The Zinc Binuclear Cluster Activator AlcR Is Able to Bind to Single Sites but Requires Multiple Repeated Sites for Synergistic Activation of the alcA Gene in Aspergillus nidulans*

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The alcA gene which is part of the recently identified ethanol regulon, is one of the most strongly inducible genes in Aspergillus nidulans. Its transcriptional activation is mediated by the AlcR transactivator which contains a DNA-binding domain belonging to the C6 zinc binuclear cluster family. AlcR differs from the other members of this family by several features, the most striking characteristic being its binding to both symmetric and asymmetric DNA sites with the same apparent affinity. However, AlcR is also able to bind to a single site with high affinity, suggesting that unlike the other C6 proteins, AlcR binds as a monomer.

In this report, we show that AlcR targets, to be functional in vivo, have to be organized as inverted or direct repeats. In addition, we show a strong synergistic activation of alcA transcription in which the number and the position of the AlcR-binding sites are crucial. The fact that the AlcR unit for in vitro binding is a single site whereas the in vivo functional unit is a repeat opens the question of the mechanism of the strong alcA transcription. These results show that AlcR displays both in vitro and in vivo a new range of binding specificity and provides a novel example in the C6 zinc cluster protein family.

The recently identified ethanol regulon in Aspergillus nidulans, localized to chromosome VII (1), comprises the structural gene alcA, encoding alcohol dehydrogenase I (2), the positive acting regulatory gene alcR (3) and at least five new identified alc genes (alcU, alcS, alcM, alcO, alcP), whose functions are unknown (1). The second structural gene necessary for ethanol utilization is alxA encoding aldehyde dehydrogenase, which maps to chromosome VIII (4). All these genes are inducible by the specific activator AlcR in the presence of an exogenous co-inducer (ethanol, the gratuitous inducer ethyl methyl ketone or other alcohols or ketones) (1, 5–7).

In the presence of glucose, the alc genes are subject to carbon catabolite repression mediated by the general repressor CreA (8, 9). Transcriptional repression can be achieved both by direct and independent repression of the trans-acting gene alcR (6) and of other alc genes such as the structural genes alcA and alLD (10), alcS, and alcP (1). Both repression and induction are mediated by binding of the regulatory proteins to their cognate targets in the alc promoters (11–13). It was proposed by Mathieu and Felenbok (10) that the two regulators AlcR and CreA compete for the same region of DNA containing both types of targets. Indeed it is the case for the alcR promoter, in which competition occurs even in non-inducing growth conditions (14).

The AlcR protein belongs to a subclass of the zinc DNA-binding family containing a zinc binuclear cluster (Zn(II),Cys(6)) found in ascomycetes (15, 16) with novel features. Previous work has identified two different types of specific targets, direct and inverted repeats with the same consensus core, 5′-CCGC-3′, separated by DNA of various lengths. Recently the consensus has been extended to 5′-CCCG(A/T)-3′ (17). In the positively autoregulated alcR gene, both direct and inverted repeat targets are closely linked (11). In the alcA promoter, three AlcR targets have been identified by gel retardation assays as well as enzymatic and chemical footprint methods. They are located within a short region of 150 base pairs very close to the transcription start (see Fig. 1). One palindrome is flanked by two tandem repeats (12). In previous experiments, we have shown by deleting the two AlcR repeated binding sites in the alcR promoter and two out of three AlcR-binding sites in the alcA promoter, that the AlcR consensus sequence should be functional in A. nidulans (11, 12). However, the deletion included both direct and inverted repeats. The alcA promoter is one of the strongest inducible promoters in A. nidulans. It is the most widely used for overexpressing proteins in A. nidulans and other filamentous fungi both for fundamental research and applied aspects in biotechnology (reviewed in Ref. 18). Therefore one intriguing question is to elucidate the molecular mechanisms of the strong alcA transcriptional activation in comparison to promoters of the other alc genes.

In contrast to other proteins of the zinc binuclear cluster family, the AlcR zinc-binding domain is able to bind to a single consensus site in vitro (17). Therefore it was important to determine if a single copy site was sufficient to induce transcription.

Here we present evidence that two sites, either in direct or inverted orientation, are necessary for full transcriptional activation. We also show that they act synergistically but not as the result of the cooperative binding of AlcR to multiple DNA sites. In addition, we show a strong effect position of the AlcR targets.

EXPERIMENTAL PROCEDURES

Strain, Media, and Growth Condition—The A. nidulans strain used as the host for transformation was alcA′ argB′ (biA1, pyroB4, argB2, alcA4951) (12).

Media and supplements were as described by Cove (19). The mycelia
for ADHI<sup>1</sup> were grown for 19 h at 37 °C on 3% lactose as sole carbon source. Induction was achieved by adding the gratuitous inducer 50 mM ethyl methyl ketone. Cells were harvested after a further 2.5 h (induced conditions).

**Polyacrylamide Gel Electrophoresis and ADHI Activity**—Gel electrophoresis and activity staining of ADHI were according to the method described by Sealy-Lewis and Fairhurst (20). Protein concentration was measured according to Bradford method (1976) (21). Concentrations of protein from different extracts were equalized before loading onto a gel and verified by Coomassie staining. Experiments were repeated at least twice and results vary by 15–20%.

**Plasmid Construction**—To create the alcA deleted mutants, a plasmid containing the alcA gene from the HindIII site (nucleotide –738) to the 3′ KpnI site (nucleotide +204) was constructed in Bluescript (bA2). Deletions have been carried out from PvuII (–423) to NsiI (–330) sites for the TA<sub>a</sub> mutant, from NsiI (–330) to MluI (–255) sites for the TA<sub>b</sub> mutant, from MluI (–255) to SplI (–167) sites for the TA<sub>c</sub> mutant. The TA<sub>a</sub>b<sub>c</sub> mutant carries a deletion from the PvuII (–423) to MluI (–255) sites.

The XbaI–XhoI fragment extracted from the pAN923–42bGII, carrying a mutant arg<sup>B</sup> allele was cloned into the Smal site of the bA2 plasmid, resulting in plasmid bA2 arg<sup>B</sup>. The plasmids mutated in the AlcR-binding sites on the alcA promoter were generated by oligonucleotide-directed mutagenesis carried out by the method of Kunkel et al. (22). An uracil-SK template containing the alcA promoter from the PvuII to SplI sites was mutagenized. The a, b, and c mutations were confirmed by sequencing.

Cloning of the mutated sequence was achieved by substituting the PvuII–SplI or PvuII–MluI fragments with the corresponding mutated sequences in the bA2 arg<sup>B</sup> plasmid. The alcA–arg<sup>B</sup>–host strain was transformed with the mutated plasmids. Southern blot analysis of the selected arg<sup>B</sup>– transformants showed that the plasmids were integrated in a single copy at the argB2 locus.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay was performed with a Mini ProteinII apparatus from Bio-Rad. The DNA binding shift assays were as described previously (10). 6% Polyacrylamide gels were run in TBE (0.25 ×) at 4 °C and 18 V/cm for 1 h. The relative apparent affinity of AlcR-tagged protein was calculated as the concentration of free AlcR protein necessary for half amount of DNA to be bound in a gel shift reaction (this value takes into account the AlcR-tagged protein purity which was estimated to be approximately 15 ± 5%). Quantification was performed on a PhosphorImager (Molecular Dynamics).

**RESULTS**

**AlcR Binds in Vitro to Three Specific Targets in the alcA Promoter**—As shown in Fig. 1 three AlcR targets, called a, b, and c, have been previously localized in the alcA promoter by footprinting experiments (12). These three AlcR targets are different to each other (see Fig. 1). Target b is a palindromic target in which the consensus motifs are separated by two nucleotides. The two sites of the tandem repeat targets a and c are separated by 7 and 8 nucleotides, respectively. In the c target region, the situation is complex since besides the two direct repeat sites (c<sub>a</sub> and c<sub>c</sub>) a third inverted site c<sub>b</sub> (with respect to c<sub>a</sub> or c<sub>c</sub>) is located 16 nucleotides downstream to c<sub>a</sub>. The 3′ last nucleotide in c<sub>b</sub> is T instead of A: 5′-CCGCT-3′.

In previous experiments, using the GST-AlcR(7:60) fusion protein, this c<sub>b</sub> site appeared to be very weakly protected in DNase I protection footprinting experiments and therefore was not further considered as a binding site (12). However, recent studies have shown that the use of glutathione S-transferase as a carrier protein introduces a bias in the binding specificity (17). We therefore repeated these gel-retardation experiments using a longer AlcR protein (1:197) oligohistidine tagged at its carboxyl terminus. Fig. 2 shows that, when the AlcR(1:197) protein concentration is increased, four complexes are formed with the fragment encompassing the three AlcR-binding sites (a, b, and c). It was necessary to explain the formation of these complexes in terms of AlcR occupancy of the individual binding sites.

Fig. 3A shows that target a, encompassing two direct repeat sites (–375 to –357) is able to form only one complex with AlcR(1:197). In contrast, for the palindromic target b, two complexes are observed with increasing AlcR concentration (Fig. 3B). The binding of AlcR to both sites of the palindromic target results in the slowest migrating complex. The amount of AlcR-DNA complex measured by scanning with a PhosphorImager increases gradually with the protein concentration which indicates that there is no cooperativity in binding. Surprisingly target c which contains three sites also forms a single complex (Fig. 4A). The simplest explanation is that one AlcR molecule binds to one site only, as will be discussed later. The apparent affinity (K<sub>d</sub>) determined from the scanning of AlcR(1:197) for the formation of one complex with targets a, b, and c, is in the same range 4 × 10<sup>−8</sup> ± 20%. A slightly lower affinity (2-fold) for the formation of the second complex with the palindromic site b, is observed. These experiments show that in vitro AlcR binds...
Mechanism of alcA Transactivation by AlcR

![Fig. 2. DNA binding of AlcR(1:197) protein to the alcA promoter. Gel mobility shift assays were performed as described under “Experimental Procedures” using a constant amount of labeled PvuII-NsiI fragment encompassing the three AlcR-binding sites and increasing concentration of AlcR(1:197) protein as indicated on the top of the gel. The concentration of AlcR(1:197) was estimated from SDS-polyacrylamide gel electrophoresis assuming that AlcR represents about 15% of the purified fraction (17). cx1, cx2, cx3, and cx4 correspond to the four complexes formed with this fragment.](image92x621 to 264x729)

![Fig. 3. DNA binding of AlcR(1:197) protein to targets a and b in the alcA promoter. Gel mobility shift assays were performed with increasing AlcR(1:197) protein concentration as indicated on the top of each gel. Arrows represent the orientation of the consensus motif (5’-CCGCA-3’). A, binding of AlcR(1:197) protein to the 32P-labeled PvuII-NsiI fragment encompassing AlcR target a. B, binding of AlcR(1:197) protein to the 32P-labeled AvaII-MluI fragment encompassing AlcR target b. cx1 and cx2 correspond to the two complexes formed with this fragment.](image358x459 to 514x729)

...to a single site in direct repeats whereas it binds to two sites in inverted repeats, in a non-cooperative manner.

**Role of the Three Sites in Target c on AlcR Binding**—It was important to understand the contribution of each of the three individual sites in target c, organized as a direct repeat (c1-c2), and c3 which is part of the inverted repeat c2-c1 or c1-c2 (see Fig. 1). Site-directed mutagenesis of the three individual sites was carried out and AlcR binding was tested in vitro in gel band shift experiments with increasing AlcR(1:197) protein concentration (Fig. 4). It is clear that in c1-c2 direct repeats only a single site binds AlcR (Fig. 4D), as with c2-c1 (Fig. 4C), in agreement with our previous results with target a (Fig. 3A).

To discriminate the site responsible for AlcR binding, the site c2 was mutagenized leaving c1 (Fig. 4F), and the two sites c1 and c2 were disrupted, leaving c3 intact (Fig. 4E). Fig. 4, E and F, show that with these two latter probes, AlcR(1:197) binding was completely impaired. Therefore it can be concluded that the c1 site is the only one which binds AlcR(1:197) with a high affinity ($4 \times 10^{-8}$ M), as shown in Fig. 4B. These data are consistent with results of footprinting experiments which exhibit interference solely with binding site c2 (17).

Each AlcR Target in the alcA Promoter Contributes Differently to the Activation of alcA Expression—As a first approach, the contribution of the three individual AlcR direct and inverted repeat targets to alcA expression was tested by deletion analysis, and then, by mutagenesis, at all times retaining two out of the three AlcR-binding sites. The effects of these modifications on the alcA expression were analyzed in monocopy alcA transformants in which the alcA plasmid was integrated at the argB locus. Three different parameters were monitored: (i) growth tests on ethanol, (ii) toxicity growth test on glycerol with allyl alcohol (allyl alcohol can be oxidized by ADHI into acrolein, a highly toxic compound), (iii) measurements of ADHI activity in non-denaturing gel electrophoresis using mycelia grown under non-induced and induced conditions. We could not analyze the alcA transformants by Northern blot analysis since the recipient strain (alcA4951) shows a normal alcA transcriptional pattern, even if the transcription is 10-fold impaired compared with the wild type (data not shown). Since the three tests were in complete agreement, only the semi-quantitative evaluations of ADHI activity are being presented here. This test allows the quantification of ADHI activity only whereas assaying the enzyme in crude cell extracts measures the activity of all alcohol dehydrogenases in the cell. As seen in Fig. 5, both deletion and mutagenesis of AlcR target a result in a 70% decrease in ADHI activity in transformants TAm and TAa, respectively. However, the 30% remaining ADHI activity is sufficient for normal growth on ethanol.

Deletion of target b in transformants results in normal growth on ethanol, whereas transformants carrying mutations in one or both sites of the target b, are unable to grow on ethanol (data not shown). ADHI activity measured in these transformants are in complete agreement with these growth tests. No ADHI activity is observed when one or both symmetric sites are mutagenized in target b transformants, TAhalfb and TAbm, respectively, whereas there is substantial ADHI activity in the b site deletion transformant TAb (Fig. 6). These results could appear contradictory. However, upon examining carefully the target b deleted alcA promoter, it appears that target a occupies the position of the deleted target b in relation to the start of transcription (240 base pair upstream). In other words, the resulting deleted promoter containing site a and c drives 25% of ADHI activity. Therefore the straightforward conclusion of these experiments is that there is a crucial position effect of site b in the transcriptional activation of the alcA promoter.

The opposite situation is observed when the AlcR site c in the alcA promoter is deleted. No growth on ethanol is observed with the corresponding transformants (TAc) and no measurable ADHI activity is found in their extracts. However, mutagenesis of this c site results in an ethanol partial utilization phenotype and in a weak but visible ADHI activity after gel staining (TAm) (see Fig. 7). Therefore deletion of site c prevents alcA transcriptional activation in part via another sequence than the AlcR-binding sites.

By comparing the ADHI activity remaining after mutagenesis of each target (TAm, TAbm, TAmc, Figs. 5–7) it can be seen that each target appears to contribute differently to alcA expression by AlcR binding, i.e. b > c > a. Therefore, we can...
conclude that target b in the alcA promoter is the most important for induced transcriptional activation.

The Functional AlcR Target c Comprises Three Sites—We have shown that, in vitro, the three AlcR consensus sites in target c do not have equivalent roles in AlcR binding, c2 being the only site directly involved in the in vitro AlcR binding. To determine which sites have a functional role in the activation of the alcA gene, different combinations of mutagenized sites in target c were analyzed. The resulting alcA promoter contains sites a and b as well as the mutagenized site c. Fig. 8 shows that mutation in site c2 results in a 25% decrease in ADHI activity compared with the wild type. Surprisingly, mutation in site c1 which leaves the inverted repeat c2-c3, gives a decrease in ADHI activity of only 2-fold compared with the wild type. In contrast, simultaneous mutations in both c1 and c3, leaving the single site c2, results in a drastic decrease in ADHI activity (20-fold), comparable to that obtained after disruption of the three sites c1, c2, and c3 (Fig. 7).

Therefore, the first conclusion is that to be functional, AlcR targets have to be organized as direct or inverted repeats. Second, in target c, the direct repeat site, c1-c2, is the most important in alcA transcriptional activation. Finally, the inverted repeat site, separated by 16 nucleotides (c2-c3), is still capable of substantial activation of alcA transcription whereas single sites are inactive. Therefore, in this case, it could be considered that c2-c3 is a palindrome rather than two separate sites. It is surprising that mutations of sites such as c1 and c3 which have no binding activity in vitro affect alcA expression in vivo. These results open the question on the definition of physiological AlcR targets, which have to be direct or inverted repeats.

Synergistic Activation of alcA by AlcR Is Mediated via Its Specific Binding Sites—We have seen that disruption of the direct repeat site a in the alcA promoter results in 70% decrease in ADHI activity (see Fig. 5). Double deletion of target sites a and b, leaving target c intact, has a drastic effect on alcA expression ADHI activity (~5%) resulting in a low noninducible level. Therefore as seen in Fig. 9, and in agreement

Fig. 4. DNA binding of AlcR(1:197) protein to AlcR target c in the alcA promoter. Gel mobility shift assays were performed with increasing concentration of AlcR(1:197) protein. Binding of AlcR(1:197) protein to the 32P-labeled MluI-PstI fragment encompassing: A, the three sites c1, c2, and c3; B, the c1 and c3 mutagenized sites; C, the c2 mutagenized site; D, the c1 and c2 mutagenized sites; E, the c1 and c2 mutagenized sites; F, binding of AlcR(1:197) to 32P-labeled oligonucleotide encompassing c1 and c2 sites in which c2 is mutagenized. cx, complex; p, probe.

Fig. 5. ADHI activity in target a disrupted strains. All mycelia were grown for 19 h at 37 °C on 3% lactose as sole carbon source and induction was achieved by adding 50 mM ethyl methyl ketone for a further 2.5 h. The amount of protein loaded per track was equalized and controlled by Coomassie staining. Independent experiments including mycelium growth and running gels were performed at least twice. Activity staining of ADHI was according to Sealy-Lewis and Fairhurst (20). The scanning diagram was normalized to a value 100, representing ADHI activity of the wild-type strain under these conditions. The numbers reported are accurate to within ±15–20%. A, ADHI activity of TAΔa strain which is deleted for the AlcR target a. B, ADHI activity of TAAma strain which is mutagenized for the AlcR target a.
Mechanism of \textit{alcA} Transactivation by AlcR

**Fig. 6.** ADHI activity in target \textit{b} disrupted strains. Growth conditions, ADHI activity staining, and further details are as described in the legend to Fig. 5. \textit{A}, ADHI activity of T\text{A}\text{D}b strain which is deleted for the AlcR target \textit{b} in the \textit{alcA} promoter. \textit{B}, ADHI activity of T\text{A}\text{m}b strain which is mutagenized for the AlcR target \textit{b} in the \textit{alcA} promoter. Strains T\text{A}\text{m}\text{A}b and T\text{A}\text{m}b show the same ADHI activity (data not shown).

**Fig. 7.** ADHI activity in target \textit{c} disrupted strains. Growth conditions, ADHI activity staining and further details are as described in the legend to Fig. 5. \textit{A}, ADHI activity of T\text{A}\text{D}c strain which is deleted for the AlcR target \textit{c} in the \textit{alcA} promoter. \textit{B}, ADHI activity of T\text{A}\text{m}c strain which is mutagenized for the AlcR target \textit{c} in the \textit{alcA} promoter.

with our results, no additive response with each binding site is observed and, in fact, a strong synergistic effect is noticed. Another important conclusion is that this synergistic activation of \textit{alcA} transcription occurs through the binding of AlcR to its cognate binding sites.

**DISCUSSION**

In this study we have investigated the molecular basis of the high induction level of the \textit{alcA} gene in \textit{A. nidulans}. The three AlcR-binding sites, occupying a short 150-base pair region in the \textit{alcA} promoter are shown to work synergistically to activate \textit{alcA} transcription. Disruption of the distal AlcR-binding site, \textit{a}, results in a 70% decrease in \textit{alcA} expression whereas combined disruption of the two AlcR sites, (\textit{a} and \textit{b}), leads to a 95% decrease, resulting in a residual noninducible ADHI activity, sufficient to allow only weak growth on ethanol.

This synergism is not explained by cooperative binding of AlcR to the three binding sites, as we observed by \textit{in vitro} DNA binding experiments. Moreover AlcR was shown to bind to single-site (17) (results herein). The same AlcR(1:197) apparent affinity was observed upon binding to a palindromic site (\textit{b}), a direct repeat (\textit{a}), or a single site (\textit{c} for example). The absence of cooperativity distinguishes AlcR from GAL4 for which cooperative DNA binding provides an explanation for the synergistic effects observed \textit{in vivo} (23, 24). We have to point out that the AlcR(1:197) protein utilized in these studies was truncated but contained a region downstream to the zinc binuclear cluster shown in most proteins of this C6 family class to contain the linker and dimerization regions (Ref. 15, reviewed in Refs. 25 and 26). Moreover it was shown using the transcription-translation reticulocyte system that no heterodimers were formed (17) and in addition with the \textit{\lambda} CI repressor system (27) no sequence in AlcR could serve as a dimerization element.2 In agreement with this result, physiological data, obtained after mutagenesis of the most proximal target (\textit{c}) to the transcription start site, have shown clearly that even a single site (\textit{c}) is necessary for full transcriptional activation of \textit{alcA}. Therefore all these data are consistent with the finding that AlcR binds DNA as a monomer. The number of AlcR-specific sites in the \textit{alcA} promoter could be one of the parameters playing a role in the synergistic activation of the \textit{alcA} gene, but not the only one. In fact, in other inducible \textit{alc} clustered genes (1), the localization and the number of AlcR-binding sites are variable and do not seem directly correlated with the strength of the \textit{alc} promoters (28).3

Another important parameter in synergistic \textit{alcA} transcriptional activation is AlcR's target position effects. The position of the upstream target \textit{a} is the least important among the three, since its deletion or its mutagenesis, does not abolish inducibility of the \textit{alcA} promoter. However, both target positions \textit{b} and \textit{c} are important, but at different levels. Half-site organization either in symmetric or asymmetric repeats, is not involved in this position effect. In fact, replacing the palindromic site \textit{b} by the direct repeat site \textit{a}, resulting in an \textit{alcA} promoter containing two direct repeat binding sites, allows transcriptional induction as with the disrupted binding site \textit{a}. In contrast, disrupting target \textit{c} sites leaving single sites, strongly prevented the \textit{alcA} induction mediated by AlcR binding to the remaining

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2 I. Nikolaev, F. Lenouvel, and B. Felenbok, unpublished results.

3 S. Fillinger and B. Felenbok, unpublished results.
targets a and b. A stronger effect was even observed when target b sites were mutated (either one or both), resulting in a total loss of alcA transcription, as if AlcR was no longer able to occupy the remaining targets a and c in the absence of target b in the alcA promoter. Therefore the position occupied by target b is crucial in the transcriptional activation process by AlcR.

The above data provide evidence that a single copy of the AlcR target is not sufficient to mediate AlcR induced transcriptional activation, and that target positions in the promoter are determinant in this process, unlike transcriptional activation with GAL4. In fact with this later activator a single copy of GAL4 palindromic site is able to activate transcription, with a wide tolerance in position in the GAL4 controlled promoter (24, 29).

Finally our results open the question of the functional organization of AlcR targets. Interestingly, to be functional, AlcR targets in the alcA promoter have to be organized in repeats, either in tandem or in palindrome. Mutations in target b, leaving a single site, result in a total loss of alcA transcription. This is also true in the case of target c which contains three sites and has a double overlapping organization with direct sites (e1-e2) and two possible invert repeats (e2-e3 and e1-e3). In fact, the direct repeat sites (e1-e2) contribute predominantly to target e functionality. However, the inverted repeat couple (e2-e3), in which the spacing is important (16 nucleotides), also have a substantial activity when the upstream site e1 is destroyed. In that case, there is an alternative utilization of the couple e2-e3 as was shown for another transactivator of A. nidulans, AreA, which could utilize alternative GATA-binding sites in the alcA promoter region (30). Our results rule out completely in vivo utilization of AlcR single sites.

These results are not in favor of the dimerization of AlcR in the presence of properly spaced DNA sites. This was described for the zinc finger protein ADR1 (31), and more recently for the zinc cluster protein HAP1 whose cooperative interaction with the DNA stabilizes dimer binding (32). A number of nuclear receptors are also monomeric in solution but bind as heterodimers, with strong cooperativity, to their cognate targets. This asymmetric dimerization interface provides the molecular basis for receptor heterodimers spacer discrimination for binding to asymmetrical half-sites (33).

Therefore to explain the necessity of two AlcR-binding sites, the involvement of another element should be hypothesized. Another nonexclusive and relevant model, other than the cooperative DNA-binding of a dimer activator to its cognate targets, that could explain synergistic transactivation, is the multiple contact model. This model was proposed by Lin et al. (34) and Carey et al. (35) and suggests that synergism is a manifestation of multiple contacts between activators and the general transcription machinery. Multiply bound copies of a single activator could contact a common target interacting with the transcriptional apparatus. In eukaryote cells, some TATA-binding protein-associated factors function as coactivators which mediate synergistic activation by multiple activators (reviewed in Refs. 36–38). However, while some of the data seem to support the multiple contact model, there is evidence supporting the cooperative DNA-binding model and discrepancies appear between results for the same GAL4 activator which are not understood (for example, see Refs. 24 and 34). It is interesting to point out that the AlcR unit for in vivo binding is a single site, whereas the in vitro functional unit is a repeat even when the spacing between the sites is 16 base pairs as for e2-e3. In addition, AlcR discriminates in vivo between these sites with the same consensus sequence, since with e1 and e3, for example, no AlcR binding is observed even when the e3 site is mutated (Fig. 4). It is possible that parts of the AlcR protein missing in our in vitro experiments are necessary for the binding to those sites. This hypothesis has been eliminated by results obtained after a complete mutagenesis of the AlcR site flanking regions to the consensus core.2 Another line of evidence against this idea is that inverted repeats both bind AlcR molecules, suggesting that the remainder of AlcR is not necessary in this case. The hypothesis of a steric hindrance preventing AlcR binding in vivo to the second site of direct repeats, has been also ruled out by site-directed mutagenesis experiments.2 In both crystallographic studies of GAL4 and PRR1-DNA complexes it has been pointed out that other factors could possibility interact with the proteins (39, 40). In PPR1, there is a cavity near the DNA-binding site which has been suggested to be a binding site for another component of the transcription machinery or a site of contact from another region of PPR1 (40).
The three-dimensional structure of the AlcR-DNA complex is under study and we do not know yet if such a cavity exists also in AlcR. The interaction of another protein with AlcR contacting the transcriptional machinery could account for both in vitro binding experiments and physiological results.

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