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Chromatin Rearrangements in the *prnD-prnB* Bidirectional Promoter: Dependence on Transcription Factors

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The *prnD-prnB* intergenic region regulates the divergent transcription of the genes encoding proline oxidase and the major proline transporter. Eight nucleosomes are positioned in this region. Upon induction, the positioning of these nucleosomes is lost. This process depends on the specific transcriptional activator PrnA but not on the general GATA factor AreA. Induction of *prnB* but not *prnD* can be elicited by amino acid starvation. A specific nucleosomal pattern in the *prnB* proximal region is associated with this process. Under conditions of induction by proline, metabolite repression depends on the presence of both repressing carbon (glucose) and nitrogen (ammonium) sources. Under these repressing conditions, partial nucleosomal positioning is observed. This depends on the CreA repressor's binding to two specific *cis*-acting sites. Three conditions (induction by the defective PrnA80 protein, induction by amino acid starvation, and induction in the presence of an activated CreA) result in similar low transcriptional activation. Each results in a different nucleosome pattern, which argues strongly for a specific effect of each signal on nucleosome positioning. Experiments with trichostatin A suggest that both default nucleosome positioning and partial positioning under induced-repressed conditions depend on deacetylated histones.

In simple eukaryotes, some genes are transcribed divergently from a common bidirectional promoter. Well-studied examples are the *GAL1-GAL10* promoter of *Saccharomyces cerevisiæ* (reviewed in references 10 and 35) and the *niiA-niaD* promoter of *Aspergillus nidulans* (37, 41, 52). Here we analyzed the nucleosome rearrangements of the bidirectional *prnD-prnB* intergenic region. This is a 1.7-kb region located between the gene coding for proline oxidase (*prnD*) and the one coding for the major, specific proline transporter (*prnB*) (29, 50). These genes are located in the *prn* gene cluster in the right arm of chromosome VII (Fig. 1). The regulation of *prnD* and *prnB* involves a multiplicity of metabolic signals. The pathway-specific transcription factor PrnA is essential for proline induction of both genes (11, 21, 22, 46). The *prnD-prnB* intergenic region is a genuine bidirectional promoter, as mutations in the two PrnA binding sites present in this region affect the transcription of both *prnD* and *prnB* (21; I. García, D. Gómez, and C. Scazzocchio, unpublished results).

Transcription of *prnB* but not of *prnD* can also be induced by amino acid starvation. This effect depends on the integrity of a canonical GCN4 binding site in the proximity of the *prnB* TATA box (56).

Repression of both *prnB* and *prnD* occurs only when both carbon (glucose) and nitrogen-repressing (ammonium) sources are present simultaneously (2, 5, 6, 21, 22). Repression acts directly on *prnB* expression, while repression of *prnD* is indirect and results from inducer exclusion (5, 16, 22). Repression necessitates both the activation of the negative regulator CreA and the inactivation of the GATA factor AreA. A model to account for this pattern of repression has been published previously (23). Figure 1 shows the *prnD-prnB* intergenic region with the *cis*-acting sites that have been shown to be physiologically relevant.

In this article we show that eight nucleosomes are positioned in the *prnD-prnB* region. Upon induction, these nucleosomes are no longer positioned, while a single nucleosome is partially positioned at a new location. In conditions of simultaneous carbon and nitrogen metabolite repression in the presence of inducer, a partial repositioning of nucleosomes occurs. We analyze in detail the role of the three transcription factors involved in *prnD-prnB* regulation, PrnA, CreA, and AreA, in this process.

MATERIALS AND METHODS

Strains. A *pabaA1* strain was used as the wild type. *creA* loss-of-function strains were *creA^{d}1 pabaA1* (48) and *creA^{d}25 pabaA1* (4). The *areA* loss-of-function strain was *areA600 biA1 sB43*. *areA600* is an early chain termination null mutation (1, 30). The *prnA* loss-of-function mutations analyzed in this work are listed in Table 1. Strains used were *prnA404 pabaA1*, *prnA15 cnxJ1 pabaA1 fwA1*, *prnA80 pabaA1*, *prnA407 pabaA1 fwA*, and *prnA442 pabaA1 alcR125 fwA1*. *alcR125* is an *alcR* loss of function (43). For definitions of the standard genetic markers, see the Glasgow Stock List at http://www.gla.ac.uk/Acad/IBLS/molgen/aspergillus/strintro.html.

Sequences of new *prnA* alleles. The sequence changes of a number of *prnA* alleles were determined (Table 1). The approximate position in the gene in relation to deletion mutations was known (11, 46). Thus, the appropriate sequence was amplified by PCR with specific primers and the newly introduced changes were checked by sequencing (automatic sequencing; MWG Biotech, Ebersberg, Germany).

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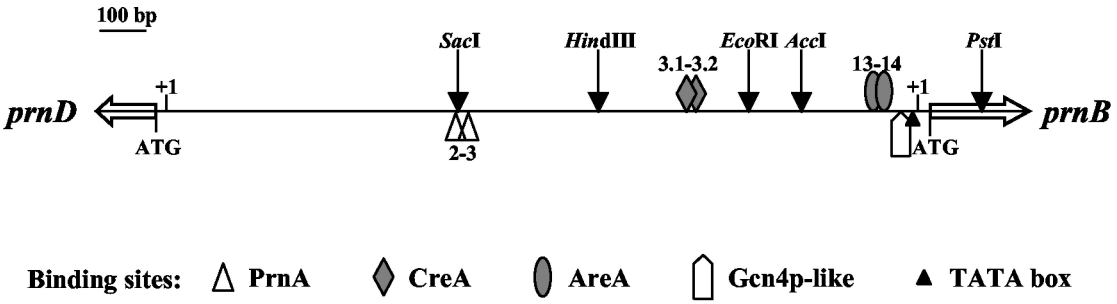

FIG. 1. *prnD-prnB* intergenic region. *prnD* encodes proline oxidase, and *prnB* encodes the specific proline transporter (20, 28, 29, 50). The CreA-binding sites 3.1 and 3.2 (grey lozenges) are essential for *prnB* and indirectly for *prnD* repression (51, 15, 16). The AreA-binding sites 13 and 14 (grey ovals) are necessary to set the maximal level of transcription of *prnB* and to integrate carbon and nitrogen metabolite repression of this gene (24). High-affinity PrnA binding sites 2 and 3 are shown by white triangles; their occupancy upon induction by PrnA in vivo has been demonstrated (23). A putative binding site for a GCN4-like factor is shown as a thick arrow (56) A black triangle indicates the *prnB* TATA box (24). The positions of the transcriptional start points (+1) and of the ATG of *prnD* and *prnB* are shown (20, 50; S. Demais and C. Scazzocchio, unpublished results). Relevant restriction sites mentioned in the text are also shown.

Growth conditions. A total of 10^6 spores of each strain per ml were inoculated at 37°C into liquid minimal medium with the appropriate supplements plus 0.1% fructose as the carbon source and 5 mM urea as the nitrogen source, except for the *areA600* strain. Mycelia were grown for 8 h at 37°C and then incubated for 2 additional hours at 37°C or repressed with glucose (1%) and ammonium (20 mM ammonium-L[+]-tartrate), or induced with 20 mM L-proline, or induced with 20 mM L-proline and simultaneously either carbon repressed (1% glucose), nitrogen repressed (20 mM ammonium-L[+]-tartrate), or carbon and nitrogen repressed and incubated 2 h at 37°C. The *areA600* strain was grown at 37°C in liquid minimal medium with the appropriate supplements plus 1% glucose and 5 mM ammonium-L(+)-tartrate for 7 h at 37°C, and then cultures were filtered and shifted to minimal medium containing the appropriate supplements and neutral carbon and nitrogen sources (5 mM urea and 0.1% fructose) without or with 20 mM proline and incubated for 2 additional hours at 37°C. An *areA*$^+$ strain was grown in parallel in the same culture conditions. Mycelia were harvested by filtration through sterile Blutex tissue, washed with sterile distilled water, and frozen in liquid nitrogen.

RNA preparation and Northern blots. Total RNA was isolated with the RNA Plus Extraction Solution (Biogen) following the manufacturer's instructions. RNA electrophoresis and Northern blot hybridizations were carried out as described previously (22, 23). *prnB*, *prnD*, and *acnA* probes were prepared as described by Gómez et al. (22).

Nucleosome positioning. Micrococcal nuclease I digestions were performed by the method adapted by Gonzalez and Scazzocchio (24). 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: *Pst*I (SC1 hybridization) or *Hin*dIII (SC2 hybridization). Probe SC1 is the 389-bp *Pst*I-*Acc*I fragment of the *prnD-prnB* intergenic region (24). Probe SC2 is the 332-bp *Eco*RI-*Hin*dIII fragment of the *prnD-prnB* intergenic region (23).

For the restriction enzyme protection assay, a method adapted from Gregory et al. (25) was used (M. Mathieu, personal communication). Probe HP is the 836-bp *Hin*dIII-*Pst*I fragment of bAN926, which contains a 907-bp fragment of the *prnD* open reading frame, the *prnD–prnB* intergenic region, and 1,512 bp of the *prnB* open reading frame (23). At least three independent experiments were carried out for each mutant and/or condition with identical results. In every case, induction and repression were checked by Northern blots made in parallel with the same mycelia.

In vivo footprinting. Binding of PrnA to sites PrnA-2 and PrnA-3 was detected by in vivo footprinting as described by Gómez et al. (23) following the technique of Wolschek et al. (59).

TABLE 1. Sequence changes of the *prnA* mutations used in this work

Mutation	Nucleotide sequence change[a]	Amino acid sequence change[a]	Reference
prnA15	T(2209)C	L(621)P	This work
prnA80	G(2319)C	A(658)P	This work
prnA407	C(2101)A	S585Y	11
prnA442	G(2744)T	AMB(819)Y+18-residue extension	This work
prnA469	Insertion of G(1911) and deletion of C(1987)	Substitution of residues 522–547	11
prnA404	Deletion of 67–1510	Deletion of residues 23–398	11, 12

[a] Numbering of nucleotides and amino acids is that of Cazelle et al. (11).

RESULTS

Chromatin rearrangements in the *prnD-prnB* bidirectional promoter. Throughout this article we compare three growth conditions: noninduced, absence of proline in a medium which contains nonrepressing carbon and nitrogen sources (under these conditions expression of *prnD* and *prnB* is minimal and virtually undetectable in Northern blots); induced, the same but in the presence of proline; and induced-repressed, where proline is added together with repressing nitrogen and carbon sources. The simultaneous presence of these repressing metabolites strongly diminishes transcription, but it does not abolish it (22, 51). While the exact experimental conditions are given in Materials and Methods, it is important to keep in mind the conceptual differences between the three conditions. When other conditions are used, these are defined in the text.

Under noninduced conditions, eight nucleosomes are positioned in the *prnD-prnB* promoter. This pattern is identical, under noninducing, nonrepressing (noninduced, defined above, Fig. 2), or repressing conditions (glucose and ammonium in the absence of proline, not shown). Upon induction (under nonrepressing conditions), nucleosome positioning is lost (Fig. 2). Between positions 904 and 1205, the pattern of micrococcal nuclease I cuts is different from that of the naked DNA and indicates that a nucleosome is partially positioned between these boundaries. The nuclease cut at position 1055 is much weaker under induced than noninduced conditions, which indicates that this nucleosome is positioned differently from either nucleosome +1 or +2 under noninducing conditions. The pattern of micrococcal nuclease I digestion does not allow us to conclude whether a new nucleosome is positioned between the cutting sites at nucleotides 510 and 725. However, the presence of a *Sac*I restriction site in position 685 allowed us to investigate the positioning of this putative nucleosome. No

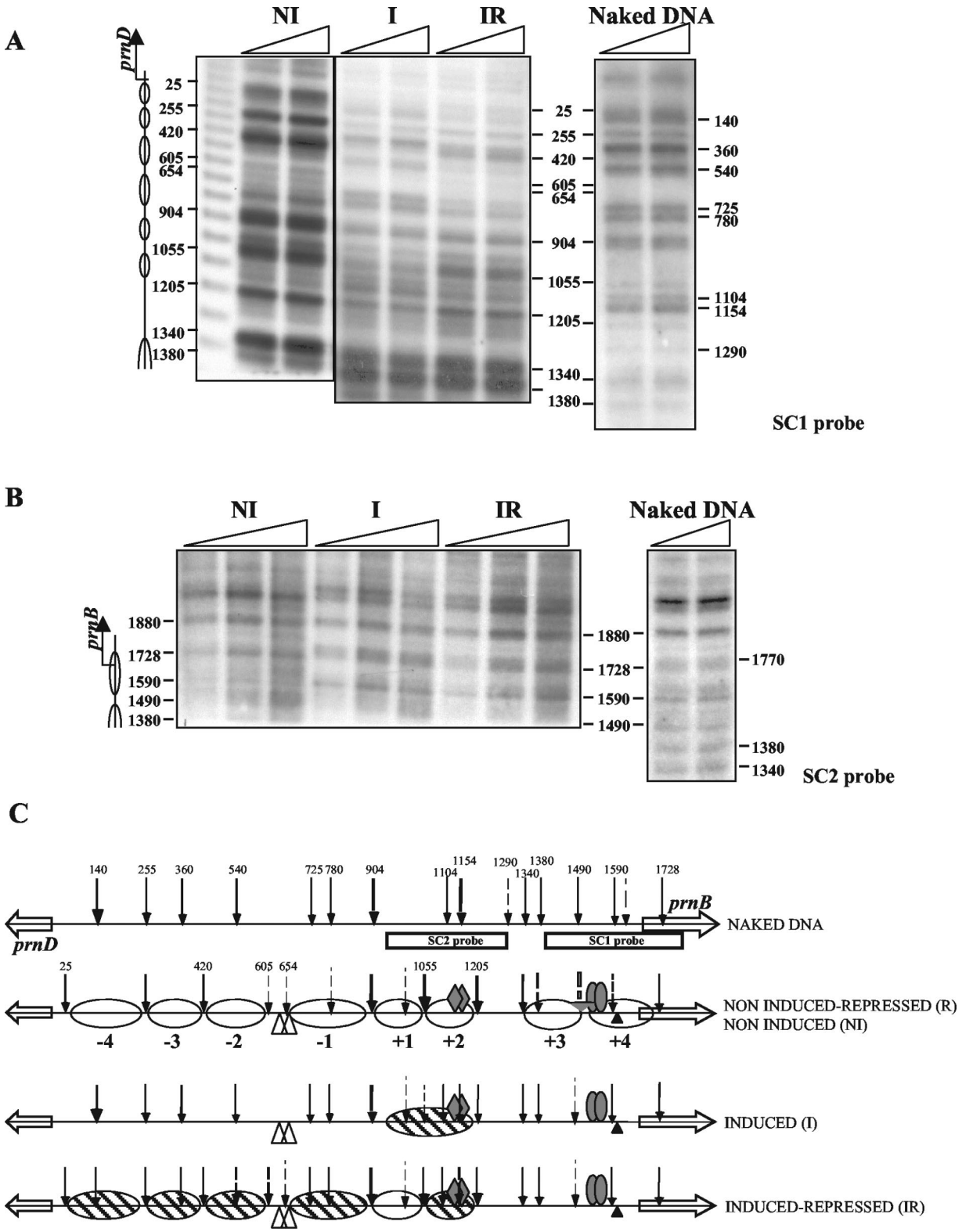

FIG. 2. Nucleosome positioning in the *prnD-prnB* intergenic region. Numbers besides the autoradiograms correspond to the positions of the main cuts relative to the *prnD* ATG. These were calculated from molecular size markers run in every gel. Three conditions are shown. NI, noninduced, mycelia grown in the absence of proline in the absence of glucose and ammonium; I, proline-induced; IR, proline-induced in the presence of glucose and ammonium. (A) Pattern obtained with the SC1 probe (revealing the *prnD* proximal pattern). (B) Pattern obtained with the SC2 probe (revealing the *prnB* proximal pattern). These patterns are partially overlapping. Triangles, increasing concentration of micrococcal nuclease I. (C) Schematic representation of nucleosome positioning. Arrows indicate micrococcal nuclease I cuts. Their thickness indicates the relative intensity of the bands in the autoradiogram. Dashed arrows indicate weakly cut sites. White ovals represent fully positioned nucleosomes, while partially positioned nucleosomes are shown by diagonally hatched ovals (see text). The positions and lengths of the probes used are also indicated. Other symbols are as in Fig. 1. Under noninduced, repressed conditions (R, see text), the nucleosome pattern is identical to the one shown in the figure for the noninduced conditions. See Materials and Methods for exact growth conditions.

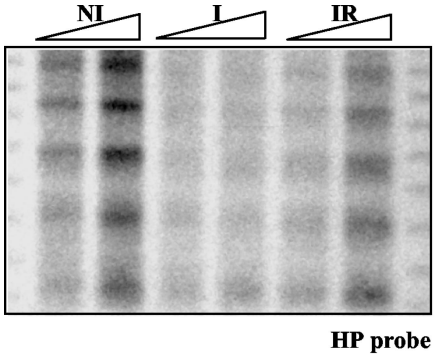

FIG. 3. Presence of nucleosomes in the *prnD-prnB* promoter, independent of positioning. We show here only the *prnB* proximal region (bp 998 to 1834 from the *prnD* ATG) NI, I, and IR are as in Fig. 2.

protection of this restriction site is seen, and thus induction does not result in the positioning of an additional nucleosome (not shown) between these boundaries.

Addition of either glucose or ammonium, which singly do not result in significant repression (22, 23), does not affect the destabilization of nucleosome positioning seen in induced cultures; the pattern is identical to that found under induced conditions in the absence of any repressing metabolite (results not shown). When both glucose and ammonium are added to an induced culture, nucleosome positioning is seen for nucleosomes +2 to −4. The pattern is, except for nucleosome +1, which is fully positioned, one of partial positioning (see above), while the *prnB* proximal nucleosomes, +3 and +4, are not positioned at all. By partial positioning we mean that the pattern observed is what would be generated by superimposing the fully positioned and the fully nonpositioned patterns. The possible significance of this finding will be discussed below.

Induction results in nucleosome delocalization, not in complete nucleosome loss. It is possible to differentiate between nucleosome delocalization and nucleosome loss if, rather than revealing micrococcal nuclease I cuts by indirect terminal labeling, one hybridizes a chromatin micrococcal nuclease I digest with a probe covering the whole region that is being analyzed. When this is done, the typical digestion ladder should be seen whether nucleosomes are positioned or not (J. L. Barra, personal communication).

The *prnD-prnB* intergenic region shows a typical nucleosome repeat under all induction and repression conditions, with a length of ≈160 bp, the length previously reported for *Aspergillus nidulans* (24, 36, 42). This is shown for the *prnB*-proximal region in Fig. 3. Note that under induction conditions the bands became fuzzy and that this effect it more pronounced the longer the polynucleosome is. This is exactly what is to be expected if in a given segment of DNA nucleosomes is present but not translationally positioned, the distribution of sizes obtained by digestion being broader the larger the number of nucleosomes. Bands are fuzzy in induced conditions, clear in noninduced conditions, and intermediate under induced-repressed conditions. The latter finding supports the data obtained (Fig. 2) with indirect terminal labeling, which corresponds to a pattern of partial positioning for the induced-repressed conditions (see Discussion). Other blots, hybridized with suitable probes, show the same pattern for the whole intergenic region (not shown). As this experiment does not distinguish individual nucleosomes, it cannot be excluded that some nucleosomes may be lost and some delocalized, nor can it be excluded that the population of nuclei be heterogeneous vis à vis these two possibilities.

PrnA is necessary for nucleosome delocalization upon induction. Figure 4 shows that in a deletion of *prnA*, all nucleosomes remain completely positioned upon induction. A number of *prnA* mutants outside the DNA binding domain have been characterized (11, 12, 46; this article). We investigated whether any of these mutants, unable to activate *prnD* and *prnB* transcription, as assessed by Northern blots, maintain the ability to delocalize nucleosomes. The sequence changes of these mutations are shown in Table 1. All mutations tested, with the exception of *prnA80*, are unable to elicit transcription and nucleosome delocalization (not shown). The binding of some PrnA mutant proteins to high-affinity sites 2 and 3 (23) was also investigated by in vivo methylation protection. The results are shown in Fig. 5. All mutations tested, with the exception of *prnA80*, resulted in inability to bind both PrnA sites 2 and 3. *prnA80* does not affect the binding to either site 2 or 3. The *prnA80* mutant was classified as cryosensitive in growth tests (46); it equally affects transcription of *prnD* at 37°C and at 25°C, while showing a cryosensitive phenotype for *prnB* transcription (Fig. 6A). At the level of chromatin, a *prnA80* strain behaves exactly like a *prnA*$^+$ strain: upon induction all nucleosomes are delocalized and a nucleosome is newly (and partially) positioned between nucleotides 904 and 1205 (Fig. 6B). The significance of these results will be discussed below.

Induction of *prnB* transcription by amino acid starvation results in loss of positioning of *prnB* proximal nucleosomes. The transcription of *prnB* (but not of *prnD*) can be elicited independently from proline induction by amino acid starvation, possibly mediated by a GCN4-like factor (56). This activation is lower, but noticeable, in a strain with *prnA* deleted (56). We used 3-amino-1,2,4-triazole, a competitive inhibitor of the histidine biosynthetic enzyme His3p, to induce amino acid starvation (as in reference 56). Under these conditions, the positioning of the *prnB* proximal nucleosomes +3 and +4 is lost (Fig. 7). All other nucleosomes remained positioned as they are under noninduced conditions. This chromatin rearrangement is identical in *prnA*$^+$ and *prnA404* strains.

AreA is not necessary for chromatin remodeling upon induction. The GATA factor AreA is necessary to achieve the maximal levels of transcription of *prnB* and *prnD* (22). We investigated here the role of AreA in nucleosome positioning upon induction. Under both inducing and noninducing conditions, the nucleosomal pattern is the same in *areA*$^+$ and *areA600* strains (Fig. 8).

CreA is essential for nucleosome positioning upon repression but is not involved in the establishment of the default chromatin structure. The *creA* loss-of-function mutant *creA*^{d}*1*, which bears a point mutation in the DNA binding domain (48), results in complete derepression of *prnB* and *prnD* (Fig. 9A). Figure 9B shows that the nucleosome positioning associated with repression depends strictly on CreA. The pattern of nucleosome positioning in *creA* mutant strains is the same under induced nonrepressed and induced-repressed conditions and identical to the wild-type pattern obtained under induced con-

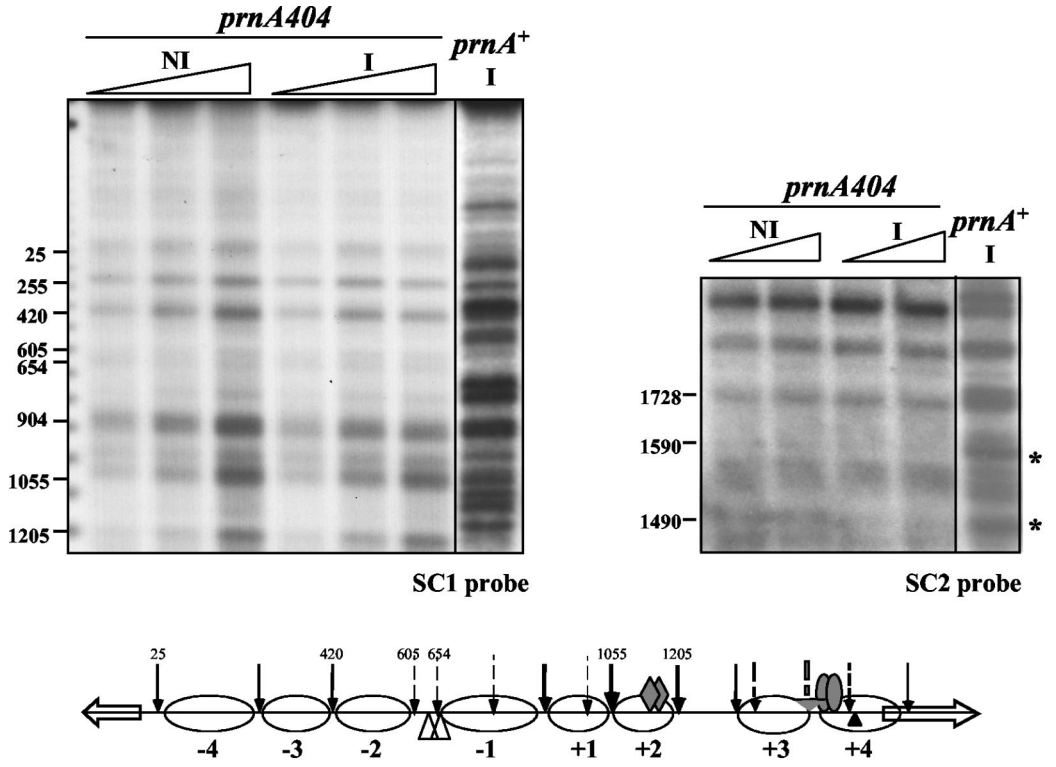

FIG. 4. Nucleosome positioning in a *prnA* deletion. For comparison, the micrococcal nuclease I protection pattern of a *prnA*$^+$ strain grown under inducing conditions is also shown. Left, SC1 probe; right, SC2 probe. Asterisks indicate the positions of the relevant changes observed with probe SC2. Symbols are as in Fig. 1 and 2. No transcription of either *prnD* or *prnB* is seen in this mutant (shown for *prnB* also in Fig. 7). Bottom panel, schematic representation of nucleosome positioning in a *prnA404* mutant, which was the same under noninduced and induced conditions.

ditions. *creA*^{d}*25*, a mutation in the carboxy terminus of the protein which results specifically in derepression of *prnB* and *prnD* but not of *alcR* and *alcA* (4; B. Cubero, M. Mathieu, B. Felenbok, and C. Scazzocchio, unpublished results) has the same effect on nucleosomal positioning as the more extreme *creA*^{d}*1* mutation (not shown). Figure 9B also shows the chromatin pattern obtained under noninduced conditions in *creA*-derepressed mutants, which are identical to the one obtained

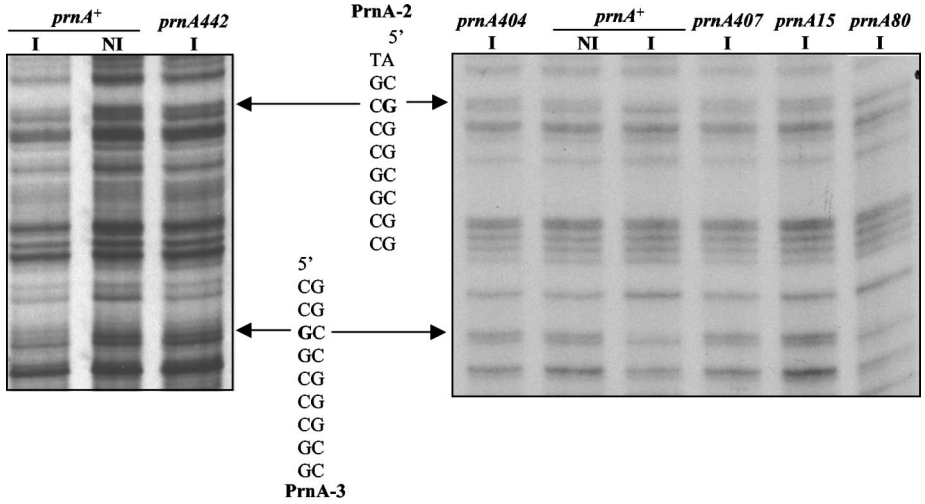

FIG. 5. In vivo footprints of a number of *prnA* mutants. Left panel, in vivo footprints of a *prnA*$^+$ and a *prnA442* strain (*prnD* coding strand shown). Right panel, in vivo footprint pattern of a number of *prnA* missense mutants. The *prnA404* deletion mutant is also included as a control. Footprints of a *prnA*$^+$ strain under both noninducing and inducing conditions are also shown. All mutant strains were grown under inducing conditions (*prnD* coding strand shown). The sequence corresponding to PrnA binding sites 2 and 3 is shown to the side of the autoradiograms. The protected G's in PrnA binding sites 2 and 3 are indicated in bold in the sequence and by arrows pointing to the autoradiogram. Symbols are as in Fig. 2. Nucleotide and amino acid changes in each mutant are shown in Table 1.

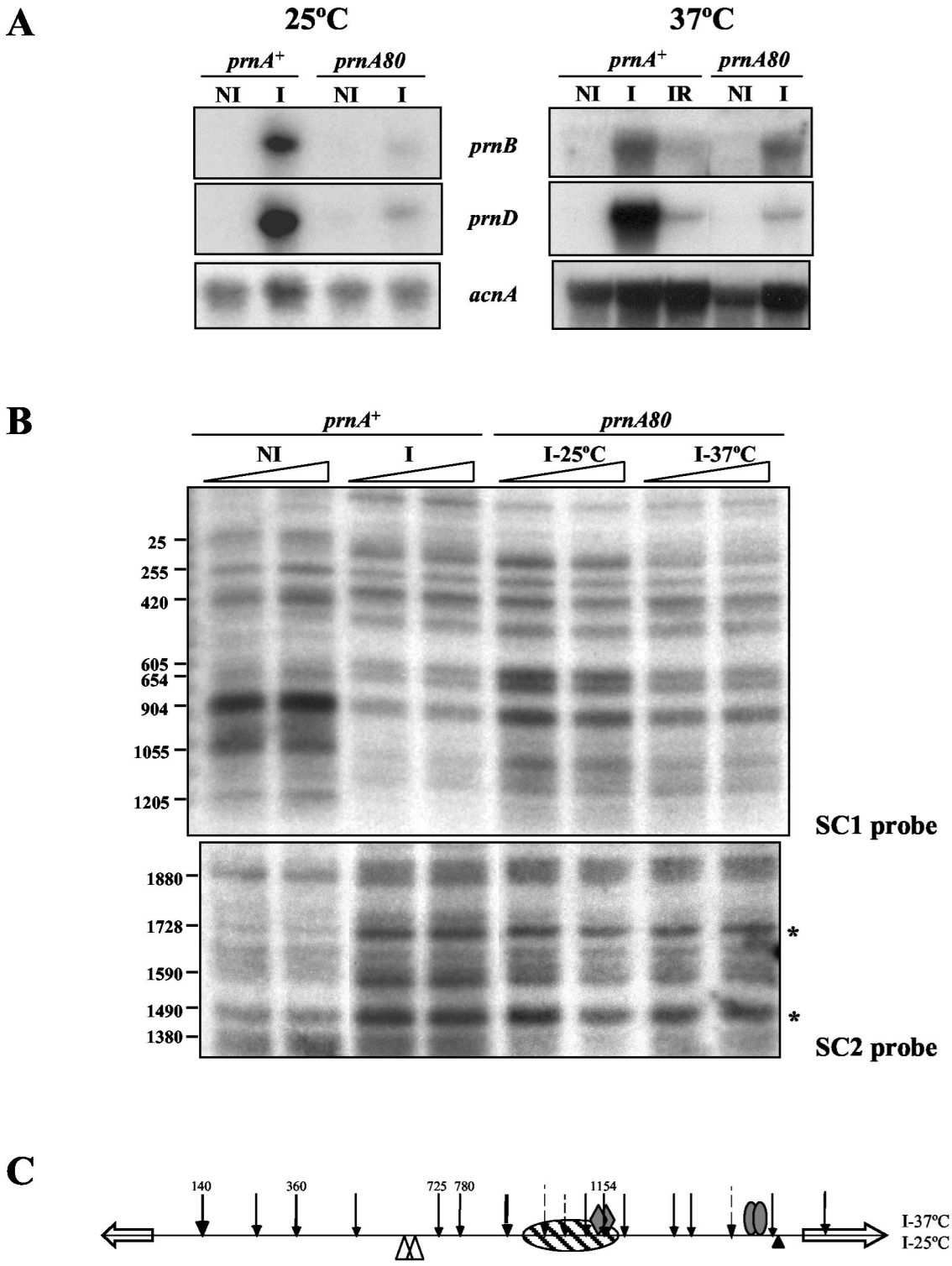

FIG. 6. Transcription and nucleosomal rearrangements in a *prnA80* mutant. (A) Northern blots of mycelia grown at 25 and 37°C. (B) Micrococcal nuclease I digestion of the *prnA80* induced mycelia at 25 and 37°C. For comparison, the *prnA*$^+$ strain grown at 25°C is shown. This is identical to the pattern obtained for *prnA*$^+$ at 37°C (Fig. 2 and Fig. 4). (C) Schematic representation of nucleosome positioning of a *prnA80* mutant grown under inducing conditions at both 25 and 37°C. Asterisks indicate the positions of the relevant changes observed with probe SC2. Other symbols are as in Fig. 2.

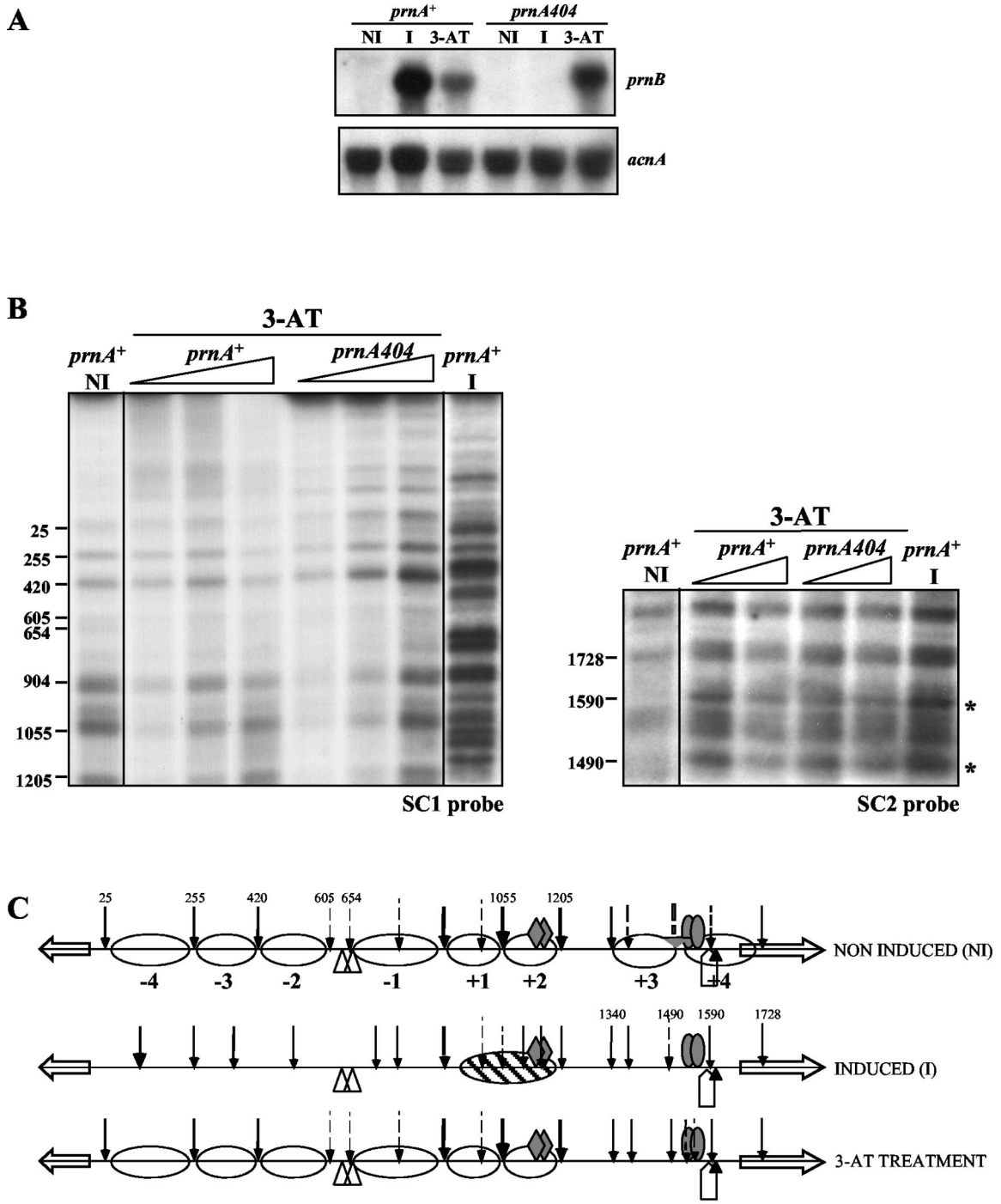

FIG. 7. Transcriptional activation and nucleosome positioning under conditions of amino acid starvation. (A) Northern blot. (B) Patterns obtained after micrococcal nuclease I treatment of mycelia grown in the presence of 3-amino-1,2,4-triazole (3-AT). For comparison, patterns of a *prnA*$^+$ strain grown in noninduced and induced conditions are also shown. All other symbols are as in Fig. 2. Asterisks indicate the bands resulting from micrococcal nuclease I cuts and revealed by the SC2 probe that appear under conditions of both proline and 3-amino-1,2,4-triazole induction and that show loss of positioning of nucleosomes +3 and +4. (C) Schematic representation of nucleosome positioning of both *prnA*$^+$ and *prnA404* strains grown in the presence of 3-amino-1,2,4-triazole.

in *creA*$^+$ strains grown in the same conditions. This demonstrates that CreA is not involved in default nucleosome positioning.

We constructed a double *creA prnA* loss-of-function mutant (*prnA404 creA*^{d}*1*). Northern blots and chromatin analysis were carried out, showing that the *prnA404* deletion is completely epistatic to a *creA*^{d}*1* mutation for both transcriptional activation and nucleosome delocalization (not shown).

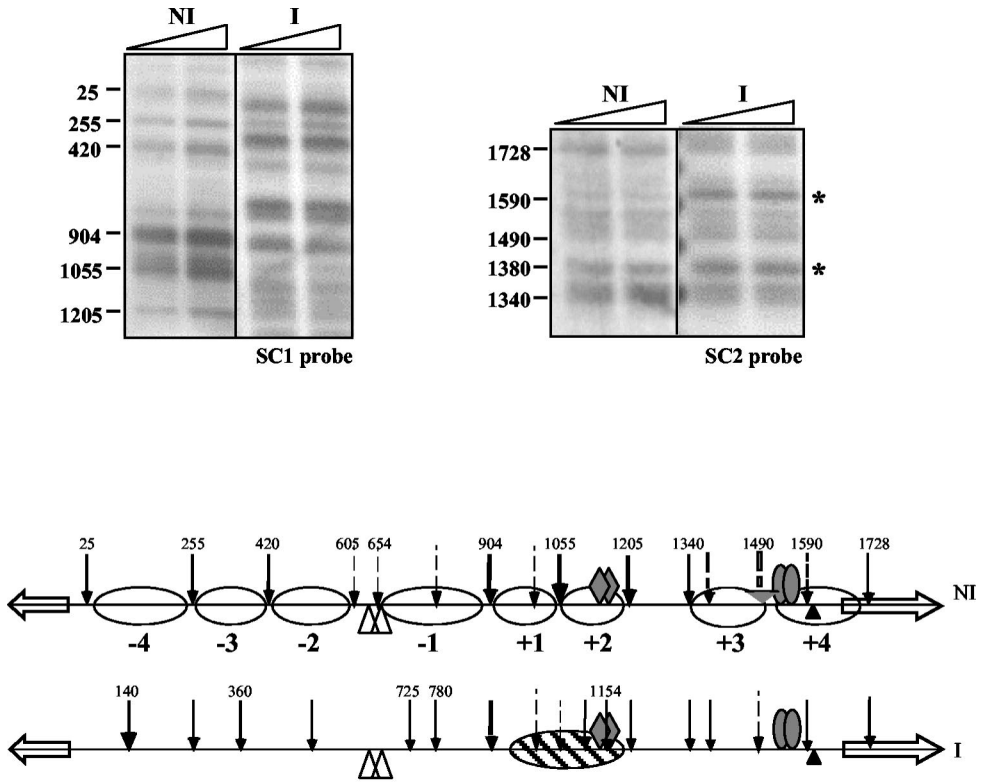

FIG. 8. Micrococcal nuclease I digestion pattern of an *areA600* mutant. Noninduced and induced conditions are shown. Symbols are as in Fig. 2. The growth conditions used to permit the growth of the *areA600* strain are different from those used in other experiments (see Materials and Methods). Under the same growth conditions, the *areA*$^+$ strain behaves exactly as shown in Fig. 2 for noninduced and induced cultures (not shown). Asterisks indicate the positions of the relevant changes observed with probe SC2. Other symbols are as in Fig. 2.

Nucleosome positioning upon repression does not occur in *cis*-derepressed mutants. Mutations in CreA-binding sites 3.1 and 3.2 (*prn*^{d}*22* and *prn*^{d}*20*, respectively) have shown these sites to be essential for CreA-mediated repression (3, 5, 15, 51). Figure 9C shows that the nucleosome positioning pattern in a *prn*^{d}*22* mutant is the same under induced and induced-repressed conditions, indicating that the partial positioning of nucleosomes −4 to −1 does not occur when this site is mutated. Experiments with *prn*^{d}*20* and the *prn*^{d}*22 prn*^{d}*20* double mutant gave identical results (not shown). Thus, mutation at these sites results in the same chromatin pattern as mutations in the CreA *trans*-acting factor.

Histone deacetylation is involved in default and CreA-promoted nucleosome positioning. *creA*^{d} mutations suppress *areA* loss-of-function mutations for the utilization of proline in the presence of a repressive carbon source (2, 8). This happens because AreA is only necessary for *prnB* transcription in the presence of an active CreA-repressing protein (2, 23, 24). We found that the presence of trichostatin A, an inhibitor of histone deacetylation (61), results in a similar phenotypic suppression of an *areA* null mutation (Fig. 10A). For analogous reasons, *creA*^{d} mutations also suppress *areA* loss-of-function mutations for the utilization of acetamide and γ-aminobenzoic acid as nitrogen sources in the presence of glucose. We thus checked whether trichostatin A results in a similar phenotypic suppression of an *areA* null mutation on the latter nitrogen sources. We could not see any phenotypic suppression on either acetamide or γ-aminobenzoic as the nitrogen source at a trichostatin A concentration identical to that used in Fig. 10A. Higher concentrations were too toxic to be tested usefully (not shown).

We then investigated the effect of trichostatin A on both transcription and nucleosome positioning (Fig. 10B and Fig. 11). The presence of trichostatin A results in an elevated basal level of *prnB* but not *prnD* transcription. Proline affords optimal induction independently of the presence of the drug. As predicted by the partial suppression of an *areA* mutation by trichostatin A, transcription of *prnB* and, to a lesser extent, *prnD* is partially derepressed when the drug is added to the culture medium. Trichostatin A results in specific changes in the pattern of nucleosome positioning. In the presence of trichostatin A in noninduced conditions, positioning of nucleosomes +1 to +4 is completely lost and nucleosomes −1, −3, and −4 are only partially positioned. It may be relevant that nucleosome +4 occludes the *prnB* TATA box. This result is shown in Fig. 11.

The most striking differences, however, are found under inducing-repressing conditions. Trichostatin A treatment results in total loss of nucleosome positioning; that is, the same result as obtained for a *creA*-derepressed mutant (Fig. 11, compare with Fig. 8). There is complete agreement between the partial phenotypic suppression of *areA600* by trichostatin A, the levels of transcription, and nucleosome positioning in

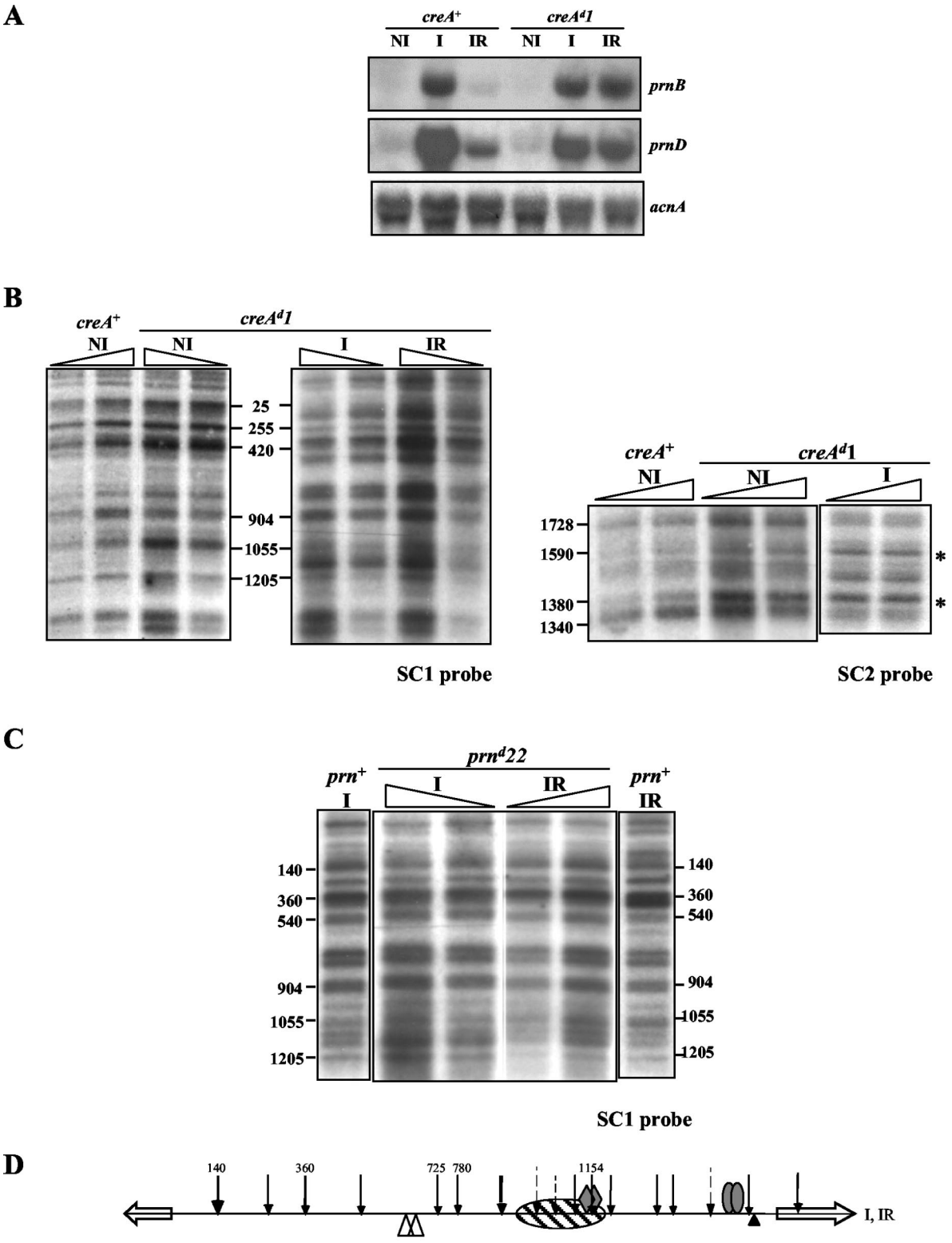

FIG. 9. Nucleosome patterns of derepressed mutants. (A) Northern blot of *prnB* and *prnD* mRNAs in *creA*$^+$ and *creA*^{d}*1* strains. (B) Micrococcal nuclease I protection pattern of the *creA*^{d}*1* strain. For probe SC2, the induced-repressed pattern is not shown, as the pattern under induced-repressed conditions does not differ from the pattern under induced conditions in this region in a *creA*$^+$ strain (see Fig. 2). The pattern of a *creA*$^+$ strain grown under noninduced conditions is also shown. (C) Micrococcal nuclease I pattern of a *cis*-derepressed mutant (*prn*^{d}*22*). For comparison, the pattern obtained for a *prn*$^+$ strain in induced and induced-repressed conditions is shown. (D) Schematic representation of nucleosome positioning in both *creA*^{d}*1* and *prn*^{d}*22* strains grown under inducing and inducing-repressing conditions. Asterisks indicate the positions of the relevant changes observed with probe SC2. Other symbols are as in Fig. 2. The *creA*^{d}*1* and the *prn*^{d}*22* mutants show the same noninduced (noninduced, repressed) position pattern as the wild type (not shown).

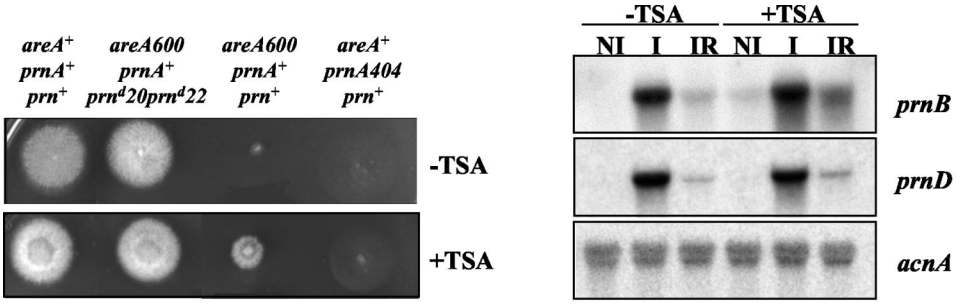

FIG. 10. Trichostatin A treatment. Left, growth tests showing partial suppression of an *areA600* mutation. The medium contains proline as the sole nitrogen source plus glucose as the sole carbon source. The relevant genotypes of strains are indicated above the growth tests. Right, Northern blots showing the effect of trichostatin A on *prnB* and *prnD* expression. −TSA, no trichostatin A; +TSA, 3.3 μM trichostatin A.

the *prnD-prnB* region. These results suggest that CreA-mediated repression acts via histone deacetylation.

DISCUSSION

Loss of nucleosome positioning in *prnD-prnB* depends on the specific activator PrnA and is independent of the GATA factor AreA. Transcriptional activation is often accompanied by a loss of nucleosome positioning. Usually, specific transcription factors are necessary for this process (7, 53, 57), the *niiA-niaD* promoter of *A. nidulans* being an interesting exception (37). In the *prnD-prnB* promoter, loss of nucleosome positioning requires an active PrnA protein. Chromatin rearrangements may be elicited directly or indirectly by transcription factors or could be merely a passive result of transcription. Of the eight nucleosomes in the *prnD-prnB* intergenic region, only nucleosomes +4 and −4 overlap the initiation of transcription. The progress of RNA polymerase has been shown to result in positive DNA supercoiling downstream and negative DNA supercoiling upstream of its site of action (34). Positive supercoiling but not negative supercoiling has been associated with nucleosome destabilization (32, 33). On the contrary, negative supercoiling has been associated with nucleosome stability (38). Thus, it is extremely unlikely that the delocalization of nucleosomes +3 to −3 could be in any way related to topological alterations in the DNA resulting passively from transcription.

In other experimental systems, mutations in the TATA box have been used to investigate the dependence of chromatin rearrangements on transcriptional activation (18, 47), but this is not possible in this promoter, because a deletion of the putative *prnB* TATA box does not abolish transcription (22) and there is no obvious *prnD* TATA box. To discriminate the transcriptional activation function of PrnA from its chromatin remodeling function, we have taken advantage of a number of mutations available outside the DNA binding domain (Table 1). All these mutants were tested for transcriptional activation and chromatin rearrangement activity. We failed to find a mutant that had completely lost transcriptional activation while maintaining chromatin remodeling. Nevertheless, the results with *prnA80* strongly suggest that these functions are indeed separable. This mutation results in greatly impaired transcriptional activation, but chromatin remodeling occurs exactly as in a *prnA*$^+$ strain. The transcription of *prnB* and *prnD* in *prnA80* mutants is as low as that found under induced-repressed conditions in the wild type. Under the latter conditions, we see an intermediate pattern of nucleosome positioning (see below). The fact that complete loss of nucleosome positioning occurs in *prnA80* strains in spite of the strongly diminished transcription of *prnB* and *prnD* argues strongly for a specific effect of PrnA on nucleosomal delocalization and, by the same token, for a specific role of CreA on nucleosome positioning under inducing-repressing conditions (see below).

prnB transcription can also be induced by amino acid starvation. While we have not shown by mutational analysis that a GCN4 homologue is directly involved in chromatin restructuring, this was previously shown for the transcriptional activation elicited by amino acid starvation (56). A mutation in a putative Gcn4p-like binding site abolishes this alternative induction process but not PrnA-mediated proline induction. There is a close homologue of GCN4 in *A. nidulans* (CpcA) (58), and thus it is very likely that this is the transcription factor involved (56). Gcn4p has been shown to be involved in destabilization of nucleosome positioning in the *HIS3* and *PHO5* promoters (31, 55). This protein interacts physically with coactivators such as Gcn5p and other proteins of the SAGA and the SWI/SNF complexes (reference 54 and references therein).

The *prnB* transcription levels elicited by amino acid starvation are considerably higher than those found in a *prnA80* mutant induced by proline, where loss of nucleosome positioning is complete. What is relevant here is that two different induction processes, mediated necessarily by different transcription factors, result in different chromatin rearrangements and that these are not correlated with the levels of transcript. A similar uncoupling of transcriptional activation and nucleosomal rearrangement has been observed in mutants resulting in derepression of the *SUC2* promoter of *Saccharomyces cerevisiae* (19).

The GATA factor AreA is totally irrelevant for nucleosome delocalization upon induction in the *prnD-prnB* promoter. This contrasts with its essential role in the *niiA-niaD* promoter (37). Ammonium repression on its own does not lead to nucleosome positioning in the *prnD-prnB* promoter. As ammonium repression prevents AreA function (30, 40) this is consistent with the fact that AreA is not necessary for nucleosome delocalization upon induction.

Partial positioning of nucleosomes upon repression depends on the CreA repressor. Positioning of nucleosomes under non-induced conditions is independent from the presence of the

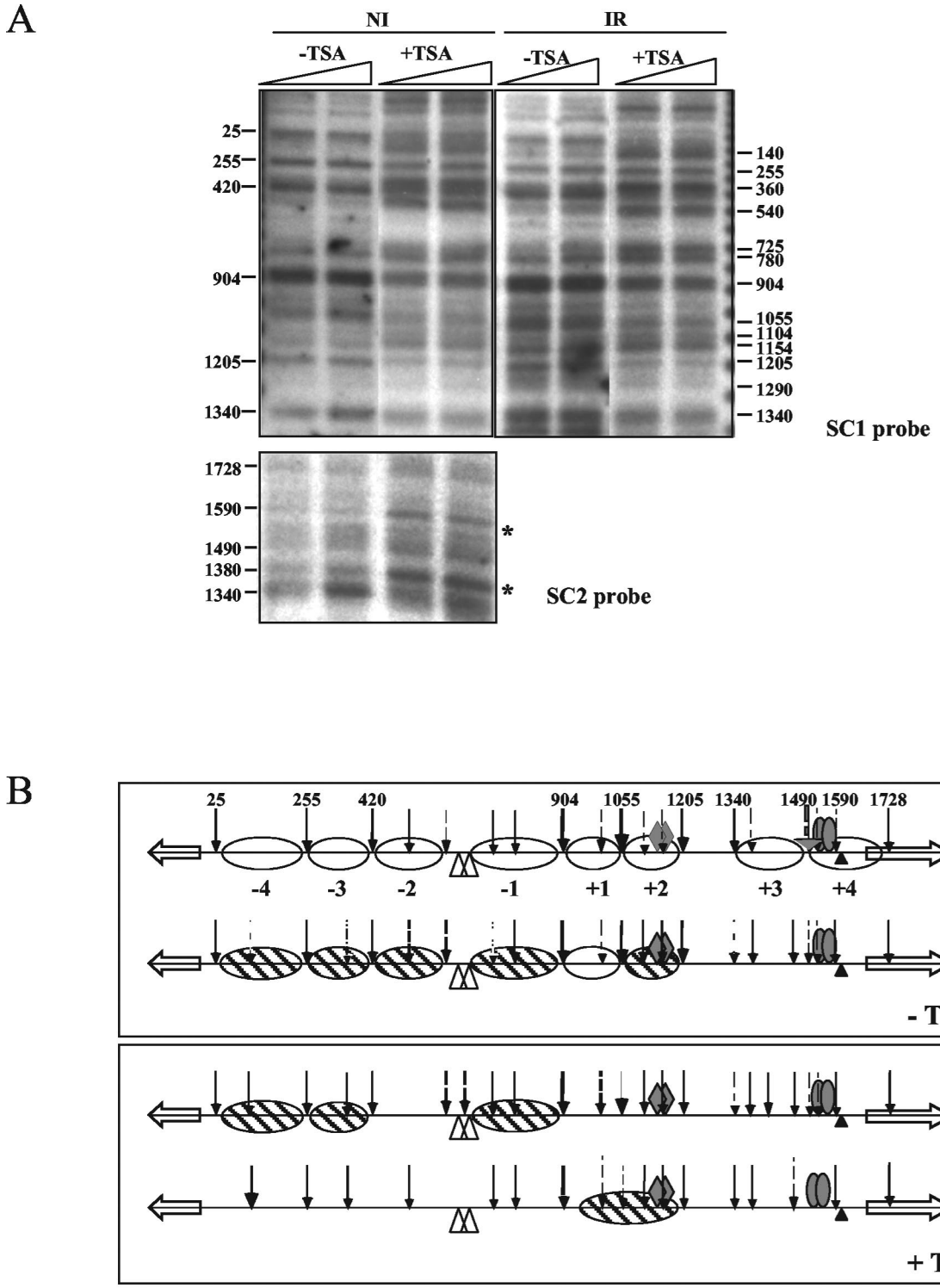

FIG. 11. Effect of trichostatin A on nucleosome positioning. (A) Micrococcal nuclease I patterns. In noninduced (NI) conditions, under trichostatin A (TSA) treatment, nucleosomes +2 and −1 to −4 are partially positioned (probe SC1), and nucleosome +3 and +4 positioning is lost (probe SC2). In induced-repressed (IR) conditions, positioning of nucleosomes −4 to +2 is lost after trichostatin A treatment, as revealed with probe SC1. For probe SC2, the induced-repressed pattern is not shown, as in this region the pattern under induced-repressed conditions does not differ from the pattern under induced conditions (see Fig. 2). Under induced (I) conditions, the patterns obtained in the presence of trichostatin A are identical to those found in its absence (Fig. 2) and thus are not shown. Asterisks indicate the positions of the relevant changes observed with probe SC2. (B) Schemes comparing nucleosome positioning under noninduced and induced-repressed conditions in the presence and absence of trichostatin A. Symbols are as in Fig. 2. −TSA, no trichostatin A; +TSA, 3.3 μM trichostatin A. As trichostatin A is prepared in dimethyl sulfoxide, controls without trichostatin A were treated with equivalent amounts of this solvent.

CreA repressor. A specific pattern of nucleosome positioning, different from the default pattern and from the fully induced pattern, is seen under inducing-repressing conditions (see below). Positioning upon simultaneous glucose and ammonium repression requires CreA. This has been shown by using mutations in both the CreA protein itself and in its cognate binding sites in the *prnD-prnB* promoter. The comparison of these results with those obtained with the *prnA80* mutant demonstrates that the partial positioning found under induced-repressed conditions is specific (see above).

We have shown by in vivo methylation protection experiments that under conditions of CreA-mediated repression, PrnA remains bound to the high-affinity sites 2 and 3 (Fig. 8 in reference 21). This implies that CreA acts by negating PrnA interactions with the transcriptional and chromatin remodeling complexes rather than by preventing its binding to DNA.

Under conditions of CreA-mediated repression, positioning of nucleosomes +3 and +4 is completely lost, nucleosome 1 is positioned, and nucleosomes −4 to −1 and +2 show a pattern of partial positioning (see Fig. 2 and Results section). A similar pattern of partial positioning has been obtained for the *PHO8* promoter in *S. cerevisae* (9). This pattern can be due either to an "oscillation" in the state of each nucleosome or to a heterogeneity in the nuclear population, in which some nuclei show an "open" and others a "closed" chromatin pattern. We favor the first alternative. Nuclear heterogeneity would imply that the intracellular concentration of CreA is limiting. It is unlikely for a protein that represses every single gene sensitive to carbon catabolite repression to be present in limiting concentrations. Limiting concentrations of transcription factors lead to codominance of loss-of-function mutations when tested in diploids with their wild-type allele (6, 14, 44, 45), while *creA*^{d} (loss of function) mutations are clearly recessive (8).

In *S. cerevisae*, Mig1, the specific carbon catabolite repressor, acts by recruiting the Tup1-Ssn6 complex, and this in its turn acts directly on chromatin structure, and specifically on H3 acetylation (17, 27; reviewed in references 49 and 60). One cannot, however, extrapolate directly from *S. cerervisae* to *A. nidulans*. CreA shows similarity to Mig1 only in its DNA binding domain. RcoA, the only clear Tup1 homologue present in the *A. nidulans* genome, is not involved in carbon catabolite repression of either the *prn* cluster or the *alc* regulon (26; I. García, M. Mathieu, B. Felenbok, and C. Scazzocchio, unpublished data). Its role will be analyzed in detail in another publication.

Both default nucleosome positioning and positioning upon repression are probably dependent on deacetylation. Trichostatin A treatment results in loss of positioning of nucleosomes +1 to +4 and very mild transcriptional activation of *prnB* in the absence of induction by proline. This implies that the deacetylation of histones plays a role in the default positioning of at least some nucleosomes in the *prnD-prnB* promoter. Among the nucleosomes delocalized by trichostatin A treatment, we find nucleosome +4, the one that occludes the *prnB* TATA box. Previous work has shown that this element is not essential for *prnB* transcriptional activation, but that its deletion leads to halving the steady-state level of the *prnB* mRNA (22). A striking effect of trichostatin A is seen under conditions of repression (induced-repressed). Here we see total loss of nucleosome positioning and partial derepression. However, the derepression observed is not nearly as drastic as that seen in a *creA*^{d} mutation. This implies that while nucleosome repositioning may be necessary for full repression, CreA can still partially repress on completely open chromatin.

The work presented here is an analysis of the chromatin structure of a region subject to a multiplicity of transcription signals in a simple eukaryote. The *prnD-prnB* promoter integrates four different signals, proline induction, amino acid starvation, and nitrogen and carbon metabolite repression. This level of complexity is higher than that found in some other well-studied promoters, such as *GAL1-GAL10* of *S. cerevisiae* and *niiA-niaD* of *Aspergillus nidulans*. We have been able to discriminate between the roles of the different transcription factors involved on nucleosome positioning. Transcription factors act on chromatin structure by recruiting remodeling and acetylation complexes (see reference 39 for a review). The availability of the complete genomic sequence of *A. nidulans* and of new methods simplifying the procedures for gene inactivation (13; K.-H. Han, Z. Hamari, J.-H. Seo, C. Scazzocchio, and J.-H. Yu, unpublished results) will permit us to study systematically the involvement of these complexes and their interaction with a multiplicity of transcription factors.

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