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# The DAL82 protein of *Saccharomyces cerevisiae* binds to the DAL upstream induction sequence (UIS)

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## ABSTRACT

**Expression of the *DAL2*, *DAL4*, *DAL7*, *DUR1,2*, and *DUR3* genes in *S.cerevisiae* is induced by allophanate, the last intermediate in the allantoin catabolic pathway. Analysis of the *DAL7* promoter identified a dodecanucleotide, the *DAL7 UIS*, which was required for inducer-responsiveness. Operation of the *DAL7 UIS* required functional *DAL81* and *DAL82* gene products. Since the *DAL81* product was not an allantoin pathway-specific regulatory factor, the *DAL82* product was considered as the more likely candidate to be the *DAL UIS* binding protein. Using an *E.coli* expression system, we showed that DAL82 protein specifically bound to wild type but not mutant *DAL UIS* sequences. DNA fragments containing *DAL UIS* elements derived from various *DAL* gene promoters bound DAL82 protein with different affinities which correlate with the degree of inducer-responsiveness the genes displayed.**

## INTRODUCTION

Expression of the allantoin catabolic pathway (*DAL*) genes of *Saccharomyces cerevisiae* is regulated by three environmental conditions. A rich supply of nitrogen efficiently inhibits expression, a physiological response known as nitrogen catabolite repression (NCR) (1, 2). Under non-repressive conditions, but in the absence of pathway precursors or metabolites, the *DAL* genes are expressed at low basal levels. In the absence of a rich nitrogen supply, but availability of allantoin or its catabolic derivatives, *DAL* gene expression is induced to high levels (1, 3). Although all of the *DAL* genes are sensitive to NCR, not all are inducer-responsive (1, 2, 4–6). Several, including *DAL3* and *DAL5*, are expressed at their highest levels in an inducer-independent manner except when down-regulated by NCR (4, 7, 8).

Previous work has identified the cis-acting elements and trans-acting proteins that mediate these environmental responses. All of the *DAL* genes possess multiple copies of the upstream activation sequence $UAS_{NTR}$ in their 5′ flanking regions (4, 7–13). The operation of $UAS_{NTR}$ requires a functional *GLN3* product, and it is through this cis-acting element that NCR is exerted (14). Detailed genetic analysis of the $UAS_{NTR}$ demonstrated it to be a dodecanucleotide with the sequence GATAA at its core (15).

All of the *DAL* genes are down-regulated through a second element, the upstream repression sequence, $URS_{GATA}$ and the DAL80 protein that binds to it (16–18). This element is also responsible for maintaining inducible *DAL* gene expression at its low, basal level when inducer is absent from the cell (16, 18, 19). In *dal80* deletion mutants all *DAL* genes are overexpressed to some degree and those that are inducible become largely inducer-independent (18,19). It was not until recently that we have had any concrete idea of the structure of the cis-acting element through which *DAL80* product regulated *DAL* gene expression even though we have known for some time that the site existed (17). Elucidation of its gross structure depended upon the finding that DAL80 is a DNA binding protein. Using electrophoretic mobility shift assays (EMSAs), the optimum $URS_{GATA}$ site tested was shown to consist of two sequences containing GATAA at their core, oriented tail to tail, 20 bp apart (17).

The third element identified upstream of the inducible *DAL* genes, the upstream induction sequence (*UIS*), mediates inducer-responsiveness (16). Saturation mutagenesis of the *DAL7 UIS* site demonstrated it to be a dodecanucleotide with the consensus sequence 5′-GAAAATTGCGTT-3′ (20). One or more copies of a sequence homologous to this one is situated upstream of all inducible *DAL* genes and the *CAR2* gene which also responds to allantoin pathway control (20). Two trans-acting gene products, *DAL81/UGA35/DURL* and *DAL82/DURM*, are required for *DAL* gene response to inducer (21–26). Since our earlier experiments suggested that the *DAL UIS* element bound one or more proteins, both of these molecules were candidates for DNA binding proteins (20). The *DAL81* product was the least likely candidate to be the *UIS* binding protein, however, because it is not allantoin pathway specific (24, 25). It is also required for response of the *UGA* genes to inducer (22, 24, 25). This left the *DAL82* product as a prime candidate to be the *DAL UIS* binding protein. Experiments described in this work were designed to test this hypothesis. A preliminary report of this work has already appeared (27).

## MATERIALS AND METHODS

### Expression of *DAL82* in *E.coli*

The strategy described in Figure 1 was used to construct plasmid pRD31 (T-7-DAL82), which contains the *DAL82* coding

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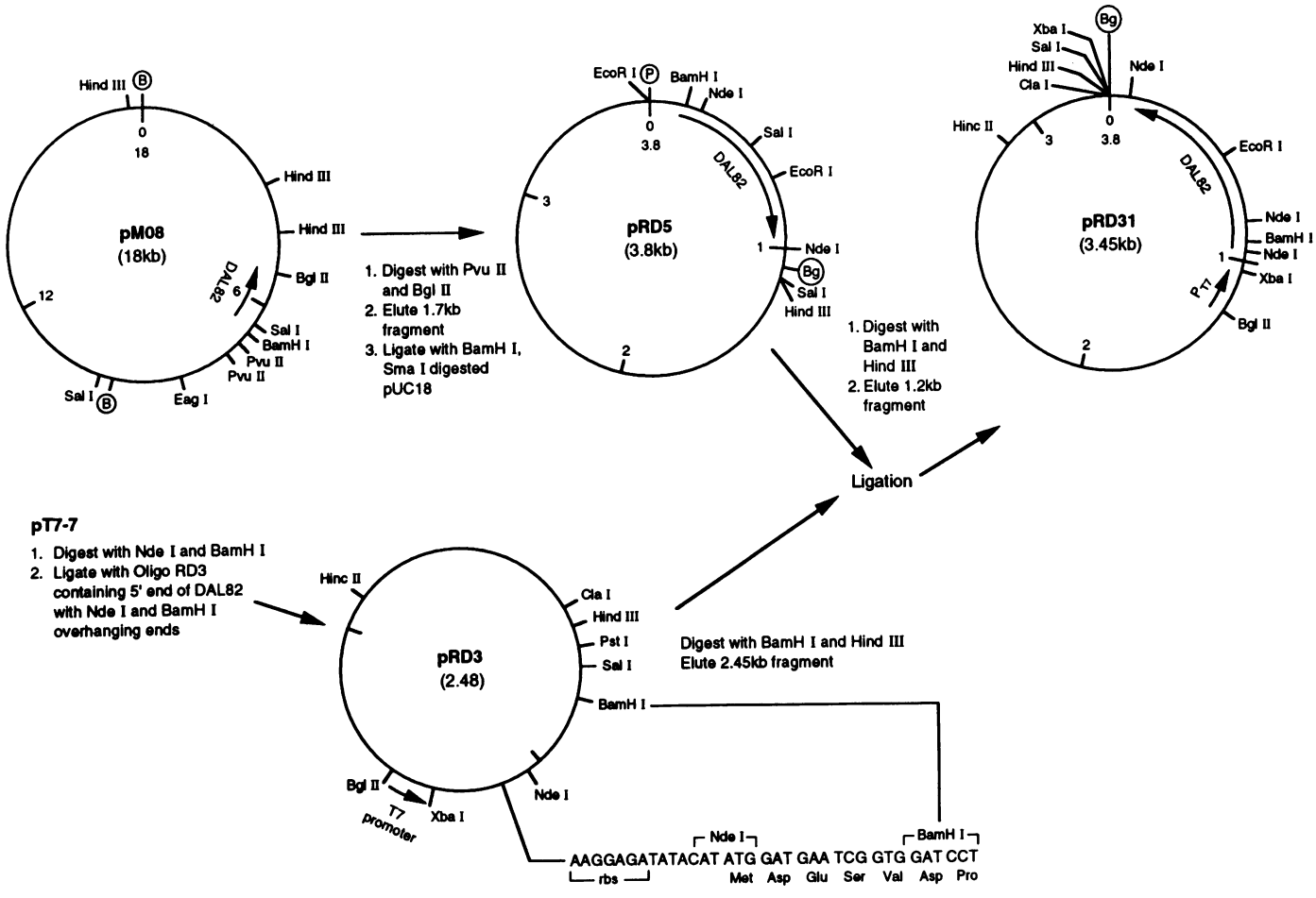


**Figure 1.** Strategy used to construct plasmids pRD31 and pRD34 which express the *DAL82* gene under control of the T7 gene 1 promoter. Arrows denote the position and orientation of the *DAL82* coding sequence and the orientation of the T7 promoter. B represents a destroyed *Bam*HI site, while Bg and P represent destroyed *Bgl*II and *Pst*I sites respectively; rbs shows the position of the T7 gene 1 ribosome binding site.

sequence derived from plasmid pM08 (26) fused behind the T7 gene 1 promoter and ribosome binding site of plasmid pT7-7 (28). Plasmid pRD41 (T-7-DAL82 Epitope) is identical to pRD31 except that a nine amino acid epitope derived from the influenza hemagglutinin protein and a four amino acid factor Xa recognition site were fused to the amino terminus of *DAL82* (oligonucleotide RD4, (Figure 2). Recombinant plasmids were prepared and maintained in strain HB101 (using standard recombinant DNA techniques (29). Plasmids pT7-7, pRD31, and pRD41 were expressed in strain BL21(DE3) to produce protein extracts essentially as described by Studier (30). Cultures were grown at 37°C (A595 = 0.4) in LB broth containing 125 mg per litre ampicillin. At this point, IPTG was added to a final concentration of 1 mM and the cells incubated for a further three hours at 37°C. After harvesting, the pellets were frozen at −80° C, and the cells were resuspended in 1/20 volume cold (4°C) sonication buffer (20 mM Tris HCl, pH 7.4, 100 mM KCl, 1 mM DTT, 10 mM $MgCl_2$, 1 mM PMSF). Cells were sonicated using a microtip (Fisher Scientific Sonic 300 Dismembrator) for 15 cycles of 10 second bursts at 35% power punctuated by 10 second periods on ice. Cell debris was removed by centrifugation at 10,000 rpm for 30 min at 4°C and glycerol was added to the supernatnant (final concentration (v/v) of 20%). Aliquots were stored at −80°C.

## SDS-PAGE and electromobility shift assays (EMSAs)

SDS-PAGE was performed as described by Sambrook et al. (29). Cells were pelleted and resuspended in 1/10 volume sample cracking buffer (62.5 mM Tris HCl pH 6.8, 2% SDS, 5% (w/v) β-mercaptoethanol, 0.005% bromophenol blue). Samples were heated to 95°C for 5 min and 20 μl were then loaded per lane. Proteins were visualized by staining with coomassie brilliant blue.

EMSAs were performed essentially as described by Luche et al (31). The sequences of oligonucleotides used in the experiments described in this work are shown in Figure 2. When used as EMSA probes, they were end-labelled with polynucleotide kinase and unless otherwise stated, approximately 50 ng of radioactive DNA probe were used per assay. Probes were incubated at room temperature with 1−2 mg total crude protein for 20 min in incubation buffer (4% glycerol, 4 mM Tris HCl [pH 8.0], 100 mM NaCl, 4 mM $MgCl_2$, 0.2 mg/ml sheared calf thymus DNA, 1 mM DTT), and the complexes separated from free DNA on 4% polyacrylamide gels. All competition assays were

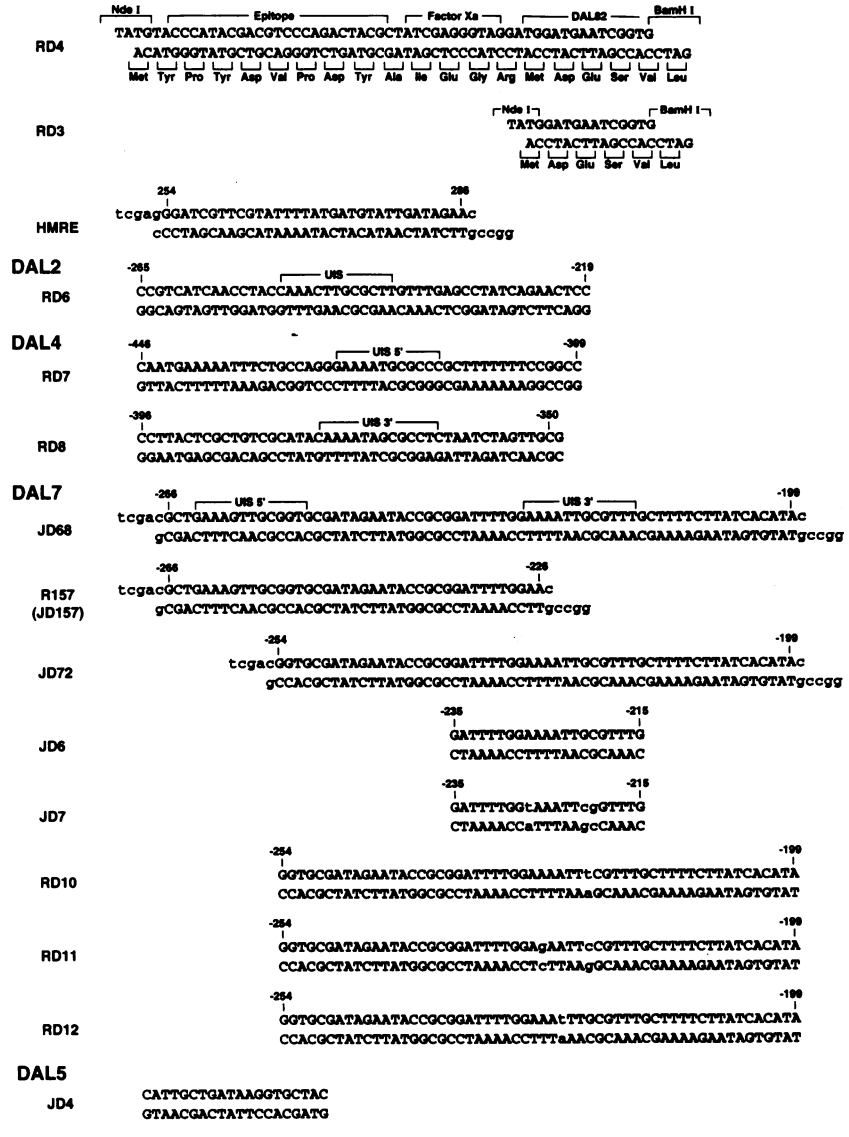


**Figure 2.** Oligonucleotides used in this study. Lower case letters within the sequence indicate mutated bases, while lower case letters at the 5′ and 3′ ends designate restriction enzyme 5′ extensions which are not part of the native sequence. All other oligonucleotides were constructed with blunt ends. In one case, fragment JD72, we used both a version that contained the restriction site extensions and one that was blunt-ended and did not contain them. The presence or absence of the restriction site extensions on fragment JD72 did not affect the EMSA results observed. The position of the *UIS*s, relevant restriction enzyme recognition sites and other features are indicated above and below the sequences.

performed in the presence of protein extracts derived from cells containing plasmid pRD31.

## RESULTS

### Expression of the *DAL82* gene in *E.coli*

To provide *DAL82* protein, devoid of other yeast proteins, we cloned the *DAL82* gene downstream of the T7 gene 1 promoter (Figure 1) and expressed it in *E.coli*. We also similarly constructed a version of the gene that encoded an epitope tag on the N-terminus of the protein (Materials and Methods). When cells containing only the vector plasmid pT7-7 (T-7) were expressed in *E.coli*, we observed the pattern of proteins depicted in Figure 3, lanes A and B. Cells expressing a plasmid containing *DAL82* (T-7-DAL82) produced the same protein profile with exception of a large amount of a protein that possessed the mobility expected of DAL82 protein (Figure 3, lanes C and D). When the epitope tagged version of the gene was expressed in *E.coli* (T-7-DAL82 Epitope), the mobility of the highly expressed protein decreased slightly (Figure 3, lanes E and F).

### *DAL82* binding to DNA fragments containing the *DAL7 UIS*s

To ascertain whether the *E.coli*-produced protein was able to bind to DNA fragments containing *DAL7 UIS* elements, the above extracts were used as the source of protein for EMSAs. As shown in Figure 3, lane G and H, use of a reaction mixture devoid of protein or one containing protein derived from *E.coli* expression of control plasmid pT7-7 (T-7), did not yield a DNA-protein complex. However, when the assay was repeated with extracts containing either plasmid T-7-DAL82 or T-7-DAL82 Epitope,

a pair of DNA−protein complexes were observed (Figure 3, lanes I and J). Careful inspection of lighter exposures of the autoradiographs depicted in Figure 3 revealed that the DNA-protein complexes appearing in lane J migrated slower than those in lane I as expected from the higher mass of the DAL82 epitope-containing protein.

To ascertain whether the DNA-protein complexes in Figure 3 displayed sequence specificity, we performed two competition EMSAs. We compared the abilities of DNA fragments JD72 and HMRE to compete with fragment JD72 for DAL82 binding. As expected, DNA fragment JD72 was an effective competitor (Figure 4, left panel, lanes A to G). In contrast, DNA fragment HMRE, which is known to contain binding sites for the ABF1 protein (32), was not an effective competitor of radioactive probe JD72 (Figure 4, left panel, lanes G to M).

Localization of the site of DAL82 protein binding upstream of the *DAL7* gene was accomplished using EMSAs with several DNA fragments containing portions of the *DAL7* upstream region (Figure 5). DAL82 protein yielded three DNA-protein complexes when incubated with DNA fragment JD68 (Figure 5, left panel, lane I); this DNA fragment contained most of the *DAL7* upstream region (−266 to −199). We next tested DNA fragments, R157 (JD157) and JD72, which roughly contained the 5′ and 3′ *UIS* elements of DNA fragment JD68, respectively (top of Figure 5). In the case of DNA fragment R157, DAL82 protein yielded only a very faint complex (Figure 5, left panel, lane F). Upon overexposure of the autoradiograph, one could see that both DAL82 complexes were present, though the higher mobility complex does not show in the photograph presented in Figure 5. The faint signal obtained in lane F raised some doubt as to whether the band was real. To more critically assess this question we determined whether DNA fragments R157 and JD72 could compete with one another for DAL82 binding. As shown in the center and right panels of Figure 4, both DNA fragments JD72 and R157 were able to compete with one another for binding to DAL82 protein, with fragment JD72 being the better competitor.

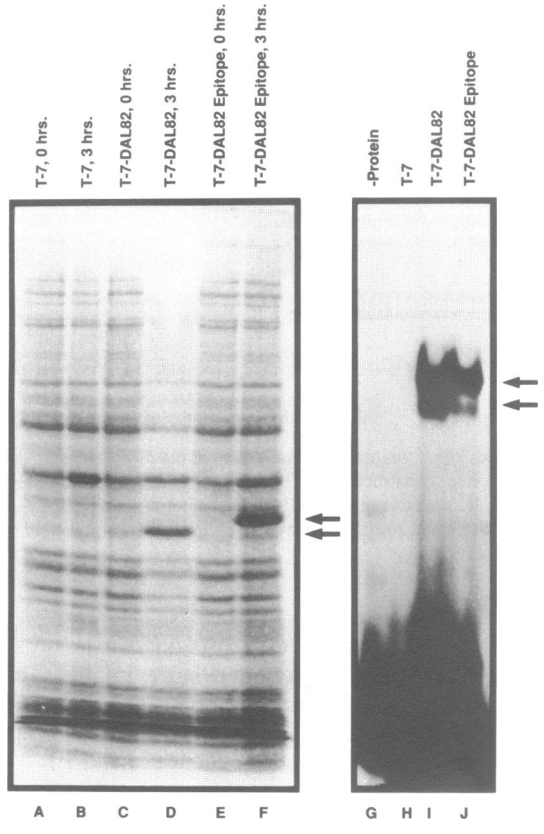


**Figure 3.** Left Panel. SDS-PAGE analysis of expression of *DAL82* in *E.coli*. Cells containing plasmids pT7-7 (T-7, lanes A and B), pRD31 (T-7-DAL82, lanes C and D) and pRD41 (T-7-DAL82 Epitope, lanes E and F) were sampled before (lanes A, C and F) and three hours after induction with IPTG (lanes B, D and F). Two arrows indicate the position of DAL82 protein (33 kD) and the epitope tagged version of DAL82 protein (35 kD). Right panel. EMSA performed with probe JD72, Lane G shows probe incubated in the absence of protein extract while lanes H, I and J samples incubated in the presence of protein extracts derived from cells containing plasmids pT7-7 (T-7), pRD31 (T-7-DAL82), and pRD41 (T-7-DAL82 Epitope), respectively. Arrows indicate the two complexes observed when DAL82 protein was present.

To more precisely locate sequences to which DAL82 protein were binding, we repeated the above EMSAs with smaller DNA fragments containing single *DAL* gene promoter elements. As shown in Figure 5, right panel, lane L, an EMSA containing DNA fragment JD4 did not yield a DNA-DAL82 complex. Fragment JD4 contained the $UAS_{NTR}$ element previously shown to be required for *DAL5* transcriptional activation (15). In contrast, when DNA fragment JD6, which contained little more than the *DAL7 UIS* element was used in the EMSA, a DAL82-specific band was observed (Figure 5, lane O). The DNA-protein complex that appeared in lane O was not observed when DNA fragment JD7, containing a highly mutated form of *DAL7 UIS*, was used in place of the wild type (Figure 5, lane R). These data suggested that DAL82 bound to DNA fragments that contained the *DAL7 UIS* element.

### *DAL82* binding to wild type and mutated *UIS* elements

Previous genetic analyses that defined the *DAL UIS* element demonstrated it to be highly sensitive to mutation. Even single mutations were capable of destroying function. To ascertain whether we could correlate representative point mutations that resulted in loss of *UIS* transcriptional function with loss of DAL82 binding, we performed several EMSAs using the wild type and mutated *DAL7 UIS* fragments. DNA fragment RD12, which contained an A to T substitution in the *DAL7 UIS* element (See Figure 2), did not destroy the element's ability to support inducer-responsive expression of a reporter gene (Figure 3, ref. 20). This fragment was also able to form a complex with DAL82 protein and to act as an effective competitor of control fragment JD72 (Figure 6, left panel, lane I; middle panel, lanes A to G; and right panel, lanes G to O). A substitution of G to T at *UIS* position −221 (fragment RD10), which resulted in loss of *UIS* transcriptional function, also resulted in formation of only a weak DNA-DAL82 complex (Figure 6, left panel, lane E). Moreover, fragment RD10 was not an effective competitor of fragment JD72 for DAL82 binding (Figure 6, middle panel). Mutations, A to G at *UIS* position −226 and G to C transversion at *UIS* position −221 (DNA fragment RD11) destroyed the ability of the fragment to form a complex with DAL82 (Figure 6, left panel, lane G). Fragment RD11 was also unable serve as an effective competitor of control fragment JD72 for DAL82 binding (Figure 6, right panel, lanes A to G). Each of these mutations separately have been shown to destroy *UIS*-mediated reporter gene induction (20).

### *DAL82* binding to *UIS* elements derived from other *DAL* genes

The *DAL UIS* element has been suggested to mediate inducer-responsive expression of all inducible allantoin pathway genes.

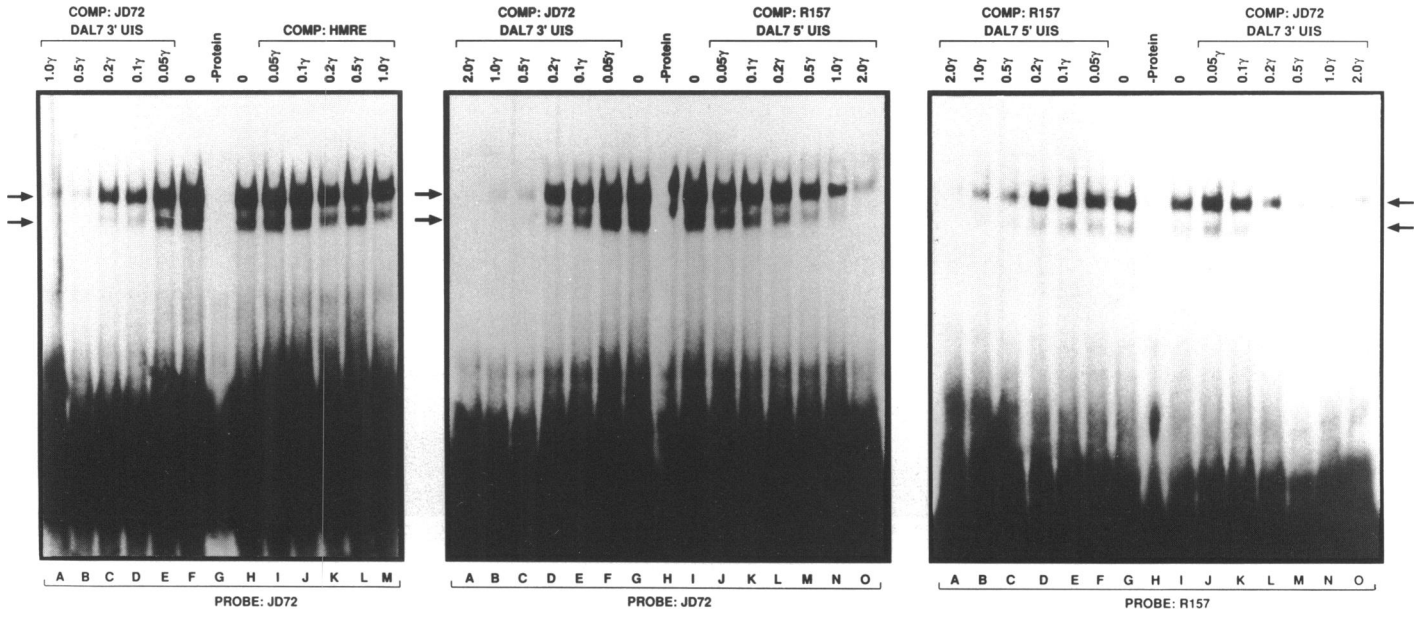


**Figure 4.** Competition between *DAL7* 3′ *UIS* fragment, JD72, the 5′ *UIS* fragment, R157 (JD157) and an HMRE fragment for binding to DAL82 protein. Left Panel. Probe JD72 was competed with increasing amounts (micrograms) of itself (lanes F to A), while lanes H to M show the effect of increasing amounts of HMRE fragment. Lane G shows probe incubated without protein extract. Center panel. Competition of probe JD72 with increasing amounts of itself (Lanes G to A), while lanes I to O show the effect of increasing amounts of fragment R157. Lane H contains probe incubated in the absence of protein extract. Right panel. DNA fragment R157 competed with increasing amounts of itself (lanes G to A), while lanes I to O contained increasing amounts of fragment JD72. Lane H shows the DNA probe in the absence of protein extract.

Therefore, DAL82 protein would be expected to bind to DNA fragments derived from those genes if they contained sequences homologous to the *DAL7 UIS* elements. As expected, DAL82 protein bound to a DNA fragment from the *DAL4* gene which contained a *DAL UIS*-homologous sequence (fragment RD7) (Figure 7, lanes F and L). However, a second DNA fragment from the *DAL4* gene (fragment RD8) and one from the *DAL2* gene (fragment RD6) did not form detectable DNA-DAL82 complexes (Figure 7, lanes I and O). Since fragments RD6 and RD8 contained *DAL UIS*-homologous sequences, it was possible that they formed complexes with DAL82 that were too weak to be detected by this assay. Therefore, we used a more sensitive assay, competition EMSAs, to test whether DNA fragments RD6, RD7, and RD8 were competitors of DNA fragment JD72 for DAL82 binding (Figure 8). DNA fragment RD7 was as strong a competitor as the positive control fragment, JD72 (Figure 8, center panel). No convincing competition could be demonstrated with fragment RD6 (Figure 8, left panel). Fragment RD8 exhibited poor competition with fragment JD72 for binding to the higher mobility DAL82-specific complex, but did not exhibit convincing competition with the control fragment binding to the slower mobility complex (Figure 8, right panel).

## DISCUSSION

Data presented in this work demonstrate that DAL82 protein specifically binds to the *DAL UIS* element which mediates inducer-responsiveness of the inducible allantoin pathway genes. Binding is specific since mutation of even a single base of the *UIS* element was sufficient to significantly diminish DAL82 binding. The functionality of an epitope tagged version of DAL82 protein suggests that the added 13 amino acids at the 5′ end of *DAL82* can be tolerated without detriment to DNA binding. Whether function diminishes has not yet been investigated.

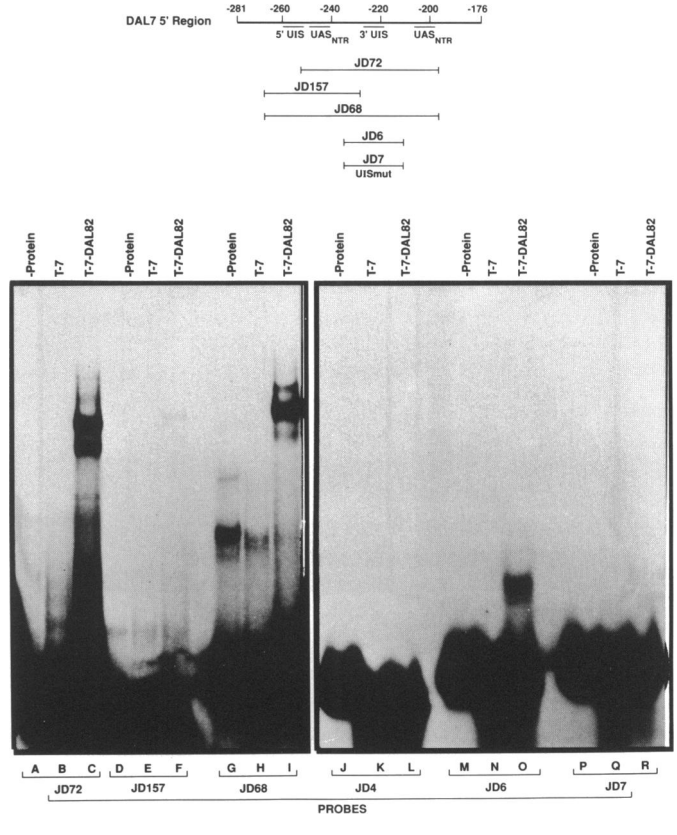


**Figure 5.** EMSA analysis of the ability of various DNA fragments from the 5′ region of *DAL7* to bind DAL82 protein. The DNA fragments used as probes (Figure 2) are indicated below and their positions in the DAL7 promoter are shown diagrammatically above. Lanes A, D, G, J, M, and P show probes incubated in the absence of protein extract. Lanes B, E, H, K, N, and Q show DNA fragments incubated with extracts derived from cells containing plasmid pT7-7 (T-7), while lanes C, F, I, L, O, and R contain extracts from cells containing plasmid pRD31 (T-7-DAL82).

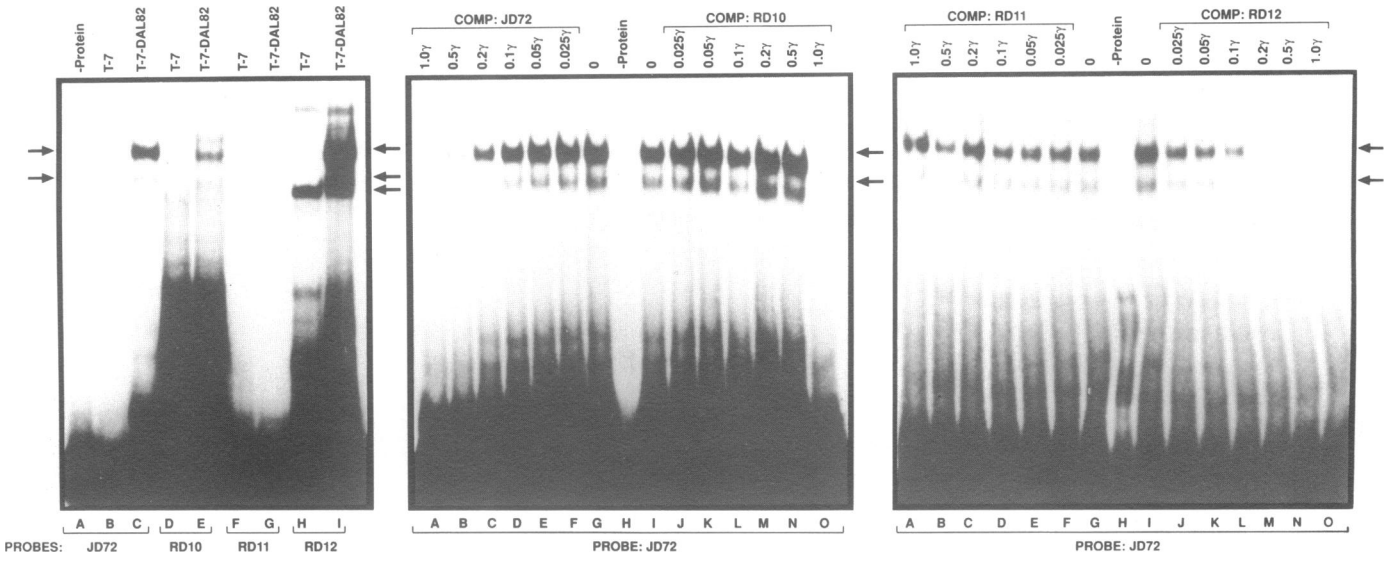


**Figure 6.** EMSAs to test the ability of wild type and mutant *DAL7* 3′ *UIS* fragments to bind DAL82 protein. Left panel. EMSA using wild type (JD72) and mutant (RD10, RD11 and RD12) 3′*UIS* fragments. Lane A shows the results obtained with DNA probe JD72 incubated in the absence of protein. Lanes B, D, F and H show the results obtained when DNA fragments JD72, RD10, RD11, and RD12, respectively, were incubated with protein extracts derived from cells containing plasmid pT7- (T-7). Lanes C, E, G and I show the effect of incubating DNA probes JD72, RD10, RD11, and RD12, respectively, with protein extracts from cells containing plasmid pRD31 (T-7-DAL82). Center panel. DNA fragment JD72 (approximately 25ng) competed with increasing amounts of itself (Lanes G to A), while Lanes to O depict the effects of incubating DNA fragment JD72 with increasing amounts of fragment RD10. Lane H shows the results obtained when the DNA probe was incubated in the absence of protein. Right panel. DNA fragment JD72 (approximately 25ng) competed with increasing amounts of fragment RD11 (Lanes G to A), while Lanes I to O depict the effects of incubating DNA fragment JD72 with increasing amounts of fragment RD12. Lane H shows the data obtained when the DNA probe was incubated in the absence of protein.

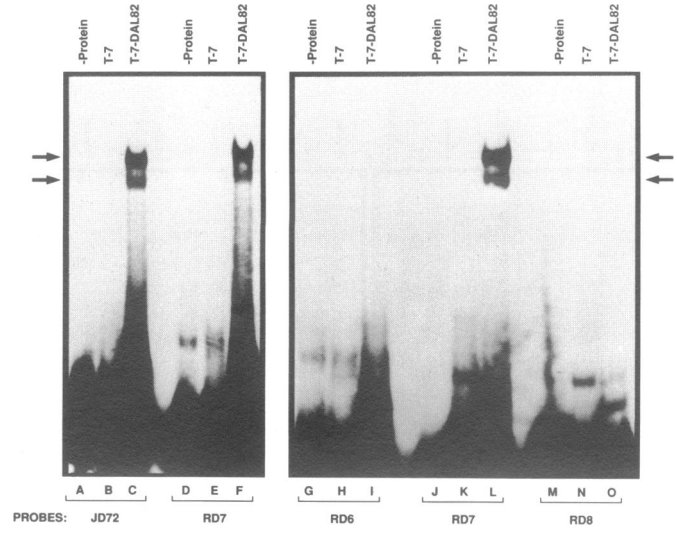


**Figure 7.** EMSA to measure the binding of various DNA fragments containing *DAL UIS* elements to bind DAL82. Left panel. EMSA with the *DAL7* 3′ *UIS* [fragment JD72] (lanes A to C) and the 5′ *UIS* of *DAL4* [fragment RD7] (lanes D to F). Lanes A and D contain the DNA probe incubated in the absence of protein, while lanes B and E and lanes C and F show probe incubated with protein extracts derived from cells containing plasmids pT7-7 (T-7) and pRD31 (T-7-DAL82), respectively. Right panel. Radioactive DNA fragments containing the *UIS* sequences from the *DAL2* gene (fragment RD6, lanes G to I) and the 5′ and 3′ *UIS*s from the *DAL4* gene (fragments RD7 and RD8, lanes J to L and M to O, respectively). Lanes G, J and M show the DNA probe incubated without protein while lanes H, K and N and lanes I, L and O show DNA probe incubated with protein extracts derived from cells containing plasmids pT7-7 (T-7) and pRD31 (T-7-DAL82), respectively.

At face value, evidence derived from the above experiments indicate that the presence of allophanate or oxalurate is not required for DAL82 protein binding to DNA fragments containing the *UIS* element. Such a conclusion, however, contains far too many caveats to be taken seriously at this point. First, *E.coli* may contain compounds with analogous chemical structures that are already bound to the protein in the crude extracts used in our experiments. Although efforts to remove such effector molecules from DAL82 protein (ammonium sulfate precipitation of the protein from the crude cell extract and overnight dialysis) did not result in an effect of oxalurate addition on formation of the DAL82 protein-DNA complex (data not shown), we feel that this question must not be considered resolved until far more detailed biochemical studies of the DAL82 protein are possible.

Two altered mobility species appear in all of the figures presented in this work. Moreover, the species with the slower mobility binds DNA fragments more tightly than the faster species. This is manifest in the differing competition profiles observed for the two species. It takes more competitor DNA to effectively compete with the probe DNA for binding to the slower species than the faster one. We considered three reasons that might give rise to these results. The first possibility is that the two species represent different degrees of DAL82 protein polymerization. Second, perhaps the DAL82 protein in the slower species is complexed to an unknown protein present in the *E.coli* extract. Third, the slower species represents intact DAL82 protein, while the lower band is generated by proteolytic degradation. By exclusion and existence of a preliminary result, we favor the third explanation. We doubt the first explanation, because there are multiple instances when only a single *UIS* site

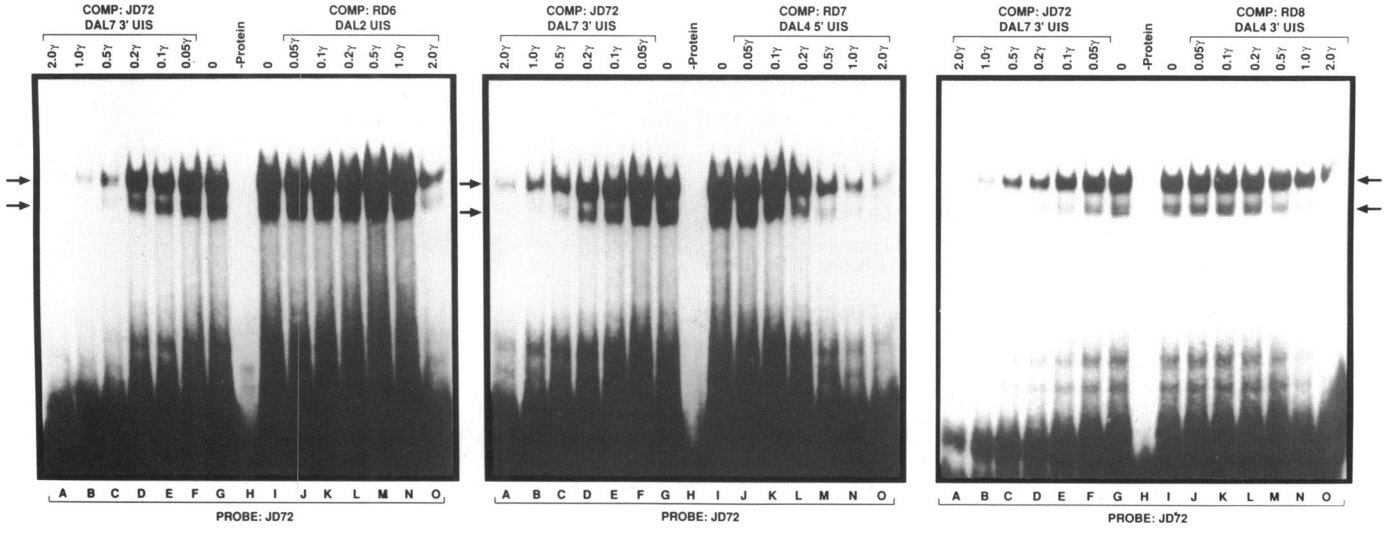


**Figure 8.** Competition of the 3′ *DAL7 UIS* [fragment JD72] with *UIS*-containing fragments from *DAL2* (RD6) and *DAL4* (RD7 and RD8). Left panel. Fragment JD72 competed with increasing amounts of itself (lanes G to A), while Lanes I to O depict the effects of incubating of DNA fragment JD72 with increasing amounts of fragment RD6. Lane H shows the results obtained when the DNA probe was incubated in the absence of protein. Center panel. DNA fragment JD72 competed with increasing amounts of itself (Lanes G to A), while lanes I to O depict the effects of incubating DNA fragment JD72 with increasing amounts of fragment RD7. Lane H shows the results obtained when the DNA probe was incubated in the absence of protein. Right panel. DNA fragment JD72 competed with increasing amounts of itself (Lanes G to A), while lanes I to O depict the effects of incubating DNA fragment JD72 with increasing amounts of fragment RD8. Lane H shows the results when the DNA probe was incubated in the absence of protein.

is present *in vivo* and there is no apparent symmetry in the sequence of the *UIS* site. Moreover, whether a DNA fragment contained one or two *UIS* elements, the same EMSA profile was observed and mutation of the element results in diminished or loss of binding, not a change in profile. We doubt the second explanation because it is more complicated; it hypothesizes the appearance of an *E.coli* protein that specifically binds to DAL82 protein. However, there are no observations that contradict its validity. We favor the third observation, because we have obtained preliminary evidence (a ladder of $^{35}$S-labelled DAL82-specific degradation products on an SDS-PAGE, data not shown) that DAL82 protein may be subject to proteolysis. The availability of purified DAL82 protein or detailed genetic analyses of the *DAL82* locus will likely be required to unambiguously distinguish among these possibilities.

Several observations of physiological interest were made during this work. Two of the genes studied, *DAL4* and *DAL7*, possessed two copies of the *DAL UIS* element in their 5′ flanking regions. In both cases, one of the two copies was a very good DAL82 protein binding site. As expected from previous genetic analyses of the *UIS* element (20), the second sites situated in both genes bound DAL82 protein less well. In addition, we could not obtain credible evidence of DAL82 protein binding to the *UIS*-homologous sequence upstream of *DAL2*. We cannot distinguish at present whether these results indicate that binding does not occur, or alternatively, occurs at a strength that is below our level of in vitro detection. It will be of interest to generate null mutations of these weak elements within the entire gene promoters to determine whether the *UIS* elements that poorly bind DAL82 protein in fact play any significant part in the inducer-responsiveness of the gene *in vivo*. Finally, the *UIS* elements of genes with the greatest inducer-responsiveness, *DAL4* and *DAL7* were also the ones that most tightly bound DAL82 protein (4, 11, 13, 16, 20).

## ACKNOWLEDGEMENTS

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