Regulation of the Aldehyde Dehydrogenase Gene (aldA) and Its Role in the Control of the Coinducer Level Necessary for Induction of the Ethanol Utilization Pathway in Aspergillus nidulans*

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Expression of the structural genes for alcohol and aldehyde dehydrogenase, alcA and aldA, respectively, enables the fungus Aspergillus nidulans to grow on ethanol. The pathway-specific transcriptional activator AlcR mediates the induction of ethanol catabolism in the presence of a coinducing compound. Ethanol catabolism is further subject to negative control mediated by the general carbon catabolite repressor CreA. Here we show that, in contrast to alcA and alcR, the aldA gene is not directly subject to CreA repression. A single cis-acting element mediates AlcR activation of aldA. Furthermore, we show that the induction of the alc gene system is linked to in situ aldehyde dehydrogenase activity. In aldA loss-of-function mutants, the alc genes are induced under normally noninducing conditions. This pseudo-constitutive expression correlates with the nature of the mutations, suggesting that this feature is caused by an intracellular accumulation of a coinducing compound. Conversely, constitutive overexpression of aldA results in suppression of induction in the presence of ethanol. This shows unambiguously that acetaldehyde is the sole physiological inducer of ethanol catabolism. We hypothesize that the intracellular acetaldehyde concentration is the critical factor governing the induction of the alc gene system.

The fungal model organism, Aspergillus nidulans, is able to grow on ethanol as the sole source of carbon (1, 2). Alcohol is oxidized via acetaldehyde into acetate, which enters mainstream metabolism in its activated form, acetyl-CoA. The two enzymes responsible for acetate formation, i.e. alcohol dehydrogenase I (ADHI) (EC 1.1.1.1) and aldehyde dehydrogenase (ALDH) (EC 2.1.2.15), are encoded by the unlinked alcA and aldA genes, respectively. Ethanol catabolism is inducible by a variety of compounds and is driven by the irreversible conversion of acetaldehyde into acetate.

Induction of ethanol catabolism further requires the action of a pathway-specific DNA-binding activator protein, AlcR, regulating both structural genes at the level of transcription (3–7). The encoding aldR gene, closely linked to alcA, concomitantly activates its own transcription. This autoactivation enables the adaptation toward ethanol conversion upon induction. Moreover, the alcR activation cascade mechanism facilitates a powerful induction; alcA and aldA are among the most highly transcribed, inducible fungal genes known to date (1, 8).

Upon addition of more preferable carbon sources, induced ethanol catabolism ceases. This phenomenon, known as carbon catabolite repression, is likewise mediated at the level of transcription via a DNA-binding protein, CreA (2, 9). Unlike induction, this negative control circuit is polytrophic since many catabolic systems are subject to carbon catabolite repression by means of the CreA repressor. CreA has been shown to act directly on both the structural alcA gene and the regulatory alcR gene, ensuring a rapid and complete shut down of ethanol conversion under repressive conditions (7, 9, 10). In the presence of both d-glucose and the gratuitous inducer 2-butanone (ethylmethyl ketone, EMK), the catabolic repression is so strong that it completely overrules the induction of all three principal genes for ethanol utilization. However, the two antagonizing control circuits allow a fine-tuning of the expression of alcR and alcA within a culture subjected to less extreme growth conditions (11). A schematic representation of the regulation of the ethanol catabolic pathway in A. nidulans is shown in Fig. 1.

To understand the underlying molecular mechanisms, we have investigated the requirements in cis for AlcR-mediated activation and CreA-mediated repression in the structural alcA gene (12, 13) and the regulatory alcR gene (7, 10). In both genes the in vivo functional cis-acting elements were identified. Multiple AlcR targets in alcA were shown to act synergistically upon transcriptional activation, which could explain the extraordinary strength of the alcA promoter upon induction, whereas alcR only contains a single activation target. Furthermore, repression and induction were shown to be mutually exclusive at the molecular level due to a competition between the two DNA-binding proteins, as their respective cis-acting elements in both the alcA and alcR promoters reside in close proximity. In addition, we have provided evidence for the coexistence of a distinct mode of action by which CreA establishes transcriptional repression of the alcR gene.

In this communication, we analyze the means by which the two regulatory circuits impose their control on the transcriptional induction of the structural aldA gene. aldA has been
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composition, supplements, and basic growth conditions are as described by Cove (22), unless otherwise stated. Ammonium chloride (10 mM) was normally added as the nitrogen source. Ethanol was used in plates at 1% (v/v), uric acid at 100 mg/liter and l-threonine at 100 mM. Generation of biomass for RNA isolation was by submerged growth in minimal medium, either for 8 h at 37 °C with 0.1% d-fructose or for 24 h at 37 °C with 3% lactose and 5 mM urea as sole carbon and nitrogen sources, respectively. Induction was achieved by adding ethanol or 2-butane (EMK, ethyl methyl ketone) at 50 mM, and biomass was harvested after a further 2.5 h of incubation (induced conditions). For repressed conditions 1% d-glucose was added simultaneously with the inducer. Noninduced biomass was allowed to grow further in the original growth medium for the aforementioned induction period of 2.5 h. Semicarbazide was administered as a freshly prepared 1 mM stock solution of the hydrochloride salt set at pH 7.

Protoplast generation and transformation of A. nidulans with plasmid DNA were performed as described by Tilburn et al. (23). Aspergillus genomic DNA was isolated according to Specht et al. (24).

**Generation of a Strain Constitutively Expressing alcR (TmgpdA:alcR)—BF001 was transformed with bAN8 (13) resulting in the L-arginine prototrophic transformant TgpdAalcR.** Southern analysis indicated that it contained two copies of the chimeric gpdAalcR gene, integrated at two ectopic loci (not shown). Subsequent Northern analysis showed that this transformant is expressing alcR constitutively at a high level under all applied growth and induction conditions.

**Generation of Strains Carrying Mutations in ALC-binding Sites in the aldA Promoter (Tma aldA)—**First, two plasmids were constructed to identify the smallest restriction fragment able to complement the aldA67 mutation. The first plasmid (pMF369) carries the 2.2-kb NheI (-220)/SspI (+1970) fragment from pAN212 (14) cloned into the XbaI and EcoRV sites of pBluescript K5+ (Strategene). The second plasmid (pMF371) harbors the larger 2.8-kb Sall (-768)/SspI (+1970) fragment cloned into the Sall and EcoRV sites of pBluescript K5+ and contains the complete upstream region present in pAN212. Both are able to complement BF111 for growth on ethanol when cotransformed with pBF39 (25) (not shown).

To generate an aldA complementation unit in which the putative Acr target has been disrupted, the 1.1-kb SauI (-768)/EcoRV (+349) fragment from pMF371 was subjected to site-directed mutagenesis according to Kunkel et al. (26). An oligonucleotide (5'-GAAAGGTCCAG-GCGGACGACCATCTTGGTAG-3') was used to introduce four base changes (underlined bases, see also Fig. 3). The mutations were subsequently verified by nucleotide sequence analysis, and the Nehl (-220)/EcoRV (+349) fragment, carrying the mutations, was re-introduced into pMF371 to yield pMF371m. Finally, the 2.3-kb ClaI/XhoI fragment from the A. nidulans urate oxidase (uaZ) gene (27) was introduced. Both pMF371 and pMF371m were digested with ClaI (+440) and XhoI (5' of the aldA insert) and ligated with the uaZ fragment to yield pMM376 (wild type aldA) and pMM377 (promoter-mutated aldA), respectively. pMM376 and pMM377 were used to transform strain BF151. The uric acid-utilizing transformants utilized in this study (TmaldA and TmaldA, respectively) contain two copies of the introduced plasmid, integrated at the uaZ locus (results not shown).

**Generation of Strains Constitutively Expressing aldA at a High Level (TgpdA:aldA)—**A plasmid (pCP352) was constructed, which carries the aldA gene fused to the gpdA promoter at its initiation codon. The 2.3-kb EcoRI/NcoI fragment from pAN52-1, carrying the A. nidulans gpdA promoter (28), was cloned in pLTMUS828 (New England Biolabs). The 5' region of the coding area of aldA was subsequently amplified with Pfu DNA polymerase (Strategene), using pAN212 as the template and two aldA-specific oligonucleotides. One oligonucleotide (5'-CCGGCT-CATCATAGTGATT-3') was used to introduce a BspHI site at the initiation codon (29), whereas the second (5'-GCTCTAGAGCGGGCCC-3') created an XbaI site 3' of the intronic PsI site (+914). The amplification fragment was digested with BspHI and XbaI and ligated into the Ncol/XbaI linearized vector containing the gpdA promoter. The polymerase chain reaction-derived insert was checked for any polymerase chain reaction-induced mutations by DNA sequencing. Finally, the 3' region of aldA was introduced by cloning the 2.6-kb EcoRV (+349)/XbaI fragment from pAN212 into the gpdAaldA fusion plasmid, opened at the intron sites, 3' of the insert, yielding pCP352. pCP352 was introduced in the recipient strain BF111 by cotransformation with pBF39, containing the A. nidulans argB gene. 1-Arginine

**EXPERIMENTAL PROCEDURES**

A. nidulans Strains, Media, Growth Conditions, and Transformation—A. nidulans strains and transformants used in this study are listed in Table I. Refer to Clutterbuck (21) for gene annotations. Media previously cloned and characterized at the nucleotide level (4, 14, 15). Our current study reveals that at the molecular level, the regulation of aldA transcription differs substantially from that of the two other principal genes of ethanol catabolism.

The control of aldA expression could be important, considering the second prerequisite for induction, a coinducing compound. It is generally accepted that ALDH can only catalyze acetaldehyde oxidation, i.e. it is unable to reduce acetate (16). This irreversible conversion drives ethanol catabolism (17). Moreover, acetaldehyde is the first intermediate common to ethanol, l-threonine, and ethylamine utilization, all processes provoking induction of the alc gene system (1, 3, 5, 18, 19). The presumed key role of ALDH in multiple catabolic pathways suggests that the expression of the encoding aldA gene is likely to be subject to tight control. Both ethanol and acetaldehyde have been proposed as physiological coinducers of the alc gene system (3, 20). However, direct evidence of the identity of the physiological inducer has not been obtained to date. To address the role of the coinducer in the induction process, we have investigated how the expression of ALDH affects the induction of the alc gene system. This approach has led to the clear identification of the physiological inducer with regard to ethanol catabolism. Furthermore, we provide evidence here indicating that a subtle control of aldA and alcA gene expression is important for the onset and maintenance of an optimal catabolic flow from ethanol.

![Diagram](image-url) - Fig. 1. Schematic representation of the regulation of the ethanol catabolic pathway in A. nidulans. In the presence of a coinducer, the AlcR activator is able to activate the two structural genes alcA and aldA encoding ADH1 and ALDH, respectively (thick arrows). In addition the alcA gene is subject to positive autoregulation that is visualized by a curved arrow. The CreA repressor, in the presence of glucose, directly represses the alcR and alcA genes (black bars). On the right, the enzymes involved in the catabolism of ethanol, which is oxidized via acetaldehyde into acetate, are depicted.
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**TABLE I**

| Name       | Genotype                                                                 | Ref. of characterized mutation or strain |
|------------|---------------------------------------------------------------------------|----------------------------------------|
| BF 001     | ya2 pantoB100 argB2 alcR125                                              | 6, 51                                   |
| BF 054     | ya2 paba1                                                                |                                        |
| BF 064     | ya2 paba1 alcR500                                                        |                                        |
| BF 097     | ya2 paba1 alcR4951                                                       |                                        |
| H 459      | biA1 nidA aldA67                                                        | 3; this work                            |
| BF 107     | ya2 paba1 aldA67                                                        | 3; this work                            |
| BF 111     | paba1 argB2 aldA67                                                      | 3; this work                            |
| BF 151     | ya2 paba1 uzo111 aldA67                                                | 3; this work                            |
| H 490      | ya2 sbs pyroA gal × acr  aldA57                                         | 3; this work                            |
| C 590      | uA3 biA1 gly× aldA57                                                   | 35; this work                           |
| Tmc3 alcR  | ya2 paba1 (ua111) UA′ × (alcR7) mC3 alcR                                 | 10                                      |
| TgpdAalcR  | ya2 pantoB100 (argB2) arg × (alcR215) gpdAalcR                           | This work                               |
| TaldA      | ya2 paba1 (ua111) UA′ × (aldA67) alda × (2 copies)                       | This work                               |
| TmaaldA    | ya2 paba1 (ua111) UA′ × (aldA67) ma alda × (2 copies)                   | This work                               |
| TgpdAalda  | paba1 (argB2) arg × (aldA67) gpdAalda                                    | This work                               |

prototrophic transformants were isolated.

Isolation of RNA and Quantitative Analysis—Total RNA was isolated from A. nidulans as described by Lockington et al. (5) and separated on gloseal agarose gels according to Sambrook et al. (29).

The 3P-labeled probes used were the entire genes cloned either into the plasmid Bluescript for alcR (19) and alcl (12), or pAN212 for alda (14), or into pSF5 (30) for actin.

The actin gene is presumed to be constitutively expressed under all conditions used and served as an internal control to normalize the amounts of mRNA present on a single blot. Autoradiographs were exposed for various times to avoid saturation of the film. Densitometric scanning was performed with a Biosoft-Orkis system. The intensities of the signals were also quantified using a PhosphorImager (Molecular Dynamics). Experiments were repeated three times.

Cross-feeding Assay—alda mutant strains were inoculated on adequately supplemented minimal medium/ethanol plates. After 2 days of incubation at 37 °C, the alcA mutant BF097 was coincubated at a distance of 1.5 cm from the alda15 and alda67 strains. Cross-feeding could be scored after a further 4 days of incubation at 37 °C.

Characterization of alda Mutants—A 2.8-kb DNA fragment, encompassing the larger alda complementation unit described above, was amplified from genomic DNA from mutant strains carrying the alda15, alda67, and alda67 alleles, respectively, with Pfu DNA polymerase and two alda-specific oligonucleotides. One oligonucleotide (5′-ATGCTCTAGATAGTGTTCAGCAAC-3′) and the second (5′-ATGCTCTAGATAGTGTTCAGCAAC-3′) contains the upstream SspI site (−788), and the second (5′-ATGCTCTAGATAGTGTTCAGCAAC-3′) introduces a unique XbaI site 3′ of the downstream SspI site (+1900). Amplified material was digested with SspI and XbaI and ligated into pBluescriptKS+.

The complete inserts were subjected to nucleotide sequence analysis. In each case, a single base change was found with respect to the wild type alda sequence (see "Results"). The three mutations were verified upon a second, independent amplification, in the case of alda67, utilizing DNA from a second mutant strain. The nucleotide data from the wild type gene and the three mutant alleles have been submitted to GenBank™ under accession numbers AF260123 (alda), AF260124 (alda15), AF260125 (alda67) and AF260126 (aldA67), respectively.

RESULTS

Induction of alda Transcription Correlates with the Intracellular Steady State Level of AlcR in the Presence of a Coincuder—It has been well established that the alda gene has an absolute requirement for a functional alcR gene and a coinciding compound for induction (3–5, 7). Furthermore, convincing evidence has been provided that alda is expressed constitutively at basal levels independently of AlcR (7, 11). Fig. 2 recapitulates these two distinct features of alda expression. A mutant completely lacking both the alcR and alcR genes (alcR500) is unable to induce alda transcription regardless of the presence or absence of a coinciding compound. The deletion strain, however, exhibits a basal level expression of alda comparable with wild type expression under noninduced conditions (Fig. 2A). The converse occurs in a strain in which the transcription of alcR is driven by the strong, A. nidulans glyceral-dehyde-3-phosphate dehydrogenase (gpdA) promoter (gpdA: alcR), as shown in Fig. 2B. In this strain, alda is overexpressed due to the constitutive presence of high steady state levels of the transactivator protein but only in the presence of a coinciding compound. As expected from our previous work, 2-butanone (EMK) is a better inducer than ethanol. Induction of alda thus correlates with the amount of AlcR present in the cell but always requires the presence of a coincuder. We confirm also that basal level expression is independent from both AlcR and coincuder. In this respect, alda expression differs significantly from that of the other structural gene necessary for ethanol utilization, alcA.

Repression of Induced alda Transcription Is Indirectly via CreA-mediated Repression of alcR—The repression of induction of alda is mediated via the transcriptional repressor CreA, as shown for the first time by Lockington et al. (5). This could be due solely to CreA-mediated repression of the transactivator-encoding gene, alcR (9). The AlcR-binding sites in the alcR promoter are shown in Fig. 3. However, results have been described suggesting that alda, like alcR and alcA, is under direct control of CreA (7, 11). To determine if alda transcription was also subject to the same control, transcriptional analysis of alda expression was performed in an alcR derepressed genetic context.

Recently, we generated strains carrying a functional alcR gene in which cis-acting elements mediating CreA repression of transcription had been disrupted (10). These derepressed transformant strains provide the proper means to investigate whether alda transcription is subject to direct control by CreA, as only the regulatory alcR gene is able to escape genuine repression. Fig. 4 shows that a transformant carrying a disruption of one of the functional CreA targets in alcR (Tmc3) induces transcription of the introduced derepressed alcR gene and the genuine alda gene, essentially to the same extent under induced and repressed conditions, i.e. regardless of the presence or absence of d-glucose. Essentially identical results were obtained with derepressed alcR transformants harboring distinct promoter mutations (disrupting either one of the two functional CreA targets in alcR) (results not shown). Therefore, unlike alcR, repression of alda induction occurs solely by CreA-mediated repression of the regulatory alcR gene.

**AlcR Controls the alda Promoter via a Unique Palindromic Site**—Considering the relevance of the AlcR transcriptional activator for both the activation and repression of induced alda expression, we set out to identify the cis-acting element(s) in the alda promoter. The functional alda gene resides on a 3.7-kb SstI fragment able to complement the stringent alda67 mutation for growth on ethanol as the sole carbon source (14).
However, the complementation of the *aldA*67 mutation also occurs using the smaller 2211-bp *Nhe*I/*Ssp*I fragment (results not shown). This complementation unit contains 220 bp upstream of the proposed initiation codon of *aldA* and 383 bp downstream of the proposed termination codon. The 5' non-coding region from the *Nhe*I/*Ssp*I fragment, depicted in Fig. 3, harbors two conjugated AlcR sites with the consensus core 5'-WGCGG-3' represented by gray triangles. The starts of transcription are marked ST; two are identified in *alcR* (ST1 and ST2). The *aldA* promoter contains a single palindromic site for AlcR, whose sequence is indicated beneath. The two AlcR-binding sites are in bold. The downward arrows indicate the nucleotide changes introduced to disrupt the AlcR target (see also “Experimental Procedures” and “Results”).

The *aldA*67 mutation provides an ideal genetic background since, as will be shown later in this paper, the mutant *aldA*67 gene yields heavily impaired transcript levels. Therefore, we can investigate AlcR-mediated induction of *aldA* at the level of transcription in this background. The putative AlcR target was modified as follows: three residues within the distal consensus site were changed, whereas the proximal site was mutated at the ambiguous first position (see Fig. 3 and “Experimental Procedures” for details). We know from previous studies (12) that a single AlcR site is not functional in vivo. Transformants with the *aldA* mutant promoter (Tma*aldA*) were unable to grow on L-threonine (or ethanol) as shown in Fig. 5A. Note that growth on L-threonine is under the positive control of the *alcR* gene. It requires ADHII as well as ALDH activities (review in Ref. 1). Transcriptional analysis of one of these transformants (Fig. 5B) shows a major loss of *aldA* induction. The residual *aldA* induction observed is the consequence of
the induction of the host aldA67 allele (which is not expressed under noninduced conditions), in addition to the two extra copies of the mutated aldA promoter gene, which are expressed at a basal level under all conditions. As expected, the aldA and alcR genes remain normally inducible. On the other hand, the control transformant expresses both the introduced aldA gene and the alcR and aldR genes at wild type levels. Hence, the results from growth plates and Northern blots are in agreement; the inverted repeat of sites constitutes the cis-acting element involved in AlcR-mediated induction of aldA and is absolutely necessary for growth on L-threonine or ethanol.

Structural Mutations in aldA Give Rise to Induction of the alc Gene System Under Normally Noninducing Conditions—Acetaldehyde is a catabolic intermediate in ethanol utilization as well as in L-threonine degradation and in ethylamine deamination (1, 3, 18). It is possible that aldA is subject to multiple pathway-specific activation circuits responding to distinct induction signals. To address the inducing signal in ethanol conversion, we have investigated the transcriptional behavior of the alc gene system in three existing, allelic mutants in aldA (3, 33). Two of these have been characterized to some extent by Pateman et al. (3). aldA67 is a stringent mutation that does not allow growth on ethanol or on L-threonine (Fig. 6C). Conversely, the aldA15 mutation shows a leaky phenotype on ethanol (Fig. 6C). Neither mutant produces detectable ALDH activity. Both are reported to exhibit some expression of ADH activity under noninduced, nonrepressed growth conditions. We have also investigated the aldA57 mutant which shows a stringent phenotype for ethanol and L-threonine comparable to that of aldA67 (results not shown).

We have determined the respective mutations within these three aldA alleles. In all cases, a single point mutation was found within the coding region. In aldA67, a G458A mutation introduces an opal termination codon at the position of Trp-131 (index position number 237). The truncated protein is unlikely to harbor enzymatic activity, and aldA67 should be considered as an absolute loss-of-function mutation. In aldA57, G1077A changes Gly-338 (index position 489) into Ser. Gly-338 is one of the few highly conserved residues found among ALDHs (34, 35). The stringent nature of the aldA57 mutation suggests that this Gly residue is indispensable for enzymatic activity. We presume that the mutant produces a full-length but inactive protein. Finally, the leaky phenotype of aldA15 is caused by C922T and A286V (index position 424) in the protein. The sequence similarity directly N-terminal of the catalytic Cys residue (index position 437) is poor even among proteins closely

3 The index position numbers refer to the ALDH superfamily sequence alignment (34).
related to *A. nidulans* ALDH (34). Human liver cytosolic ALDH1 and mitochondrial ALDH2 both carry an Ala at index position 424. However, the deduced amino acid sequences of the ALDHs from *Alternaria alternata* and *Cladosporium herbarum* specify a Val (36).

The residual enzymatic activity of the mutant ALDH15 protein is sufficient to allow delayed growth and sporulation on ethanol plates as shown in Fig. 6C. Under such conditions acetaldehyde produced from ethanol is excreted into the medium, giving rise to a cross-feeding phenomenon of structural mutants in *alcA* when coinoculated on the same plate (33). *alc* mutants show typical nutrient-starved growth on ethanol plates, sparse mycelial outgrowth and very poor conidiation, similar to that of wild type strains on agar plates without carbon source (37). Cross-feeding is visualized by an increased mycelial density and sporulation of the *alcA* mutant in the vicinity of the *aldA15* mutant, which provides the acetaldehyde supporting this local growth (Fig. 6A). Conversely, *aldA67* mutants do not grow at all on ethanol and are unable to cross-feed the *alcA* mutant (Fig. 6B). Note that mutants in *alcR* cannot be cross-fed (results not shown), because the *aldA* gene is not inducible in such strains (cf. "Results").

Fig. 7A shows the transcriptional behavior of the three principal *alc* genes in the structural *aldA* mutants. Interestingly, lesions in *ALDH* lead to induction of the *alc* gene system under normally noninducing conditions. The level of this noninduced expression appears to correlate with the nature of the *aldA* mutant. The leaky *aldA15* mutant exhibits a modest but clear transcription of *alcA* under noninducing conditions. Induction of *aldA* and *alcR* over their respective basal level expression seen in the wild type strain is likewise apparent. Conversely, the *aldA67* absolute loss-of-function mutant expresses *alcA* and *alcR* to virtually the same levels in the presence or absence of ethanol.

The stringent *aldA57* mutant provides an intermediate response. An intracellular accumulation of a coinducing compound during growth on the noninducing carbon source lactose could explain the acquired constitutivity in structural *aldA* mutants. This typical phenomenon is termed pseudo-constitutive expression. The inducer involved has to be an in *vivo* substrate of ALDH. We presume that this inducer is acetaldehyde formed for instance by l-threonine turnover (38) or by constitutive pyruvate decarboxylase activity (39). Acetaldehyde is the main substrate for *A. nidulans* ALDH (17). A similar situation occurs in case of the *A. nidulans* purine utilization pathway. Loss-of-function mutations in the urate oxidase (*uacZ*) gene exhibit a pseudo-constitutive expression of the structural genes in this pathway due to an accumulation of uric acid (27, 40).

The direct correlation between the level of pseudo-constitutive expression and the stringency of *aldA* mutations suggests that *alc* gene expression depends on the intracellular concentration of the coinducing compound. This could be confirmed in *vivo* using the aldehyde scavenger semicarbazide (41). Fig. 7B shows that the level of pseudo-constitutive expression in *aldA67* reduces progressively with increasing amounts of semicarbazide in a concentration range that does not affect transcription of the actin gene.

One would expect that the mutated *aldA* genes are induced concomitantly with the *alc* genes under both growth conditions in all *aldA* mutants. However, the nonsense mutation in *aldA67* not only results in a truncated protein but also in severely reduced transcript levels (Fig. 7A). This feature enabled us to use *aldA67* as a recipient background in the identification of the functional cis-acting element mediating *aldA* induction, described above. The transcript levels in the *aldA67* mutant are comparable with that in the wild type under non-induced conditions but do not represent a basal level in *aldA67*. The mutant gene is regulated normally by the AlcR transactivator because no *aldA* transcript can be detected in *alcR/aldA67* double mutants (results not shown). It is most likely that the mutant messenger is subject to nonsense-mediated mRNA decay under all tested growth conditions (42, 43).

**Constitutive Overexpression of aldA**

**Results in a Suppression of the Induction of the*alc* Gene System in the Presence of Ethanol**—To address further the apparent relationship between the intracellular coinducer concentration and ALDH activity, we have investigated the effects of a fully constitutive overexpression of the *aldA* gene on the induction of the *alc* gene system in the presence of ethanol. For this purpose we constructed a plasmid in which the *aldA* gene is under the control of the strong constitutive and nonrepressible *gpdA* promoter (for details, see "Experimental Procedures"). Transformants (*TgpdA::aldA*) that were able to grow on l-threonine as the sole carbon source, implying that *aldA* function had been restored, were selected.

Fig. 8 shows a transcriptional analysis of one of these transformants under two different induced conditions: ethanol and 2-butanol (EMK), respectively. Interestingly, the induction on ethanol of the *alcR* and *alcA* genes, found in the wild type, is drastically suppressed in the *gpdA::aldA* transformant. Constitutive overexpression of *aldA* apparently reduces intracellular accumulation of acetaldehyde, which normally induces the
physiological inducer of ethanol catabolism. by itself but that acetaldehyde formed from ethanol is the retained showing that ethanol is not inducing the transformant. Hence, for the first time direct evidence is obtained for ALDH and retains its inducing capacity in the aldA15 structural genes, by converting the acetaldehyde initially formed from ethanol into acetate. Constitutive overexpression of aldA suppresses the induction of the aldA expression reside within 220 bp upstream of the start of the gene (the structural gene encoding aldehyde dehydrogenase). We have established that all elements required in cis for the transcriptional induction of the aldA gene system on ethanol. The aldA gene was expressed from the constitutive derepressed gpdA promoter (TggpA:aldA) (see “Experimental Procedures”). Total RNA was extracted from mycelia either induced with ethanol or with EMK, of the wild type and of the gpdA: aldA transformant. Manipulations and legends were as described in Fig. 2. Results with one multicopy transformant are shown. The results are representative among four independent experiments.

DISCUSSION

In this paper we have investigated the requirement of the pathway-specific AlcR transactivator and a coinducing compound for the transcriptional induction of the A. nidulans aldA gene (the structural gene encoding aldehyde dehydrogenase). We have established that all elements required in cis for proper aldA expression reside within 220 bp upstream of the start of translation of the aldA gene. We have identified within that promoter the sole functional target mediating the activation of transcription by AlcR. Like all other AlcR activation targets identified in vivo so far (6, 10, 12), it consists of two adjacent AlcR consensus sites. The target in aldA is organized as an inverted repeat of consensus sites, both with an A at the ambiguous first position (44). Furthermore, by using a transformant strain carrying a derepressed functional alcR gene, we have shown now that aldA itself is not subject to repression by means of the general carbon catabolite repressor CreA. Unlike alcA, the other structural gene required for ethanol utilization, CreA-mediated repression of the regulatory alcR gene completely accounts for aldA transcriptional repression. The sole putative CreA consensus sequence within the aldA promoter, although tentatively eclipsing the single functional AlcR target, is not sufficient to subject aldA directly to carbon catabolite repression. Most physiologically relevant CreA targets characterized in A. nidulans to date consist of two adjacent CreA consensus sites (10, 13, 45, 46).

We have now elucidated the means by which the two antagonizing control circuits, pathway-specific induction and general carbon catabolite repression, impose regulation on the three principal genes of ethanol catabolism (see Fig. 3). In both alcR and aldA, activation is mediated via a single AlcR inverted-repeat target, whereas in alcA three functional targets are present. The targets in alcA have been shown to act in synergy (12), and this could well account for the strength of the alcA promoter upon induction of the alcA gene system. However, the aldA promoter should be considered at least as powerful as that of alcA, despite the fact that it contains only one activation target. The direct involvement of the CreA repressor in the expression of alcA and alcR could explain this. Previously, evidence was presented that suggests that the repressor and activator compete for binding on the same promoter region in both these genes under all growth conditions (11). Carbon catabolite repression is a phenomenon that depends on the carbon source catabolic flow. In other words, carbon catabolite repression is never totally absent. As a result, disruption of functional CreA targets in both the alcR and alcA genes does not only lead to a derepression in the presence of D-glucose but also results in overexpression under induced conditions (7, 10, 13). Induction of aldA transcription is regulated in a more straightforward manner since the genuine aldA promoter is only subject to AlcR-mediated activation. The expression of
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aldA thus reflects the true force of the AlcR activation cascade mechanism mediated via a single cis-acting element.

Additionally, the differences in promoter strength could depend on the composition and the context in which the various functional AlcR targets reside. Previous studies have shown that the core context, spacing between the two half-sites of a target as well as orientation of the half-sites, influence AlcR binding in vitro (32, 44). It has been shown recently that one basic residue (Arg-6) in AlcR, outside the AlcR zinc binuclear cluster, is involved not only in DNA binding but also in transcriptional regulation. The rationale for the reduced transcription of aldA lies in the lack of binding of AlcR to the inverted repeat target when this basic residue is mutated (44). Another possible important consideration is the distance between the activation target and the site where the transcription machinery assembles, whereby the aldA promoter may be more accessible to the general transcription machinery. Indeed, it has been shown recently that nucleosome rearrangement can occur in A. nidulans in response to an induction signal (47). Finally, considering the involvement of ALDH in multiple catabolic pathways, the existence of a second, yet unidentified transactivator for aldA should not be excluded.

The second major finding reported in this paper is that acetaldehyde is the physiological inducer of ethanol utilization in A. nidulans. Our experiments with the aldA mutants indicate that when acetaldehyde (formed from regular cellular metabolism under noninducing growth conditions) is not sufficiently converted into acetate, the alc gene system is induced (cf. Fig. 7A). In the aldA mutants the expression level of alcR and aldA mRNAs in noninduced growth conditions reflects the stringency of the aldA mutation, i.e. the expected accumulation of acetaldehyde. Consistent with this, we recently showed that acetaldehyde can evoke induction when added externally to pregrown cultures.4 Ethanol utilization represents an example of a degradation pathway induced by a catabolic intermediate and not by the growth substrate itself.

Acetaldehyde is considered to be highly toxic (48, 49). Our results pose a challenging new question; how can the cells resolve the inducing and the toxic properties of this intermediate? As noted earlier, aldA67 mutants can still grow on carbon sources like lactose despite the apparent lack of ALDH activity. Therefore, the acetaldehyde accumulated in this mutant might be detoxified by other means. One possibility would be by reduction to ethanol, catalyzed by the aldA-encoded ADHI that is expressed pseudo-constitutively in aldA mutants. Acetaldehyde has been shown to be an excellent substrate for ADHI in vitro; reduction by ADHI is considerably faster than oxidation by ALDH (50). One has to note however that other mechanisms of detoxification may be operational, such as the presence of another ALDH activity that would not be sufficient by itself to support growth on ethanol. In the presence of ethanol as the sole carbon source, limiting amounts of coinducer would trigger the formation of more acetaldehyde since the ADHI reaction equilibrium is fully shifted toward oxidation of ethanol. One of the most prominent features of modest pseudo-constitutive expression in aldA15 is the presence of a considerable alcA transcript level. A preferential induction of alcA in the early stages of the induction process could enable enough accumulation of acetaldehyde to permit an optimal catabolic flow from ethanol. With the increase in intracellular acetaldehyde, the activation system can adapt the expression profile of the responsive genes until a steady state acetaldehyde concentration (below the toxic level) is reached. The presence of three synergistically acting AlcR targets in the alcA promoter together with differences in affinity among the functional AlcR targets in alcA, alcR and aldA could contribute to a preferential alcA induction in response to limiting amounts of acetaldehyde. In support of this, we have previously obtained evidence indicating that the proximal AlcR targets in the alcA promoter are predominant in transcriptional activation (12). Recently, we have shown that mutants, carrying a functional alcR gene but which is not subject to autoactivation (due to the disruption of the AlcR functional target), are perfectly able to grow on ethanol as the sole carbon source (10) even though the induction of the alcR responsive genes is far weaker than that in the wild type. This puts the autoregulation of the transactivator-encoding alcR gene into another perspective. The adaptation in response to increasing coinducer levels apparently does not require induced alcR expression but is facilitated by its endogenous regulation.

Another important question arising from the finding of acetaldehyde as the coinducer concerns the onset of induction. Induction requires acetaldehyde whose production requires ADHI. But expression of aldA by AlcR via acetaldehyde. To enable initial induction of alcA, a constitutive basal level expression of the alcR gene is required, and indeed, alcR basal transcription is substantially and is clearly observed in alcR loss-of-function mutants (7, 10, 11, 31). For the onset of the induction process, the level of acetaldehyde should further increase beyond the conversion capacity of the constitutively present ALDH. Changes in acetaldehyde concentration are important in determining the onset and maintenance of the induction process. Clear evidence is provided by transformants expressing aldA from the strong constitutive gpdA promoter (Fig. 8). Prominent expression of aldA in the early phases of induction, as is the case in such transformants, slows down the build-up of intracellular acetaldehyde. As a consequence, the induction process is suppressed due to a failure to induce alcA sufficiently. This implies that the apparent in situ level of ALDH plays an important role in the transduction of the coinducer signal to the transcriptional activator AlcR.

A subtle control of both aldA and alcA gene expression, in response to differential intracellular concentrations of acetaldehyde, could therefore be essential for the onset and maintenance of an optimal catabolic flow from ethanol.

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