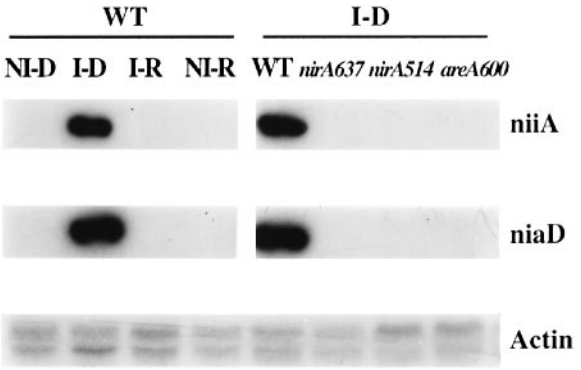

Fig. 2. *niaD* and *niiA* mRNA steady-state levels of wild-type and mutant strains. The left hand panel show the steady-state levels of *niaD* and *niiA* mRNAs of the wild-type mycelia grown under the four different conditions which are summarized in the text and described fully in Materials and methods. The right hand panel shows the levels of the wild-type and mutant strains transferred to induced–derepressed conditions. Both panels correspond to the same Northern blot and have been separated to facilitate the relevant comparisons. NI-D, non-induced–derepressed; I-D, induced–derepressed; I-R, induced–repressed; NI-R non-induced–repressed.

expression to ~20% of that found in the wild-type promoter in an *areA*$^+$ background. However, most noticeably for *niaD*, expression is considerably lower in an *areA* null mutant (*areA600*, tested with the wild-type promoter), which implies that other GATA sites could be acting additively with the four central sites or in an accessory capacity when the central sites are mutated.

The crucial GATA site 5 is occupied by AreA even under repressing conditions

We then investigated occupancy by AreA of a number of GATA sites by *in vivo* methylation protection (Wolschek *et al.*, 1998). We have investigated protection of sites 3, 4, 5, 6, 7 and 8 under induced–derepressed conditions. Only protection of the G corresponding to binding site 5 is seen (Figure 3, only the region corresponding to sites 5 and 6 is shown). Possible reasons for the failure to visualize binding to other GATA sites will be dealt with in the Discussion.

Figure 3A shows an experiment carried out with the wild-type under the four conditions described for Figure 2 and a comparison of an *areA*$^+$ and an *areA600* strain transferred to fully inducing conditions. Protection is evident under both non-induced–derepressed and induced–derepressed conditions. It is interesting that protection is not lost even under conditions of complete repression. Figure 3B shows a more sophisticated kinetic experiment. This figure shows that even after 1 h under repressing conditions, protection of site 5 is still clearly visible. RNA was extracted from mycelia grown under conditions identical to those used to generate the samples analysed in Figure 3B and analysed by Northern blots. Complete repression of transcription is seen when nitrate and ammonium are present simultaneously [induced–repressed conditions (I/R)] and after 45 min when mycelia are transferred from induced–derepressed conditions to induced–repressed conditions (I+R) (data not shown). However, even after 1 h transfer to induced–repressed conditions, considerable protection of site 5 is still seen.

Table I. Reporter gene expression driven by the wild-type and mutant bidirectional promoters

	β-Galactosidase (*niiA*)				β-Glucuronidase (*niaD*)			
	NI	I	I/R	R	NI	I	I/R	R
Host strain: *areA*$^+$								
Plasmid[a]								
pTRAN3-1A	8.3	100.0	2.1	1.4	0.4	100.0	0.8	2.0
pTRAN3-G5	2.0	43.6	2.9	1.8	3.1	55.0	2.0	4.0
pTRAN3-G6	7.8	83.0	2.4	0.5	2.7	103.4	3.2	1.5
pTRAN3-G5/6	1.8	40.7	3.3	1.9	1.3	38.9	0.7	0.0
pTRAN3-G7/8	4.3	62.2	2.0	2.2	1.5	59.2	1.4	1.7
pTRAN3-G5/6/7/8	1.7	18.9	3.3	0.6	1.3	16.5	0.7	0.8
Host strain: *areA600*								
Plasmid[a]								
pTRAN3-1A	1.6	6.2	2.5	1.1	0.7	0.7	0.3	0.3

Enzyme activities are expressed as percentages of the activities obtained for the pTRAN3-1A transformant in an *areA*$^+$ background, grown under induced–derepressed conditions (I). All strains were pre-grown in non-induced–derepressed conditions and, after harvesting and washing, as indicated in Materials and methods, mycelia were transferred to the appropriate nitrogen sources. NI, non-induced–derepressed (5 mM urea); I, induced–derepressed (10 mM $NaNO_3$); I/R, induced–repressed [10 mM $NaNO_3$ and 5 mM ammonium D-(+)-tartrate]; R, non-induced–repressed [5 mM ammonium D-(+)-tartrate]. For all strains analysed, means of three replicate experiments are shown, standard errors were always <15%.
[a]The plasmids indicated are those used to introduce into an *areA*$^+$ or an *areA600* background a *niiA–niaD* intergenic region driving the two reporter genes. pTRAN3-1A indicates a plasmid containing a wild-type intergenic region, pTRAN3-G5, G6, G5/6, G7/8 and G5/6/7/8, indicate plasmids containing *niiA–niaD* intergenic regions mutated as indicated in the text for GATA sites 5; 6; 5 and 6; 7 and 8; and 5, 6, 7 and 8, respectively.

areA600 was used as a control. No protection is seen in this null mutant. *areA18*, a second null mutant, gave results identical to *areA600* (not shown). The guanine corresponding to site 6 is visible in this gel, but no protection is evident.

niiA–niaD expression is associated with a drastic nucleosomal reorganization

We studied the *in vivo* structure of the *niiA–niaD* promoter by both DNase I and micrococcal nuclease (MNase) digestion. The results of this analysis are shown in Figures 4 and 5A and schematized in Figures 5B and 6. The four central GATA sites whose function has been shown to be essential (see above) are contained in a DNase I-hypersensitive region (Figure 4). This hypersensitivity is apparent in all culture conditions except under conditions of expression (induced–derepressed). MNase digestion shows that the DNA of the *niiA–niaD* intergenic region is packaged in a positioned array of nucleosomes under both non-induced–repressed and induced–repressed conditions. Areas protected from MNase cleavage define the positions occupied by the nucleosomes; MNase-sensitive sites (Figure 5A) define nucleosome boundaries. The length of the protected areas is compatible with the presence of core particles and ranges from 160 to 190 bp. A nucleosome-free region of ~170 bp, which corresponds exactly to the hypersensitive region observed by DNase I digestion, interrupts the nucleosomal array.

Six positioned nucleosomes have been mapped and numbered divergently from the nucleosome-free region (Figure 5B). The crucial NirA-binding site 2 is at the boundary of the nucleosome-free stretch, while the other three NirA-binding sites are included in nucleosomes –2, +2 and +3 (Figure 6). Besides the central GATA sites present in the nucleosome-free region, six additional GATA sites are included in pairs in nucleosomes –2, –1 and +4 (Figure 6). Under conditions of expression (induced–derepressed conditions), the MNase digestion pattern characteristic of this positioned nucleosome array is lost. The profile observed in these conditions is explained by the destabilization of at least nucleosomes –2, –1, +1 +2 and +4. This is shown by the appearance of a number of MNase-sensitive sites. This is illustrated schematically in Figure 5B (appearance of bands numbered 2, 3, 6, 8, 11, 13 and 14).

This experiment does not establish the fate of nucleosome +3. The absence of any preferential cutting at the sequences cognate to this nucleosome could be due either to the nucleosome conserving its positioning or, trivially, to the absence of any MNase-sensitive sites at the level of the DNA sequence corresponding to its position. It seems reasonable to suppose the nucleosome +3 suffers the same fate as the whole nucleosomal array in this region. These changes imply an extensive chromatin rearrangement of the whole region. The DNase I digestion profiles (Figure 4) support this interpretation. Under induced–derepressed conditions, the loss of nucleosomal positioning makes the whole promoter equally sensitive to DNase I, and thus the appearance of a DNase I-hypersensitive region in conditions of non-expression is only an apparent paradox, reflecting the complete protection of the flanking sequences. The DNase I-hypersensitive region is in fact a diagnostic feature of the stable positioning of nucleosomes –1 and +1. This is supported by the fact that secondary DNase I-sensitive bands, corresponding to linker sequences identified by MNase digestion, are seen in all conditions except when the genes are expressed (induced–derepressed conditions). A loosening of the positioning of nucleosomes +2 and –2 seems to occur in conditions of derepression, in the absence of induction. However, these changes are minor and may not be significant. The experiment shown in Figure 5A has been repeated several times. The drastic nucleosomal rearrangement found under conditions of expression is absolutely reproducible and clear-cut, while the appearance of micrococcal cutting sites within nucleosomes +2 and –2 found in non-induced–derepressed conditions is somewhat variable and thus will be not discussed further.

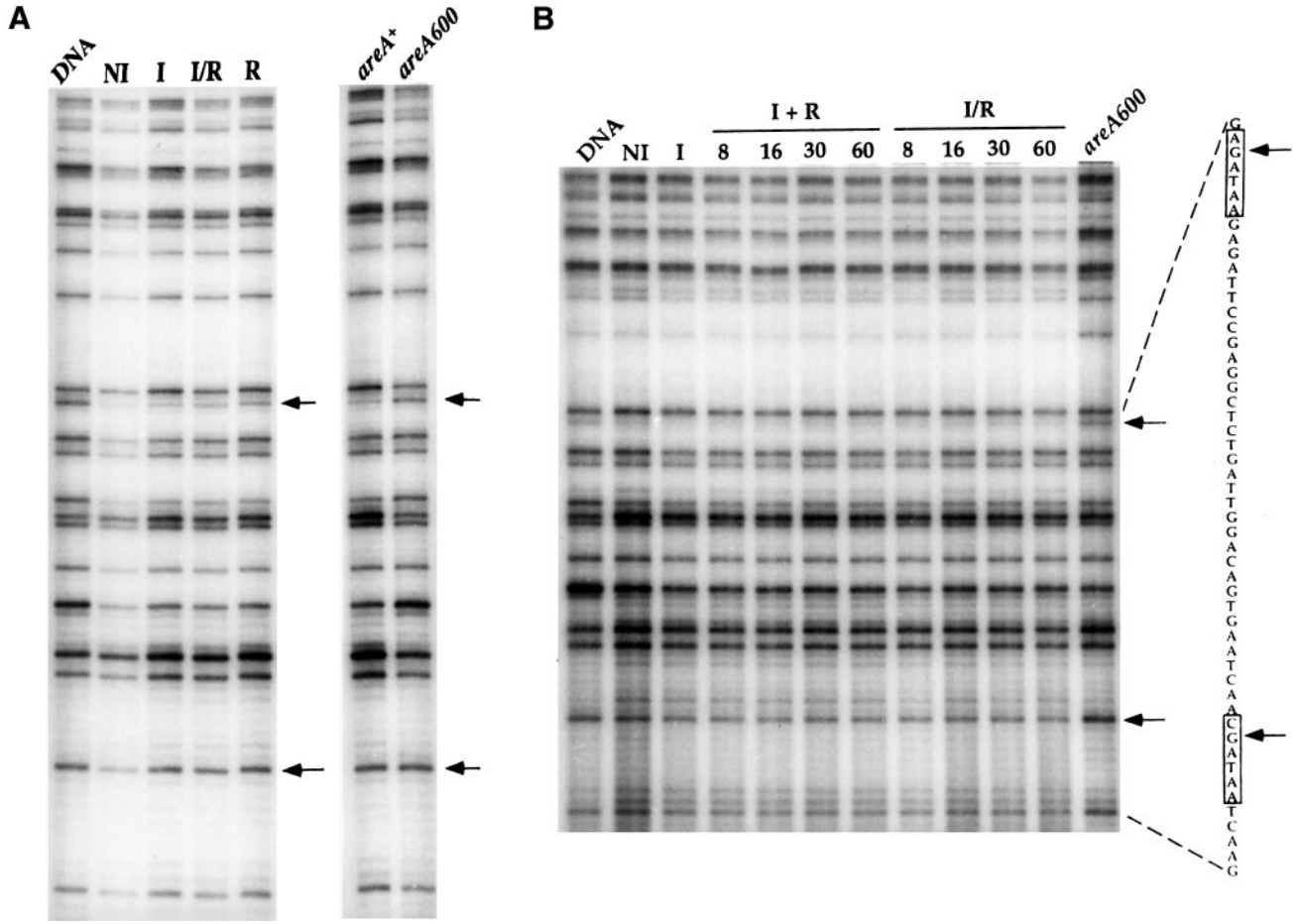

Fig. 3. *In vivo* methylation protection of AreA-binding sites 5 and 6. A relevant section of the sequence is shown to the right of (B). The top site is site 5, and the bottom site is site 6. The strand is the *niaD* coding strand. Arrows indicate the Gs corresponding to the two sites. The G corresponding to site 5 is protected, the G corresponding to site 6 is not. In both (A) and (B), DNA indicates control mycelial DNA extracted and treated with DMS after extraction followed by an amplification procedure identical to that used for the samples methylated *in vivo.* (**A**) NI, I, I/R and R protection experiments carried out with wild-type mycelia grown under exactly the same conditions as the Northern blot shown in Figure 2. *areA*$^{+}$ and *areA600* show the pattern of protection of the wild-type and a null mutant strain transferred in parallel to induced–derepressed conditions. We have compared by densitometry the protected G with the G immediately above it, outside the GATA site. The corresponding ratios are: DNA, 0.97; NI, 0.41; I, 0.43; I/R, 0.48; R 0.54; *areA*$^{+}$ 0.33; *areA600* 0.97. Two independently run gels gave almost identical results. (**B**) All samples except the last are from a wild-type (*areA*$^{+}$) strain. NI: non-induced; after pre-growing, harvesting and washing as indicated in Materials and methods, mycelia were transferred to non induced–derepressed conditions (5 mM urea) and grown for an additional 16 min. I: induced, as above but transferred to induced–derepressed conditions (10 mM sodium nitrate). I+R: the samples were grown for 16 min under induced–derepressed conditions and then under induced–repressed conditions [i.e. ammonium D-(+)-tartrate, 5 mM was added], and subsequently grown for 8, 16, 30 and 60 min as indicated. I/R: the samples are transferred directly to media containing both nitrate and ammonium (induced–repressed) as indicated above. *areA600* indicates a track where the sample is that of an *areA600* mutant, transferred exactly as indicated for the wild-type induced–derepressed.

Chromatin repositioning is absolutely dependent on AreA

We have investigated the role of the AreA GATA factor by repeating both the MNase and DNase I experiments in a strain carrying an *areA600* null mutation. The absence of *niiA* and *niaD* mRNAs in the *areA600* mutant strain is shown in Figure 2. Figure 7 shows that in the absence of AreA, the positioned nucleosome array persists in conditions that elicit expression in the wild-type. Under induced–derepressed conditions, the DNase I digest shows the typical strong hypersensitive region seen in the wild-type under non-induced–repressed conditions. Thus, AreA function is essential for the nucleosome destabilization observed in conditions of induction and derepression. None of the changes observed occur in an *areA* null background. This is consistent with the fact that, in the wild-type, AreA-dependent derepression is a pre-requisite for all changes in nucleosomal positioning. The same experiment shows that the maintenance of the positioned nucleosome state is independent of the presence of a functional AreA protein.

Chromatin reorganization depends on induction but only marginally on the NirA transcription factor

The NirA transcription factor is essential for the expression of the *niiA* and *niaD* genes (Pateman and Cove, 1967; Cove, 1979; Burger *et al.*, 1991a; Hawker *et al.*, 1992). This is shown further in Figure 2. NirA is supposed to mediate specific nitrate induction of the expression of *niiA*, *niaD* and the nitrate permease gene, *crnA* (Pateman and Cove, 1967; Unkles *et al.*, 1991). Therefore, we studied the role of NirA protein in chromatin remodelling. We have analysed nucleosome positioning in two *nirA*

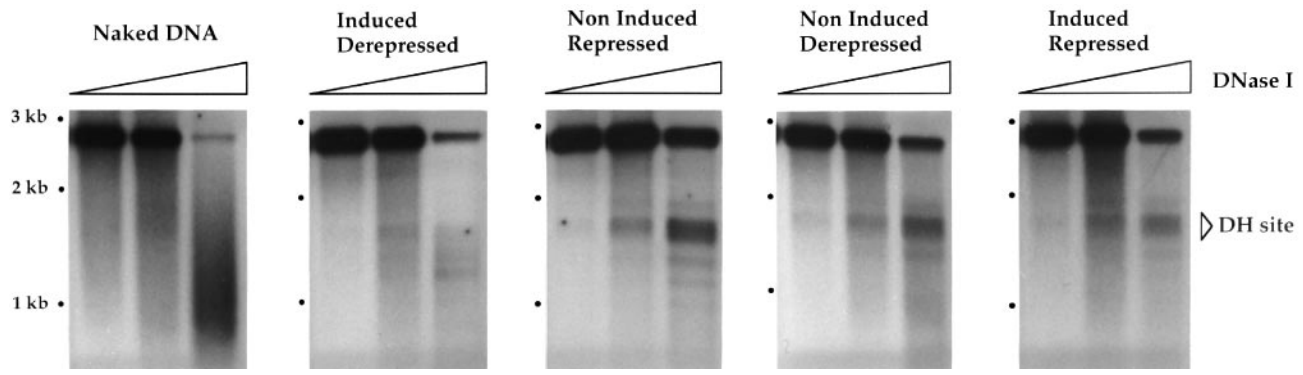

Fig. 4. Chromatin structure of the *niiA–niaD* intergenic region in a wild-type (*areA*$^{+}$ *nirA*$^{+}$) strain. DNase I digestion. To the left of the panel, the digestion pattern of the naked DNA is shown. Increasing quantities of enzyme were used as described in Materials and methods. Growth conditions are as in Figure 2. To the left of each inset, the position of markers corresponding to the 1 kb DNA ladder (Gibco-BRL) used in these experiments is shown. The triangle indicates the DNase I-hypersensitive region and corresponds to the pre-set region discussed in the text. In this and following experiments, chromatin extracted from different mycelial samples was run in parallel and in the same gel, within the limits of the capacity of the gel. When more than one gel had to be run, accurate comparisons were made as described in Material and methods.

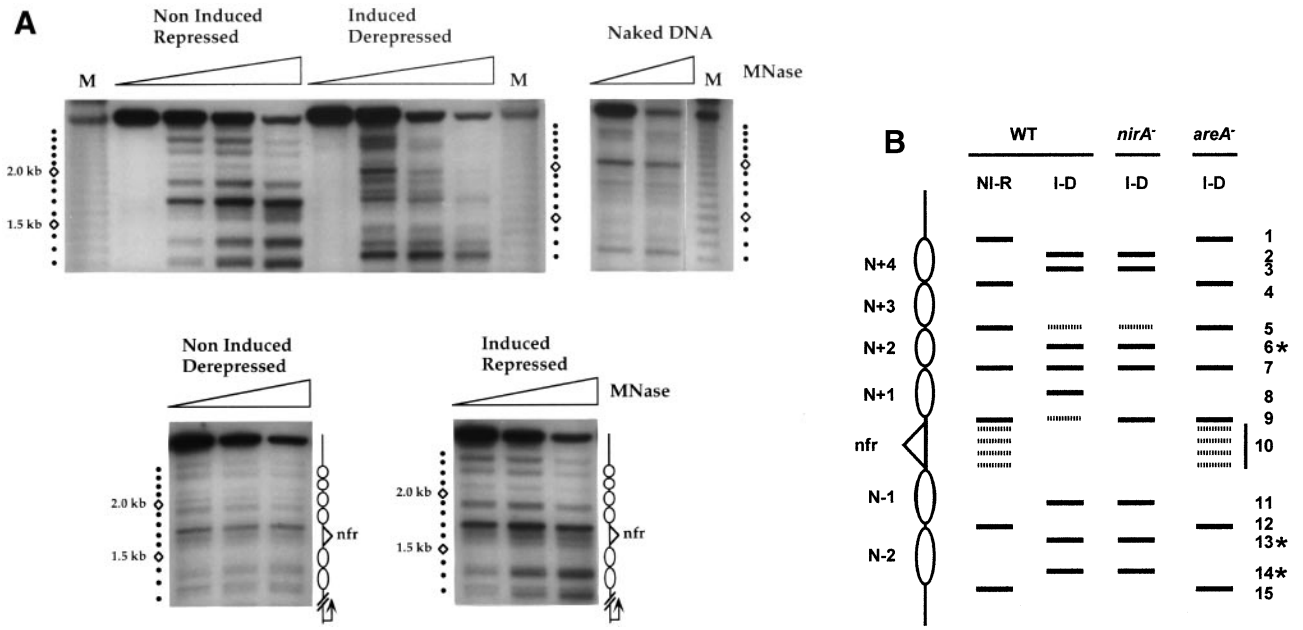

Fig. 5. Chromatin structure of the *niiA–niaD* intergenic region in a wild-type (*areA*$^{+}$ *nirA*$^{+}$) strain. (**A**) MNase digestion. To the right of the panel, the digestion pattern of the naked DNA is shown. Increasing quantities of enzyme were used as described in Materials and methods. Growth conditions are as in Figure 2. To the left of each inset, the position of markers corresponding to the 100 bp ladder from Pharmacia is shown. For the insets in the top half of the figure, these markers are actually included (M). We show to the right the nucleosome structure deduced from the MNase digest; the arrow in the nucleosome schemas represents the probe used to reveal the nucleosome structure by indirect end labelling. No nucleosomes are positioned under induced–derepressed conditions, and the chromatin structure of the promoter under non-induced–repressed, induced–repressed and non-induced–derepressed conditions is virtually identical (see B) and is schematized only for the latter two. The triangle indicates the nucleosome-free region. This corresponds to the pre-set region discussed in the text. (**B**) Schematic representation of the changes in the MNase digestion patterns for the wild-type strain (A) grown under conditions of non-expression (non-induced–repressed) and expression (induced–derepressed), and under conditions of expression (induced–derepressed) for *areA600* (shown as *areA*$^{-}$), *nirA637* and *nirA514* (shown collectively as *nirA*$^{-}$) strains (Figures 7 and 8). NI-R, non-induced–repressed; I-D, induced–derepressed. To indicate the increase or decrease of sensitivity to nuclease digestion, we have represented bands as solid (strong) or dashed (weak). Bands resulting from nuclease digestion have been numbered from the top (*niaD* initiation codon) to the bottom (*niiA* initiation codon). Solid bars also represent bands whose intensity is constant under all conditions. The smear resulting from the MNase sensitivity of the nucleosome-free region is shown schematically as four neighbouring dashed bars numbered collectively as band 10. This figure was constructed from densitometry readings of experiments carried out with a Pharmacia 100 bp ladder (for MNase experiments) run in parallel. The size of the bands was then estimated using the Molecular Analyst software from Bio-Rad. Two to five different independent experiments were carried out for each lane with completely consistent results. Asterisks indicate bands that reveal partial DNA cleavage seen under non-induced–derepressed conditions in some experiments and which may imply a destabilization of nucleosomes +2 and –2 (see text).

null mutations. One, *nirA637*, is an in-phase 566 bp deletion, removing 153 residues from the N-terminus of the protein, including the DNA-binding domain. The second, *nirA514*, is a chain termination mutation in codon 82, 12 residues C-terminal to the DNA-binding domain. Both mutations result in complete loss of utilization of nitrate and nitrite as nitrogen sources and in undetectable *niiA* and *niaD* mRNA levels even under the conditions that elicit maximal expression in the wild-type strain (Figure 2). Figure 8 shows that the MNase digestion

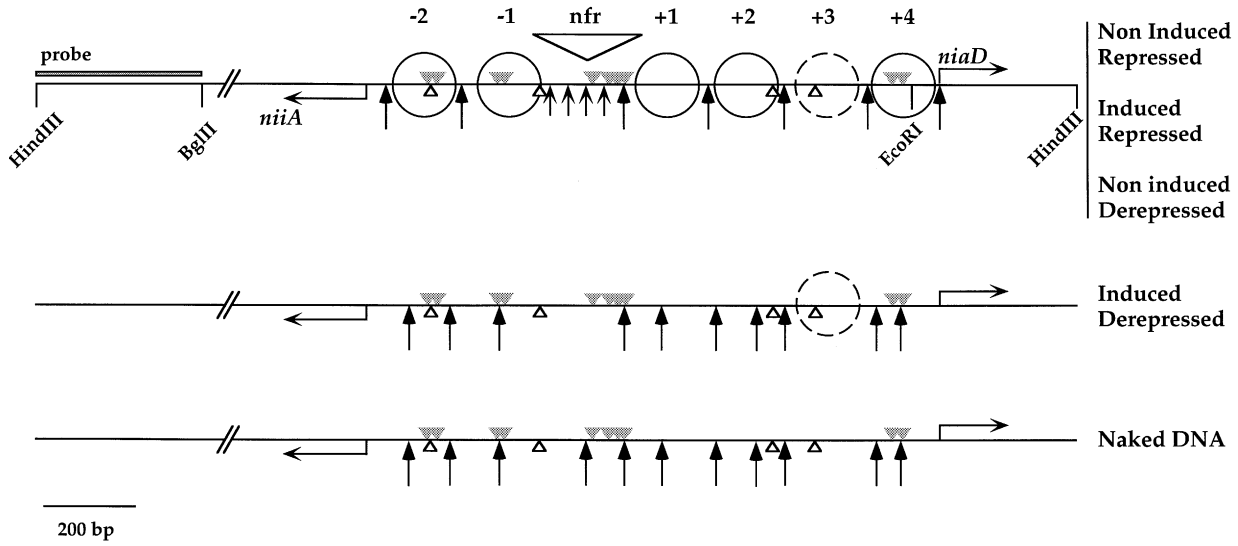

Fig. 6. Chromatin structure under different growth conditions. These are indicated to the right of each schematic representation. Positioned nucleosomes are shown as continuous circles. A broken line circle indicates the nucleosome whose fate cannot be established, as no MNase sites are found within its position. Grey triangles above the line indicate AreA-binding sites and open triangles below the line show NirA-binding sites. The nucleosome-free region (nfr) is indicated by an open triangle above the line. Long arrows indicate sites which are highly sensitive to MNase. Short arrows indicate less sensitive sites. The position of the nucleosomes in the *niiA–niaD* intergenic region was confirmed by repeating the experiment shown in Figure 5 with a probe (*Hin*dIII–*Eco*RI) overlapping the *niaD* open reading frame (results not shown). The minor changes that may occur under non-induced–derepressed conditions (see text) are not shown.

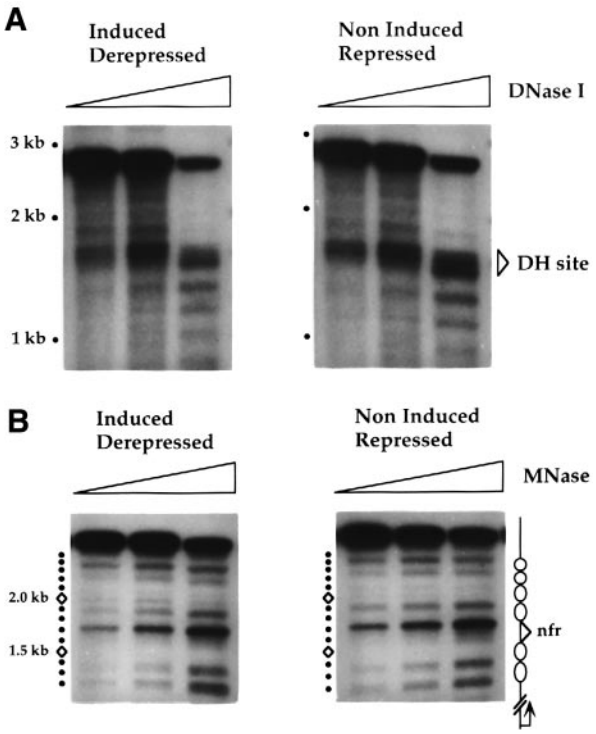

Fig. 7. Chromatin structure of the *niiA–niaD* promoter in a strain that carries an *areA600* mutation. (**A**) DNase I digestion; (**B**) MNase digestion. All symbols are as in Figures 4 and 5.

patterns of *nirA637* are very similar (but see below) to those of the wild-type in all the culture conditions tested. Identical results were obtained for *nirA514* (not shown). Thus the presence of a functional NirA protein is irrelevant to the gross chromatin restructuring of this region. There is, however, a specific change, which is indeed dependent on NirA. Under induced–derepressed conditions in a *nirA*$^-$ background, the appearance of a clear MNase cutting site within nucleosome +1 does not occur (band 8, corresponding to an MNase-sensitive site within nucleosome +1 is present only in the wild-type; compare relevant tracks of Figures 5A and 8; schematic representation in Figure 5B). Moreover, MNase cleavage at the nucleosome-free region proximal boundary of nucleosome +1 is not reduced in the transition from the non-induced–derepressed to the induced–derepressed state, as it is in a *nirA*$^+$ strain (band 9 in Figure 5B). These features are true for both *nirA*$^-$ strains tested; only *nirA637* is shown. These results imply that NirA is necessary to destabilize nucleosome +1.

As transcription is strictly dependent on NirA (see Figure 2), the nucleosome rearrangement observed upon induction (with the possible exception of destabilization of nucleosome +1) is not dependent on transcription of the *niiA–niaD* cluster. In Figure 5B, we compare, schematically, together with the MNase digestion patterns of the wild-type strain, those of *nirA637* (and *nirA514*) and *areA600* strains in induced–derepressed conditions. This pattern is almost identical for the wild-type and *nirA*$^-$ strains (see above) and radically different from that found for *areA600*. The latter is identical to the pattern seen for the wild-type in non-induced–repressed conditions.

Chromatin remodelling can occur when the four central GATA binding sites are mutated

In order to analyse the role of the four central GATA sites in chromatin remodelling, we have investigated the chromatin status in the wild-type and mutated bidirectional *niiA–niaD* promoter when this promoter drives the expression of *lacZ* and *uidA* reporter genes. The resident promoter was monitored as a control in the same experiment, and it behaves exactly as in the wild-type strain (not shown). For the construction carrying no mutations in the bidirectional promoter, we did find, as expected, that the bidirectional promoter driving the transgenes suffers

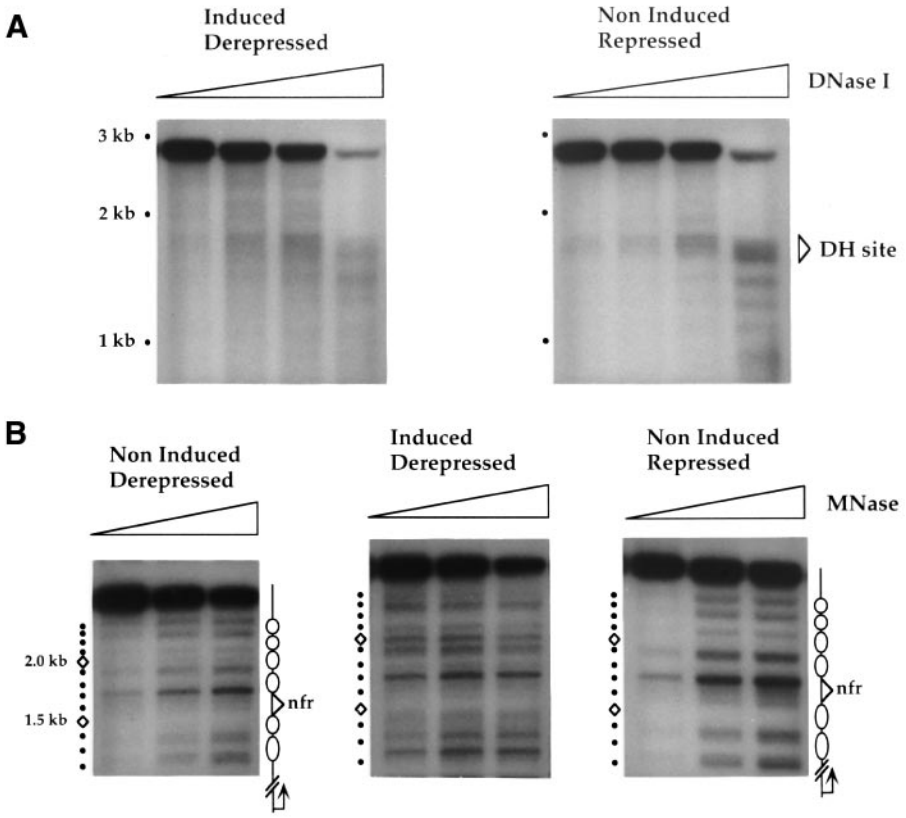

Fig. 8. Chromatin structure of a *niiA–niaD* promoter in a strain that carries a *nirA637* mutation. (**A**) DNase I digestion; (**B**) MNase digestion. Identical results were obtained with a strain carrying *nirA514* (see text). All symbols are as in Figures 4 and 5.

the same nucleosomal rearrangements associated with induction as does the resident promoter. What was not expected was that in a strain where the four central GATA-binding sites are eliminated by point mutations, the chromatin rearrangement is almost identical to that which occurs in a wild-type promoter (Figure 9). There is, however, one difference between the mutant and the wild-type promoter. This difference involves the MNase-sensitive site seen upon induction within the position of nucleosome +1 (band 8 in Figure 5B). This site is absent in the mutant promoter. This absence could be a genuine difference or an artefact. The artefact could be due to the fact that the mutations in the transgene result in the appearance of two new strongly MNase-sensitive sites. The mutations introduced a change of GATA sequences to TATA, and it has been reported already (Dingwall *et al.*, 1981; Hörz and Altenburger, 1981) that MNase preferentially cuts TA sequences. The new MNase-sensitive sites are clearly visible in the naked DNA (Figure 9B); they are adjacent to the sequence occupied by nucleosome +1 and thus may mask the MNase site that appears in induced conditions in this DNA stretch in the wild-type promoter.

Discussion

The role of the central GATA sites in the activation of transcription

We demonstrate that the four central GATA sites act bidirectionally, and collectively are responsible for >80% of the transcriptional activity.

The fact that we visualize, by *in vivo* footprinting, the occupation of site 5 but not that of the other GATA sites tested deserves some comment. We have found that not all binding sites which both bind to a given transcription factor *in vitro* and are clearly shown by mutation studies to be important, or even essential, *in vivo* can be revealed by *in vivo* methylation protection footprinting. This is true in this and in other systems (e.g. the *prnB–prnD* bidirectional promoter; B.Cubero, J.Strauss and C.Scazzocchio, unpublished results) studied by us. This is a clear limitation of the methodology. The fact that some physiologically important sites are clearly protected *in vivo* while others are not might reflect different half-lives of each of the protein–DNA complexes *in vivo*. This problem may occur more generally, and thus caution must be exercised in interpreting negative *in vivo* footprinting results in systems where no mutational evidence is also available. It is satisfying that the AreA site which is revealed makes the larger contribution to transcriptional activation. It is interesting that considerable protection is seen also under conditions of repression. That ammonium (and glutamine) repression acts by preventing the binding of a GATA factor to DNA has been proposed for *A.nidulans* and *N.crassa* (Platt *et al.*, 1996; Marzluf, 1997). Our results suggest that nitrogen metabolite repression, at least for site 5 of the *niiA–niaD* promoter, may be operating by some other mechanism. Results to be presented elsewhere suggest that a possible target is the stability of a NirA–AreA complex (F.Narendja C.Scazzocchio and J.Strauss, unpublished, see below).

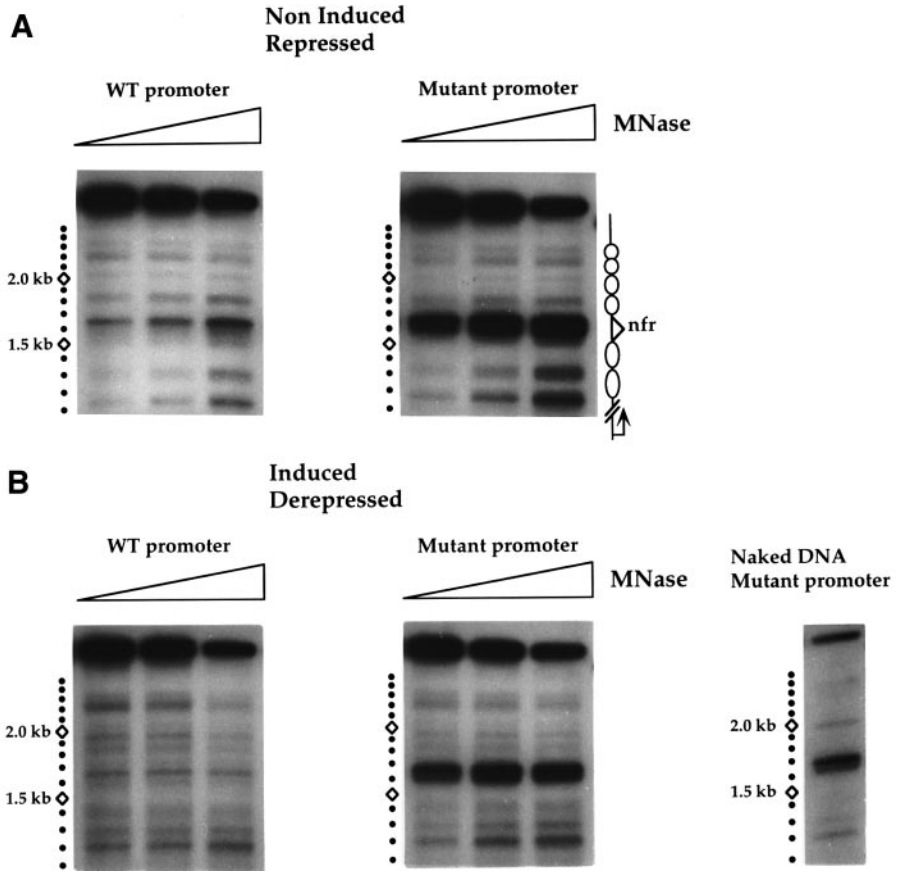

Fig. 9. Chromatin structure of the transgenic promoter which drives the *lacZ* and *uidA* reporter genes. The resident promoter was monitored in parallel (not shown, identical to Figure 5). Wild-type promoter is the transgenic promoter as shown in Figure 1, and mutant promoter is the transgenic promoter where the four central AreA-binding sites have been mutated from GATA to TATA (see Materials and methods). These mutations result in the appearance of two new MNase-sensitive sites, clearly seen in the naked DNA (on the right of B). Comparison of the wild-type and mutant transgenic promoters under (**A**) non-induced–repressed conditions and (**B**) induced–derepressed conditions. Note the near identity (see text) of the MNase pattern of the wild-type and mutant promoters in both conditions, with the exception of the MNase-sensitive bands mentioned above.

A drastic chromatin remodelling

We show in the present work that six nucleosomes are positioned in the *niiA–niaD* bidirectional promoter. This nucleosome organization is completely disrupted under conditions of transcriptional activation, but this rearrangement also occurs in the absence of transcription (see below).

Simultaneous nitrogen metabolite derepression and nitrate induction, both necessary for transcriptional activation of the *niiA–niaD* promoter, result in loss of nucleosomal positioning. NirA-binding sites 1, 3 and 4 are within positioned nucleosomes. Site 3 is a minor bidirectional site, while deletion of site 1 has a drastic effect only on *niiA* transcription (Punt *et al.*, 1995). Site 4 is within nucleosome +3. The absence of MNase-sensitive sites in the DNA stretch occupied by this nucleosome does not permit the assessment of its status after derepression and/or induction, but it is very likely that it is destabilized under induced–derepressed conditions as are all other nucleosomes in this region.

The role of AreA in chromatin remodelling

Remodelling of the chromatin structure in the *niiA–niaD* promoter is strictly dependent on the GATA factor AreA. This major rearrangement does not require transcription (with the possible exception of the loosening of the +1 nucleosome) as it occurs in a *nirA*$^-$ background and in a transgene mutated for the four crucial GATA sites. The chromatin of the *niiA–niaD* intergenic region in an *areA*$^-$ null mutant background is always in the fully structured, repressed state. GATA factors have been shown to mediate chromatin changes in other systems. GATA-1 is involved together with NF-E2 in the induction of the erythroid-specific DNase I-hypersensitive site 4 (HS4) of the human β-globin LCR and in the chicken $\beta^{A/\varepsilon}$-globin gene (Stamatoyannopoulos *et al.*, 1995; Boyes and Felsenfeld, 1996).

There are 10 AreA-binding sites in the *niiA–niaD* bidirectional promoter. We have shown that four central GATA sites (5, 6, 7 and 8) account for >80% of the transcriptional activation. These sites are in a region of DNA which is always nucleosome-free, even in non-induced–repressed conditions. It may be of some importance that the crucial NirA-binding site 2 (see below) is at the boundary of the nucleosome-free region. Similar nucleosome-free regions have been observed in well-characterized yeast promoters such as *PHO5* and the bidirectional *GAL1–GAL10* promoter (recently reviewed in Lohr, 1997; Svaren and Hörz, 1997). In the *PHO5* promoter, a Pho4p-binding site, UASp1, is found in the hypersensitive region, while the second site, UASp2, lies on the surface of a positioned nucleosome. Mutation of

UASp1 results in a promoter in which the nucleosome positioning is not disrupted by induction. In this mutant promoter, there is no binding of Pho4p to UASp2 either (Venter *et al*., 1994). This suggests that binding of Pho4p to the nucleosome-free UASp1 site induces disruption of adjacent nucleosomes and promotes binding to UASp2. In the *GAL1–GAL10* promoter, the four Gal4p-binding sites lie within a nucleosome-free region, suggesting again that such a region is required for activation and chromatin remodelling. In these two promoters, it has also been demonstrated that the ability of Pho4p and Gal4p, respectively to activate transcription correlates directly with their ability to promote chromatin remodelling (Axelrod *et al*., 1993; Svaren *et al*., 1994). This contrasts with the situation found by us in the *niiA–niaD* bidirectional promoter, where mutation of the four central and transcriptionally crucial GATA sites does not prevent chromatin remodelling. These results dissociate, at least partially, the chromatin remodelling function and the transcriptional activating function of AreA, suggesting a dual role for this protein, and highlight an interesting difference with the *PHO5* and *GAL1* systems. The implication is that binding of AreA to sites other than 5, 6, 7 and 8 is sufficient to remodel chromatin into the active state. The results shown in Table I already suggested that other GATA sites might be functional *in vivo*. This result also implies that a nucleosome-free sequence is not an obligatory pre-requisite for AreA binding. An alternative, albeit far-fetched, interpretation of these results is that binding to DNA is not necessary for AreA-mediated chromatin remodelling.

In some systems, a structured nucleosome is unable to bind specific and general transcription factors (Beato and Eisfeld, 1997). In other systems, transcription factors were found to bind mononucleosomes (Steger and Workman, 1996; Beato and Eisfeld, 1997). Felsenfeld and colleagues have shown recently that the erythroid factor GATA-1 is able to bind to reconstituted nucleosomes *in vitro* (Boyes *et al*., 1998). DNase I and MNase analyses show that GATA-1 binding causes extensive breakage of the histone–DNA contacts and generates a complex very similar to that formed by GATA-1 with free DNA. The authors suggest that formation of these complexes may be a step in the generation of fully hypersensitive regions *in vivo*. In the *niiA–niaD* promoter, all GATA sites outside the central region are within nucleosomes that are positioned under non-induced–repressed conditions (Figure 6). It is possible that binding of AreA to sites other than the four central GATAs promotes chromatin remodelling, while binding to the latter is involved in transcriptional activation. This is consistent with the fact that the occupancy of site 5 is maintained under conditions that prevent both transcription and chromatin remodelling. Kingston *et al*. (1996) have discussed possible mechanisms by which transcription factors can destabilize nucleosomes, either directly or indirectly through recruitment of histone acetylases or of protein complexes able to disrupt the histone–DNA interactions (SWI/SNF, NURF, CHRAC, etc.). These mechanisms require the presence of a binding site for a 'disrupting factor' on the surface of a positioned nucleosome. It has been proposed that some zinc finger-containing histone acetylases might be targeted to specific genes by means of contacts between their zinc fingers and other DNA-bound zinc finger proteins. (Mackay and Crossley, 1998). It would be premature to propose a mechanism by which AreA could promote nucleosome disruption, but the position of sites able to mediate this process within nucleosomes rather than in the nucleosome-free central sequence may not be irrelevant to it.

In vivo studies and the transcriptional activation of the niiA–niaD promoter. The role of NirA in question

Our *in vivo* studies highlight the crucial role of the AreA protein and cast doubts on the traditional role of the NirA protein as the unique mediator of nitrate induction (Pateman and Cove, 1967; Cove, 1979; Scazzocchio and Arst, 1989). The work presented here characterizes nitrate induction as a complex physiological process leading finally to transcriptional activation. That the NirA protein is a specific transcriptional activator for the genes of the nitrate assimilation pathway has been established without any doubt by numerous studies (Burger *et al.*, 1991a; Hawker *et al.*, 1992; Punt *et al.*, 1995; Strauss *et al.*, 1998; Figure 2, this study). That it mediates nitrate induction is now more doubtful. It is involved only subtly in chromatin remodelling (see below) and it does not mediate nitrate induction of the striking chromatin rearrangement that occurs in the *niiA–niaD* promoter. Whether NirA mediates nitrate induction of transcription at all remains an open question.

These results leave open the problem of which protein interacts with nitrate. It could be, for example, AreA or AreA complexed with an unknown protein. NirA and AreA seem to act on the *niiA–niaD* promoter in a highly interactive manner. Genetic data strongly suggested that NirA and AreA interact in a complex (Rand and Arst, 1978; Tollervey and Arst, 1981). Recent data from Marzluf's laboratory show a direct *in vitro* interaction between NIT2 and NIT4 (Feng and Marzluf, 1998), and similar data have been obtained for AreA and NirA (M.I.Muro-Pastor and C.Scazzocchio, unpublished data). The studies presented here reinforce this picture. Chromatin rearrangement, absolutely dependent on AreA, seems necessary to recruit NirA as a specific transcription factor. In the absence of ammonium and the presence of nitrate, AreA makes a major contribution to chromatin remodelling, and this is independent of *niiA* and *niaD* transcription. In contrast, the contribution of NirA to chromatin remodelling is highly specific. It is limited to the loosening (in an *areA*$^{+}$ background) of the positioning of nucleosome +1, and this may be related to the onset of transcription. As no NirA-binding sites are adjacent or within this nucleosome, this implies either that NirA can promote nucleosome destabilization at a distance or that the loosening of nucleosome +1 (but not of any other nucleosome) is an indirect result of the onset of transcription promoted by NirA. This is supported by the fact that nucleosome +1 destabilization does not seem to occur in the transgene when the four central GATA sites are mutated (but see Results for a possible alternative interpretation of the latter).

The results presented here demonstrate a crucial role for AreA in chromatin remodelling and transcription as well as in direct or indirect sensing of both ammonium and nitrate. The study of the mechanisms by which AreA

is able to carry out these functions will require a detailed analysis of its molecular partners.

Materials and methods

Strains, plasmids and genetic techniques

The following *A.nidulans* strains were used: a *biA1* strain was used as wild-type; *argB2*, *inoB2* was used as recipient strain for transformation with pTRAN3-1A derivatives; *argB2*, *pabaA1*, *areA600* was used in crosses to obtain strains containing pTRAN3-1A in an *areA600* background. *biA1*, *sb43*, *areA600*; *pabaA1*, *nirA637*; and *yA2*, *biA1*, *pantoB100*, *prnB110*, *nirA514* are the complete genotypes of the strains carrying null mutations as described in the text. *areA600* is a chain termination mutation in codon 646 (Al-Taho *et al*., 1984; Kudla *et al*., 1990). *nirA514* is a spontaneous mutation isolated by chlorate resistance; it has a chain termination mutation in codon 82 (GGA to TGA), 12 residues C-terminal to the DNA-binding domain. *nirA637* is an in-phase 566 bp deletion eliminating 153 amino acids residues after the first methionine of the protein. This mutant was constructed as follows: a *Cla*I–*Xba*I fragment was amplified from the *nirA* locus using appropriate primers starting at positions 262 and 3626, respectively, according to Burger *et al*. (1991b). The primer starting at position 262 contains a *Cla*I restriction site not present in the *nirA* sequence (ATCGAG original sequence, ATCGAT primer sequence). The amplified fragment was cloned into pBluescriptII SK(+), the plasmid was cut with *Nco*I and *Eco*RV (positions 2320–2888), the *Nco*I receding end was filled in using Sequenase Version 2.0 (Amersham) and the plasmid religated. The *Cla*I–*Xba*I fragment (missing the *Nco*I–*Eco*RV fragment) was released from the plasmid and used to transform the *A.nidulans pabaA1* strain. Transformants containing the *nirA* deletion were selected as chlorate-resistant strains and checked by Southern blot for loss of the *Nco*I site. The presence of the deletion was checked by PCR. *biA1*, *inoB2*, *sb43*, *argB2*, *pantoB100* and *pabaA1* are standard auxotrophic markers for biotin, inositol, thiosulfate, pantotenic acid, arginine and *p*-aminobenzoic acid, respectively (Clutterbuck, 1993). *prnB110* is a mutation in the major proline permease (Arst *et al*., 1981). Genetic techniques for *A.nidulans* followed Pontecorvo *et al*. (1953).

pTRAN3-1A is a twin-reporter vector containing two divergently oriented reporter genes, encoding *Escherichia coli* β-glucuronidase (*uidA*) and β-galactosidase (*lacZ*), described by Punt *et al*. (1991). pAN302 contains a 2.7 kb *Eco*RI–*Eco*RI fragment, comprising a 1500 bp fragment of the *niiA* open reading frame and 1214 bp of the *niiA–niaD* intergenic region (Johnstone *et al*., 1990; de Lamotte-Malardier, 1991); this plasmid was used to obtain the *Hin*dIII–*Bgl*II 360 bp probe for indirect end labelling.

Escherichia coli strain DH5α: F^-, *endA1*, *hsdR17* (mk^+,rk^-), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, D*lacU169* (*f80d-lacZDM15*) was used for routine plasmid preparation. Strain CJ236: *dut-1*, *ung-1*, *thi-1*, *relA-1*; pCJ105(Cm^r) was employed in the *in vitro* mutagenesis experiments.

Northern blots

Mycelia of *A.nidulans* grown as described above (see Chromatin structure analysis) were collected by filtration, washed with sterile water and immediately frozen in liquid nitrogen. Total RNA isolation and Northern hybridization were carried out as described in Mach *et al*. (1996). The probe for detecting *niaD* transcripts was amplified by PCR using primers *niaD-F* (position 341: 5′-CACAATGTCTACAACCGTCA) and *niaD-R* (position 1680: 5′-TGGTAGTTAGGAAGCGTACA) from chromosomal DNA. The probe for detecting *niiA* transcripts was amplified using primers *niiA-F* (position 921: 5′-CATCATGCCGTTGCTGGACG) and *niiA-R* (position 2242: 5′-GCTTAGGTCCGGCCTGAAG). Numbering of sequences is as in Johnstone *et al*. (1990). The probe for detecting the actin transcript was derived from plasmid pSF5 (Fidel *et al*., 1988). Probes were labelled by random priming.

Constructions of the mutant derivatives of expression vector pTRAN3-1A by site-directed mutagenesis

To obtain pTRAN3-1A derivatives mutated in the central GATA sites of the *niiA–niaD* intergenic region (GATA5, GATA6, GATA7 and GATA8, according to Punt *et al*., 1995), the complete intergenic region provided with flanking *Not*I sites (Punt *et al*., 1995) was isolated from pTRAN3-1A and cloned into pBluescriptII KS(+). Mutagenesis was carried out as described in Strauss *et al*. (1998). The following oligonucleotides were used as mutated priming oligodeoxynucleotides; numbers in parentheses refer to nucleotide positions in the *niiA–niaD* intergenic region (Punt *et al*., 1995), and the bold characters indicate mutated bases: GATA5 (486–517), 5′-CTTCTTCCCACCA**T**A**T**ATAA-GAGATTCCGAGG 3′; GATA6 (526–557), 5′-GGACAGTGAATC-AAC**T**ATAATCAAGCCCTATC-3′; GATA7/8 (540–578), 5′-CGATAA-TCAAGCCCTAT**A**T**A**TATA**T**ATGAGACACGATCG-3′.

The putative mutated clones were confirmed by sequencing the whole intergenic region. The different mutant intergenic regions were isolated as *Not*I fragments and were used to substitute the wild-type region in the pTRAN3-1A plasmid. Mutated plasmids carrying a mutation in more than one site were generated by successive cycles of mutagenesis of the desired GATA sites. The resulting derivative plasmids were designated pTRAN3-G5 (mutant for GATA5), pTRAN3-G6 (mutant for GATA6), pTRAN3-G5/6 (mutant for GATA5 and GATA6), pTRAN3-G7/8 (mutant for GATA7 and GATA8) and pTRAN3-G5/6/7/8 (mutant for GATA5, GATA6, GATA7 and GATA8).

Isolation of A.nidulans strains carrying single copies of the various pTRAN3-1A derivatives

Reporter vectors were introduced into the *argB2*, *inoB2 A.nidulans* recipient strain by transformation, carried out according to Tilburn *et al.* (1983). The use of a mutant *argB* selection marker (Punt *et al*., 1990), contained in pTRAN3-1A and derivatives, ensures that all transforming sequences are integrated by homologous recombination at one precise place in the genome, the *argB* gene. Screening of the transformants carrying pTRAN3-1A and derivatives was carried out on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Punt *et al*., 1991), and nitrate as the sole nitrogen source. Even the quadruple GATA site mutant has enough LacZ residual activity to be identified by this technique. Southern analysis of these transformants was used to select those that carried a single copy of the pTRAN3 vector. For the analysis of reporter gene expression in an *areA600* background, the transformants containing pTRAN3-1A were crossed with a *pabaA1*, *argB2*, *areA600* strain by standard genetic techniques. As no reporter gene expression could be expected in the plates containing X-gal as described above, $argB^+$ segregants carrying a pTRAN3-1A plasmid were identified by PCR amplification, using two primers corresponding to the *lacZ* and *uidA* reporter genes, respectively.

Reporter enzyme assays

To determine reporter gene expression, single-copy transformants were grown for 7 h at 37°C in minimal medium (Pontecorvo *et al*., 1953) with appropriate supplements plus 1.25 mM ammonium D-(+)-tartrate as nitrogen source. This medium allows the growth of all strains, including *areA600*. The mycelia were then harvested by filtering through sterile blutex tissue, washed with sterile water and transferred to the same medium without any nitrogen source. Incubation was continued for 20 min in this medium when the following nitrogen sources were added: 5 mM urea (non-induced–derepressed conditions); 10 mM $NaNO_3$ (induced–derepressed conditions); 10 mM $NaNO_3$ and 5 mM ammonium D-(+)-tartrate (induced–repressed conditions); or 5 mM ammonium D-(+)-tartrate (non-induced–repressed conditions). Mycelia were incubated for an additional 3 h. The β-galactosidase and β-glucuronidase activities in mycelial extracts were determined as previously described (Punt *et al*., 1991). The reporter activities are expressed as percentages of the activities of the pTRAN3-1A transformants (wild-type for the intergenic region and all relevant regulatory genes) under induced–derepressed conditions ($NaNO_3$). Specific activities of 290 ± 30 nmol of *p*-nitrophenol/min/mg of protein for β-glucuronidase and 450 ± 15 nmol of *o*-nitrophenol/min/mg of protein for β-galactosidase were found in repeated assays with pTRAN3-1A transformants grown under these conditions. For all strains analysed, means of three replicate assays are shown; standard errors were always <15%.

Chromatin structure analysis

DNase I and MNase sensitivity analyses were performed by the indirect end labelling technique, following the method described for filamentous fungi by Gonzalez and Scazzocchio (1997). Strains were grown for 7 h at 37°C in minimal medium with appropriate supplements plus 1.25 mM ammonium D-(+)-tartrate as nitrogen source. The mycelia were then harvested by filtering through sterile blutex tissue, washed with sterile water and transferred to the same medium without any nitrogen source. Incubation continued for 20 min in this medium and, after this period, the following nitrogen sources were added: 5 mM urea (non-induced–derepressed conditions); 10 mM $NaNO_3$ (induced–derepressed conditions); 10 mM $NaNO_3$ and 10 mM ammonium D-(+)-tartrate (induced–repressed conditions); or 10 mM ammonium D-(+)-tartrate (non-induced–repressed conditions). Mycelia were incubated for an additional 2 h.

DNase I digestion profiles were obtained using 10–200 U of the enzyme per gram of mycelium, and for MNase digestion 1–25 U of the enzyme were used. After nuclease treatment, DNA was digested with *Hin*dIII and hybridized with the *Hin*dIII–*Bgl*II 360 bp probe (Figure 1), or digested with *Eco*RV and hybridized to an *Eco*RV–*Cla*I 288 bp probe derived from *lacZ* sequences (Figure 1). Samples from the same strain grown under different conditions were run on the same gel, within the limits of the capacity of the gel. A Pharmacia 100 bp ladder (for MNase experiments) or a Gibco, 1 kb BRL ladder (for DNase I experiments) was included in every gel. This allowed us to make completely accurate comparisons of samples run on different gels. These comparisons were facilitated by the fact that the Pharmacia 100 bp ladder hybridized non-specifically with the probe used. In all cases, gels were scanned and the size of the bands estimated using the Molecular Analyst software from Bio-Rad and the appropriate ladder as a standard.

In vivo footprint studies

For Figure 3A, all strains were grown in exactly in the same conditions as described for the Northern blots and chromatin structure analysis. For panel B, all strains except *areA* loss-of-function strains were pre-grown for 12 h at 30°C in minimal medium appropriately supplemented (Pontecorvo *et al*. 1953) with 5 mM urea as sole nitrogen source. For *areA*$^{-}$ strains, 2.5 mM ammonium D-(+)-tartrate was used together with 2.5 mM urea. Mycelia were harvested by filtration, washed extensively with ice-cold sterile water and resuspended in supplemented minimal medium without any nitrogen source. The concentration of the mycelial suspension was adjusted to ~0.1 g wet weight/ml, and 18 ml of this suspension were aliquoted into 100 ml Erlenmeyer flasks. For the *in vitro* methylated control DNA, one aliquot was harvested by filtration without further incubation. From this sample, the DNA was extracted and methylated *in vitro* as described elsewhere (Cubero and Scazzocchio, 1994). For non-induced–derepressed conditions, urea was added to a final concentration of 5 mM. Samples were incubated in a shaking water bath at 37°C for 16 min. Mycelial suspensions of each sample were then treated with dimethylsulfate (DMS) as described elsewhere (Wolschek *et al*., 1998). For induced–derepressed conditions, 10 mM $NaNO_3$ was added to an aliquoted suspension. Samples were incubated for 16 min. Northern blots show that full induction of the *niiA* and *niaD* transcript is achieved after this time. Each sample was then treated with DMS. For conditions of induction followed by repression (I+R), 10 mM $NaNO_3$ was added to five aliquoted suspensions which were incubated in the shaker at 37°C for 16 min to obtain full induction of the structural genes (as seen by Northern analysis). After 16 min, one sample was treated with DMS (this constitutes the fully induced sample) and 5 mM ammonium D-(+)-tartrate was added to the remaining four aliquots which were incubated further for 8, 16, 30 and 60 min, respectively and then treated with DMS. For induced–repressed conditions (I/R), 10 mM $NaNO_3$ and 5 mM ammonium D-(+)-tartrate were added simultaneously to four aliquots which were incubated for 8, 16, 30 and 60 min, respectively and then treated with DMS. In all samples, DMS was added immediately after the prescribed incubation time. DMS-treated samples were then processed as described by Mueller and Wold (1989) and Garrity and Wold (1992) as modified for use in filamentous fungi (Wolschek *et al*., 1998).

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