The basal level of transcription of the *alc* genes in the ethanol regulon in *Aspergillus nidulans* is controlled both by the specific transactivator AlcR and the general carbon catabolite repressor CreA

Sabine Fillinger, Cristina Panozzo, Martine Mathieu, Béatrice Felenbok*

Institut de Génétique et Microbiologie, Bâtiment 409, Université Paris-Sud, Centre d'Orsay, F-91405 Orsay Cedex, France

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Abstract **In the *A. nidulans* ethanol utilization pathway, specific induction is mediated by the transactivator AlcR which is subject to strong positive autogenous regulation and activates the transcription of the two structural genes *alcA* and *aldA*. Carbon catabolite repression is mediated by CreA which represses directly the transacting gene *alcR* and the two structural genes. We show here that the basal expression of the *alcR* and *alcA* genes is also controlled by the two regulatory circuits, positively by the transactivator AlcR and negatively by the repressor CreA, the *aldA* gene being subject only to the control of the CreA repressor.**

Key words: Transcriptional regulation; Specific induction; Glucose repression; *A. nidulans*

1. Introduction

The ethanol utilization pathway of *Aspergillus nidulans* is a model system for studying pathway-specific transcriptional activation and wide domain glucose repression [1,2]. Induction of the ethanol regulon genes is mediated by the positive acting gene *alcR* in the presence of a co-inducer, ethanol or a gratuitous inducer, ethyl methyl ketone (induced growth conditions, I). The AlcR protein activates the expression of the two structural genes *alcA* and *aldA*, encoding alcohol dehydrogenase I (ADHI) and aldehyde dehydrogenase respectively (AldDH) [3], and other genes, whose functions are unknown, closely linked to *alcA* and *alcR* on chromosome VII (S. Fillinger and B. Felenbok, unpublished). The *alcR* gene is subject to strong positive autogenous regulation [4]. The transcriptional activation mechanism of the *alc* genes was shown to occur through the binding to specific targets of the AlcR DNA binding-protein, which contains in its N-terminal part a zinc binuclear cluster, Cys6/Zn2, identified in diverse ascomycetes [5,6]. These binding sites were shown to exist in the *alcR* [7] and in the *alcA* [8] promoters in two different orientations, direct and inverted repeats with the same consensus core. They were demonstrated to be both upstream activation sequences (UASalc).

Glucose repression in *A. nidulans* is controlled by the negative acting gene *creA* ([9]; see review in [10]). It encodes a zinc finger protein which is closely related to the MIG1 glucose repressor in yeast [11] and mammalian proteins known as GC-box binding proteins [12,13]. Indeed, CreA is able to bind to GC-rich sequences which are localized in the promoters of both the regulatory gene *alcR* and of the structural genes *alcA* and *aldA* [14,15]. This specific binding results in direct and independent repression of these three genes in the presence of glucose (repressed growth conditions, IG). Recent work has shown that CreA is a major negative controlling element acting under both repressing and non-repressing conditions. It was also shown that the AlcR activator and the CreA repressor compete for the same region of the *alcR* and probably of the *alcA* promoters which contain close or overlapping binding sites [16]. The disruption of just one CreA binding site overlapping with an AlcR target in the *alcR* promoter results not only in the derepression of the *alcR* gene (in glucose growth conditions), but also in a striking increase in *alcR* transcription under induced and non-induced conditions. The expression of the two structural genes *alcA* and *aldA* is unchanged in this mutant under non-induced conditions but is partially derepressed when glucose is added. Indeed when a *creA* loss-of-function mutant (*creA^{d}30*) [17] was analysed, besides the expected derepression observed with the three *alc* genes (in glucose growth conditions), an increased transcription is also observed under inducing and non-inducing conditions [16]. This points out the fact that the growth conditions used in these experiments (0.1% fructose, in the absence of glucose) are partially repressing for the expression of the *alc* genes, since a strong basal level of CreA-dependent repression is at work. The observation that the two structural genes *alcA* and *aldA* are able to be expressed under non-inducing conditions raises the question whether this basal level is also controlled by the specific transactivator AlcR. In other words, is the AlcR protein active in the absence of an exogenous inducer?

To answer this question we looked for a non-repressing C-source, and analysed the transcription of *alcA*, *alcR* and *aldA* genes in an *alcR-creA* double mutant background, under non-repressing growth conditions. We conclude that the involvement of the AlcR protein in determining basal levels of transcription, differs among the three genes.

2. Materials and methods

2.1. Strains, media and growth conditions

The *A. nidulans* strains used in this study were wild type, *yA2 panto B100* and *paba A1*; *alcR125 argB2* [4]; *creA^{d}30* [17]; *creA* TE [12]; the *alcR125 creA^{d}30* strain (*creA^{d}30*, *alcR125*, *biA1*) was isolated by intercrossing *alcR125* with *creA^{d}30*. Media and supplements were as described by Cove [18], 5 mM urea was used as nitrogen source.

The mycelia were grown at 37°C on 0.1% fructose or on 3% lactose as sole carbon source (non-induced growth conditions, NI). After 8 h of growth, induction was achieved by adding the gratuitous inducer (50 mM) ethyl methyl ketone (EMK) (induced growth conditions, I). Cells were harvested after a further 2.5 h (induced conditions). Under repressing conditions, 1% glucose was added simultaneously with the inducer (repressed growth conditions, IG).

*Corresponding author. Fax: (33) (1) 6941-7808.
E-mail: felenbok@igmors.u-psud.fr

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2.2. Isolation of RNA and quantitative analysis

Total RNA (25–50μg) was isolated from *A. nidulans* as described by Lockington et al. [4] and separated on glyoxal agarose gel as described by Sambrook et al. [19]. The probes used were the entire genes of *alcR*, *alcA* and *aldA* cloned into the Bluescript plasmid [20–22]. The membranes were hybridized with a restriction fragment corresponding to 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. Experiments were repeated three times.

3. Results and discussion

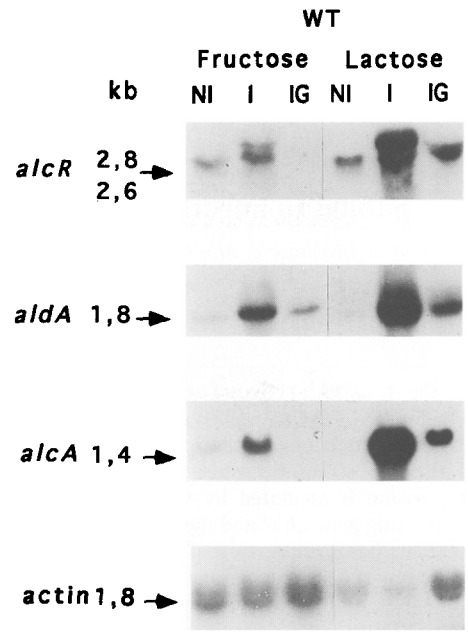
3.1. A transcriptional derepression of the alc genes occurs in poor C-sources

Recent work has shown that CreA partially represses the *alc* genes in 0.1% fructose, a C-source previously considered as non-repressing [16]. We tested if other typical non-repressing C-sources such as lactose, could be less repressing than fructose. Fig. 1 shows the levels of the mRNAs of *alcR*, *alcA* and *aldA* on two different carbon sources, one partially repressing (0.1% fructose), the other less repressing (3% lactose). It is clear that much higher induced mRNAs levels are obtained for the three genes when the mycelium has been grown on lactose than fructose in the presence of an inducer (induced growth conditions, I). More strikingly, in the latter conditions, even the addition of the strong repressing sugar glucose does not shut off transcription totally. In fact, the levels found on lactose for mycelia where glucose was added simultaneously with the inducer (repressed growth conditions, IG) are of the same order as the levels found on fructose under induced conditions in the absence of glucose.

Interestingly, when no inducer is added in the medium (non-induced conditions, NI), the *alcR* transcriptional basal level is considerably increased in lactose, compared to fructose growth conditions. This latter observation is in agreement with a strong control by CreA. It clearly appears that lactose is a good non-repressing C-source compared to 0.1% fructose which allows a subtantial repression of the three *alc* genes, results in agreement with those obtained for the *ipnA* gene [23]. Glycerol, another poor C-source was tested with comparable results (data not shown). It should be pointed out here that it does not exist an absolute non-repressing C-source, and even lactose should also be considered as a partially repressing C-source.

3.2. CreA represses the basal level of the alc genes

In order to ascertain the role of the CreA repressor on the transcription of the three *alc* genes under non-induced and induced grown conditions, a transcriptional analysis of the *creA* multicopy transformant, *creA* TE (17 copies) [12] was carried out in comparison with the wild-type strain. Results in Fig. 2 show clearly that the *alcA* and *aldA* mRNAs levels are noticeably repressed under non-induced growth conditions, mainly for the *alcA* gene. Even in induced conditions, transcription of the two genes *alcA* and *aldA* is strongly repressed as a consequence of the high amount of CreA in this transformant, that of *alcR* is less impaired. Nevertheless, the low levels of *alcA* and *aldA* mRNAs found in induced growth conditions are sufficient for the *creA*TE strain to grow on ethanol as a sole carbon source via the alcohol dehydrogenase I (ADHI) and the aldehyde dehydrogenase (Fig. 3, lane B). But as a consequence, the low ADHI activity confers resistance to the toxic effect of



	alcR			alcA			aldA		
	NI	I	IG	NI	I	IG	NI	I	IG
Fructose	2	10	0.05	0.3	10	0.15	0.3	10	1
Lactose	5	400	10	0.3	300	10	0.3	200	5

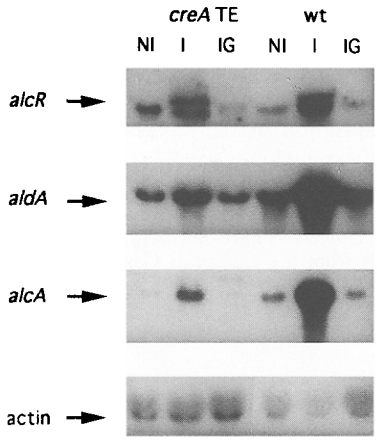
Fig. 1. Northern blot of RNA from *A. nidulans* grown in a derepressing C source. Total RNA (25–50 μg) was extracted from the mycelia of the wild-type strain, separated on agarose gels and hybridized to ^{32}P-labelled probes *alcR*, *alcA* and *aldA* as described in section 2. The mycelia were grown for 8 h at 37°C on 0.1% fructose or 3% lactose as sole carbon source (NI). Induction (I) was achieved by adding the gratuitous inducer (50 mM) ethylmethylketone during 2.5 h after 8 h of growth. Under repressed conditions (IG), 1% glucose was added simultaneously with the inducer. An actin probe was used as an internal control. The amounts of hybridized mRNAs were quantified by scanning densitometry of the Northern autoradiograms. Values were corrected for the total amount of RNA by using as an internal control the amount of actin RNA. mRNA steady state levels of the wild type strain grown on 0.1% fructose in induced conditions were normalized to 10 as indicated in the table at the bottom. nd signifies not detectable mRNA. The values in the table represent the steady state amounts of mRNA of the wild-type lactose grown conditions relative to the normalized induced mRNA levels of the wild type fructose grown conditions. Experiments were performed in triplicate and vary by 20–30% amongst the various hybridizations.

a low concentration of allyl alcohol in the presence of fructose as a carbon source whereas the wild-type strain is sensitive, as seen in Fig. 3, lane C.

These results clearly indicate and confirm that CreA represses directly the expression of the *alc* genes even in the absence of glucose (except for the *alcR* mRNA in non-induced conditions).

3.3. AlcR controls differently the basal level of the alc *genes*

The fact that in *creA^{d}30* under non-induced conditions, *alcA* and to a lesser extent *aldA* were transcribed [16] did not rule out the possibility of an additional positive control by the spe-



	alcR			alcA			aldA		
	NI	I	IG	NI	I	IG	NI	I	IG
WT	2	10	0.05	0.3	10	0.3	2	10	2
creA TE	2	3	<0.02	<0.02	1	<0.02	0.5	1	0.4

Fig. 2. Northern blot analysis of a *creA* multicopy transformant. Total mRNA was extracted from wild type and from *creA*TE, a multicopy transformant [12]. The mycelia were grown for 8 h at 37°C on 0.1% fructose in different conditions (NI, non-induced; I, induced; IG, repressed) as described in section 2 and in the legend to Fig. 1. Three ^{32}P-labelled probes, *alcR*, *alcA* and *aldA* were used. Values indicated in the table represent the relative amounts of mRNA of the *creA* TE strain compared to those of the wild-type strain (wt) as described in Fig. 1.

cific transcriptional activator AlcR on the basal level in non-induced conditions. This hypothesis was tested in the double mutant *alcR125 creA^{d}30*.

It has already been observed [4] that in the putative non-sense *alcR125* mutant [24], no induction of *alcR* transcription occurs, since the *alcR* gene is positively autoregulated. Fig. 4 shows that in non-induced conditions, the decrease in the *alcR* basal transcript level of *alcR125* is lower compared to the wild-type (lanes NI). This decrease could be explained by two non-exclusive hypotheses: (i) the expression of the *alcR* basal level needs also a functional AlcR protein; (ii) and/or the presence of a non sense mutation in *alcR125* mRNA, could reduce its stability as described in some other systems (review in [25]).

The transcriptional analysis in induced conditions (Fig. 4, lanes I) of the double mutant *alcR125 creA^{d}30* compared to the *creA^{d}30* mutant, shows clearly the drastic effect of the *alcR* mutation on the transcription of the three *alcR, alcA* and *aldA* genes. When glucose is added (lanes IG), the amount of mRNAs is nearly the same as in induced conditions for the three genes. As expected in the absence of both active AlcR and CreA proteins in glucose medium, no induction and no repression are observed. Perhaps the most significant result is that, under non-inducing growth conditions, there is a 5–10-fold decrease in the basal level of both *alcR* and *alcA* genes whereas that of *aldA* remains unchanged compared to the *creA^{d}30* strain. Therefore the direct consequence of the *alcR125* loss-of-function mutation is a decrease in the basal level only of *alcR* (5 fold) and *alcA* (10-fold).

The inescapable conclusion is that the positive acting gene *alcR* not only controls its own basal level, but also that of *alcA* and not that of *aldA*. Since in non-induced conditions, the steady state amounts of *alcR* and *alcA* mRNAs are less in the *alcR125 creA^{d}30* strain than in the *creA^{d}30* mutant, we can conclude that the *alcR125* mutation is epistatic to the *creA^{d}30* mutation.

To our knowledge, the control of the basal transription in non-induced conditions, of a pathway specific activator in inducible carbon catabolite pathways has not been thoroughly analysed before in eukaryotic microorganisms. In fact, in most of these pathways, the transactivator is constitutively transcribed and induction of the structural gene is triggered through several possible mechanisms (binding to a repressor, i.e. GAL_4-GAL_{80}, post-translation modification, i.e. ADR1, induced conformational changes of the protein (review in [26]). However, in the *A. nidulans* purine catabolism pathway, a loss-of-function mutation in the specific activator UaY results in a reduced level of the expression of the *uaZ* structural gene, suggesting a positive control of UaY on the basal level in non-induced conditions [27].

Our results show that AlcR can by-pass the presence of an exogenous inducer. We could suppose that in *A. nidulans* metabolites could exist, albeit at low concentration, which could serve as co-inducer. Besides ethanol, threonine and ethylammonium are also inducers of ADHI and AldDH, and are metabolised via acetaldehyde which might be the physiological inducer, which has not been shown formally yet [3,28]. The low *alcR* transcription in non-induced growth conditions might be sufficient to drive transcription of the *alc* genes. However the basal transcription of the *aldA* gene was clearly shown not to be under *alcR*'s control. Therefore, if such endogenous co-inducer is present it should act differently via the AlcR protein on *alcR*, *alcA* than on *aldA*. Interestingly, in the *creA^{d}30* strain, an increase in the intracellular concentration of some polyols as glycerol [29] which could serve as a weak inducer for *alcR* and *alcA* was observed (S.F., M.M. and B.F., unpublished results; [3,30]). However, this hypothesis should not rule out the direct role of CreA on the basal expression of the *alc* genes,

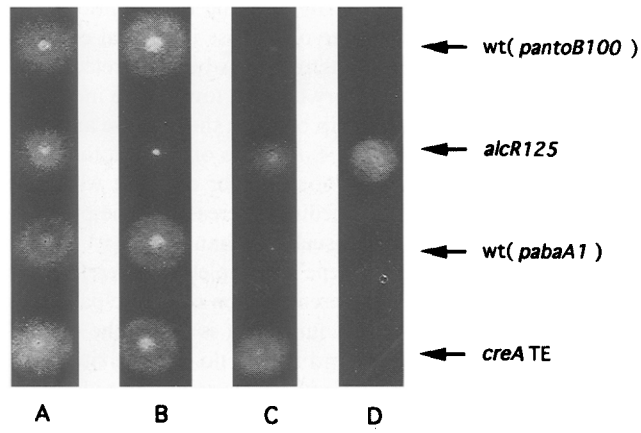
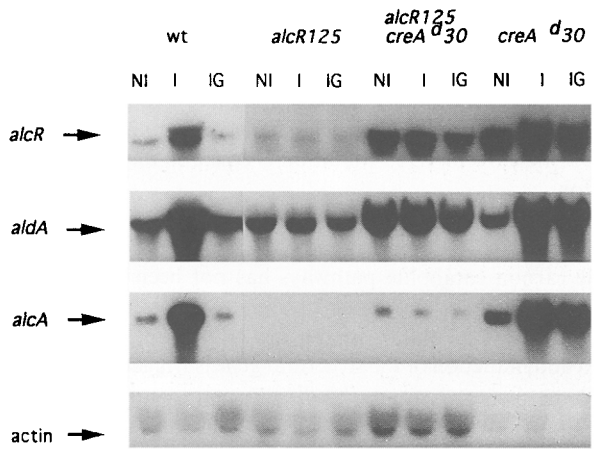


Fig. 3. In vivo ADHI activity in the *creA*TE multicopy transformant. The *creA*TE multicopy transformant [12] was compared for growth on ethanol and fructose in the presence of allyl alcohol with *A. nidulans* control strains; wild-type, *alcR125* [24] strains. The order of the different strains is indicated. (A) 0.1% fructose; (B) 1% ethanol; (C) 0.1% fructose and 200 μM allyl alcohol; (D) 0.1% fructose and 10 mM allyl alcohol.



	alcR			alcA			aldA		
	NI	I	IG	NI	I	IG	NI	I	IG
creAd30	50	100	80	20	100	80	20	100	90
alcR125	2	2	2	<0.1	<0.1	<0.1	10	10	10
alcR125 creAd30	10	10	10	2	2	2	20	20	20

Fig. 4. Northern blot analysis of a double mutant *alcR125 creA^{d}30*. Total RNA was extracted from *alcR125* [4,24], *creA^{d}30* [17] and from the double mutant *alcR125 creA^{d}30*. The mycelia were grown on 0.1% fructose as described in Fig. 2. The RNA loading of *alcR125 creA^{d}30* was increased compared to *creA^{d}30*, in order to try to visualise the *alcA* mRNA hybridization signals. Three ^{32}P-labelled probes *alcR*, *alcA* and *aldA* were used. Values indicated in the table represent the relative amounts of mRNA of the *alcR125* and the *alcR125 creA^{d}30* strains compared to those of *creA^{d}30*, whose induced level was normalized to 100.

clearly demonstrated in Northern blot analyses in the *creA* TE transformant compared to the wt strain.

One can question the physiological significance of this strong double antagonist control on the expression of the *alc* genes. This dual control should allow on one hand a completely turned off expression of the *alc* genes in repression conditions and, on the other hand, a very high level of activation in induced conditions via the binding of the regulators to their cognate targets on the DNA. In fact, the transcriptional increase observed in the presence of glucose in several contexts (mutants and growth conditions), can now be interpreted as the shift between the two regulatory circuits toward the induction process. Indeed this relationship between the positive and negative feedbacks is triggered in *A. nidulans* only when there is a change in the avaibility of C-sources, for example when the glucose concentration in the medium decreases in the presence of an inducing carbon source such as ethanol.

The situation of the *aldA* gene is completely different. This gene is at a cross between different carbon catabolic pathways (ethanol, threonine, ethylammonium). It is under the control of AlcR and CreA, but in its promoter, no binding sites were found for AlcR when several CreA binding sites were identified [14]. The fact that the basal transcription of *aldA* in non-induced conditions, is only controlled by CreA is consistent with several non exclusive hypotheses: the *aldA* promoter could present an AlcR independent promoter activity and/or *aldA* could be subject to a more complex regulation in which other genes may be involved.

Interestingly, the *alcR* and *alcA* genes are organised in a cluster with several other *alc* genes on chromosome VII, all being subject to the two regulatory circuits (Fillinger and Felenbok, unpublished results). The *aldA* gene is on chromosome VIII. We do not know if there is a physiological advantage of the cluster organisation of the *alc* genes in setting in their different level of expression.

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