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Nucleosome positioning and histone H3-acetylation are independent processes in the *Aspergillus nidulans prnD-prnB* bidirectional promoter.

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Abstract

In *Aspergillus nidulans*, proline can be used as carbon and nitrogen source and its metabolism requires the integration of three signals including proline induction, and nitrogen and carbon metabolite de-repression. We have previously shown that the bidirectional promoter in the *prnD-prnB* intergenic region undergoes drastic chromatin rearrangements where proline induction leads to loss of positioned nucleosomes whereas simultaneous carbon and nitrogen metabolite repression results in partial re-positioning of these nucleosomes. In the proline cluster, inhibition of deacetylases by trichostatin A (TSA), leads to partial de-repression and is associated with a lack of nucleosome positioning. Here, we investigate the effect of histone acetylation in the proline cluster using strains deleted for essential components of putative *A. nidulans* histone acetyl transferase (HAT) complexes, namely *gcnE* and *adaB*, the orthologues of the *S. cerevisiae GCN5* and *ADA2* genes, respectively. Surprisingly, GcnE and AdaB are not required for transcriptional activation and chromatin remodelling but for repression of *prnB* and *prnD* and for re-positioning of nucleosomes in the divergent promoter region. Chromatin immunoprecipitation (ChIP) directed against histone H3 lysines K9 and K14 revealed that GcnE and AdaB participate in increasing the acetylation level of at least one nucleosome in the *prnD-prnB* intergenic region during activation, but these activities do not determine nucleosome positioning. Our results are consistent with a function of GcnE and AdaB in gene repression of the proline cluster, probably an indirect effect related to the function of CreA, the DNA-binding protein mediating carbon catabolite repression in *Aspergillus nidulans*.

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Introduction

In eukaryotic cells, acetylation of histones is correlated with both transcriptional activation and the chromatin rearrangements usually associated with it. A turning point in our understanding of the mechanism of histone acetylation was the demonstration of the intrinsic acetylase activity of the *Tetrahymena pyriformis* p55 protein (7), the homologue of the *Saccharomyces cerevisiæ* Gcn5p protein, the latter already known to be involved in the transcriptional activation of a number of genes in this organism (reviewed in (33). Homologues, possibly orthologues, of Gcn5p, are universally present in eukaryotes. Gcn5p and its homologues interact with chromatin in large multi-protein complexes such as ADA and SAGA (17). SAGA is a multiprotein complex whose Gcn5p subunit posses histone acetyltransferase activity. Gcn5p acetylates several lysine residues on histones N-termini including K9 and K 14 on histone H3 and K8 and K16 on histone H4 (21). The Gcn5 containing complexes share several subunits, such as Ada2p, Ada3p, Spt3p and Tra1p (24) and it has been estimated that the yeast SAGA complex regulates the expression of ~ 10% of *S.cerevisiae* genes, approximately one-third of them being negatively regulated (23). Gcn5p forms a ternary complex with Ada2p and Ada3p, a complex which is conserved in SAGA and ADA (2). The proteins of the complex modulate the acetylating activity of Gcn5p (8, 17, 35) in patterns that are not identical for different promoters or different transcriptional activators. (9, 33). In the context of transcriptional regulation the SAGA complex can have different functions. The role of SAGA has been extensively studied in the S. *cerevisiae GAL1* promoter and has been shown to be essential for *GAL1* transcription. In the case of *GAL1*, the first step in transcriptional activation is binding of the specific Gal4p activator which in turn recruits SAGA to the upstream activating sequence (UAS) and the UAS-bound SAGA

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then promotes the binding of TATA-binding protein and assembly of pre-initiation complex (5, 22). In contrast to the *GAL1* system, in the yeast *ADE* regulon SAGA is not recruited by the specific activators Bas1p and Pho2p (nor is SWI/SNF, another chromatin remodelling complex recruited in this promoter). Instead, in promoters of this regulon SAGA function is required for recruitment and efficient binding of the specific activators (19). In the yeast nitrogen-carbon utilization regulatory interface, different components of SAGA have been shown to have different functions. The expression of both paralogous glutamate dehydrogenase genes, *GDH1* and *GDH3*, require different SAGA components, depending whether glucose or ethanol is the carbon source. *GDH1* expression requires Ada2p and Ada3p on ethanol as carbon source, but only Ada3p on glucose. In both cases, *GDH1* expression is *GCN5* independent (32). In contrast, *GDH3* expression and chromatin remodelling activities in its cognate promoter, which is only seen under carbon de-repressing conditions (ethanol), are dependent on the SAGA components *GCN5, ADA2, ADA3* and *SPT3*.

There is little information on SAGA function in filamentous fungi. Recent work in *Neurospora crassa* established that blue light-induced transcription of the early light-inducible genes *al-3* and *vvd* depends on increased acetylation of histone H3 at lysine K14 in the promoters of these genes (18). The authors showed that acetylation and transient gene activation requires the *N. crassa GCN5* homologue *ngf-1* and it was suggested that the specific activator, White Collar-1, is required for the recruitment of NGF-1 to *al-3* and *vvd* promoters.

We have recently described the relationship between the transcriptional activation of a number of *A. nidulans* promoters driving genes of primary metabolism and their chromatin architecture (3, 12, 25, 27-29). The *prn* cluster of *A. nidulans* comprises all

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the genes involved in proline utilisation as sole nitrogen and/or carbon source. We have studied in detail the bidirectional *prnD-prnB* promoter, driving respectively the genes encoding proline oxidase and the specifc proline transporter (10, 11, 13-15, 34). The proline metabolic genes are induced by proline and repressed when preferred carbon (glucose) or nitrogen (ammonium, glutamine) sources are available in the medium. A graphical representation of regulatory proteins and their *cis*-acting recognition motifs involved in the regulation of the bidirectional promoter is shown in Figure 1A. In this promoter eight nucleosomes lose their positioning upon induction while simultaneous carbon and nitrogen metabolite repression results in partial nucleosome repositioning. Chromatin re-structuring is strictly dependent on the PrnA pathway-specific activator but not on the wide-domain nitrogen regulator AreA which was proven to be essential for chromatin opening in the *A. nidulans* nitrate regulon (3, 27). AreA in the proline cluster is only required in the presence of glucose, which is consistent with its proposed function which is to counteract the repressive effect of the global carbon repressor CreA. A functional CreA protein and two CreA binding sites in the *prnD-prnB* bidirectional promoter were shown to be essential for nucleosome re-positioning under repression conditions (12). A first indication that histone acetylation is involved in the CreA-mediated repression of the *prnD-prnB* bidirectional promoter process came from the observation that Trichostatin A, an inhibitor of histone deacetylases, mimics the effect of a CreA loss-of-function mutation or of *cis*-acting CreA binding site mutations (termed *prn*^{d}). Nucleosome positioning after repression and transcriptional repression is completely lost when CreA is non functional and also partially lost when TSA is present in the culture medium (12).

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To investigate in more detail how acetylation affects nucleosome positioning in this system, we deleted two genes coding for members of the putative SAGA/ADA acetylation complexes in *A. nidulans*. In this article we describe the effect of *gcnE* and *adaB* deletions, homologues of the *S. cerevisiae GCN5* and of *ADA2* genes, a putative histone acetylase and adaptor protein, respectively. We studied the effects of these deletions on the transcriptional competence, the chromatin structure and the acetylation status of histone H3 lysine K9 and K14 in the *prnD-prnB* bidirectional promoter. Unexpectedly, low levels of histone H3 acetylation in the deletion strains were found not to affect transcriptional activation, but are paradoxically associated with partial derepession of *prnB and prnD.*

Materials and Methods

Strains and growth conditions.

A. nidulans strains used throughout the study are listed in Table 1. A total of 10^6 spores of each strain per ml were inoculated into liquid minimal medium (30) with the appropriate supplements plus 0.1% fructose as the carbon source and 5 mM urea as the nitrogen source. Mycelia were grown for 12 h at 37°C on a rotary shaker with continuous shaking at 180rpm and then harvested by filtration. For condition-specific growth, aliquots of these pre-cultures were then further incubated for two hours at 37°C under following conditions: non induced, NI: 0,1% fructose, 5mM urea; induced, I: 20 mM proline; induced repressed, IR: 20 mM proline plus 1% glucose and 20 mM ammonium-L[+]-tartrate. Finally, mycelia were harvested by filtration for RNA isolation, MNase analysis and chromatin immunoprecipitation. For microscopic observations, cultures were grown on solid complete media (30) until sporulation

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occurred (roughly 7-10 days for the *adaB* and *gcnE* deletion strains and around 3 days for the wild type control strains) and surface samples were prepared for microscopy.

Cloning of *gcnE* and *adaB* and construction of disruption vector.

Using the *S.cerevisiae* Gcn5p protein in a BLAST search of the *A.nidulans* genome database (www.broad.mit.edu) we identified gene AN3621.3 as GcnE, a putative orthologue of yeast Gcn5p with an overall identity of 66% between the *A. nidulans* and the *S. cerevisiae* proteins (for predicted domain organisation and BLAST results, see http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/).

A deletion cassette was constructed by DJ-PCR. Deletion of the *gcnE* open reading frame (ORF) was achieved by replacing it with the *A. fumigatus pyrG* gene. In this cassette, the *pyrG* gene is flanked by upstream and downstream *gcnE* ORF sequences. The 3'flanking fragment of *gcnE* was amplified with primers G1F (5' TAGCAGACCCTGATGCATCAAAC 3') and G2632R (5' TAAAGAGCTTTCAGTAAGTAATGATACTCG 3'), and the 5' flanking fragment of *gcnE* was amplified with G3330F (5' GAAACATCCCCGAGTGGTCGGTAAGTACAA 3') and G5950R (5' CTCTCAAATACTAAGCCAAGTGGTACCAAG 3'), from *A. nidulans* wild type (*pabaA*1) genomic DNA . *A. fumigatus pyrG* was amplified from *A. fumigatus* wild type genomic DNA with primers GCNPYRF (5' GCGTGCTACCTCTGCGAGTATCATTACTTACTGAAAGCTCTTTAGGACTGAA TTCGCCTCAAACAATGC 3') and GCNPYRR (5' ATTGTACTTACCGACCACTGGGGATGTTTCGAATCTGCTGCCACATGAAGGA

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ATTCTCAGTCCTGCTCC 3'). Nested primers GCNESTF (5' GAAAATTCAACTGTCGATTCG 3') and GCNESTR (5' TGGGTGAAAAGTGGATTGTATG 3') were used to amplify the complete assembled molecule containing 5´*gcnE-pyrG*-3´*gcnE*. This *gcnE* deletion cassette was used to transform a *pyrG*89 *argB*2 *pantoB*100 *riboB*2 *yA*2 strain. Transformants were selected on minimal media with appropriate supplements lacking uracil and uridine.

To identify the strains deleted for *gcnE*, a Southern blot with genomic DNA digested with *KpnI* was hybridized with a ^{32}P labeled probe derived from a 2,6 kb PCR product amplified with primers G3330Fand G5950R. A single 3 kb band confirmed *gcnE* deletion and single integration of the deletion cassette. The same membrane was stripped and hybridized with a ^{32}P labeled probe derived from a 800 pb PCR product amplified by GCNORFF (5' TCGTTGGAGGTATAACCTACCG 3') and GCNORFR (5' GTAGGGTGTGTTCTCGTTATTATACC 3') corresponding to the *gcnE* ORF. No signal was detected for the *gcnE* deleted strain (not shown).

Using the *S.cerevisiae* Ada2p protein in a BLAST search of the *A.nidulans* genome database (www.broad.mit.edu) we identified gene AN5974.2 (changed to gene number AN10763.3 in the latest annotation) as a putative orthologue of yeast Ada2p with a similarity score of 3x10e^{-79} (for predicted domain organisation and BLAST results, see http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/). Transcription start and termination points were determined empirically by 5´ and 3´ RACE, respectively, and were found to correspond to the sites shown in the *A. nidulans* database. Plasmid pMS12 (Fungal Genetics Stock Center, http://www.fgsc.net) containing the *argB* gene was used to insert PCR fragments derived from the *adaB* 5´ region upstream of *argB* and from the *adaB* 3´region downstream of *argB*. The *adaB*

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5´and 3´ sequences were obtained by PCR amplification using the sequence information from the *A.nidulans* sequence database (www.broad.mit.edu). Roughly 700 bp of the *adaB* 5´region was amplified with primers 5´forward: 5´GATGCAAGACCGCGGCCGCCACAGCAGTCAGC 3´ (NotI site introduced by mutating three bases is underlined) and 5´reverse: 5´CATCGATCCCGAGAGCCTTTCATCCTCCGCG 3´). The fragment was cloned as NotI-ClaI fragment into the vector. Around 1,4 kb of the *adaB* 3´region was amplified with primers 3´forward: 5´CGAATCATCACGTCGACTTGAATTTAGAGGCC 3´ (SalI site introduced by mutation of the HindIII restriction site, positioned 50 bp downstream of the empirically determined poly adenylation site, is underlined) and 3´reverse: 5´GCGAGTTGACTGAGCTCGAGAAGGATCACCCTC 3´. The fragment was cloned as Sal I-Xho I fragment into the vector. The resulting 7,2 kb vector was called p5´-3´adaB::argB. Strain MH 9233 (26) (gift from Michael Hynes, The University of Melbourne) was used as recipient strain for the knock out construct. To verify the insertion of the *argB* gene at the *adaB* locus, Southern hybridisation was carried out and the putative deletion strains (showing absence of the 4,5 kb *adaB* band but instead, a single 3,5 kb band hybridizing with the 3´*adaB* Hind III-Xho I probe) were verified for the lack of the *adaB* ORF and additional *argB* insertions. The strain was crossed to a wild type *pabaA*1 strain to obtain an *adaB*Δ strain with only one additional marker for further studies. *adaB*Δ *biA*1 was recoverd from the progeny and for further studies, a wild type *biA*1 strain was used as reference strain.

RNA preparation and Northern blots. Total RNA was isolated with the RNA Plus Extraction Solution (Biogen) following the manufacturer's instructions. RNA

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electrophoresis and Northern blot hybridizations were carried out as described previously (13). *prnB*, *prnD*, and *acnA* probes were prepared as described by (14).

Nucleosome positioning. Micrococcal nuclease I digestions were performed by the method adapted by Gonzalez *et al* (16). Micrococcal nuclease was used at concentrations ranging between 0.5 and 2.5 U/g of mycelium. DNA was digested with an appropriate restriction enzyme. Probes SC1 and SC2 for the *prnB-D* intergenic region were prepared as described previously (12)

The position of each MNase cut was calculated by running in each gel a scale of molecular size markers (100 Base-Pair Ladder, Amersham Pharmacia Biotech., Piscataway, NJ). The values reported in the scale adjacent to each gel represent the position of the MNase cut from the ATG of the relevant gene. For each mutant and growth condition the experiments were performed in triplicate.

ChIP assays

Chromatin immunoprecipitation assays (ChIP) were carried out following our published protocol (4). Antibodies for ChIP analysis of acetylated histone H3 were purchased from Upstate Biotechnology, Charlottesville, VA, recognizing acetylated K9 and K14 of histone H3 (#06-599). Rabbit polyclonal antibody recognizing the C-terminus of histone H3 (#ab1791) was purchased from abcam (Cambridge, UK). Latter antibody was used to normalize the acetylation status of H3. 2 µl of antibody solution were used to incubate 200 µg of protein.

Quantitative real-time PCR used a BIO-RAD (Hercules, CA) MyiQ Cycler with the Platinum® SYBR® Green qPCR SuperMix-UDG from Invitrogen (Karlsruhe,

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Germany) for amplification. Chromosomal DNA from a wild-type strain was used as external standard for setting up the calibration curve. Primers which amplified Fragment **a** (see Figure 4B) had the following sequence: prnB+2for: 5´TGAGGATCCCATTAGTCAAGG3´ and prnB+2rev: 5´GGATCAGGTTCCCTAAGATCAG3´. PCR was calibrated by a dilution series of total DNA extracted from a wild-type strain (3.3 µg μl^{-1}). For each sample the absolute amount of the specific DNA fragment in the immunoprecipitated sample was divided by the amount of this fragment in the sample before precipitation. Each PCR reaction was replicated (technical repetition). The resulting ratio of the precipitation with antibody H3-acetyl was divided by the resulting ratio of the antibody H3 C-term precipitation and the resulting ratio of the WT strain treated for non inducing conditions was set to one. Two biological repetitions were performed for each condition and standard deviations were calculated.

Results

Phenotypes of the *gcnE* and *adaB* deletions

Both the *gcnE* and *adaB* deletion strains show a strongly reduced growth rate and conidiation on solid medium. Both deletions resulted in similar morphological alterations of the conidiophore. Supplementary Figure S1 shows a selection of pictures obtained from microscopic observations of the *adaB* deletion strain. The strain produces stalks and vesicles, but metulae are missing. Short stalks with vesicle heads and a few phialides repeatedly emerge directly from vegetative mycelia. None of the known conidiation mutants of *A. nidulans* displays this phenotype.

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We tested for specific effects of the *gcnEΔ* and *adaBΔ* strains on the utilization of various carbon and nitrogen sources. Compared to the isogenic wild type strains, both deletion strains displayed a reduced growth on all the nutrients tested, including complete media and proline as sole carbon or nitrogen source (data not shown).

prnD-prnB expression is partially de-repressed in _gcnEΔ_ and _adaBΔ_.

García and co-workers (2004) have shown that expression of proline catabolic genes and chromatin rearrangements in the *prnD-prnB* intergenic region are affected by trichostatin-A mediated inhibition of deacetylases. Thus we investigated expression of *prnB* and *prnD* in a *gcnEΔ* and *adaBΔ* background. Figure 1B shows Northern blots from isogenic *gcnE*$^{+}$ and *gcnEΔ* (right panel) as well as isogenic *adaB*$^{+}$ and *adaB*Δ strains (left panel) grown under non-inducing (NI, fructose, urea), inducing (I, proline) and inducing-repressing (IR, proline, glucose, ammonium) conditions. The results of deleting *gcnE* or *adaB* on the expression of *prnB* and *prnD* are surprising. There is no effect on the non-induced (undetectable) and induced levels. However, a partial de-repression of *prnB* and *prnD* is seen in both deletions strains. Previous work (10) has shown that only the repression of *prnB* is direct, the repression of *prnD* being the result of inducer exclusion. The latter easily accounts for the fact that the mild de-repression of *prnB* results in an elevated de-repression of *prnD*, particularly pronounced in the *gcnEΔ* strain. Interestingly, neither deletion significantly alters the induced levels of *prnB* or *prnD* transcripts, suggesting that the induction process could be independent of the histone acetylation status.

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Chromatin organisation in the *prnB-prnD* promoter is affected in SAGA/ADA component deletion strains.

Figure 2A shows the results of a micrococcal nuclease (MNase) digestion assay for *adaB*$^{+}$ and *adaB*Δ under the three standard conditions investigated. The results of MNase digestions in the *gcnEΔ* strain (not shown) are identical to those obtained for the *adaB* deletion. In both deletion strains, the only difference to the corresponding isogenic wild type control in the nuclease digestion pattern is observed under induced-repressed conditions (IR). In an *adaB*$^{+}$ *gcnE*$^{+}$ strain all nucleosomes are re-positioned under IR conditions (12), whereas in the *adaB*Δ and *gcnEΔ* strains, this re-positioning is defective. In the deletion strains the IR nucleosome pattern is identical to that seen under induced conditions which is also identical to the pattern obtained under induced conditions in the wild type strain. Hence, the derepressed expression of *prnB* and *prnD* observed in the *adaB* and *gcnE* deleted strains is associated with and could depend on defective nucleosome re-positioning. The SC1 probe shown here allows the detection of the region including from nucleosome -4 to nucleosome +2. The probe detecting the region including nucleosomes +3 and +4 (probe SC2) did not reveal any differences between the deletion strains and the corresponding wild type strains (data not shown). A graphical representation of nucleosome positioning in the *prnB-prnD* promoter region is shown in Figure 2B. Nucleosome positioning in the *adaB*Δ strain under IR conditions and a de-repressed phenotype for *prnB* and *prnD* expression closely resembles results obtained when the de-acetylase inhibitor trichostatin A is used in the wild type or when MNase patterns are analysed in a *creA*^{d}1 strain (10). We thus used ChIP to determine the acetylation status of histone H3 in the region of nucleosome +2, the only

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nucleosome that remains positioned under these conditions in the *adaB*Δ and *gcnEΔ* strains.

The acetylation status of histone H3 in the region of nucleosome +2 depends on physiological conditions and on the function of GcnE and AdaB.

We chose to look at the region of nucleosome +2, as MNase digests indicate that a nucleosome is present in this region under all conditions, whether completely positioned (non induced conditions) or partially positioned (induced and induced repressed conditions) (12). The acetylation status of lysines 9 (H3K9) and 14 (H3K14) in histone H3 is a critical mark for gene activation. To investigate the acetylation status of H3K9 and H3K14 in nucleosome +2 we employed chromatin immunoprecipitation (ChIP) assays using an antibody recognising both H3K9ac and H3K14ac followed by amplification of a fragment encompassing the region covered by nucleosome +2 (fragment **a** in Figure 4B). The ratio between acetylated and total H3 (precipitated with an antibody directed against a H3 C-terminal epitope) is shown in Figure 3. Both acetylation patterns in *adaBΔ* and *gcnEΔ* strains are very similar, but, in contrast to the MNase digests, they are not identical and are therefore presented here for both strains.

In the *adaB*$^+$ (wild type) strain, isogenic to the *adaBΔ* strain, acetylation of K9 and K14 in H3 increases with induction indicating a correlation between H3 acetylation, chromatin rearrangements (loss of positioning) and transcriptional activation. Upon repression, the acetylation status of H3K9 and H3K14 does not change significantly, which indicates that de-acetylation of these residues in H3 is not required to trigger gene repression and re-positioning of nucleosome +2. In contrast to *adaB*$^+$, in the *adaB*Δ strain, acetylation of H3K9 and K14 is strongly reduced under all conditions and despite

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considerable induction of *prnB* and *prnD* transcription, an increase in acetylation of H3 is not seen in these strains. A qualitatively very similar pattern is observed when the *gcnE*$^{+}$ (wild type) control strain is compared to the isogenic *gcnEΔ* strain under the different growth conditions. As in the *adaBΔ* strain, acetylation of H3K9 and K14 is strongly reduced in the *gcnE* deletion strain under all conditions.

Discussion

Often, enhanced gene expression correlates with loss of positioning and increased acetylation of nucleosomes (33). In the *prnD-prnB* bidirectional promoter, we see increased acetylation of lysines 9 and 14 in histone H3 under inducing conditions in the region corresponding to partially positioned nucleosome +2. However, this increased acetylation is not required for efficient transcription or chromatin remodelling. In the strains lacking essential components of the putative SAGA/ADA complexes, i.e. GcnE or AdaB, we detect only low levels of H3 lysine acetylation, but induction and remodelling of chromatin are not affected. This is not without precedence as in *S. cerevisiæ*, SAGA/ADA-dependent and independent activators are known. Activation of transcription requires SAGA or ADA complexes in the GAL1 promoter where GAL4 recruits the SAGA complex, and this in turn is necessary for the recruitment of the mediator complex (20). Our results indicate that, while a putative SAGA or ADA complex might be involved in determining the acetylation levels in this region, PrnA-mediated activation and chromatin remodelling of the *prnD-prnB* promoter does not depend on AdaB and GcnE, putative members of *A. nidulans* SAGA/ADA complexes.

It should be noted that in our ChIP experiments we have only tested histone H3 acetylation at lysines 9 and 14, residues known to be acetylated by SAGA and ADA

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complexes (9). Other covalent modifications not detected by the antibody used here, such as acetylations of H2A, H2B and H4 at diverse lysines in their N-termini could also lead to hyperacetylation of nucleosomes in the *prnD-prnB* promoter thus providing the signal for effective chromatin remodelling. In filamentous fungi, it is completely unknown which mechanisms direct the cross-talk of histone modification. In a study focussing on the different roles of H3 and H4 acetylation in the yeast *Saccharomyces cerevisiae,* it was shown that H4 hyperacetylation in promoters of Adr1p-dependent genes does not compensate for low H3 acetylation in a *gcn5* mutant strain (1).

The observed partial de-repression of *prnB* and *prnD* expression in the *adaB* and *gcnE* deleted strains was unexpected. However, in *Neurospora crassa*, mutation of the GCN5 homologue *ngf-1* results in loss of repression of the blue light-responsive *al-3* gene in the dark (18). A *ngf-1*^{RIP1} mutant was found to express *al-3* constitutively suggesting that the histone acetyltransferase is not only involved in blue-light induction but also in repression of *al-3* in the absence of light. Another example of HAT activities being involved in both, gene activation and repression is provided by the yeast GAL system. Glucose repression requires ADA2p and ADA3p/NGG1p, an ADA-complex protein functionally associated with GCNp5 and ADA2p (6) and it has been suggested that ADA2p/ADA3p directly inhibits the activation domain of GAL4p. Similarly, in the yeast arginine metabolic system GCN5 function and increased histone acetylation are associated with transcriptional repression. In this case, Gcn5p-mediated increase of H3 acetylation correlates with binding of the arginine repressor complex ARGR/MCM1 (31).

However, our nucleosome positioning and ChIP studies lead to a different picture. Although histone H3 acetylation increases during transcriptional activation, it is neither

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required for transcriptional activation (compare induced conditions in *gcnE*$^{+}$/*adaB*$^{+}$ with *gcnEΔ* or *adaBΔ* in Figures 1 and 3) nor loss of nucleosome positioning (compare Figure 2). Additionally, transcriptional repression and re-positioning of nucleosomes are not accompanied by de-acetylation of histone H3 at K9 or K14 (see Figure 3, IR conditions). These results suggest that differences in histone acetylation are associated with, but not necessary for transcriptional activation and nucleosome positioning processes in the *prnD-prnB* intergenic region. It was therefore surprising to see that both, hyper-acetylation by TSA inhibition of deacetylases and hypoacetylation by deletion of *adaB* or *gcnE* leads to partial de-repression of the proline catabolic genes and simultaneous lack of nucleosome re-positioning.

Transcriptional repression and nucleosome re-positioning upon simultaneous glucose and ammonium repression requires a functional CreA protein. This has been shown by mutations in both the CreA protein itself (*creA*^{d}1) and in its cognate binding sites (*prn*^{d}20 or *prn*^{d}22) in the *prnD-prnB* promoter (12). The simplest hypothesis integrating our experimental results would therefore propose that both, hyperacetylation by TSA treatment and hypoacetylation by *adaB* or *gcnE* deletion impair CreA function. Several observations support this view. Firstly, nucleosomal patterns obtained in the *prnD-prnB* region by TSA treatment and in *adaBΔ* and *gcnEΔ* under IR conditions are identical to those found in *creA*^{d}1, *prn*^{d}20 or *prn*^{d}22 mutant strains (13). Secondly, de-repression under TSA treatment or in *adaBΔ* and *gcnEΔ* is only partial whereas de-repression is complete in *creA* loss-of-function strains. This also implies that while nucleosome re-positioning may be necessary for full repression, CreA can still partially repress on completely open chromatin, probably by directly interfering with the activating function of the pathway-specific activator PrnA.

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Thirdly, the function of CreA, but not the acetylation status of histones, defines whether nucleosomes are positioned or not. In summary, nucleosomal rearrangements, transcriptional activity and histone acetylation are clearly distinct processes in the *prnD-prnB* region and therefore a direct influence of the acetylation status on nucleosomal positioning and transcription is highly unlikely. Further studies will reveal at which level, transcriptional and/or post-transcriptional, CreA function is impaired by TSA treatment or lack of histone acetyl transferase activities.

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1 Table 1: *Aspergillus nidulans* strains used throughout this work

Strain	**Genotype**	**Reference**
gcnE^{+}	*pyrG*89 *argB*2 *pantoB*100 *riboB*2 *yA*2	This work
*gcnE*Δ	*pyrG*89 *argB*2 *pantoB*100 *riboB*2 *yA*2 *gcnE*Δ::*pyrG*$^{+}$	This work
a*daB*$^{+}$	*bi A1*	FGSC[1] strain *#A26*
*adaB*Δ	*biA1 argB::trpCΔB* *adaBΔ::argB*$^{+}$	This work
MH 9233	*wA3* *biA1* *argB::trpCΔB* *pyroA4 riboB2*	(26)

2 [1]Fungal Genetics Stock Center: http://www.fgsc.net

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Figure Legends

Figure 1. Regulation of proline utilization in *A. nidulans*. **A.** Overview of proline cluster regulation. The *prnD-B* intergenic region is shown. *prnD* encodes the proline oxidase, and *prnB* encodes the specific proline transporter (12, 14). The pathway-specific transcription factor PrnA is essential for proline induction of both genes. In the absence of preferential carbon (glucose) and nitrogen (ammonium) sources and the presence of proline, PrnA and the GATA factor AreA bind to their cognate sites in the intergenic region resulting in the expression of *prnD* and *prnB*. Repression requires both glucose activation of the negative regulator CreA and ammonium inactivation of AreA. Full repression occurs only in the simultaneous presence of glucose and ammonium. Repression acts directly on *prnB* expression, *prnD* repression is indirect and results from inducer exclusion. Activation processes are represented by arrows and repression by terminals.

B. Effect of *adaB* and *gcnE* deletion on *prnB* and *prnD* transcription. Strains (see Table 1) were pre-grown in liquid minimal medium under non-inducing conditions (5 mM urea, 0,1 % fructose) with the appropriate supplements, harvested, divided into aliquots und further incubated for two hours under conditions indicated. NI, 5 mM urea and 0.1% fructose; I, induced by 20mM proline, IR, 20 mM proline and repression by 1% glucose and 20 mM ammonium-L(+)-tartrate (IR). Expression levels (bottom) were quantified using phosphoimaging and ImageQuant software analysis. Normalised signals were obtained by comparison of specific signals with actin gene (*acnA*) expression signals. The induced level in the $adaB^{+}$ and $gcnE^{+}$ strains is given in every

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case the arbitrary value of 100, filled columns represent *prnB* and open columns *prnD* expression.

Figure 2. Nucleosome positioning in the *prnD-prnB* intergenic region in *adaB*$^+$ and *adaBΔ* strains. **A**. Indirect end-labelling micrococcal nuclease I (MNaseI) analysis of the *prnD-prnB* promoter region using probe SC1. This probe reveals nucleosomes +2 to -4. MNase analysis of nucleosomes +3 and +4 are not shown as the pattern obtained for the mutant strains are identical to the one extant in the wild type. Growth conditions (NI, I, IR) were identical to those described in Figure 3. Numbers besides the autoradiograms correspond to the positions of the main cuts relative to the *prnD* ATG. These were calculated from molecular size markers run in every gel. Asterisks indicate the position of the relevant changes observed. Triangles indicate increasing concentration of MNaseI. nDNA, naked DNA. To the left of the *adaBΔ* NI lanes a schematic representation of the nucleosome structure is shown. The wild type pattern under all conditions have been previously published (12), we only include in the same gel, for comparison, the pattern obtained under IR conditions, the only one where an *adaB* deletion pattern differs from that of the wild type

B. Schematic representation of nucleosome positioning (based on figure 2A and reference (12)). All eight nucleosomes of the intergenic region are drawn. Arrows indicate MNase I digests. Their thickness indicates the relative intensity of the bands in the autoradiogram shown in figure 2A and in other auoradiograms (not shown) covering nucleosomes +3 and +4. Dashed arrows indicate weakly cut sites. Under non-induced (NI) and induced (I) conditions, the nucleosome patterns are identical in strain *adaB*$^+$ and *adaBΔ*. White ovals represent fully positioned nucleosomes, while partially

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positioned nucleosomes are shown by diagonally hatched ovals. The interpretation and significance of this partial positioning is discussed in the text and in references (12, 25). Symbols: white lozenges, CreA-binding sites 3.1 and 3.2, which are mutated in the *prn*^{d}20 and *prn*^{d}22 strains and result in derepression (see text); grey ovals, AreA-binding sites 13 and14, shown to be the physiologically relevant AreA binding sites (15); white triangles, high-affinity PrnA binding sites 2 and 3 (14); black triangle, TATA box (15); The fragment amplified in the ChIP analysis (**a**) is shown as solid line below nucleosome +2. nDNA, pattern obtained with naked DNA, to the right of the scheme we indicate to which strain(s) a given nucleosome pattern correspond.

Figure 3. Chromatin immunoprecipitation (ChIP) assay comparing acetylated H3 K9 and K14 in nucleosome +2 of the *prnD-prnB* intergenic region between *adaB*$^{+}$ and *adaBΔ* (panel A) and between *gcnE*$^{+}$ and *gcnEΔ* (panel B) strains (see Table 1 for genotype details). The ratio between total H3 (C-terminal epitope) and H3-acetyl (recognizing H3 K9/K14) in the wild type strains (*adaB*$^{+}$ or *gcnE*$^{+}$) grown under non-inducing conditions (NI) was set to 1. Error bars are indicating the standard deviation of four experiments (two biological and two technical repetitions per condition). Growth conditions: NI, non-inducing; I, inducing; IR, simultaneously inducing-repressing.

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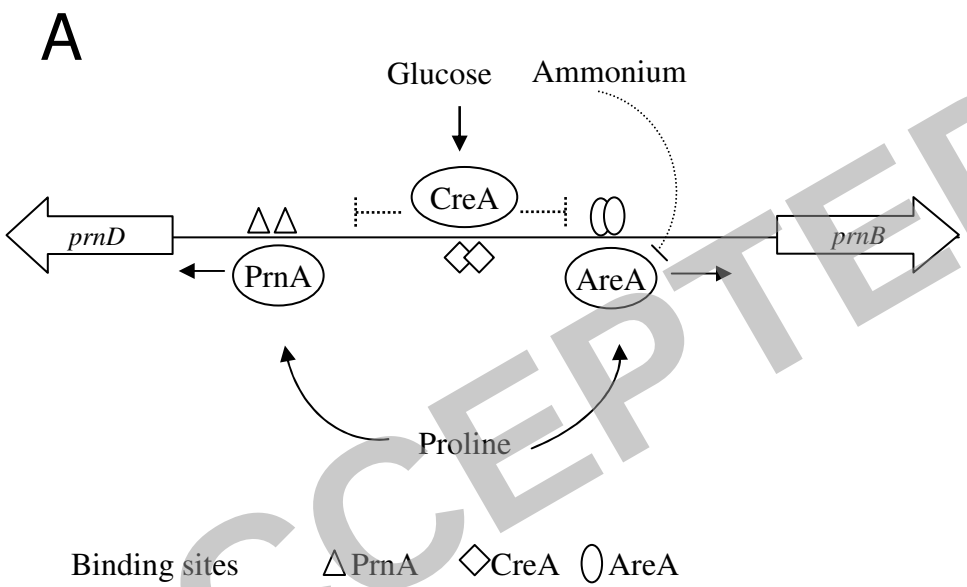

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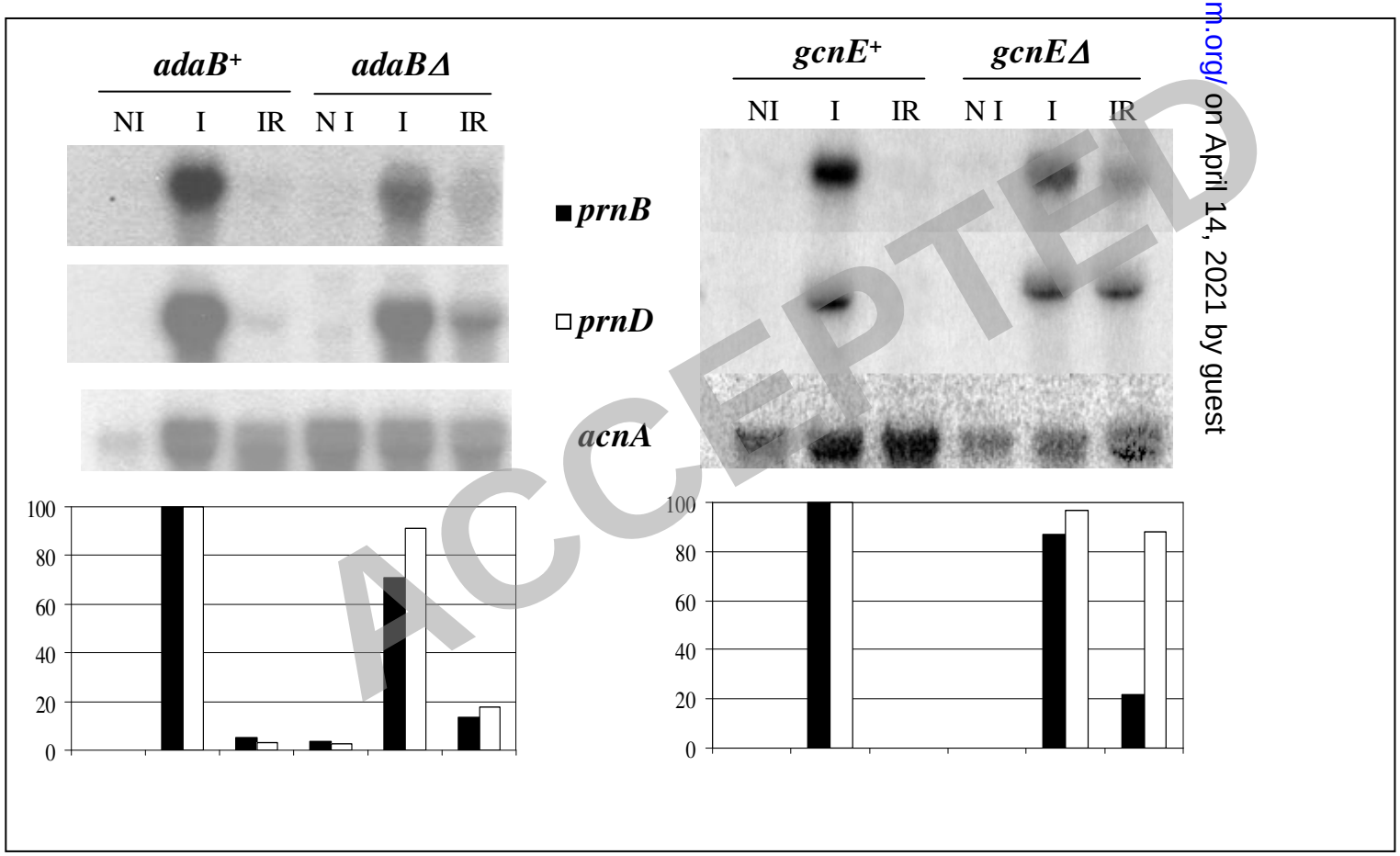

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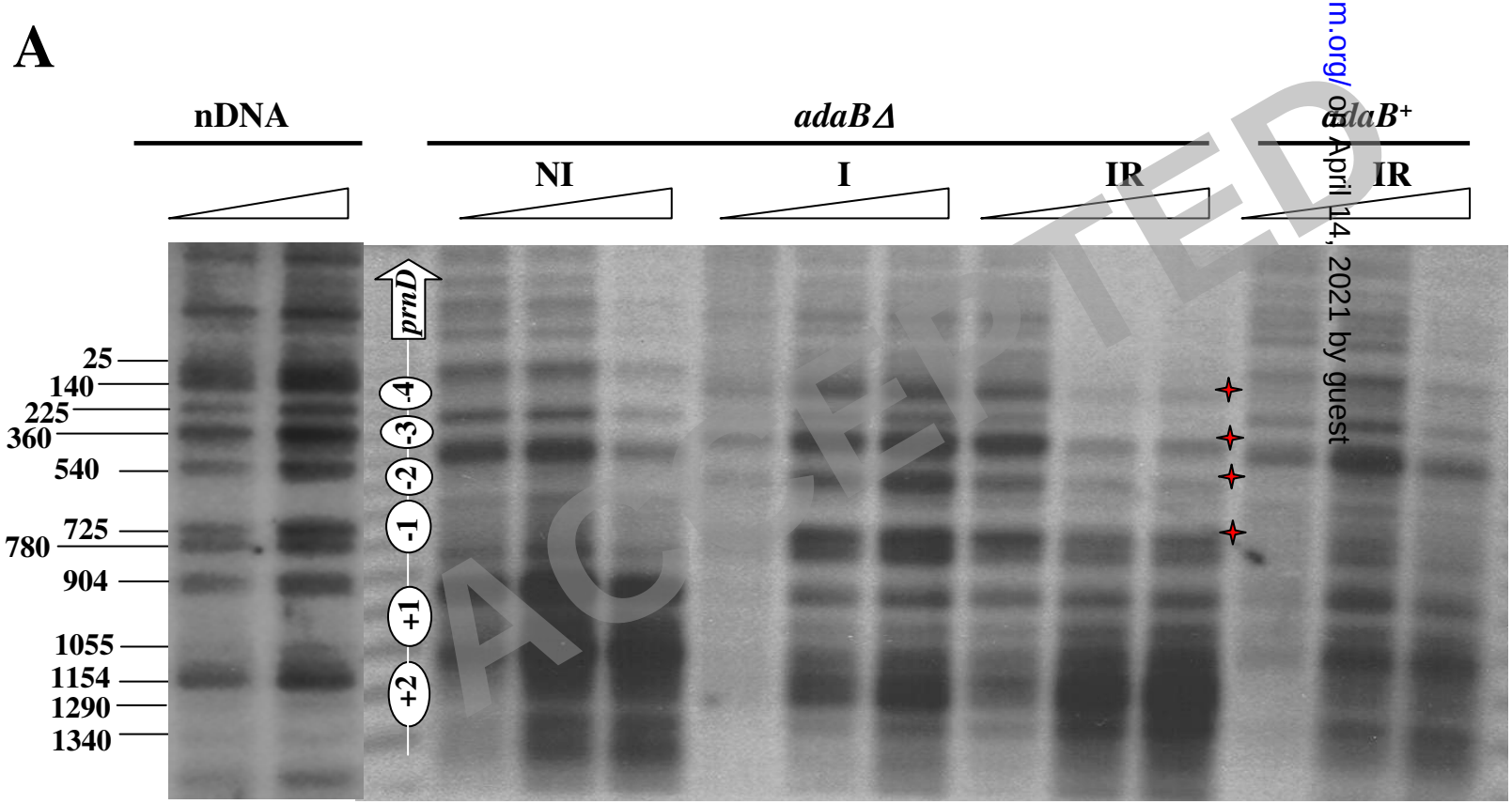

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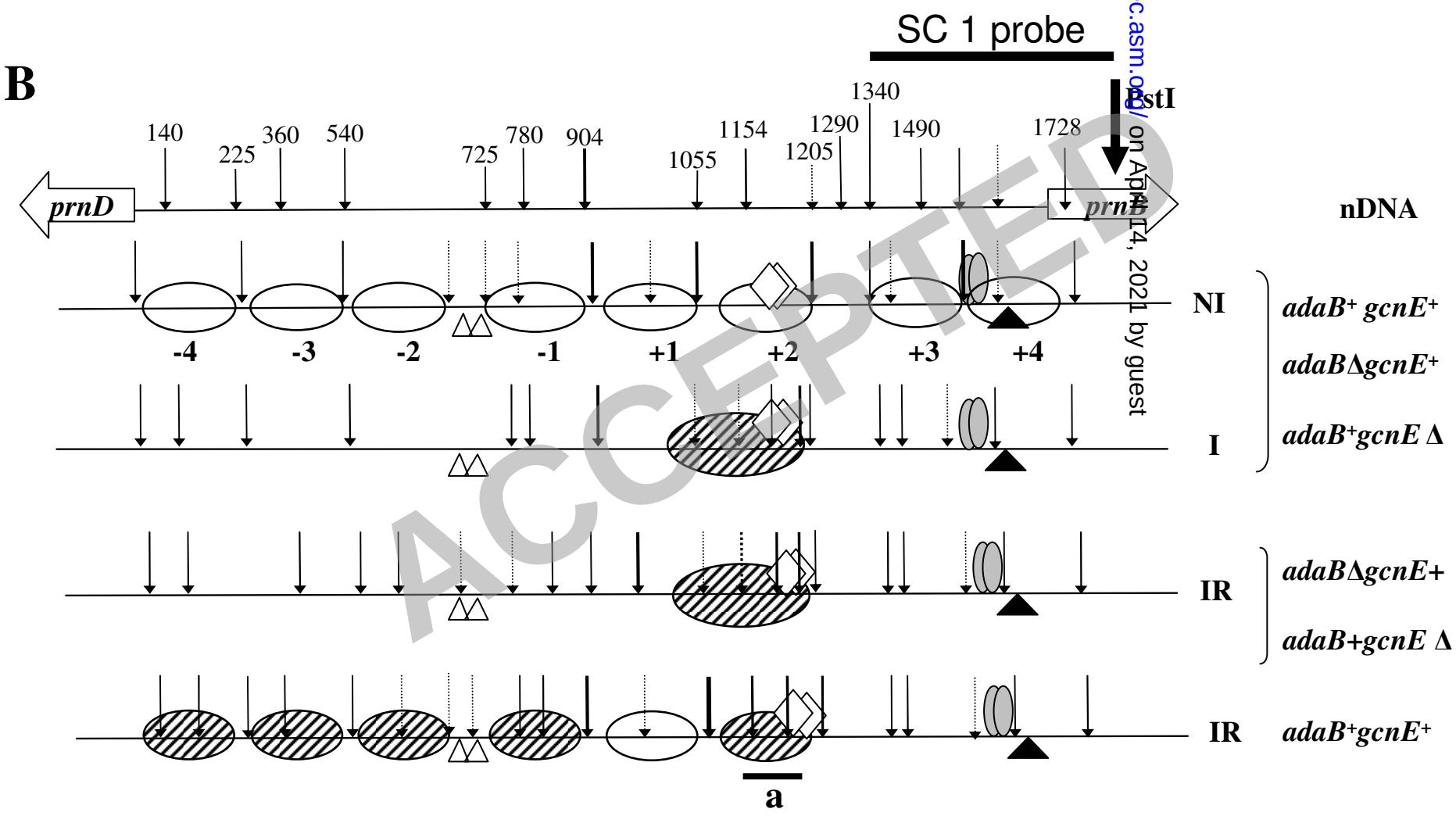

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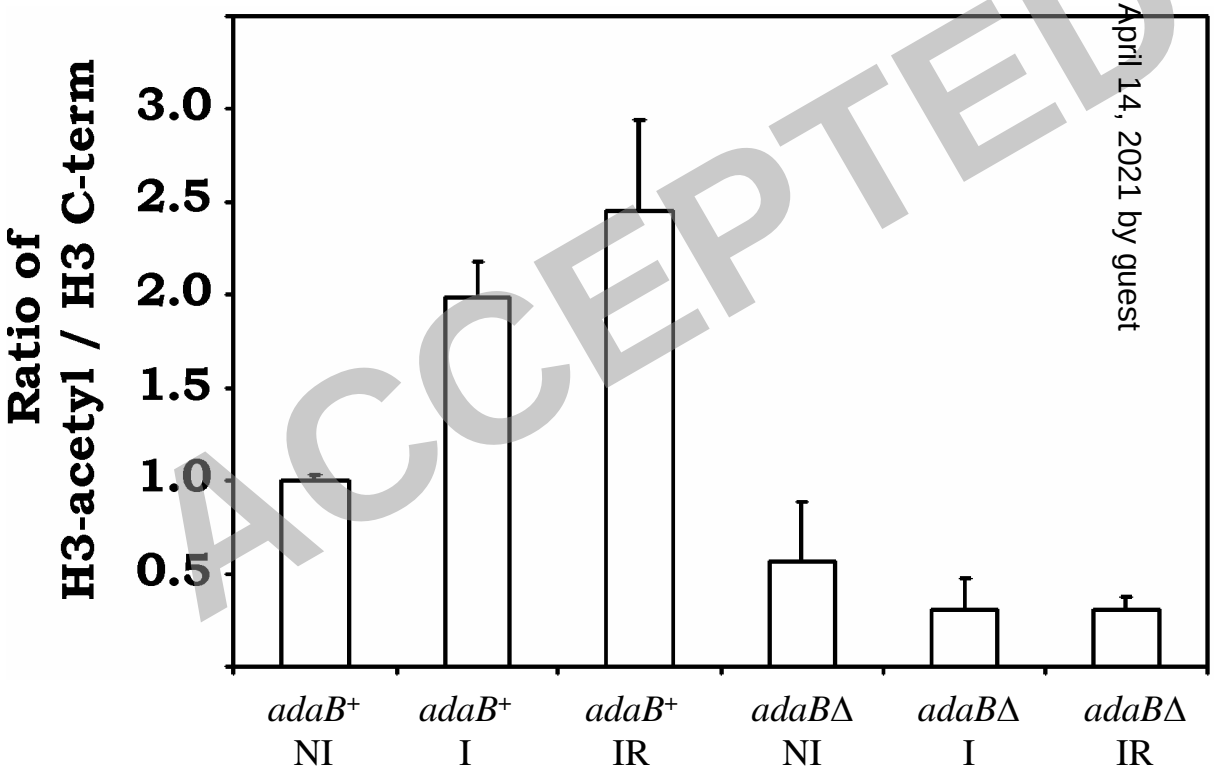

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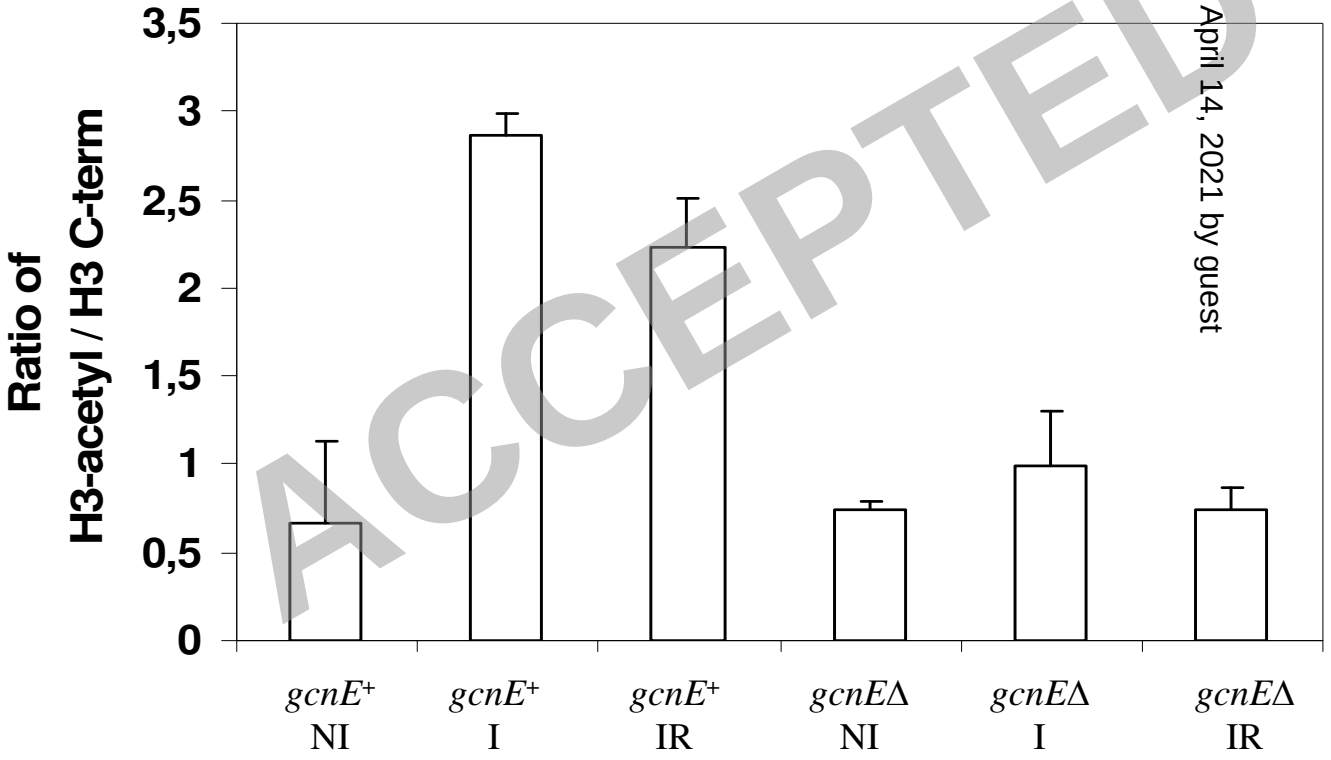

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