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Upstream Induction Sequence, the *cis*-Acting Element Required for Response to the Allantoin Pathway Inducer and Enhancement of Operation of the Nitrogen-Regulated Upstream Activation Sequence in *Saccharomyces cerevisiae*

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Expression of the *DAL2*, *DAL4*, *DAL7*, *DUR1,2*, and *DUR3* genes in *Saccharomyces cerevisiae* is induced by the presence of allophanate, the last intermediate of the allantoin degradative pathway. Analysis of the *DAL7* 5′-flanking region identified an element, designated the *DAL* upstream induction sequence (DAL UIS), required for response to inducer. The operation of this *cis*-acting element requires functional *DAL81* and *DAL82* gene products. We determined the DAL UIS structure by using saturation mutagenesis. A specific dodecanucleotide sequence is the minimum required for response of reporter gene transcription to inducer. There are two copies of the sequence in the 5′-flanking region of the *DAL7* gene. There are one or more copies of the sequence upstream of each allantoin pathway gene that responds to inducer. The sequence is also found 5′ of the allophanate-inducible *CAR2* gene as well. No such sequences were detected upstream of allantoin pathway genes that do not respond to the presence of inducer. We also demonstrated that the presence of a UIS element adjacent to the nitrogen-regulated upstream activation sequence significantly enhances its operation.

All genes that participate in the allantoin degradative pathway (*DAL* and *DUR*) of *Saccharomyces cerevisiae* are sensitive to nitrogen catabolite repression (NCR) (5, 7). This global regulation is exerted through a common nitrogen-regulated upstream activation sequence (UAS_{NTR}) (3, 4, 7, 19, 26, 27). This element is necessary and sufficient for sensitivity to NCR (7) and has been observed in multiple copies upstream of every gene for which there is unambiguous evidence demonstrating sensitivity to NCR. In addition to NCR, some of the allantoin pathway genes (*DAL2*, *DAL4*, *DAL7*, *DUR1,2*, and *DUR3*) are inducible; their expression is markedly increased by the presence of the last pathway intermediate, allophanate, or its analog; oxalurate (OXLU) (5, 6, 8, 27, 28). For the others (*DAL1*, *DAL3*, and *DAL5*) expression in strain Σ1278b is independent of inducer (18, 26, 28).

The composition of *cis*-acting elements present in the 5′-flanking regions of the *DAL* and *DUR* genes correlate with their physiological responses. All of the genes are NCR sensitive and found to have multiple UAS_{NTR} elements upstream of their coding sequences (3, 4, 7, 19, 25–27). Only the UAS_{NTR} element has been found upstream of the allantoin pathway genes expressed in an inducer-independent manner (3, 19, 26). In contrast, the inducible genes are found to possess two additional *cis*-acting elements. The first of these elements is an upstream repression sequence (URS) that has been suggested to maintain gene expression at a low level when inducer is absent (25) but which as yet is not well characterized. The second element is the upstream induction sequence (UIS) that has been demonstrated to be required for response to inducer (25). The UIS element alone in single or multiple copies, however, will not mediate significant transcriptional activation of a heterologous reporter gene and hence probably does not function as a UAS (25).

Two recessive, unlinked genes are associated with the function mediated by the *DAL7* UIS. The *DAL81* locus was first defined genetically by mutations that resulted in loss of ability to use allantoin as nitrogen source and lacked the normal increased urea amidolyase production in response to addition of urea or oxalurate to the culture medium (1, 2, 23). The *DAL81* gene was cloned and found to be expressed in an inducer-independent, NCR-insensitive manner (1). Deletion of the gene permitted us to demonstrate that *DAL81* is not required for UAS_{NTR} function but is required for response to inducer and function of the UIS element in the induction process (2). The 109-kDa gene product contains $Zn(II)_2Cys_6$ and helix-turn-helix motifs (2). DAL81 product is not, however, a prime candidate for the factor that binds to the *DAL7* UIS element because it is required for induced synthesis of a number of unrelated genes, including *UGA1*, which participates in γ-aminobutyrate metabolism (2) and hence may be a more general regulatory factor of transcription.

The *DAL82* locus encodes a 29-kDa protein specifically required for *DAL7* UIS function (17). The phenotype of *dal82* mutations is identical to that of *dal81* with an important exception; *UGA1* gene expression is not affected by loss of the *DAL82* gene product (24). Hence, *DAL82* appears to be allantoin pathway specific. The deduced structure of *DAL82* protein does not contain any of the motifs usually reported to be associated with DNA binding proteins except a coiled-coil motif (17).

A first step towards elucidating the functions of *DAL81* and *DAL82* proteins and unraveling potential interactions of all proteins required to mediate induced allantoin system gene expression is careful identification of the *cis*-acting sites involved in the process. Therefore, we began our genetic analysis with a DNA fragment that was similar to one shown earlier to contain the *DAL7* inducer-responsive UIS element and to support induced reporter gene expression (23). The UIS portion of the fragment was subjected to saturation

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TABLE 1. Strains used

Strain	Relevant genotype
S. cerevisiae	
RH218	*MATa trp1 mal suc2 CUP1 gal2*
M1682-19b	*MATa ura3-52 trp1-289*
PB200	*MATa ura3-52 trp1-289 Δdal81::hisG*
TCY1	*MATα ura3 lys2*
M02	*MATa ura3 dal82-1*
M970	*MATa lys5*
	MATα lys2
E. coli HB101	*hsdR hsdM recA13 supE44 lacZ24 leuB6 proA2 thi-1* Sm^r

mutagenesis to identify those nucleotides required for response to inducer. As a result of this work, the *DAL7* UIS site has been clearly defined. Data which are consistent with the suggestion that proteins associated with the *DAL7* UIS element significantly enhance the operation of UAS_{NTR} are also presented.

(Preliminary reports of this work have already appeared [23a, 23b].)

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this work are shown in Table 1. Cultures used for β-galactosidase assays were grown in medium containing yeast nitrogen base without amino acids or ammonium sulfate (Difco Laboratories) supplemented with glucose to a final concentration of 2%. L-Proline was provided as the sole nitrogen source at a final concentration of 0.1%. Oxalurate (OXLU) was added to a final concentration of 0.25 mM where indicated. Media used for *S. cerevisiae* transformation were YEPD for cell growth and Difco yeast nitrogen base plus 0.1 or 0.5% ammonium sulfate for selective growth following transformation. Medium used for growth of *Escherichia coli* HB101 cells was Luria broth either with or without ampicillin (Sigma; technical grade, 0.2 mg/ml). Bacterial cells were grown in M-9 medium (0.7% Na_2HPO4, 0.3% KH_2PO4, 0.05% NaCl, 0.1% NH_4Cl, 1.2 mM $MgSO_4$, 2.5 mg of thiamine per liter, 50 mg of thymine per liter, 0.4% $CaCl_2$, 0.5% Casamino Acids, 2% glucose) supplemented with 0.2 mg of ampicillin per ml. Chloramphenicol (Sigma) was added at a concentration of 0.2 mg/ml for plasmid amplification.

***S. cerevisiae* and bacterial transformation.** Strain RH218 was transformed by using the spheroplast method of Hinnen et al. (10) as described earlier (21). Strains M1682-19b, PB200, TCY1, and MO2 were transformed by the lithium acetate method of Ito et al. (11). Bacteria were transformed by using the Tschumper and Carbon (22) modification of the Mandel and Higa procedure (15).

Plasmid constructions. All synthetic oligonucleotide sequences described in this work were cloned into the *Sal*I and *Eag*I sites of *CYC1-lacZ* expression vector pNG15 (Fig. 1) (4). This vector was constructed from and is identical to plasmid pHY100 (25) with two exceptions: (i) a polylinker was engineered into plasmid pNG15 at the *Xho*I site, whereas plasmid pHY100 possesses only a single *Xho*I site at the same position; and (ii) plasmid pNG15 contains a 14-bp deletion of DNA between positions −242 and −228 (relative to the *CYC1* ATG), which is not present in plasmid pHY100. We have never detected any phenotypic difference in β-galactosidase expression supported by these two vectors. All plasmid insert structures, including restriction sites

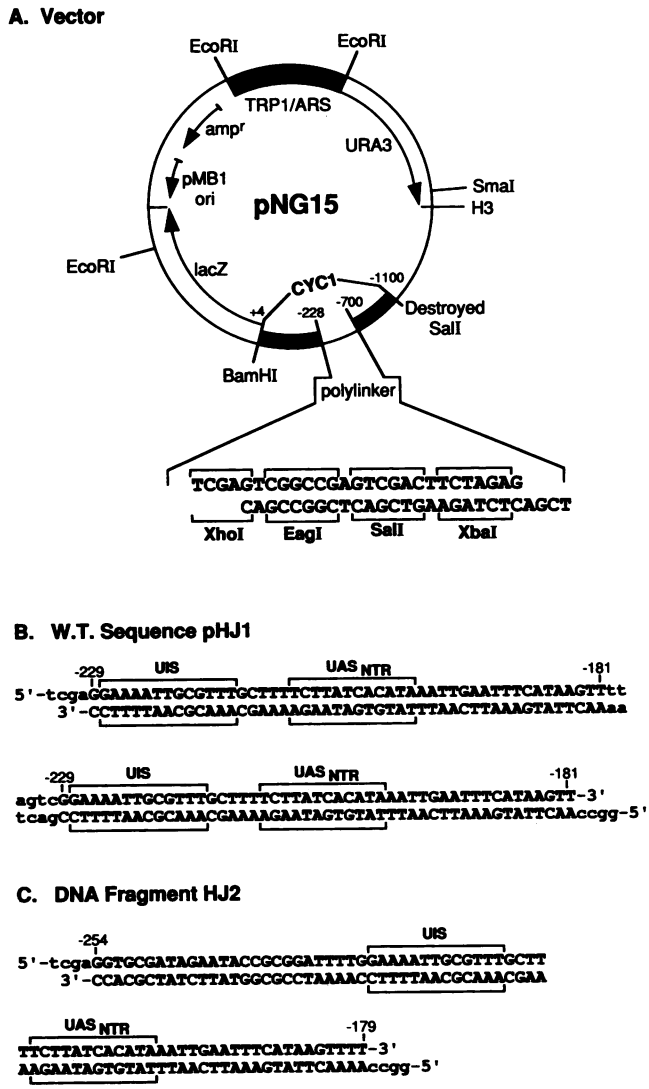

FIG. 1. Vector (A) and wild-type sequence (B) used for the saturation mutagenesis experiments. The UIS and UAS sequences are within brackets. (C) DNA fragment used for the footprint experiment. Lowercase letters indicate restriction site linkers used for cloning.

used for cloning purposes, were verified by using the dideoxy sequencing method of Sanger et al. with modified phage T7 polymerase (20).

The starting construction used for mutational analysis and the deletions in Table 2 was plasmid pHJ1, which was similar but not identical to plasmid pHY174 (25). The insert of

TABLE 2. Deletion of sequences flanking the *DAL7* UIS[a]

Plasmid	Sequences deleted	β-Galactosidase	
		Pro	Pro + OXLU
pHJ1	None	15	280
pHJ1-Δ1	−229 to −225	7	7
pHJ1-Δ2	−229 to −223	5	8
pHJ1-Δ3	−218 to −220	9	10

[a] Strain RH218 was used as the transformation recipient in this experiment.

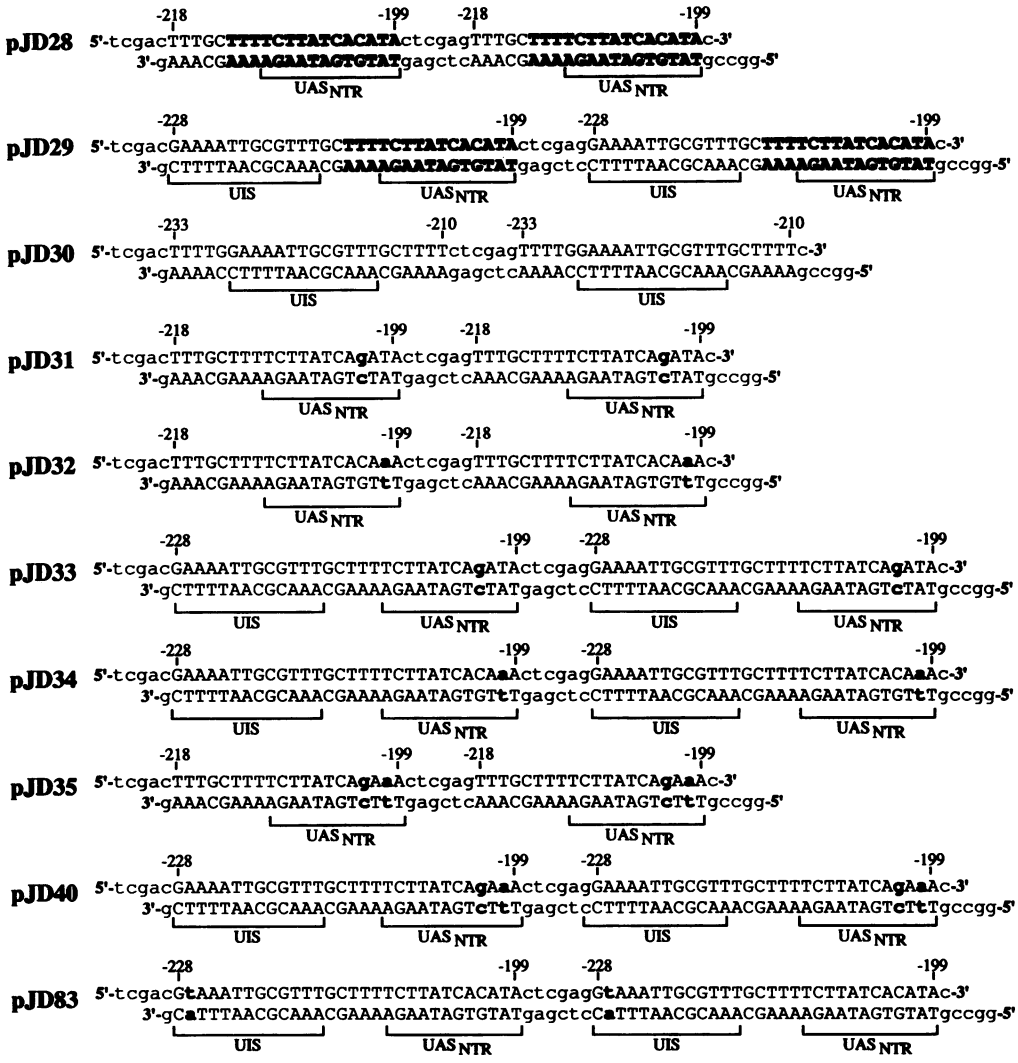

FIG. 2. Sequences of inserts contained in representative plasmids used in this work. Coordinates are those from the *DAL7* upstream region, the source of these sequences. UIS and UAS_{NTR} elements appear in brackets. Regions in bold letters indicate sequences that were substituted for one of the experiments. Lowercase letters indicate linkers that were placed between native sequences or at their termini for cloning. Bold lowercase letters indicate positions of single or double mutations.

plasmid pHJ1 (Fig. 1) contained two copies of *DAL7* sequences −229 to −181 separated by linker sequence 5′-TTAGTC and flanked by the 5′ extensions of the *Sal*I and *Eag*I recognition sequences, such that cloning the insert into the *Sal*I and *Eag*I sites of vector pNG15 resulted in destruction of these sites in the finished construction. In plasmid pHY174, the 5′ extensions of *Sal*I linkers were used for cloning; they too are destroyed as a result of cloning.

The *ARS1* element contained in most of the plasmids used in this work was replaced in a representative set of plasmids with a DNA fragment containing *CENIV-ARS1*. This was accomplished by substituting the *Nde*I-*Xmn*I DNA fragment derived from plasmid YCp50 for the *Eco*RI fragment containing *TRP1* and *ARS1* of plasmid pNG15 to yield plasmid pHP41 (17a). Then the *Xma*I-*Bam*HI fragments from each of the representative plasmids was exchanged for the *Xma*I-*Bam*HI fragment of plasmid pHP41.

To determine the ability of UAS_{NTR}-homologous sequences in the *DAL7* promoter to support UAS function in the absence or presence of an adjacent UIS element (see Table 4), we used synthetic oligonucleotides that were identical to those contained in plasmids pJD28 and pJD29 (Fig. 2), except that each of the UAS_{NTR}-homologous sequences to be tested and shown in the table were substituted for the UAS-homologous sequences (Fig. 2, boldface print) in plasmids PJD28 and pJD29. Again, the plasmid inserts contained two copies of the UAS_{NTR} sequences because we have previously shown that two copies of the UAS_{NTR} sequence are required for function (19). These inserts were also cloned into the *Sal*I and *Eag*I sites of plasmid pNG15, but the linker separating the two sets of duplicated elements was *Xho*I rather than TTAGTC used in plasmid pHJ1.

Constructions used in Table 6 were prepared as follows. A synthetic oligonucleotide fragment containing only *DAL7* UIS sequences and *Xho*I-compatible 5′ extensions, one of which would generate a nonsite after ligation, was cloned into the *Xho*I site of plasmid pNG48, whose detailed structure is described elsewhere (4). Following confirmation of the correct sequence and orientation of the UIS, the entire *Bam*HI-*Xho*I fragment was excised and used to replace the

corresponding fragment of each of the plasmids containing the mutant UAS_{NTR} sequences chosen for analysis.

Enzyme assays. β-Galactosidase activities were determined by using the method of Guarente and Mason (9). The medium used for assay was YNB-proline. Activities were reported as Miller units, except that 10 ml of cells were harvested rather than 1 ml (16). Many of the plasmids used in this work contained an autonomously replicating sequence (*ARS1*). Therefore, we took the same precautions we have used successfully in the past (4, 13, 19, 25) to avoid potential problems that might result from varying plasmid copy number. In addition, we assayed β-galactosidase activity supported by *CENIV* plasmids containing representative inserts identical to those used in this analysis and obtained the same patterns of activity as with the ARS plasmids (4; this study). The most significant difference was that the *CENIV* plasmids supported much lower β-galactosidase activities, as we have reported in previous analyses of this type (4; this study). For example, the values obtained for the wild-type insert contained on ARS plasmid pHJ1 and the same insert cloned into an equivalent *CENIV* plasmid (pJD134) were 36 and 398, versus 26 and 232 units in the absence and presence of inducer, respectively, for the two plasmids (strain M1682-19b was used for this experiment).

The following protocol was rigidly adhered to for all assays performed in this work. Following transformation, yeast transformants were incubated on selective media (YNB) at 30°C for 3 days. Under no circumstances were transformants frozen, subcultured, or stored for subsequent use, and all of the plasmids listed in a given table were transformed into the same sample of competent cells. After 3 days of growth, single transformants were patched onto an additional selective plate and again incubated at 30°C for 2 days. These patches were then used to inoculate 50 ml of YNB-proline medium, and the cells were grown at 30°C to cell densities (A_{600}) of 0.5 to 0.8 as measured on a Gilford Response spectrophotometer. Cells (10 ml) were harvested and assayed for production of β-galactosidase. Each assay was repeated at least twice, and each transformant was assayed in duplicate. Values for transformants containing the same plasmid construction and assayed on different days generally differed by not more than 20%, and duplicate assay values differed less than 5%. The pattern of the activities obtained for a set of plasmids on different assay dates always remained the same.

DNA binding experiments. Protein extracts for the footprinting experiments were prepared as described earlier (19). To prepare the DNA fragment for footprinting, the 5′ terminus of the coding strand of DNA fragment HJ2 (an 80-mer distinct from plasmid pHJ2) (Fig. 1C) was radioactively labeled by using the polynucleotide kinase reaction. The complementary strand of this DNA fragment was combined with the labeled one, heated to 95°C for 10 min, and slowly cooled to room temperature. The annealed DNA fragment was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. DNase digestion was performed as described earlier (19). The treated DNA was then resolved by electrophoresis on a 10% polyacrylamide gel containing 7 M urea. After electrophoresis, the radioactive species were visualized by autoradiography.

RESULTS

Saturation mutagenesis of the *DAL7* UIS element. Previously reported experiments indicated that the 5′ terminus of a *DAL7 cis*-acting element required for response to inducer (UIS) was situated at or 3′ to position −228 (25). In that study a DNA fragment extending from positions −228 to −183 supported OXLU-inducible reporter gene expression (plasmid pHY174 [25]). In contrast, a DNA fragment covering only sequences −218 to −203 (plasmid pHY129) was sufficient to support transcriptional activation of the reporter gene, but in an inducer-independent fashion (25). Together, these observations suggested that the UIS element was likely situated somewhere between nucleotides −228 to −212; the latter position has been shown by saturation mutagenesis to be a terminal nucleotide of the UAS_{NTR} (4). To ascertain whether or not additional sequences could be deleted, several additional deletion plasmids were constructed (Table 2). Deletion of nucleotides −229 to −225 or −229 to −223 resulted in loss of OXLU-inducible reporter gene expression (Table 2). Similarly, deletion of nucleotides −218 to −220 generated the same phenotype. Therefore, we chose the sequence −229 to −215 as the minimum to be analyzed.

To conduct the saturation mutagenesis of the *DAL7* UIS, we synthesized a set of mutant DNA fragments and cloned them into expression vector pNG15 (Fig. 1A) as described in Materials and Methods. The wild-type sequence (insert to plasmid pHJ1) cloned into plasmid pNG15 is shown in Fig. 1B. The sequences from −229 to −181 were duplicated in the insert of this plasmid, because of the requirement for two copies of UAS_{NTR} for function (19). Each synthetic oligonucleotide plasmid insert contained a single UIS mutation in both copies of the UIS element, and each position was mutated to all possible alternatives. These plasmids were used to transform strain RH218, and the transformants were assayed for β-galactosidase activity. As shown in Table 3, the expression vector without an insert (plasmid pNG15) supported low levels of reporter gene expression. When the wild-type fragment was inserted into the vector (plasmid pHJ1), 16-fold induction was observed. Mutations (such as the one carried in plasmid pHJ11) reduced induction to basal level or less, while others exhibited little effect (for example, the mutation carried in plasmid pHJ36).

Figure 3 shows the induced values obtained for each mutant sequence as percentages of the wild-type-induced level of β-galactosidase activity supported by the wild-type sequence (plasmid pHJ1) tabulated as a function of the UIS position mutagenized and the mutant substitution. Mutation of position −229 had no significant effect upon response to inducer. In fact, the G-to-C transversion (Table 3, plasmid pHJ9) resulted in a nearly fourfold increase in the induced level. The G-to-C transversion at −228 had no effect on activity, but substitution of either an A or T was quite detrimental to UIS function. The next three nucleotides (positions −227 to −225) were completely specific, i.e., no mutation could be tolerated. Either an A or a T functioned effectively at position −224, but G or C did not. Position −223 exhibited no significant specificity. Position −222 exhibited reasonable specificity for T, since substituting G or C here resulted in loss of 60 to 70% of the activity, respectively, and an A at this position resulted in nearly complete loss of activity. The next three positions, −221 to −219, were completely specific with no mutation being tolerated. Either pyrimidine functioned well at position −218. Purines at this position, however, were not functional. High specificity for T was found at position −217. The final two positions analyzed exhibited quite low specificity. Only A was detrimental at positions −216 and −215 (56 and 50% loss of activity at the two positions, respectively).

Protein binding to a DNA fragment containing the *DAL7*

TABLE 3. Mutational analysis of the *DAL7* UIS

Plasmid	Mutation	β-Galactosidase		Induction (fold)[a]
		Pro	Pro + OXLU	
pNG15	None	8	9	1
pHJ1	None	19	313	16
pHJ9	G-229→C	90	1201	13
pHJ25	G-229→T	15	274	18
pHJ24	G-229→A	40	496	12
pHJ10	G-228→C	18	310	17
pHJ27	G-228→T	6	73	12
pHJ26	G-228→A	9	70	8
pHJ11	A-227→T	5	6	1
pHJ29	A-227→C	6	16	3
pHJ28	A-227→G	12	42	4
pHJ12	A-226→T	10	9	1
pHJ31	A-226→C	4	5	1
pHJ30	A-226→G	12	11	1
pHJ13	A-225→T	15	32	2
pHJ33	A-225→C	10	12	1
pHJ32	A-225→G	5	21	4
pHJ14	A-224→T	24	358	15
pHJ35	A-224→C	9	48	5
pHJ34	A-224→G	8	55	7
pHJ15	A-223→A	39	560	14
pHJ37	T-223→C	13	199	15
pHJ36	T-223→G	28	476	17
pHJ16	T-222→A	8	45	6
pHJ39	T-222→C	79	96	1
pHJ38	T-222→G	20	127	6
pHJ17	G-221→C	21	20	1
pHJ40	G-221→A	9	9	1
pHJ41	G-221→T	13	15	1
pHJ18	C-220→G	5	5	1
pHJ42	C-220→A	5	3	1
pHJ43	C-220→T	6	9	2
pHJ19	G-219→C	19	13	1
pHJ44	G-219→A	4	5	1
pHJ45	G-219→T	9	8	1
pHJ20	T-218→A	7	13	2
pHJ47	T-218→C	45	416	9
pHJ46	T-218→G	7	8	1
pHJ21	T-217→A	7	23	3
pHJ49	T-217→C	10	47	5
pHJ48	T-217→G	13	51	4
pHJ22	T-216→A	10	139	14
pHJ51	T-216→C	89	1058	12
pHJ50	T-216→G	292	567	2
pHJ23	G-215→C	30	396	13
pHJ52	G-215→A	13	157	12
pHJ53	G-215→T	84	890	11

[a] Fold induction is defined as the quotient of induced level of β-galactosidase activity divided by the uninduced level of activity. Strain RH218 was used as the transformation recipient in this experiment.

UIS. The genetic analysis presented above identified a highly specific dodecanucleotide required for response to the allantoin pathway inducer. Such a site would be expected to bind one or more proteins in order to effect this response. To test this expectation, we ascertained whether or not protein was bound to a DNA fragment containing the *DAL7* UIS element just described. As shown in Fig. 4, a clear footprint was observed over DNA sequences containing the UIS. The bound protein also protected nucleotides adjacent to the element.

UIS element enhances UAS_{NTR} operation. In previous studies of the *DAL7* UIS element and the *DAL81* gene, we observed that when a DNA fragment containing both the UIS and UAS_{NTR} elements was cloned into a *CYC1-lacZ* expression vector, the construct supported 1.2- to 2.4-fold-greater reporter gene expression than did an analogous construct lacking the UIS element (compare results obtained with plasmids pHY129 and pHY162 in Fig. 7 of reference 25 and Fig. 8 of reference 1 [note that different strains were used for the two experiments]). Moreover, this effect was observed even in the absence of inducer. In other words, the presence of the UIS element appeared to enhance operation of the UAS_{NTR} element. To establish whether or not this was a spurious characteristic of the two plasmid constructs assayed earlier or a reflection of a more general function mediated by the UIS element and its associated proteins, we repeated the experiment. In this case, however, we successively substituted each of the seven UAS_{NTR}-homologous sequences situated upstream of *DAL7* for the UAS contained in plasmids that were similar to those we originally assayed (pHY129 and pHY162) and used to identify this phenomenon (1, 25). As shown in Table 4, the presence or absence of a UIS element adjacent to a UAS_{NTR}-homologous *DAL7* sequence, which alone was unable to support reporter gene expression, had little effect on the level of reporter gene expression observed. All of the values obtained were near background levels of β-galactosidase production (plasmids pJD59 and pJD60, pJD55 and pJD56, and pJD63 and pJD64). In contrast, UAS function supported by the remaining four *DAL7* UAS_{NTR} homologous sequences, those which alone did support clearly measurable reporter gene expression, was enhanced 23- to 40-fold by the presence of the UIS element (plasmids pJD57 and pJD58, pJD28 and pJD29, pJD61 and pJD62, and pJD73 and pJD74). Note that this experiment was carried out in the absence of inducer and also that the construction did not contain a URS element. The lack of a URS element accounts for the ability of the UAS to function in the absence of inducer. (For a detailed description of the role the URS element plays and the effects of its loss on *DAL7* expression, see reference 25.) The values in parentheses were those obtained when the experiment was repeated with a set of analogous plasmids containing a centromere along with the ARS as opposed to containing only an ARS alone. The effect of the UIS element on UAS function was the same in centromere-containing plasmids as it was in those with only an ARS although the absolute values were lower as expected.

Introducing UIS elements adjacent to the UAS elements as occurred in the plasmids assayed in Table 4, resulted in two changes: (i) addition of the UIS element, and (ii) a change in the spacing between the UAS elements due to insertion of the UIS elements (compare appropriate sequences in Fig. 1B or sequences for the inserts of plasmids pJD28 and pJD29 in Fig. 2). Therefore, the experiment presented in Table 4 contained two variables, each of which might reasonably be expected to have an effect upon the results we observed. To eliminate the contribution of a change in UAS spacing from consideration, we assayed reporter gene expression supported by three plasmids (Table 5). The first plasmid (pJD28) contained a poor UAS_{NTR}, while the second plasmid (pJD29) contained this UAS_{NTR} along with a UIS element adjacent to it, as described above. The third plasmid (pJD83) was identical to plasmid pJD29, except that its UIS elements contained a point mutation (an A-to-T transversion at *DAL7* position −227) shown in Fig. 3 to destroy the ability of the sequence to serve as a functional UIS site. Therefore, UAS_{NTR} spacing in plasmids pJD29 and pJD83 was identical; the UIS element was removed in plasmid pJD83 by point mutation rather than deletion. As

	−229 G	G	A	A	A	A	T	T	G	C	G	T	T	T	−215 G
T	88	23	2	3	10	114	100	100	5	3	3	100	100	100	284
C	384	98	5	2	4	15	64	31	6	100	4	133	15	338	127
A	158	22	100	100	100	100	179	14	3	1	2	4	7	44	50
G	100	100	13	4	7	18	152	41	100	2	100	3	16	181	100
	N	G C	A	A	A	A T	N	T	G	C	G	T C	T	T C G	N

FIG. 3. Percentage of the induced wild-type (plasmid pHJ1) β-galactosidase activity supported by each of the mutant DNA fragments. The wild-type sequence appears across the top of the figure. Substitutions appear down the left side and the *DAL7* UIS sequence that supports more than 50% induced β-galactosidase production appears at the bottom.

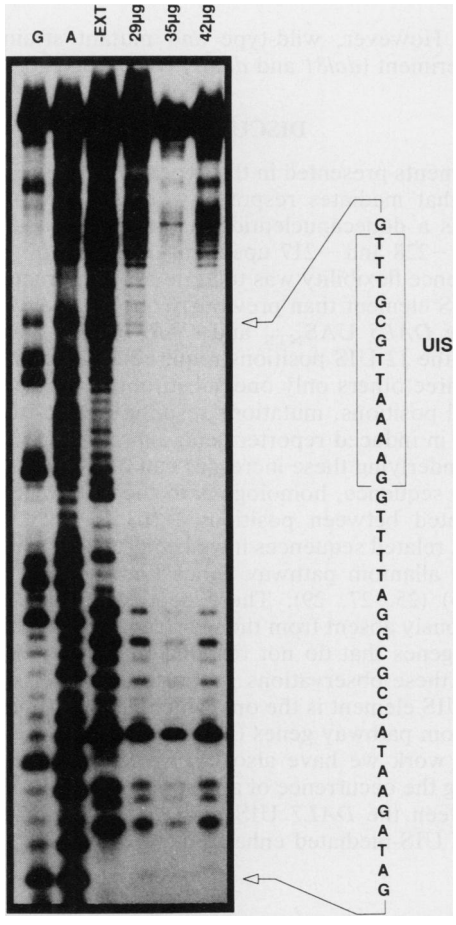

FIG. 4. DNA footprint analysis of the *DAL7* UIS sequence. The lanes from left to right contained the Maxam-Gilbert sequence reaction products from the G and A specific reactions, a reaction mixture from which cell extract was omitted (−EXT), and increasing amounts of cell extract (29, 35, and 42 μg). Optimum reaction conditions were found to be the same as those used earlier (19). The wild-type strain used in this experiment was M970 grown in minimal glucose-proline medium as described earlier (19). A portion of the sequence of the DNA fragment used in the experiment is shown on the right side of the figure with the UIS element in brackets.

shown in Table 5, mutation of the UIS element resulted in loss of its ability to mediate enhancement of UAS_{NTR} activity. Since the spacing of UAS_{NTR} elements in plasmids pJD29 and pJD83 was identical, the data obtained argued that altered UAS_{NTR} spacing was not a significant contributing factor to UIS-mediated enhancement of UAS activity.

The above results pointed out a marked limitation in interpretation of the previously reported mutational analysis of the UAS_{NTR} element (4). In that work we mutated each nucleotide of the UAS_{NTR} and ascertained the effects of these changes on its ability to serve as a UAS. Such analysis would be sufficient for genes such as *DAL5*, which possessed only UAS_{NTR} elements, i.e., their promoters were devoid of UIS elements. On the other hand, it would not be sufficient for analysis of the UAS element from a gene such as *DAL7*, which possessed both UAS_{NTR} and UIS elements. This limitation can be seen in the data in Table 4. Only the UAS in plasmid pJD28 would have been scored as being functional and then just barely so. However, as mentioned above, the UAS elements in plasmids pJD58, pJD62, and pJD74 were also capable of supporting reasonable levels of reporter gene expression when the UIS element was present as it is in the *DAL7* promoter, the source of these sequences. We performed one more experiment to evaluate the extent of this limitation relative to mutational analysis of the UAS_{NTR} reported previously (4). This experiment was identical to the one described in Table 4, except that the UAS elements used were mutated forms of the one used in the original UAS_{NTR} mutational analysis previously reported (4). In each case, the presence of the UIS element markedly enhanced the UAS activity supported by the construction (Table 6).

UIS-mediated enhancement of UAS_{NTR} function in *dal81* and *dal82* mutant strains. The *DAL81* and *DAL82* gene products have been shown to be required for response to the allantoin pathway inducer and operation of the UIS element in the induction process (1, 2, 17). Therefore, we ascertained whether mutations of these genes had any effect upon UIS-mediated enhancement of UAS activity supported by a *DAL7* UAS_{NTR} element. This was accomplished by using two sets of plasmids. One set of plasmids (pJD28, pJD32, pJD31, and pJD35) contained only UAS_{NTR}-related sequences based on the *DAL7* UAS_{NTR} sequence in plasmid pJD28 (*DAL7* positions −218 to −199). This plasmid supported 230 U of β-galactosidase production, an amount that was 16-fold over background but still low when compared with the level of production supported by the *DAL5*

TABLE 4. Ability of homologous *DAL7* sequences to support transcriptional activation in the presence and absence of the UIS element

Plasmid	UAS coordinates	Element composition		β-Galactosidase activity[a] Pro
		5'-UAS sequence-3'	UIS sequence	
pJD59	−366 to −352	TTGTATTATCATCTA	−	4
pJD60			+	4
pJD55	−290 to −275	TACGATTATCGACAT	−	5
pJD56			+	15
pJD57	−254 to −240	GGTGCGATAGAATAC	−	13
pJD58			+	520
pJD28	−213 to −199	TTTTCTTATCACATA	−	164 (94)
pJD29			+	4,535 (672)
pJD61	−180 to −166	TTGGCTTATCTTGGA	−	34 (26)
pJD62			+	690 (250)
pJD63	−172 to −158	TCTTGGATAAGGTAC	−	5
pJD64			+	40
pJD73	−138 to −124	GATTGGATAGTTTCG	−	8
pJD74			+	181
pNG15	No insert	None	−	13 (11)

[a] Values in parentheses were obtained with the *CENIV-ARS1* expression vector. Wild-type strain M1682-19b was used as the transformation recipient in this experiment.

UAS_{NTR} (19). Using results from saturation mutagenesis of the *DAL5* UAS_{NTR} as a guide (4), we altered, in stepwise fashion, the UAS sequence in plasmid pJD28 to make it more closely coincide with that of *DAL5* UAS_{NTR} (Table 7). Not surprisingly, reporter gene expression supported by each successively modified plasmid was higher than the previous one (Table 7, compare plasmids pJD28, pJD32, pJD31, and pJD35). Adding a UIS element upstream of the UAS elements in each of these plasmids resulted in markedly increased UAS activity. The fold increase was a function of the strength of the UAS element in the absence of the UIS. When a poor UAS_{NTR} element was used (plasmid pJD28), 15-fold enhancement was observed (Table 7). Enhancement decreased to only 2.5-fold when the most active UAS_{NTR} was assayed (plasmids pJD35 and pJD40). Deletion of *dal81* had no significant effect upon enhancement of *DAL7* UAS activity by the UIS element. Moreover, the fold enhancement was the same in both cases and was the same whether or not the plasmid contained a centromere (Table 7, values in parentheses). We noted, however, that all values obtained with the *dal81* mutant were 1/3 to 1/2 lower than in the wild type. Whether or not this observation is physiologically significant is not known. A different result was obtained with the *dal82* mutant. UIS-mediated enhancement of UAS_{NTR} function was all but lost in the *dal82* mutant. Elevenfold enhancement was observed in the wild type compared with only 1.6-fold in the *dal82* mutant (Table 7, compare values for plasmids pJD28 and pJD29). A similar pattern of results was observed whether or not the plasmid contained a centromere. Note also that the wild-type strains used in the two above-described experiments (Table 7) were different. However, wild-type and mutant strains within each experiment (*dal81* and *dal82*) were isogenic.

DISCUSSION

Experiments presented in this work identify the *cis*-acting element that mediates response to the allantoin pathway inducer as a dodecanucleotide sequence situated between positions −228 and −217 upstream of the *DAL7* gene. Far less sequence flexibility was tolerated in the structure of the *DAL7* UIS element than previously observed during analyses of the *DAL5* UAS_{NTR} and *CAR1 URS1* sites (4, 14). Seven of the 12 UIS positions required a unique nucleotide and for three others only one substitution was permissible. In several positions, mutations resulted in two- to fourfold increases in induced reporter gene expression. The mechanism(s) underlying these increases can only be conjectured. A second sequence, homologous to the UIS analyzed here was situated between positions −263 to −251 (Fig. 5). Similarly, related sequences have been reported upstream of all of the allantoin pathway genes known to be inducible (Figure 5) (25, 27, 29). These sequences are, however, conspicuously absent from the upstream regions of allantoin pathway genes that do not respond to inducer (3, 19, 26). Together these observations support our earlier suggestion that the UIS element is the one which mediates induction of the allantoin pathway genes (25).

In this work we have also discovered genetic evidence suggesting the occurrence of a synergistic interaction occurring between the *DAL7* UIS and UAS_{NTR} elements. The degree of UIS-mediated enhancement of UAS_{NTR} function

TABLE 5. Effects of a UIS point mutation on the element's ability to enhance UAS activity[a]

Plasmid	Insert composition	β-Galactosidase activity Pro
pNG15	Vector alone—no elements	11
pJD28	UAS (TATGTGATAAG) without UIS	172
pJD29	UAS (TATGTGATAAG) + UIS (GAAAATTGCGTTT)	4,817
pJD83	Mutant UIS (GTAAATTGCGTTT) + UAS (TATGTGATAAG)	144

[a] Strain M1682-19b was used as the transformation recipient in this experiment.

TABLE 6. UIS-mediated enhancement of UAS activity supported by mutant alleles of UAS_{NTR}[a]

Plasmid	UAS sequence	UIS sequence	Pro
pNG58	TaTGCTGATAAGGTGC	−	231
pRR204		+	1,259
pNG62	TTTGCaGATAAGGTGC	−	54
pRR205		+	562
pNG63	TTTGCTcATAAGGTGC	−	17
pRR206		+	113
pNG64	TTTGCTGtTAAGGTGC	−	4
pRR207		+	124
pNG65	TTTGCTGATtAGGTGC	−	10
pRR208		+	178
pNG68	TTTGCTGATAAcGTGC	−	199
pRR209		+	2,429
pNG77	TTTGCgGATAAGGTGC	−	32
pRR210		+	1,365
pNG79	TTTGCTGcTAAGGTGC	−	8
pRR211		+	151
pNG81	TTTGCTGATCaGGTGC	−	11
pRR212		+	273

[a] Strain M1682-19b was used as the transformation recipient in this experiment. Lowercase letters indicate mutated bases.

DAL7	**5'-G A A A G T T G C G G T G-3'**
	5'-G A A A A T T G C G T T T-3'
DAL2	**5'-C A A A C T T G C G C T T-3'**
DAL4	**5'-G G A A A A T G C G C C C-3'**
	5'-C A A A A T A G C G C C T-3'
DUR1,2	**5'-G A A A T G T G C G T T T-3'**
DUR3,4	**5'-G A A A A A T G C G T A T-3'**
CAR2	**5'-A A A A A A A G C G C G T-3'**
CAR2	**5'-C A A A T G T G C G C A C-3'**

FIG. 5. Sequences with strong homology to the UIS sequence analysed in this work that were observed in the 5′ regions of other genes which have been shown to respond to induction by allophanate or OXLU.

diminished, however, as the strength of the UAS_{NTR} increased. This suggests that strong UAS_{NTR} elements, such as those upstream of the inducer-independent *DAL5* gene, have little need for the UIS element. In contrast, it appears that without such interaction, the UAS_{NTR} elements upstream of *DAL7* would not support high-level transcriptional activation (Table 4). A potentially important physiological implication of these interactions may be a tighter coupling between transcriptional activation of the inducible allantoin pathway genes and induction, since it is the UIS element that has also been shown to be required for response to inducer (2, 25; this work). We must emphasize that inducer was not required for any of the observations reported in this work. The presence of inducer increased enhancement an additional twofold, as previously observed (1, 25). It is possible that the presence of inducer merely strengthens whatever biochemical events that mediate UAS_{NTR} enhancement in its absence. If the above discussion is correct, it suggests that each of the inducible allantoin pathway genes would be expected to possess UAS_{NTR} elements that function at high level only when in association with UIS elements, while the UAS_{NTR}s associated with the pathway genes expressed in an inducer-independent fashion would be expected to function alone.

That potential associations similar to those described in this report may occur more generally as a means of accomplishing regulated gene expression is supported by the observation that *STE3* transcription appears to exhibit similar characteristics of regulation. This gene has been reported to contain a P box that is incapable of supporting transcriptional activation unless a second element, the Q box, is present as well (12). A further parallel between the two systems is the existence of P-box versions that are able to support transcriptional activation alone.

Data presented in Table 7 lead us to conclude that the *DAL82* product is required for enhancement of UAS_{NTR} function mediated by the *DAL7* UIS element. We cannot at present ascertain that the *DAL82* protein requirement is absolute, because we observed one- to twofold residual enhancement in the *dal82* mutant strain (Table 7). This residual enhancement may have derived from residual *DAL82* gene product activity, since the mutant strain used in this work was not a deletion and we have been unsuccessful thus far in our attempts to obtain one. In this regard, it is important to note that UIS function in the induction process is completely lost in this strain (see Fig. 4 of reference 17). If, on the other hand, the point mutation used did not possess residual *DAL82* product activity, the small amount of enhancement observed may reflect participation of one or more additional proteins to generate the observed effects. These possibilities are only easily distinguishable when a deletion of *dal82* becomes available. The loss of UAS_{NTR} function enhancement observed in *DAL82* mutants is a second characteristic that distinguishes this gene's function from that of

TABLE 7. Enhancement of UAS_{NTR} function by the UIS element in wild-type and mutant strains of *S. cerevisiae*

Plasmid	Element composition[a]	UIS sequence	β-Galactosidase activity[b] Pro			
			WT (M1682-19b)	*dal81*Δ (pB200)	WT (TCY1)	*dal82* (MO2)
pNG15	Vector alone—no element		14 (5)	10 (5)	10 (9)	13 (4)
pJD30	UIS alone		79	78	15	10
pJD28	UAS (TATGTGATAAG)	−	230 (62)	104 (30)	128 (114)	125 (120)
pJD29		+	3,482 (602)	3,953 (393)	1,449 (650)	202 (262)
pJD32	UAS (TtTGTGATAAG)*	−	544	277	319	222
pJD34		+	5,175	4,474	1,973	554
pJD31	UAS (TATcTGATAAG)	−	1,303	852		
pJD33		+	6,092	5,508		
pJD35	UAS (TtTcTGATAAG)	−	2,783	1,913		
pJD40		+	7,024	5,471		

[a] Lowercase letters indicate the altered base(s).
[b] WT, wild type.

DAL81, which is also required for induction of allantoin pathway gene expression as well as that of GABA metabolism (2).

There are, at this point, no data to delineate the physical characteristics or biochemical mechanisms through which the UIS-mediated enhancement of UAS_{NTR} function occurs. Although there was a very small amount of reporter gene expression observed with a construction carrying only the UIS element (Table 7), it was only 1 to 2% of that observed when a UAS_{NTR} element was added to the construction. Such low activity does not permit us to conclude that the UIS element can independently support transcriptional activation that is physiologically significant, i.e., we cannot conclude that the UIS element is in fact an inducer-dependent UAS.

The fact that one or more proteins have been shown to bind to the *DAL7* UIS element (Fig. 4) prompts the question of their identity. Two candidate proteins are encoded by the *DAL81* and *DAL82* genes (1, 2, 17, 23). Both of these genes have been demonstrated to be required for induction (1, 17, 23), and for the functioning of the UIS element rather than UAS_{NTR} (2, 17). However, because of the potential interaction of proteins associated with the very closely spaced UAS and UIS sites, the obvious gel shift experiments involving mutant forms of the *DAL81* and *DAL82* proteins have not thus far been interpretable (19a). This may be due to protein-protein interactions such as those just discussed and/or the presence of an additional *cis*-acting site(s) and *trans*-acting factor(s) not identified thus far. Consistent with this suggestion is the observation that at least 10 nucleotides, not required for UIS function in vivo, are protected from DNase digestion in the DNA footprint experiment (Fig. 4). It is conceivable that these nucleotides represent such a previously unreported site. Since it appears that multiple proteins function in close association with the UAS_{NTR} and UIS sites as well as any we have not yet discovered, deletion mutants of all of the genes encoding these putative proteins will likely be required to unravel the biochemical relationships that occur. Equally probable is the suggestion that some of the proteins and their cognate genes remain to be identified.

A paradox appears when comparing data obtained with plasmid pJD28 used in this work and plasmid pHY129 reported in Fig. 7 of reference 24. These plasmids contain the same UAS element. Yet, in the earlier report, the UAS element was demonstrated to support significantly higher level UAS activity than observed with plasmid pJD28 in the present work. Although different strains (RH218 and M1682-19b) were used, which accounts for a two- to threefold difference in UAS activity in the two experiments, there is an additional subtle difference between the experiments. In the earlier experiment (25) we had no idea of the limits of the putative UAS element and used a DNA fragment with the sequence 5′-TTTGCTTTTCTTATCA-3′. Genetic analysis of the UAS_{NTR} reported recently indicated that this fragment was one base too short (4). Addition of the restriction site to the sequence used (to permit cloning into the expression vector) resulted in a mutation that made the insert of plasmid pHY129 a quantitatively better UAS element. This error in selecting the precise sequence to clone and assay did not alter the conclusions reached in the earlier work, however, because current data obtained with plasmids pHY129 and pJD28 differ only quantitatively.

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