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The *Aspergillus nidulans* CREA protein mediates glucose repression of the ethanol regulon at various levels through competition with the ALCR-specific transactivator

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Carbon catabolite repression in *Aspergillus nidulans* is mediated by a negative-acting protein coded by the *creA* gene. We have investigated how CREA controls the expression of the ethanol regulon genes. CREA is a major component of the control of this regulon. Its presence in the cell results in a permanent, albeit partial, repression of the *alc* genes under all physiological growth conditions, even when the fungus is grown on carbon sources considered to be non-repressing. A crucial step in the control processes is the repression of the positive-acting specific regulatory gene *alcR*, by the binding of CREA on its cognate target sites on the *alcR* promoter. The removal of one of these targets, URS_A, results in a 50% derepression of the *alcR* gene. Furthermore, the presence of this sequence contributes directly to the low *alcR* expression under non-repressing conditions and reduces *alcR* promoter function by at least 100-fold. CREA acts both on the regulatory gene *alcR* and directly on the two structural genes *alcA* and *aldA*, as glucose repression of the latter genes occurs in strains where *alcR* transcription is driven by a strong constitutive and derepressed promoter. *In vivo* and *in vitro* competition experiments show that CREA acts by competing directly with the binding of the ALCR activator for the same region of the *alcR* promoter, a region which encompasses overlapping targets for both regulatory proteins. These data are consistent with a model in which the activating and repressing regulatory proteins compete to regulate expression of the ethanol regulon genes.

Key words: Aspergillus nidulans/filamentous fungus/glucose repression/transcriptional regulation

Introduction

In the filamentous fungus *Aspergillus nidulans* growth on glucose results in the repression of the synthesis of a large number of enzymes required for the utilization of alternate carbon sources, including those required for ethanol metabolism (Bailey and Arst, 1975). The ethanol utilization regulon is regulated at two levels: (i) ethanol strongly induces the transcription of the *alc* genes required for ethanol oxidation; and (ii) glucose severely represses their transcription (Pateman *et al.*, 1983; Lockington *et al.*, 1987; reviewed in Felenbok, 1991).

The genes of the ethanol regulon comprise the *trans*-acting regulatory gene *alcR* (Felenbok *et al.*, 1988), *alcA* (encoding alcohol dehydrogenase I; Gwynne *et al.*, 1987), *aldA* (encoding aldehyde dehydrogenase; Pickett *et al.*, 1987) and other genes of unknown function (*alcM*, *alcO*) (S.Fillinger, M.Mathieu, D.Sequeval and B.Felenbok, unpublished results). Activation of the *alc* genes requires ALCR, a positive regulatory protein of the zinc binuclear cluster class (Kulmburg *et al.*, 1991). This protein is necessary for its own transcriptional induction, the *alcR* gene being positively autoregulated (Lockington *et al.*, 1987). ALCR binds to specific sites on the *alcA* and *alcR* promoter regions, which are direct and inverted repeats with the same core (5′-CCGCA-3′) sequence. These sequences were shown to correspond to upstream activating sequences, UAS_{alc} (Kulmburg *et al.*, 1992a,b, 1993).

Glucose repression of the *alc* genes depends on CREA, the general repressor mediating carbon catabolite repression in *A.nidulans* (Bailey and Arst, 1975; Pateman *et al.*, 1983; Lockington *et al.*, 1987). The mechanism by which CREA represses the expression of the *alc* genes has been investigated and considerable progress has been made in our understanding of it. We have shown that the *alcR* gene is under the direct transcriptional control of the *creA* gene product (Lockington *et al.*, 1987). In fact, for each of the *alc* genes there are two ways in which the CREA repressor could operate: (i) by direct repression of the ALCR activator, which would result from a cascade mechanism in almost undetectable expression of the *alcA*, *aldA* and other *alc* genes, or (ii) by a double repression mechanism in which CREA represses independently both the *trans*-acting *alcR* gene and any one of the *alc* genes (Felenbok *et al.*, 1989).

The sequencing of the *creA* gene (Dowzer and Kelly, 1991) has shown that this protein contains two zinc fingers of the C_2H_2 class which are very similar to the zinc fingers of MIG1, the repressor involved in glucose repression of the *GAL* genes and *SUC2* in *Saccharomyces cerevisiae* (Nehlin and Ronne, 1990; Nehlin *et al.*, 1991). CREA was shown to be a DNA binding protein able to bind to several GC-rich-specific targets on the promoter regions of both the *trans*-acting gene *alcR* and of the structural gene *alcA* (Kulmburg, 1991; Kulmburg *et al.*, 1993) and *aldA* (Kulmburg, 1991). Previous data have shown that the deletion of one of these CREA binding sites in the *alcR* promoter results in a partial derepression of the transcription of the *alcR* gene (Kulmburg *et al.*, 1993).

In this study we show that in mycelia grown on glucose, CREA binding to sequences localized in the promoters of both the regulatory gene *alcR* and of the structural genes *alcA* and *aldA* results in direct and independent repression of these three genes. The ALCR activator and the CREA repressor compete for the same region of the *alcR* and probably of the *alcA* promoters which encompass close or overlapping binding sites. Finally, we have shown that

CREA represses the transcription of both the regulatory gene *alcR* and the structural genes *alcA* and *aldA* even in derepressed growth conditions (in the presence or absence of the specific inducer). In addition, our results indicate that glucose repression does not proceed through inducer exclusion, and that it is at least partially active *in vivo* in the absence of glucose under growth conditions generally considered to be non-repressing. Our data support a model in which both regulatory proteins mediating specific induction and carbon catabolite repression compete to regulate the expression of the *alc* genes.

Results

Repression by glucose affects directly both the regulatory gene alcR and the structural gene alcA

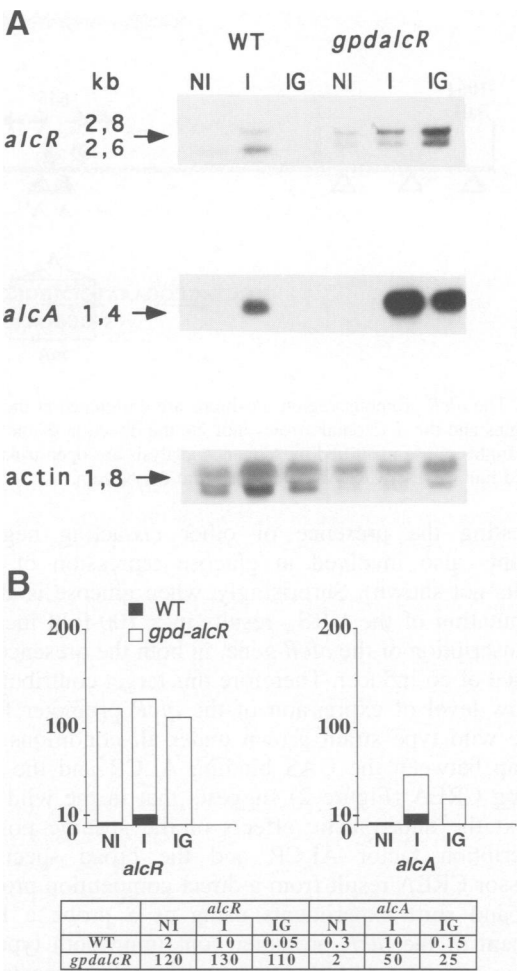
The induction of *alcA* and *aldA* is absolutely dependent on the ALCR protein and on the presence of a co-inducer (Lockington *et al.*, 1987). Thus, glucose repression may act simply by preventing the transcription of *alcR*. A strain was constructed where the coding sequence of *alcR* is driven by the strong promoter of the glyceraldehyde-3-phosphate dehydrogenase-encoding gene. This promoter is independent of ethanol induction and carbon catabolite repression (Punt *et al.*, 1988). As expected (Figure 1), the transcription of *alcR* in the *gpdA:alcR* strain is constitutive and derepressed and occurs at a high level.

In this strain the transcription of *alcA* is 5-fold higher than in the wild type grown under fully inducing conditions. It is noticeable that, not withstanding the high levels of *alcR* mRNA, a total absence of *alcA* transcript is observed when this is grown under non-inducing conditions. This confirms data obtained previously with an *alcR* multi-copy transformant (Lockington *et al.*, 1987). When glucose is added together with the co-inducer, the *alcR* transcription is lowered by ~50%. This confirms previous preliminary results (Felenbok *et al.*, 1989) which indicated that carbon catabolite repression also acts directly on the expression of the *alcA* gene.

Identification of an alcR promoter element involved in glucose repression

Figure 2 shows that several CREA binding sites are present in the *alcR* promoter. All the sites, with one exception, agree with the consensus sequence 5′-G/CPyGGGG-3′ (Kulmburg *et al.*, 1993). The exception is the CREA A′ site (5′-GCGGAG-3′). This site is protected in DNaseI footprint experiments (Kulmburg *et al.*, 1993). An identical sequence is active *in vivo* in the carbon catabolite repression process of the *prn* regulon of *A.nidulans* (Cubero and Scazzocchio, 1994).

To test the direct involvement of the CREA A binding site in glucose repression of the *alcR* promoter we destroyed this site by substituting the last two Gs of the sequence by As. *In vitro* gel band shift experiments show that this mutagenized A site (mA) does not bind the CREA fusion protein (results not shown). We examined the effects of this disruption on the transcription of *alcR* and on the two structural genes *alcA* and *aldA*. Figure 3 shows that in the disrupted *alcR*TmA strain under glucose-grown conditions there is a very high increase of *alcR* transcription (10 000-fold compared with the wild type) and a 2-fold repression is observed compared with the



	alcR			alcA		
	NI	I	IG	NI	I	IG
WT	2	10	0.05	0.3	10	0.15
gpdalcR	120	130	110	2	50	25

Fig. 1. Effect of the constitutive expression of *alcR* on the transcription of *alcR* and *alcA*. The *alcR* gene was expressed in *A.nidulans* from the constitutive derepressed *gpdA* promoter. Total RNA was extracted from the mycelia of the wild type and of the *gpdA:alcR* transformant separated on agarose gels and hybridized to ^{32}P-labelled probes. *alcR*, *alcA* and *aldA* probes were utilized as indicated in (**A**). The growth conditions utilized were: NI (non-induced), I (induced), IG (glucose in the presence of the inducer), as indicated in Materials and methods. Autoradiographs corresponding to different strains were developed at various times as described in Materials and methods. In the wild type strain, two *alcR* mRNAs are observed at 2.8 and 2.6 kb (Lockington *et al.*, 1987). In the *gpdA:alcR* strain, as expected from the start of *gpdA* transcription (Punt *et al.*, 1987), the *gpdA:alcR* mRNA was also at 2.8 kb. The 2.6 kb band corresponds to the other *alcR* mRNA of the *alcR125* recipient strain. The amounts of hybridized mRNA were quantified by scanning densitometry of the Northern autoradiograms. Values were corrected for the total amount of RNA by using the amount of actin mRNA as an internal control. mRNA levels of the wild type strain in induced conditions were normalized to 10, as indicated in (**B**). The values indicated in (B) represent the amounts of mRNA of the *gpdA:alcR* strain relative to the normalized induced mRNA level of the wild type strain. A table at the bottom of each histogram shows these relative values. Experiments were performed in triplicate and results vary by 20–30% amongst the various hybridizations.

induced level. Therefore, this target is an upstream repressing sequence, URS_A, and plays a key role in the repression of the *alcR* gene. Northern blot experiments, performed in parallel with the deleted CREA A *alcR* strain (*alcR*TΔA) (Kulmburg *et al.*, 1993) and the mutagenized CREA mA *alcR* strain (*alcR*TmA), have shown that the derepression in this latter strain is significantly lower,

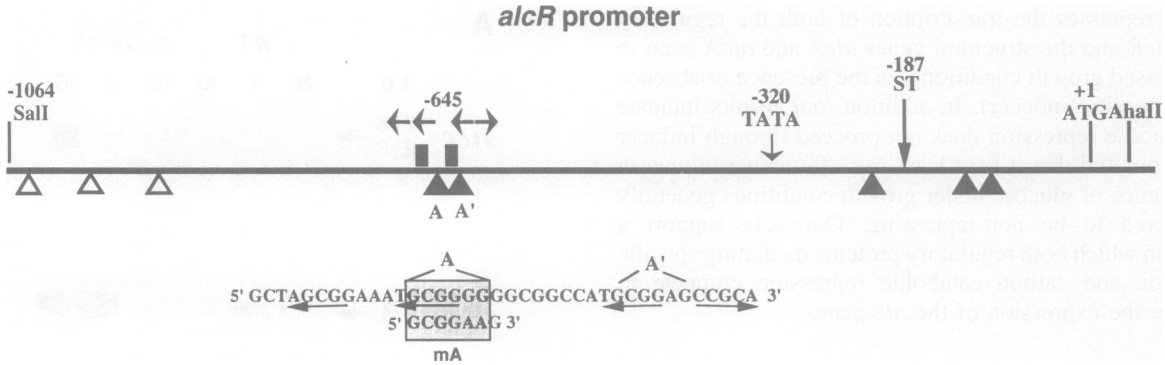
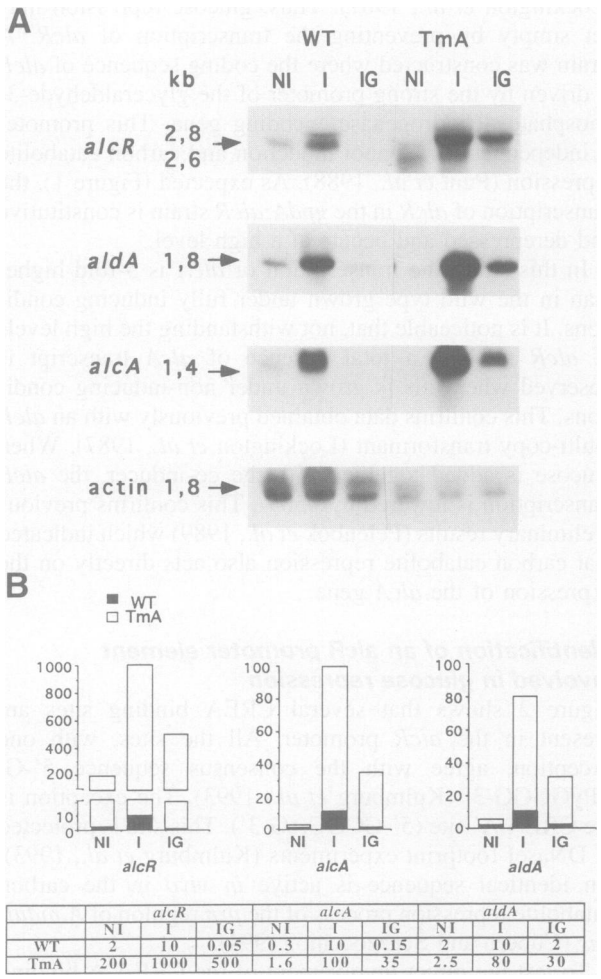


Fig. 2. The *alcR* promoter region. Positions are designated at the top in base-pairs. Locations of the ALCR binding sites are represented by rectangles and the horizontal arrows indicate the direction of the ALCR half site. The CREA binding sites identified by footprinting experiments are full triangles, those identified by sequence analysis are open triangles. The sequences of the CREA A, the CREA A′ binding sites and the site-directed mutagenesis CREAmA are shown at the bottom.

suggesting the presence of other *cis*-acting negative elements also involved in glucose repression of *alcR* (results not shown). Surprisingly, when glucose is absent the mutation of the URS_A results in a 100-fold increase of transcription of the *alcR* gene, in both the presence and absence of co-inducer. Therefore this target contributes to the low level of expression of the *alcR* promoter found in the wild type strain grown under all conditions. The overlap between the UAS binding ALCR and the URS binding CREA (Figure 2) suggests that in the wild type context the antagonistic effects of the specific positive transcription factor ALCR and the broad specificity repressor CREA result from a direct competition process. Gel band shift experiments using as a probe a DNA fragment of the *alcR* promoter containing both types of binding site (the ALCR direct and inverted repeats and the CREA A and A′ binding sites) show that competition actually occurs *in vitro*. As seen in Figure 4, when the concentration of the CREA fusion protein increases, the binding of the latter fusion protein increases greatly whereas the binding of the ALCR fusion protein decreases (lanes 3–5). Conversely, increasing the concentration of ALCR fusion protein caused a decrease in the binding of CREA (results not shown).

As a consequence of the increased *alcR* transcription, the *alcA* and *aldA* transcript-induced steady-state levels are increased in the *alcR*TmA strain (10- and 8-fold respectively) (Figure 3). In the presence of glucose there is a 65% repression of *alcA* and *aldA* transcription. These results indicate that the derepression of *alcR* overcomes only partly the glucose repression of these two genes. Competition between the two regulators ALCR and CREA could also occur here, since in the *alcA* promoter two CREA binding sites overlap or are close to the ALCR induction sites (Kulmburg *et al.*, 1993). Finally, in the *alcR*TmA strain grown under non-induced conditions, despite the presence of a high amount of *alcR* mRNA, *alcA* is not transcribed (Figure 3) and the basal level is observed for *aldA*, in agreement with the results obtained with the *gpdA:alcR* strain (Figure 1).



	alcR			alcA			aldA		
	NI	I	IG	NI	I	IG	NI	I	IG
WT	2	10	0.05	0.3	10	0.15	2	10	2
TmA	200	1000	500	1.6	100	35	2.5	80	30

Fig. 3. Effect of the disruption of the CREA A binding site in the *alcR* promoter on the transcription of *alcR*, *alcA* and *aldA* genes. Total RNA was extracted from mutagenized *alcR*TmA strain carrying the mutation in the CREA A binding site. Northern blots (**A**), scanning diagrams (**B**) and legends are as described in Figure 1, except that the values indicated in (B) represent the relative amounts of mRNA of the *alcR*TmA strain (TmA) compared with those of the wild-type strain (wt).

The two structural genes alcA and aldA are under the control of CREA

To assess whether glucose repression mediated directly by CREA acts independently on the regulatory and the structural genes of the ethanol regulon, transcriptional analyses of the *alc* genes were performed on a loss-of-function *creA* mutant. A total deletion of the *creA* gene

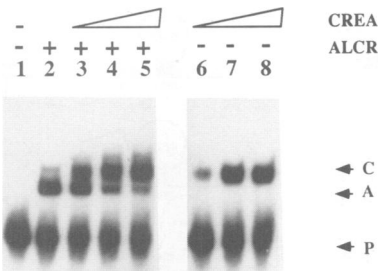
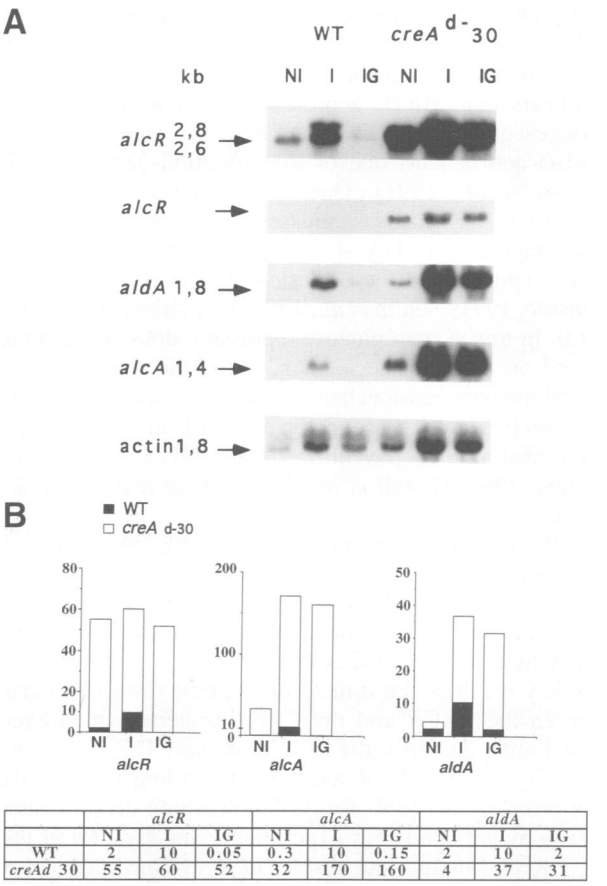


Fig. 4. Competition between the ALCR and the CREA fusion proteins for DNA binding to the *alcR* promoter. Gel mobility shift assays were performed as described (Sequeval and Felenbok, 1994) with the ^{32}P-labelled *Nhe*I−*Eco*RV restriction fragment (−668 to −578) using a constant amount of GST−ALCR protein (300 ng) and increasing amounts of GST−CREA protein (300–1500 ng). Lane 1, free probe; lane 2, 300 ng of GST−ALCR; lanes 3–5, 300 ng of GST−ALCR with 300, 900 and 1500 ng of GST−CREA; lanes 6–8, 300, 900 and 1500 ng of GST−CREA. p, free probe, C and A, DNA−CREA and DNA−ALCR complexes, respectively.

results in extremely impaired growth. Therefore an extreme *creA* allele was chosen, *creA^{d}30*, in which an inversion separates the zinc finger domain from the rest of the protein (Arst *et al.*, 1990). However, this mutation cannot be considered as a total loss of function, as many examples have been described of extremely truncated proteins containing the zinc finger region which retain some activity (Hollenberg *et al.*, 1987; and results herein). Figure 5 shows that in the *creA^{d}30* mutant grown under induced conditions, a significant increase in transcription of the three genes, *alcR* (6-fold), *alcA* (17-fold) and *aldA* (4-fold), is observed. An almost complete derepression is noticed for the three genes in the *creA^{d}30* mutant. But the most striking result is that transcription of the three genes is increased significantly even in the absence of inducer (*alcR* mRNA, 25-fold; *alcA* mRNA, 100-fold; *aldA* mRNA 2-fold).



	alcR			*alcA*			*aldA*		
	NI	I	IG	NI	I	IG	NI	I	IG
WT	2	10	0.05	0.3	10	0.15	2	10	2
creAd 30	55	60	52	32	170	160	4	37	31

Fig. 5. Effect of the loss of function of *creA* on the transcription of *alcR*, *alcA* and *aldA*. Total RNA was extracted from the *creA^{d}30* strain. Northern blots (**A**), scanning diagrams (**B**) and legends are as described in Figure 1, except that the values indicated in (B) represent the relative amounts of mRNA of the *creA^{d}30* strain. Two exposures are presented with the *alcR* probe to visualize the wild type transcription pattern.

Discussion

The wide domain repressor CREA mediates glucose repression of the *alc* genes by binding to targets located on the promoters of the *alcR* and *alcA* genes (Kulmburg, 1991; Kulmburg *et al.*, 1993). In this paper we present evidence that CREA is a major controlling element acting under both non-repressing and repressing conditions at three different and independent levels.

One important level of control is the drastic repression of *alcR* transcription by the binding of CREA to its cognate targets. Several CREA binding sites have been localized in the *alcR* promoter (Figure 2) (Kulmburg *et al.*, 1993). The disruption of one of these (CREA A) results in derepression of the transcription of the *alcR* gene (50%) and, in addition, in a considerable increase in its transcription (100-fold) (Figure 3) under non-repressing conditions. Therefore this upstream repressing sequence (URS_A) plays a crucial role in setting the level of expression of *alcR* and accounts for the low *alcR* expression found even under non-repressing and inducing growth conditions.

The second effect of glucose occurs through the direct repression of the structural genes *alcA* and *aldA* independently of the abundance of *alcR* mRNA and presumably of ALCR protein. It is strongly suggested [by the fact that in both the *gpd:alcR* construction (this work) and *alcR* multi-copy transformants the transcription of the *alcA* gene is increased considerably (review Felenbok, 1991)] that the increase of *alcR* mRNA also results in a corresponding increase in ALCR protein.

Two different lines of evidence support the independence of these mechanisms. In non-induced growth conditions, CREA represses directly the *alcA* and *aldA* structural genes. This can be seen by comparing the transcription of the *alcA* and *aldA* genes in the *creA^{d}30* mutant with the two derepressed *alcR* strains (*gpdA:alcR*, *alcR*TmA; Figures 1 and 3, lanes NI). In the two latter strains no transcription of *alcA* could be detected and that of *aldA* remained at the basal level. In contrast, in the *creA^{d}30* mutant the basal levels of the *alcA* and *aldA* transcripts are increased greatly (Figure 5, lanes NI). These results demonstrate that CREA represses the basal level of these two structural genes, not withstanding the increased expression of *alcR*. The second line of evidence comes from Northern blots of the *alcR* derepressed strains (*gpdA:alcR* and *alcR*TmA) grown on glucose in the presence of inducer. In these two strains partial repression of *alcA* and of *aldA* was observed. Therefore, in a context in which ALCR is expressed at a high level and is active

in the presence of the co-inducer, direct repression by CREA occurs on the *alcA* and *aldA* genes.

For comparison, in the repression of the *GAL* regulon it appears that MIG1, a protein homologous to CREA, mediates glucose repression of both the regulatory gene (*GAL4*) and at least one of the structural genes (*GAL1*) (Nehlin *et al.*, 1991). One of the major mechanisms controlling the glucose-repressed levels of *GAL1* is the down-regulation of GAL4 which has only a partial effect on the expression of the *GAL* structural genes (Griggs and Johnston, 1991; Nehlin *et al.*, 1991; Lamphier and Ptashne, 1992). In this system glucose repression does not seem to depend on a single mechanism, but is the summation of several independent mechanisms in which many genes are involved (reviewed by Johnston and Carlson, 1992; Griggs and Johnston, 1991; Nehlin *et al.*, 1991; Lamphier and Ptashne, 1992; Brandl *et al.*, 1993; Stone and Sadowski, 1993).

In the *alc* system a single mechanism, the repression of the *alcR* gene, can account for the repression of the *alc* structural genes. In addition to this mechanism, there is a significant direct repression of *alcA* and *aldA*, with CREA mediating both these processes.

A key result is the direct competition shown to occur between the ALCR and the CREA proteins which exert mutual antagonism in the *alcR* promoter. The very close proximity of the ALCR and CREA binding sites on the *alcR* promoter accounts for this competition demonstrated *in vitro* by gel band shift experiments. Inactivation of just one CREA binding site results in a striking increase of *alcR* expression under both non-induced and induced conditions. If we assume that transcription reflects ALCR binding, this result implies that the apparent dissociation constant of the *alcR* promoter for active ALCR protein decreases drastically in the mutated promoter. An equivalent conclusion is that there is direct competition between the ALCR and CREA proteins. The proximity between the binding sites for the two regulatory proteins on the *alcA* promoter may also favour the binding of the protein regulator which is the more abundant and/or has a higher affinity for its cognate target. However, these results do not eliminate the putative role of other CREA binding sites found in both promoters which might independently bind CREA, as suggested for the *aldA* gene.

Another point to be considered is the positive feedback loop resulting from positively autoregulated *alcR* transcription. Small changes in regulation would be amplified by this positive feedback loop, contributing to the 100-fold increase in *alcR* expression (*alcR*TmA strain) mediated by the disruption of one CREA binding site. In the absence of this positive feedback, the effect of glucose on the structural genes *alcA* and *aldA* is only 2- to 3-fold (in the *alcR*TmA and *gpdA:alcR* strains). However, the interpretation of these data on the structural genes is complicated by the existence of the negative feedback loop resulting from the ever present repression by CREA on the *alcA* and *aldA* promoters. Therefore, this feedback might also be used in the *alc* system to amplify the original repression.

Lastly, CREA exerts a repression of the *alc* genes under all physiological growth conditions. In other words, to be active CREA does not absolutely require the presence of exogenous glucose *per se* or a glucose-dependent activation process. This result could be explained by supposing

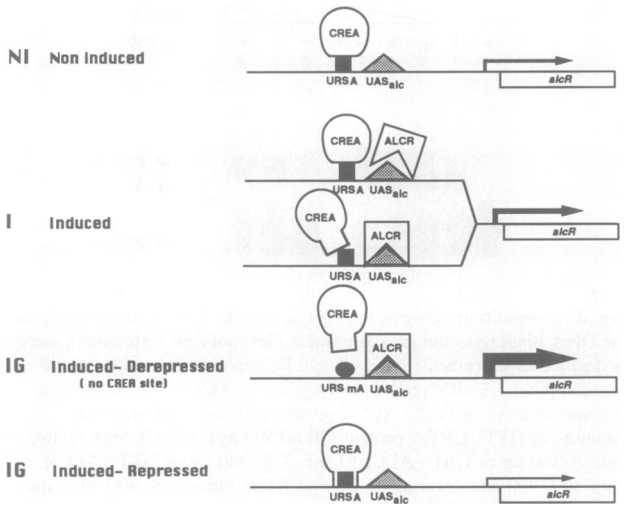


Fig. 6. Model for the role of the CREA A target on *alcR* regulation. In non-induced conditions (NI) CREA binds to the CREA A binding site (URS_A) with a low affinity (indicated in the figure by the positioning of CREA on its targets), resulting in a repressed basal transcription of the *alcR* gene. In induced conditions (I) a competition occurs between the transactivator ALCR and the repressor CREA for the same region of the DNA encompassing both types of target, resulting in an induced–repressed *alcR* phenotype. When the CREA A binding site is knocked out (induced–repressed growth conditions, IG), an induced–derepressed *alcR* phenotype is observed. Finally, in glucose-repressed conditions (IG) CREA binds to its targets with a high affinity (indicated in the figure by the embedding of CREA on its targets), preventing the transcription of the *alcR* gene.

that a co-repressor is formed, albeit at low concentration, when the organism is grown on carbon sources generally considered as non-repressing. Alternatively, CREA may have some repressing activity in the absence of a co-repressor. The disruption of CREA's targets has a stronger derepressing effect than any change in growth conditions and even than a *creA^{d}30* mutation, the most extreme *creA* mutation with which it is practical to work. The physiological significance, if any, of this 'permanent' repression is unknown. Overexpression of *alcR* is not deleterious for *A.nidulans* (Felenbok *et al.*, 1988), in contrast with many other activators (Gill and Ptashne, 1988).

Our data suggest a model, summarized in Figure 6, in which direct competition between CREA and ALCR for the same region of the *alcR* promoter accounts for all the data presented in this article.

Materials and methods

Strains, media and growth conditions

The *A.nidulans* strain used as the host for transformation was *alcR125 argB2* (Lockington *et al.*, 1987); *creA^{d}30* (Arst *et al.*, 1990).

Media and supplements were as described by Cove (1966). The mycelia were grown for 8 h at 37°C on 0.1% fructose as sole carbon source. Induction was achieved by adding the gratuitous inducer, 50 mM ethylmethylketone, after 8 h of growth. Cells were harvested after a further 2.5 h (induced conditions). Under repressed conditions 1% glucose was added simultaneously with the inducer. The times of induction and repression are short enough to allow a comparison between the different growth conditions (non-induced, induced and induced–repressed).

Construction of plasmids

The constitutive and derepressed p*gpdA:alcR argB* plasmid was constructed as follows (Kulmburg, 1991). The plasmid pAN52-1 (Punt

et al., 1987), containing the promoter of the *gpdA* gene (p*gpdA*) and the *trpC* terminator, was modified by adding an adaptor at the junction of the *trpC* terminator to generate *Eco*RI/*Bam*HI restriction sites. The *Pst*I restriction fragment encompassing p*gpdA* and *trpC* was cloned into the unique *Pst*I site of the pFB39 plasmid (Upshall *et al.*, 1986) containing the *argB* gene, resulting in the p*argB*−p*gpdA* plasmid. The next step was the cloning of the *alcR Aha*II−*Aha*II (1081–2522) restriction fragment of the *alcR* gene (Felenbok *et al.*, 1988) after filling-in at the *Eco*RI site of the adaptor which also contained a *Bam*HI site in the p*argB*−*gpdA* plasmid. The last step was the substitution of the restriction fragment containing the *alcR Bam*HI site (1712) to the *Bam*HI adaptor with the *Bam*HI restriction fragment (1712–3664). Analyses by Southern blot of the transformants (*gpdA:alcR*) of the *alcR125 argB2* strain with this p*gpdA:alcR argB* plasmid indicate that the integration was ectopic and in single copy (data not shown).

The p*alcR*mA plasmid, mutated in the CREA A binding site of the *alcR* promoter, was generated by oligonucleotide-directed mutagenesis carried out by the method of Kunkel *et al.* (1987). A uracil-M13 template containing the CREA A binding site from *Sal*I to *Aha*II (1–1081) (Felenbok *et al.*, 1988) was mutagenized with the mutated 26mer oligonucleotide 5′-CCGCATGGCCGCCAACCGCATTTCCG-3′. The mutation was confirmed by DNA sequencing of the p*alcR*mA plasmid. Cloning of the mutated plasmid in the *alcR* sequence was achieved by substituting the *Sal*I−*Hin*dIII (1–844) restriction fragment with the corresponding mutated sequence in the *alcR* Bluescript plasmid.

The host strain *alcR125 argB2* was co-transformed with p*alcR*mA and a Bluescript *argB* plasmid (Upshall *et al.*, 1986). The analysis by Southern blot of the selected $alcR^+$ $argB^+$ transformant (*alcR*TmA) showed that the plasmid was integrated at an ectopic locus in single copy (results not shown).

Isolation of RNA and quantitative analysis

Total RNA was isolated from *A.nidulans* as described by Lockington *et al.* (1987) and separated on glyoxal agarose gels as described by Sambrook *et al.* (1989). The probes used were the entire genes of *alcR*, *alcA* and *aldA* cloned into the Bluescript plasmids (Gwynne *et al.*, 1987; Pickett *et al.*, 1987; Felenbok *et al.*, 1988). The membranes were hybridized with a plasmid containing the actin gene as an internal control to monitor the amount of specific mRNAs relative to that of actin mRNA. Autoradiographs were developed at various times to avoid saturation of the film. Densitometric scanning was performed with a system Biosoft-Orkis. The intensities of mRNA bands were also quantified with a Betascope analyser (Sofi). Experiments were repeated three times.

It should be mentioned that the quantification of mRNA reflects a steady-state level of mRNAs. There is a possibility that changes in carbon source and therefore growth rate could affect, for example, mRNA stability. To avoid these possible effects, the times of induction and repression in different carbon sources were shortened (see above).

Gel mobility shift assays

DNA-binding assays were performed as described previously (Sequeval and Felenbok, 1994) in 20 µl reaction mixes containing 25 mM Tris−HCl, pH 8.0, 100 mM KCl, 1 µg/ml polyd(I-C)$_n$, 10% glycerol, 0.4 mM spermidine, 1 ng end-labelled DNA probe and 300–1500 ng protein purified on glutathione Sepharose as indicated in Sequeval and Felenbok (1994). The GST−CREA (35–240) fusion protein (49 kDa) showed a pattern of bands as described in Kulmburg *et al.* (1993). The GST−ALCR (7–60) fusion protein (33.7 kDa) was 80% homogeneous (Kulmburg *et al.*, 1992a). Following incubation for 10 min at 25°C, the binding reaction was subjected to electrophoresis for 2 h at 14 V/cm at 4°C on a 6% polyacrylamide gel in 0.25× TBE buffer (90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3).

The gels were dried on Whatman 3MM chromatography paper and subjected to autoradiography for 1–16 h at −80°C with Fuji film (RX) and Dupont Plus intensifying screens.

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