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The *Saccharomyces cerevisiae DAL80* Repressor Protein Binds to Multiple Copies of GATAA-Containing Sequences (URS_{GATA})

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Induced expression of the allantoin (*DAL*) catabolic genes in *Saccharomyces cerevisiae* has been suggested to be mediated by interaction of three different types of promoter elements. First is an inducer-independent upstream activation sequence, UAS_{NTR}, whose operation is sensitive to nitrogen catabolite repression. The *GLN3* product is required for UAS_{NTR}-mediated transcriptional activation. This site consists of two separated elements, each of which has a GATAA sequence at its core. Response of the *DAL* genes to inducer is mediated by a second type of *cis*-acting element, *DAL UIS*. The *DAL82* and *DAL81* genes are required for response to inducer; DAL82 protein is the *UIS*-binding protein. When only the UAS_{NTR} and *UIS* elements are present, *DAL* gene expression occurs at high levels in the absence of inducer. We, therefore, hypothesized that a third element, an upstream repressor sequence (URS) mediates maintenance of *DAL* gene expression at a low level when inducer is absent. Since the *DAL* and *UGA* genes are overexpressed and largely inducer independent in *dal80* deletion mutants, we have suggested *DAL80* protein negatively regulates a wide spectrum of nitrogen-catabolic gene expression, likely in conjunction with a URS element. Here we show that *DAL80* protein binds to *DAL3* and *UGA4* upstream DNA sequences, designated URS_{GATA}, consisting of two GATAA-containing sites separated by at least 15 bp. The preferred orientation of the sites is tail to tail, but reasonable binding activity is also observed with a head-to-tail configuration. URS_{GATA} elements contain the sequence GATAA at their core and hence share sequence homology with UAS_{NTR} elements.

Allantoin catabolic system (*DAL*) genes in *Saccharomyces cerevisiae* have been shown to possess multiple positively and negatively acting elements in their upstream regions (8, 21, 44). The integrated action of multiple proteins binding to these elements is likely responsible for regulating the expression of these gene systems. There are two patterns of *DAL* gene expression. *DAL3* and *DAL5* are expressed in an inducer-independent manner (32, 45). *DAL1*, *DAL2*, *DAL4*, *DAL7*, *DUR1,2* and *DUR3* respond to the presence of inducer (5, 16, 18, 32, 42, 44, 45, 46). All of the above genes, as well as most other nitrogen-catabolic genes, contain multiple copies of the nitrogen-regulated upstream activation sequence UAS_{NTR} consisting of two separated dodecanucleotide sites with the sequence GATAA at their cores (6, 14, 34, 42). In addition, UAS_{NTR} has been shown to be the *cis*-acting element required for transcriptional activation of six *DAL* genes (15a, 16a, 34, 42). Operation of protein(s) bound to this element is highly sensitive to nitrogen catabolite repression and requires a functional *GLN3* product (9, 10, 29).

The inducer-dependent *DAL* genes possess two additional types of elements in their upstream regions. The *cis*-acting element that mediates response to the allantoin pathway inducer, allophanate, is a dodecanucleotide designated *DAL UIS* (38). The *DAL81* and *DAL82* genes are required for inducer response (1, 3, 4, 18a, 29a, 37, 39); the latter of these genes encodes the *DAL UIS* DNA-binding protein (15). The *DAL81* (*UGA35*) product has been suggested to operate in a more general way, because it is also required for induced *UGA* gene expression (4, 39).

When only the UAS_{NTR} and *DAL UIS* elements are present in an expression vector, reporter gene expression is independent of inducer (38). We therefore hypothesized that a third element, an upstream repression sequence (URS), maintained *DAL* gene transcription at a low, basal level in the absence of inducer (42). Presently, little is known about the structure of this *cis*-acting element beyond its existence (7, 42).

Mutations or deletions of the *DAL80* locus result in high-level *DAL* gene expression that is largely inducer independent (7, 14). It is likely that DAL80 protein similarly regulates the *UGA* genes, since *dal80* (*uga43*) mutations affect *UGA* gene expression in the same way (11, 12, 14, 39). DAL80 protein also down-regulates a number of inducer-independent genes associated with nitrogen metabolism, such as *DAL3*, *CAN1*, *DAL80*, and *GAP1* (14, 19, 43). We have, therefore, suggested that DAL80 protein negatively regulates a wide spectrum of nitrogen-catabolic genes, likely in conjunction with a URS element (12, 14).

We recently cloned and sequenced the *DAL80* gene and found it encodes a protein whose deduced primary structure contains a zinc finger motif with extensive homology to the metazoan GATA-binding family of transcriptional activators (12, 25, 30, 40). The *GLN3* protein, required for UAS_{NTR} operation (9), also contains a zinc finger motif similar to the one in *DAL80* and the metazoan GATA-binding proteins (12, 27). These protein homologies suggested that the *DAL80* and *GLN3* products might also bind to GATA sequences (14). Although this was a reasonable suggestion, attempts to verify it experimentally have so far been frustrated. Using yeast extracts derived from cells containing wild-type, *dal80* deletion, *gln3* deletion, or *dal80 gln3* double-deletion alleles, we have been unable to show that either DAL80 or GLN3 proteins specifically bind DNA by conventional footprinting or electrophoretic mobility shift assays (EMSAs) (31). In

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fact, the only biochemical datum that exists for GLN3 protein binding is the observation that antibody against it can be used to immunoprecipitate a DNA fragment containing a GATA-related sequence (27). There is no biochemical information about the DAL80 protein's function.

If GLN3 and DAL80 proteins both bind to the same sites, as the protein structural homologies suggest, then yeast genes requiring GLN3 protein for expression should also be sensitive to regulation by the DAL80 protein; the converse should also be true. This has clearly been shown experimentally not to be the case (14). *DAL5* expression exhibits a very strong requirement for the GLN3 protein but is minimally affected by deletion of the *DAL80* gene (14). Conversely, *UGA1* expression is sensitive to DAL80 protein regulation but is not affected by deletion of *GLN3* (14). These considerations argued that it was not prudent for us to assume the structure of the DAL80 protein-binding site on the basis of protein sequence homologies. Therefore, we decided upon a strategy that involved first determining whether *DAL80* encoded a DNA-binding protein and then, if so, determining without prejudice the nature of its binding site. The results of experiments addressing these issues are presented in this report. A preliminary report of this work has already appeared (13).

MATERIALS AND METHODS

Plasmid constructions. Construction of plasmid pT-DAL80 is shown in Fig. 1. One strand of the double-stranded oligonucleotide used to construct plasmid pT-DAL80E, which is identical to plasmid pT-DAL80 except for the addition of a 9-amino-acid epitope tag (influenza hemagglutinin protein [20]) at the N terminus of the DAL80 protein, is as follows: 5′-T ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT ATG GTG CTT AGT GAT TCG TTG AAG CTG CCC TCG CCT ACA CTT TCA G-3′. Standard procedures were employed for cloning and transformation (23).

EMSAs and protein extract preparation. Oligonucleotides used for EMSAs were prepared as described earlier (21, 22) except that a Klenow fragment-mediated fill-in reaction employing [^{32}P]dCTP was used to radioactively label the DNA fragment (23). Protocols used for EMSAs were essentially those of Kovari and Cooper (22). The amount of protein used in the EMSAs was empirically determined for each batch of protein (33) and ranged from 2.5 to 5 μg per reaction (2). Protein extracts were prepared with strain BL21(DE3) (35) containing either plasmid pT7-7 (pT-7) (36) or pT-DAL80. *Escherichia coli* cultures were grown to a cell density with an A_{600} of 0.40 (Zeiss spectrophotometer) at 37°C in 100 ml of LB medium (26) containing 125 mg of ampicillin per liter. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added (1 mM final concentration), and growth continued for 3 h. Cells were harvested by centrifugation and frozen at −80°C. Cell pellets were thawed, resuspended in 5 ml of sonication buffer (20 mM Tris HCl [pH 7.4], 100 mM KCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride), and sonicated (microtip at 35% power) for 15 cycles of 10 s on, 10 s off. Samples were kept on ice. After the extract had been clarified by centrifugation (10,000 × *g* for 30 min), glycerol was added (final concentration, 20% [vol/vol]) and the solution was divided into aliquots that were stored at −80°C. Radiolabelling of the DAL80 protein with [^{35}S]methionine was accomplished by the procedures of Margolin and Howe (24).

RESULTS

Expression of *DAL80* protein in *E. coli*. To obtain DAL80 protein devoid of other yeast proteins, we cloned the coding portion of the *DAL80* gene downstream of the T7 gene 10 promoter (Fig. 1) for expression in *E. coli*. We assessed expression of the protein by inhibiting *E. coli* protein synthesis with rifampin (24, 36). No radioactive proteins were produced by rifampin-treated cells containing only expression vector plasmid pT-7 (Fig. 2, lane B). Cells containing plasmid pT-DAL80 produced a major protein with a mobility expected of a protein the size of DAL80 and several lower-molecular-weight species characteristic of proteolytic degradation products (Fig. 2, lane C). We have not investigated the nature or composition of the lower-molecular-weight species. Fig. 2, lane A, depicts the protein profile of ^{35}S-labelled crude *E. coli* extract resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

DAL80 binding to DNA fragments derived from the *DAL3* gene. To assess whether *DAL80* protein was capable of binding to sequences upstream of *DAL3*, we performed EMSAs with these protein preparations. We chose to analyze the *DAL3* promoter because of its *DAL80* sensitivity and lack of response to inducer, i.e., its regulation was the most simple of any gene responding to *dal80* deletion mutations. The first DNA fragment we tested contained sequences −184 to −86 (*DAL3*-4 or *DAL3*-3 [the *DAL3*-3 fragment differed from *DAL3*-4 by deletion of 18 bp at its 3′ terminus; the two oligonucleotides were used interchangeably throughout this work]) (Fig. 3). When the EMSA was performed with extract from cells containing plasmid pT-DAL80, two bands of altered mobility were observed (Fig. 2, lane F; Fig. 4, DAL3-3, lane I, and DAL3-4, lane L [arrows A and B]). These complexes were not observed when extracts from cells containing vector plasmid pT-7 or a reaction mixture devoid of protein was used (Fig. 2, lanes D and E; Fig. 4, DAL3-3, lanes G and H, and DAL3-4, lanes J and K). To demonstrate that DAL80 protein was responsible for the shift, we repeated the experiment but used an epitope-tagged (9 amino acids) version of *DAL80* fused to the T7 promoter (plasmid pT-DAL80E) in place of plasmid pT-DAL80. The presence of the epitope tag resulted in two complexes of larger apparent molecular weights consistent with a 9-amino-acid addition (Fig. 2; compare lanes F and G).

DAL80 protein shares strong homology with metazoan proteins reported to bind GATA-containing sequences homologous to the yeast nitrogen catabolite repression-sensitive UAS_{NTR} element (25, 40). These observations suggested that *DAL80* protein might analogously bind GATAA-containing sequences (12). Therefore, to test the specificity of the two DNA complexes in the EMSA, we selected several DNA fragments containing multiple UAS_{NTR}-homologous (GATAA-containing) sequences and little else. We determined whether these fragments were effective competitors of DNA fragments *DAL3*-3 or *DAL3*-4 for DAL80 binding. A 58-bp DNA fragment derived from positions −296 to −237 of *CAN1* was as effective a competitor of *DAL3*-4 DNA binding as the *DAL3*-4 molecule was itself (Fig. 5; compare upper left panel, lanes A to H, with lanes H to O). The *CAN1* fragment contains little other than four tandem UAS_{NTR}-homologous sequences. A DNA fragment (*DAL3*-5) derived from *DAL3* positions −155 to −86 containing three tandem UAS_{NTR}-homologous sequences was an equally effective competitor (Fig. 5; compare upper left panel, lanes A to H, with upper right panel, lanes A to H). A similar competition

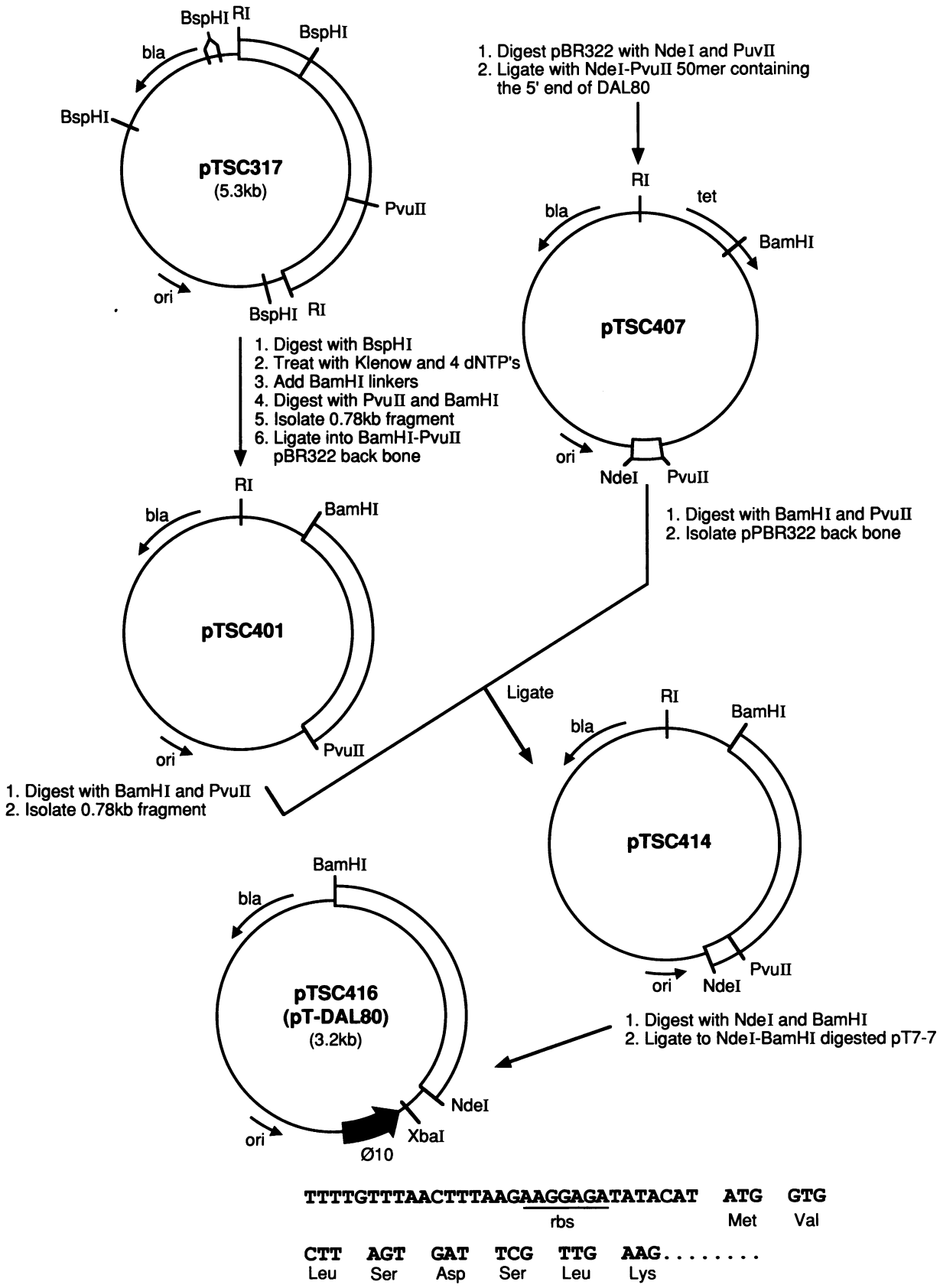

FIG. 1. Construction of plasmid pT-DAL80. Yeast sequences are denoted by double lines and pBR322 sequences are denoted by single lines. Plasmid maps are not drawn to scale.

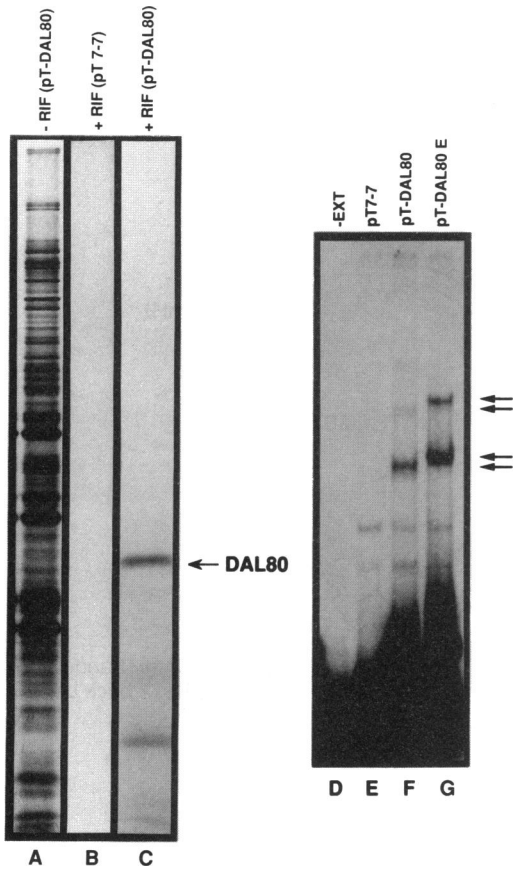

FIG. 2. (Left panel) SDS-PAGE analysis of [^{35}S]methionine-labelled proteins using the T7 expression system. Proteins were labelled as described in Materials and Methods and resolved in an SDS-polyacrylamide gel. Lane A shows radiolabelled total protein from strain MH9216/pT-DAL80 generated in the absence of rifampin, lane B shows protein from strain MH9216/pT7-7 treated with rifampin, and lane C shows MH9216/pT-DAL80 treated with rifampin. The arrow indicates the DAL80 protein (calculated molecular weight = 30,166). (Right panel) EMSAs employing protein preparations derived from *E. coli* BL21(DE) containing plasmids pT7-7 (lane E), pT-DAL80 (lane F), or pT-DAL80E (lane G). The DNA probe was oligonucleotide *DAL3*-4. Lane D did not contain protein extract (−EXT). The arrows indicate DAL80 specific complexes in lanes F and G.

experiment employing DNA fragment LK81, derived from *CAR1* UAS_{C1} and known to contain multiple protein-binding sites (21), was not an effective competitor (Fig. 5; upper right panel, lanes G to M). Note that both DAL80-specific DNA-protein complexes (indicated by arrows) were similarly diminished by increasing amounts of DNA fragments containing UAS_{NTR}-homologous sequences, though the lower-mobility complex was inhibited at lower concentrations of competitor.

The observations described above suggested that UAS_{NTR} elements containing GATAA sequences were involved in DAL80 protein binding. However, the possibility that single GATAA sequences constituted a physiologically significant DAL80 binding site seemed to us remote, because we previously showed that the *DAL5* gene, which contains nine UAS_{NTR}-homologous GATAA sequences, is not strongly down-regulated by DAL80 protein (33). Therefore, we did not make assumptions about the structure of the *DAL80* binding site but rather used EMSAs to localize the site(s) by using DNA fragments *DAL3*-3 and *DAL3*-4 as the parent fragments to be analyzed. First, we labelled DNA fragment *DAL3*-1, in which the 3′ GATAA cluster of the *DAL3*-3 fragment was deleted (Fig. 3). This fragment did not form a detectable complex with DAL80 protein (Fig. 4; DAL3-1, lanes A to C). Since the 5′ half of the parent *DAL3*-3 DNA fragment did not possess an effective DAL80 binding site, we determined whether the 3′ half of this fragment could form the DNA-DAL80 complex. DNA fragment *DAL3*-5, containing the three clustered 3′ GATAA sequences (Fig. 3), was capable of forming the higher-mobility DNA-DAL80 complex but did so less effectively than had the parent fragment (Fig. 4; compare DAL3-4 and DAL3-5, lanes L and O). Addition of one 5′ GATAA sequence to the three 3′ sequences contained on DNA fragment *DAL3*-5 resulted in greater levels of the higher-mobility DAL80-DNA complex than observed with DNA fragment *DAL3*-5 (Fig. 4; DAL3-6, lanes P to R [arrow B]). A lower-mobility DAL80-DNA complex (arrow A) was also observed with DNA fragment *DAL3*-6; it was, however, present at a much lower concentration than observed with DNA fragments *DAL3*-3 or *DAL3*-4 (compare DAL3-4, lane L, and DAL3-6, lane R).

Note that several lower-mobility complexes are present when extract from cells containing plasmid pT-DAL80 was used as the source of protein (Fig. 4; DAL3-6, lane R [arrow C]). Upon overexposure of the autoradiograms, complexes with this and similar mobilities were also observed with plasmid pT-7 and hence are not DAL80 specific (data not shown). They are neither designated nor considered further in this work. Only the complexes indicated by arrows A and B were found to be DAL80 specific in overexposures of the autoradiographs.

A hypothesis consistent with the observations described above was that multiple sequences containing the GATAA motifs constituted the DAL80 binding site. Deletion of the most 3′ GATAA sequence from DNA fragment *DAL3*-3 (DNA fragment *DAL3*-2) decreased but did not destroy DAL80 binding (Fig. 4; DAL3-2, lanes D to F). We next tested a DNA fragment (*DAL3*-7) in which the two most 3′ GATAA sequences were deleted. No DAL80 protein binding could be detected (Fig. 4; DAL3-7, lanes D to F). When we overexposed the autoradiograph, however, a small amount of DAL80-DNA complex was observed (Fig. 4; DAL3-7, lane I [arrow B]). Next we deleted one of the two 5′ GATAA sequences contained on DNA fragment *DAL3*-7 and produced fragment *DAL3*-8. No detectable complex formation was observed with this DNA fragment, even in overexposures (Fig. 4; DAL3-8, lanes J to L).

The small amount of DAL80-DNA complex observed with DNA fragment *DAL3*-7 left some doubt as to whether the signal we observed was real. Therefore, DNA fragment *DAL3*-7 was used as a competitor in an EMSA with DNA fragment *DAL3*-4 as probe. DNA fragment *DAL3*-7 was a competitor of the *DAL3*-4 DNA fragment but was significantly (10-fold) less efficient than was DNA fragment *DAL3*-6 (data not shown). Similar competition experiments were conducted with DNA fragments *DAL3*-2, *DAL3*-4, *DAL3*-5, and *DAL3*-6 as competitors. DNA fragments *DAL3*-4 and *DAL3*-6 were equally good competitors. DNA fragments *DAL3*-2 and *DAL3*-5, on the other hand, were about equally effective competitors but significantly less effective than DNA fragments *DAL3*-4 and *DAL3*-6 (Fig. 5, lower panels).

To more precisely characterize the DAL80 protein-binding site, we focused on DNA fragment *DAL3*-5, which was

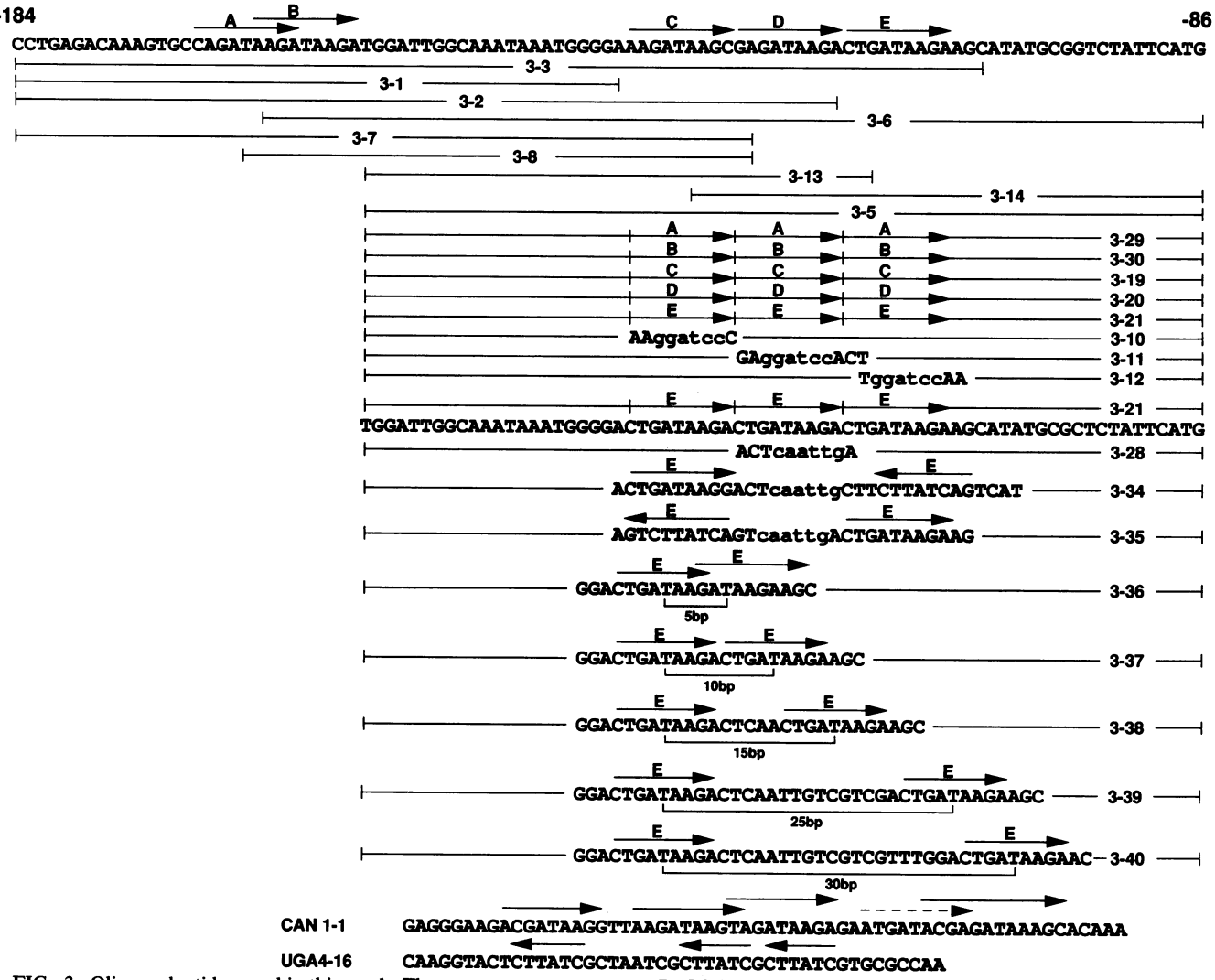

FIG. 3. Oligonucleotides used in this work. The top sequence represents *DAL3*-4, and below are the relevant constructions derived from it. Mutant sequences are denoted by lowercase letters. Labelling was accomplished by filling in the *Eag*I (GGCC) and *Sal*I (TCGA) extensions present on the double-stranded oligonucleotides. The extension sequences are not shown. DNA fragments are designated by the gene from which they were derived, e.g., *DAL3*, and a specific number, e.g., -5. The GATA sequences are designated A through E, respectively.

the smallest DNA fragment yielding a clearly visible DAL80-DNA complex. We first deleted either the most 5′ or the most 3′ GATAA sequence from DNA fragment *DAL3*-5 (Fig. 6; upper left panel, *DAL3*-13 and *DAL3*-14). Neither DNA fragment *DAL3*-13 nor *DAL3*-14 yielded a detectable DAL80-DNA complex (Fig. 6; upper left panel, lanes A to I). This result suggested that the two terminal GATAA sequences of DNA fragment *DAL3*-5 were necessary for complex formation. This experiment did not address whether the middle GATAA sequence was required. Therefore, we mutated each of the three GATAA sequences (designated 5′-C, D, E-3′ in Fig. 6) in turn and determined the effects of these mutations on DAL80-DNA complex formation. Mutating the C or E element resulted in loss of detectable complex formation, whereas mutation of the center element (D) had little effect (Fig. 6, upper right panel). These observations indicated that two copies of the GATAA sequence were required for effective complex formation. Although mutation of GATAA element C or E resulted in loss of a complex in the EMSA, a DNA fragment containing a mutated C element remained able to act as a competitor of DNA fragment *DAL3*-3 for DAL80 binding. It was, however, a much less (5-fold less) effective competitor than was the *DAL3*-5 DNA fragment (data not shown).

Data obtained with DNA fragment *DAL3*-5 and its derivatives raised the question of whether the observed results derived from the spacing of the GATAA sequences or their precise sequences. To address the sequence issue, we synthesized five DNA fragments; each contained three identical copies of one of the five GATAA sequences originally contained in DNA fragment *DAL3*-4. The duplicated sequence was 9 bp long. All five DNA fragments were able to form a complex with DAL80 protein, albeit at different levels; the fragment containing the E elements appeared to function best (Fig. 6, lower panels).

Since the DNA sequences of the *DAL3* GATAA elements we assayed did not appear to be the controlling factor in DNA-DAL80 complex formation, the spacing of the elements was considered. We tested DNA fragments in which two GATAA elements were separated by 5, 10, 15, 20, 25,

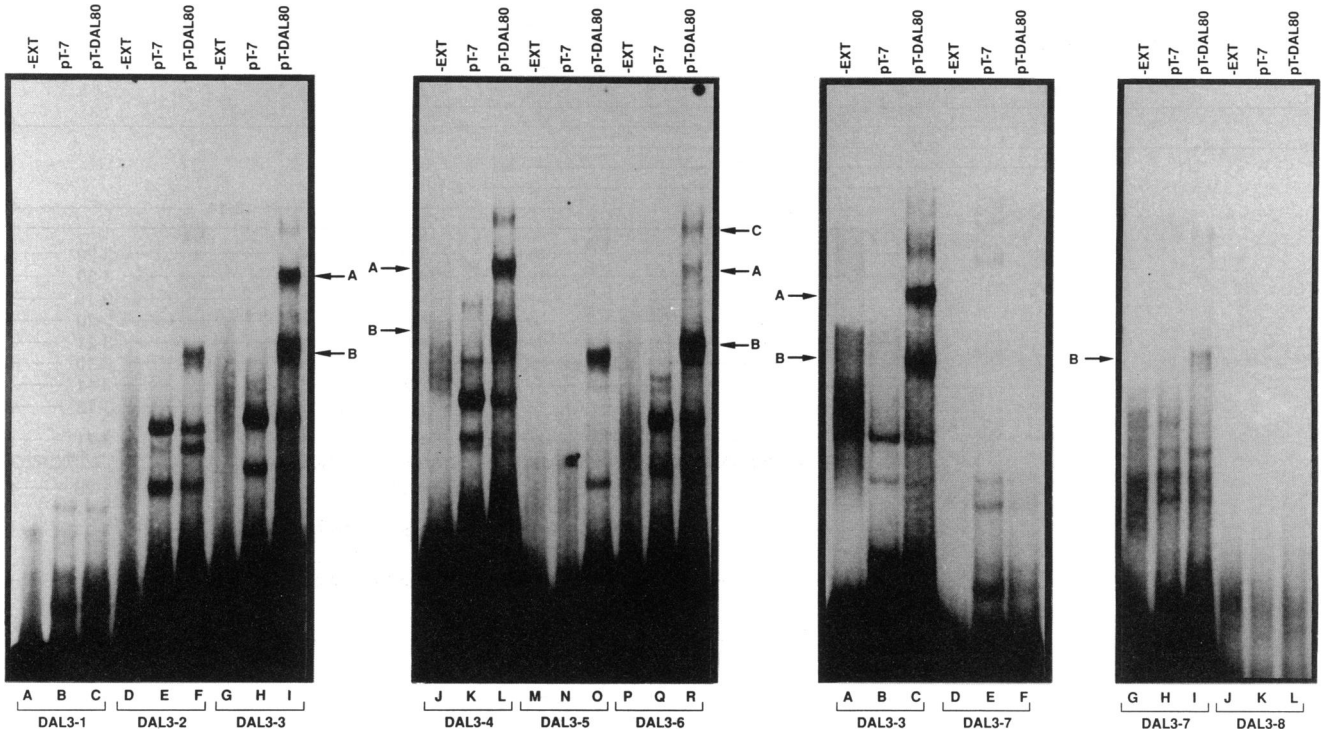

FIG. 4. EMSAs employing DNA probes derived from the *DAL3* promoter region. Lanes A, D, G, J, M, and P contained no protein extract (−EXT); lanes B, E, H, K, N, and Q contain reaction mixtures with extracts produced by cells containing plasmid pT7-7; and lanes C, F, I, L, O, and R contain reaction mixtures with extracts produced by cells harboring plasmid pT-DAL80. DNA probes are indicated at the bottom. It should be noted that autoradiograph exposure times were empirically determined to maximize the resolution of all lanes. Overexposure results in the enhancement of the complex marked A for probe *DAL3*-5 (see Fig. 4, lane N, and Fig. 6).

and 30 bp of plasmid pBR322 DNA. Extremely weak complex formation was observed when the GATA elements were spaced less than 15 bp apart (Fig. 7; left panel, lanes A to F). Although the strongest signal was observed with a fragment in which the elements were spaced 20 bp apart, bands were also clearly observed when fragments containing elements separated by 15, 25, and 30 bp were used (Fig. 7; left panel, lanes G to I, and center panel, lanes D to I).

If two separated copies of the GATAA sequence were required for DAL80 binding, did they possess an orientation requirement? We tested three DNA fragments, each one containing two GATAA elements (E) spaced 20 bp apart and in all possible orientations. The most intense bands were obtained with the fragment in which the two elements were oriented tail to tail (Fig. 7; right panel, lanes G to I). Less effective complex formation was observed when the elements were head to tail (Fig. 7; right panel, lanes A to C), and the weakest signals occurred when the elements were oriented head to head (right panel, lanes D to F).

The results described above suggested that we should be able to crudely identify DAL80 protein-binding sites by their overall structure. To test this, we determined whether a DNA fragment (Fig. 3) derived from the *UGA4* promoter region, which possessed a sequence that fulfilled the known structural requirements of a DAL80 binding site, was indeed capable of binding DAL80 protein. As shown in Fig. 8, the DNA fragment was able to bind DAL80 protein, thereby supporting the predictive value of the information derived from our experiments.

DISCUSSION

This work demonstrates that DAL80 protein binds to a DNA site optimally consisting of two UAS_{NTR}-homologous GATAA sequences oriented tail to tail or head to tail, 20 bp apart. DAL80 protein binding to DNA exhibited remarkable flexibility. Although preferred spacing and orientation requirements were demonstrated, all orientations of the GATAA elements and element spacings greater than 15 bp supported DAL80-DNA complex formation to some degree. Even single copies of the wild-type GATAA sequence were able to serve as competitors and hence bind to DAL80 protein, albeit very weakly. The physiological import of DAL80's binding promiscuity is seen in the steady-state mRNA levels of various nitrogen-catabolic genes: mutation of *DAL80* results in zero- to manyfold increases in RNA levels, depending upon the gene in question (14). The lack of severe orientation or spacing constraints on DAL80 protein binding helps to explain the wide-ranging responses.

Detailed sequence requirements of the DAL80 binding site have thus far been only superficially investigated because of the lack of pure DAL80 protein and quantitative DNA-binding assays. The GATAA core sequence is important, and the binding element exchange experiment suggests a minimal half-binding site of no more than 9 bp (since this was the length of DNA exchanged). However, careful mutagenic analysis will be required to establish its true limits. The GATAA core alone is unlikely to be an effective binding site. If this was the only requirement, then any gene with functional UAS_{NTR} elements should be regulated by DAL80

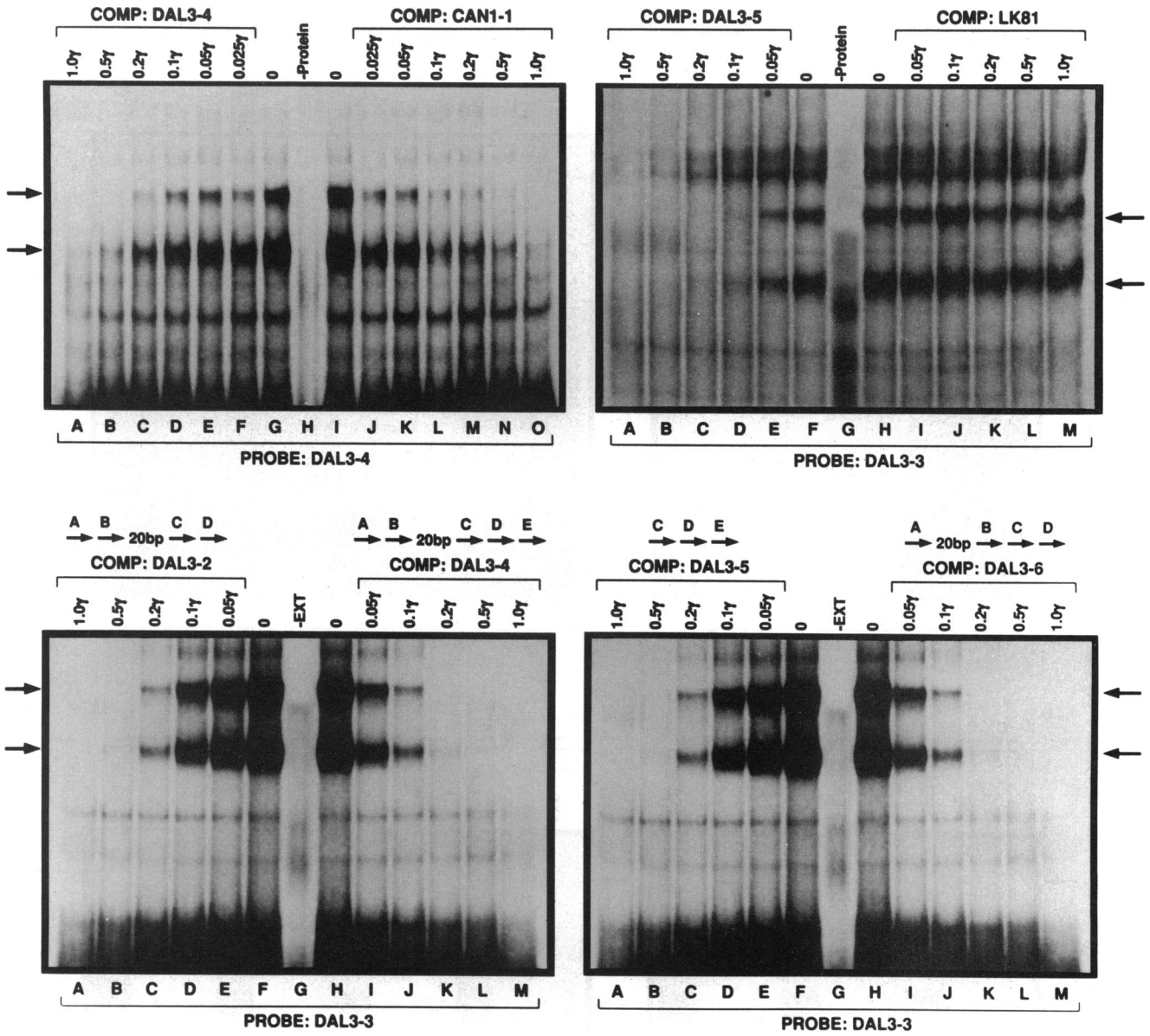

FIG. 5. Competition EMSAs between DNA fragments derived from the *DAL3*, *CAN1*, and *CAR1* promoters (upper panels) and between various *DAL3* promoter fragments and DNA fragment *DAL3*-3 (lower panels) for DAL80 protein binding. (Upper panels) ^{32}P-end-labelled oligonucleotides (*DAL3*-4 and *DAL3*-3 for the left and right panels, respectively) were used as DNA probes. Lanes F to A contained increasing amounts of unlabelled *DAL3*-4 oligonucleotide (left panel) or of unlabelled *DAL3*-5 oligonucleotide (right panel), and lanes J through O contained increasing concentrations of unlabelled *CAN1*-1 oligonucleotide (left panel) or of unlabelled insert from plasmid pLK81 (right panel). For both upper panels, lane H had no extract in the reaction mixture and lanes G and I had no competitor added. (Lower panels) Lanes E to A contained increasing concentrations of unlabelled oligonucleotide *DAL3*-2 (left panel) or of unlabelled oligonucleotide *DAL3*-5 (right panel), and lanes I to M contained increasing concentrations of *DAL3*-4 (left panel) or of unlabelled oligonucleotide *DAL3*-6 (right panel) in the reaction mixtures. For both lower panels, lane G contained no added extract (−EXT) and lanes F and H contained no added competitor. The probes used are listed under the lanes. For both upper and lower panels, large arrows indicate the DAL80 protein-specific complexes. COMP, competitor. Arrows designate DAL80 protein-specific complexes.

protein and respond to *dal80* deletions. Expression of the *DAL5* gene, which has nine GATAA-homologous sequences (33, 34), is only minimally (1.5- to 2-fold) increased in *dal80* deletion strains (12, 31). Similarly, the *GLN1* gene, which has several UAS_{NTR}-homologous sequences (28), does not respond to *dal80* deletion (14).

The clear requirement of two GATAA elements for DAL80 binding suggests that DAL80 dimerization may be involved in the protein-DNA complex. The presence of a leucine zipper in the deduced *DAL80* protein secondary structure and its requirement for DAL80 function are consistent with this suggestion (11, 12). The observation of two DNA-DAL80 protein complexes, especially prominent when DNA fragment *DAL3*-3 or *DAL3*-4 was used as the DNA probe, indicates that DAL80 forms more than one type of complex. The lower-mobility complex requires at least one

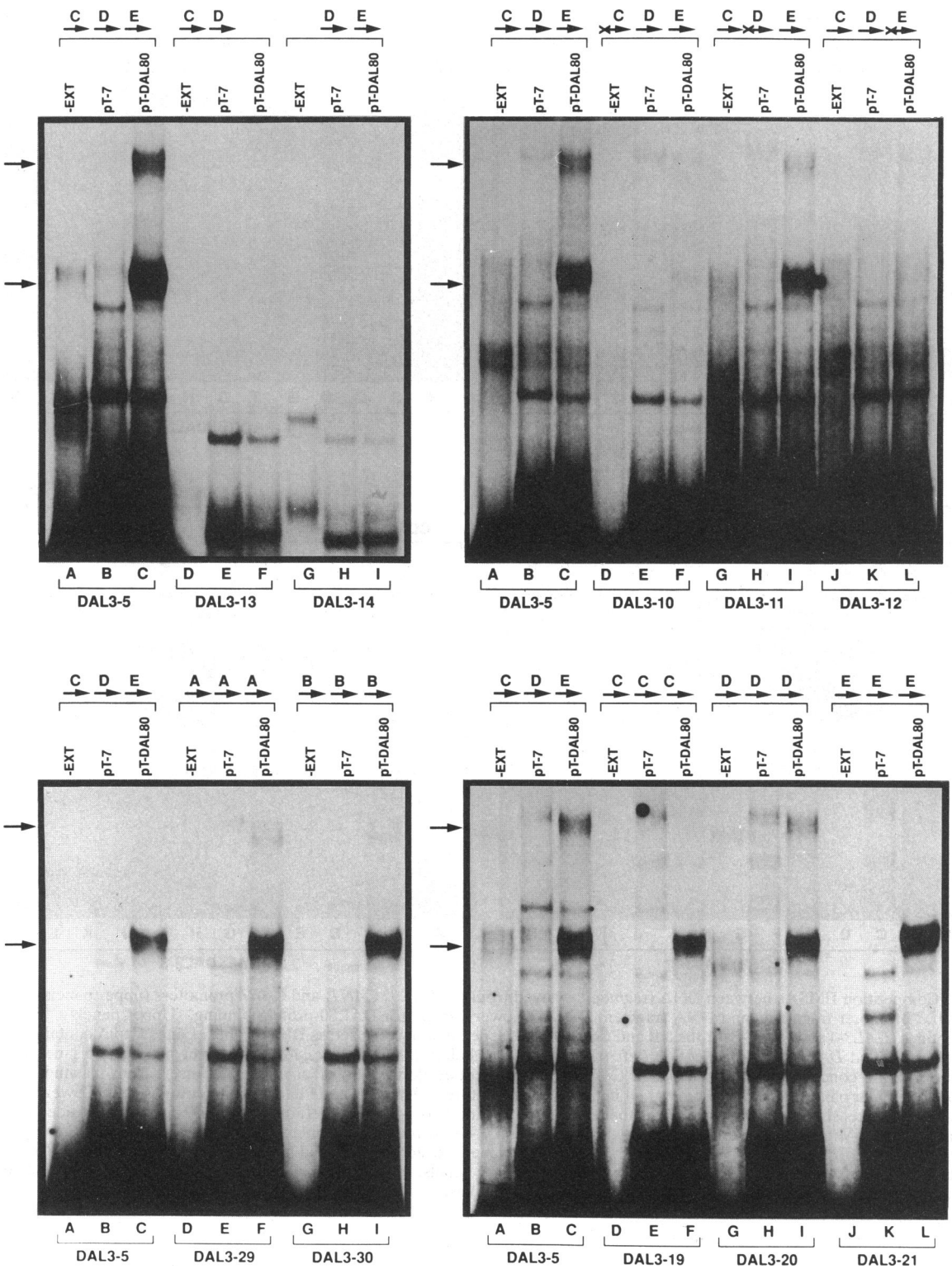

FIG. 6. (Upper panels) DAL80 protein binding to wild-type and mutant derivatives of the GATAA cluster of DNA fragment *DAL3*-5. The left panel shows an EMSA of DNA fragments *DAL3*-5 (lanes A to C), *DAL3*-13 (lanes D to F), and *DAL3*-14 (lanes G to I), and the right panel shows an EMSA of *DAL3*-5 and *DAL3*-5-derived mutant DNA fragments. The structures of all DNA fragments are shown in Fig. 3. Extracts are as described in the legend to Fig. 2, right panel. (Lower panels) Determination of whether DAL-80 protein binding is sequence specific. For both upper and lower panels, sequences of URS_{GATA} replacement oligonucleotides (designated A through E above the gels) are given in Fig. 3, and assays were performed as described in Materials and Methods with the extracts described in the legend to Fig. 2, right panel. Arrows to the left of the gels indicate the DAL80 protein-specific complexes.

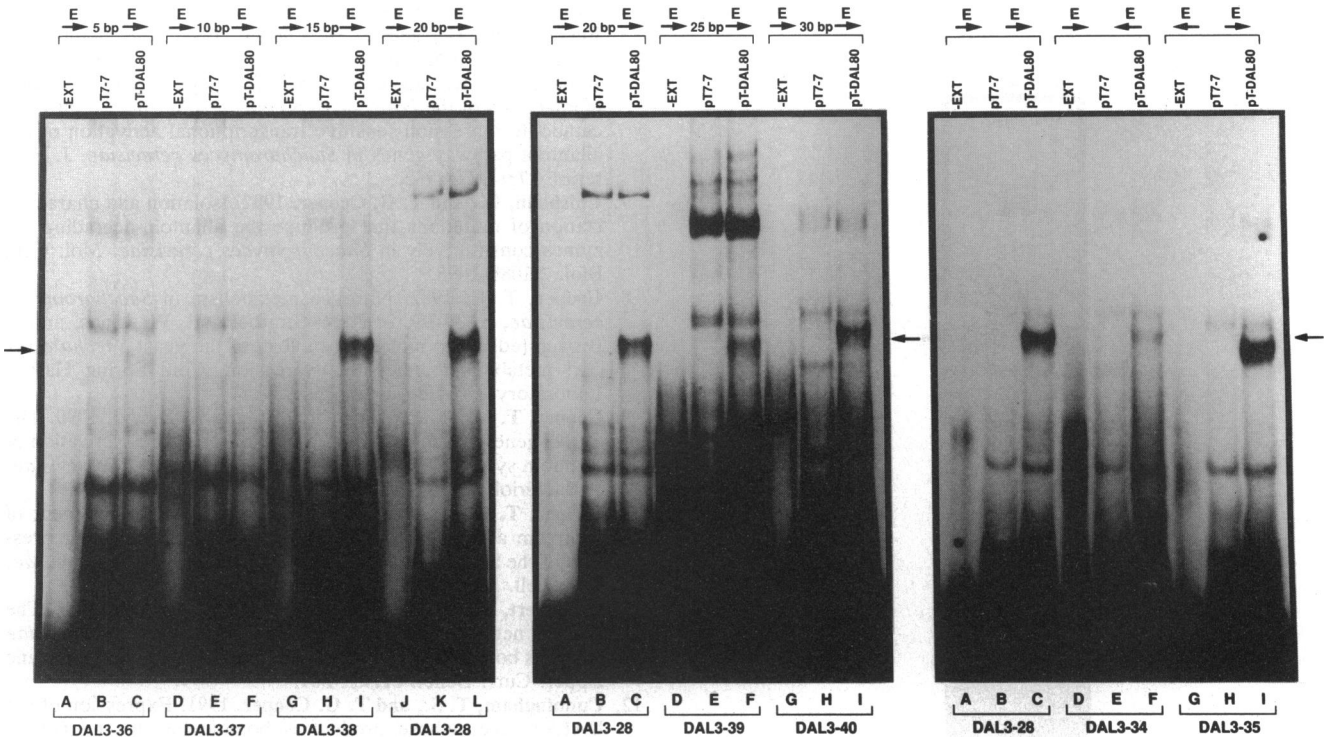

FIG. 7. (Left and center panels) Determination of the effects of element spacing on DAL80 protein-DNA complex formation. The sequences of the oligonucleotides used in this experiment are given in Fig. 3. To bring the elements in closer proximity, bases were deleted from oligonucleotide *DAL3*-28. To increase element spacing, pBR322 DNA sequences (positions 3631 to 3641) were added in 5-bp increments to *DAL3*-28. (Right panel) The effects of the orientation of URS_{GATA} elements on their ability to bind DAL80 protein. For all panels, extracts and assay conditions were as described in the legend to Fig. 2, right panel. The lower arrows indicate the B DNA complexes formed in EMSAs with oligonucleotide *DAL3*-5 and the upper arrows indicate the orientation of the paired E elements (separated by 20 bp).

of the 5′ GATAA elements in addition to those found with the minimal *DAL3*-5 fragment (Fig. 4). The decreased mobility of the complex observed with DNA fragments *DAL3*-3 and *DAL3*-4 probably results from a higher level of DAL80 polymerization than occurs with DNA fragment *DAL3*-5. There also appears to be cooperativeness in the formation of these complexes. With fragments capable of supporting formation of both the high- and low-mobility complexes, the rapidly migrating complex is much stronger than it is when a fragment, such as *DAL3*-5, which cannot as readily form the slow complex, is used as probe (Fig. 4; compare DAL3-5 and DAL3-6, lanes M to R).

Footprinting experiments are normally associated with reports that document the binding sites of DNA-binding proteins. Such experiments were performed in this work as well (31), but the results were not reported here because of an unavoidable complication. In addition to DAL80 and GLN3, there is at least one more yeast protein capable of binding GATAA sequences (31). The existence of this protein was first recognized by the observation that *UGA1* gene expression is *DAL80* regulated and nitrogen catabolite repression sensitive but does not require the *GLN3* product (14). We also find that the *DAL5* UAS_{NTR} still binds protein in a footprint assay even though *dal80 gln3* double-deletion strains were used to prepare the extract (31). We could not perform the footprint assay with *E. coli*-expressed DAL80 protein, because *E. coli* appears to possess one or more GATAA-binding proteins as well.

Thus far there is only limited experimental evidence that GLN3 protein binds to UAS_{NTR} elements, i.e., antibody against GLN3 protein can immunoprecipitate a DNA fragment containing a GATAA sequence (27). However, the zinc finger homology shared by the DAL80 and GLN3 proteins and the metazoan GATA-binding family of transcriptional activators suggests that DAL80 and GLN3 should both possess binding sites with some shared homology. In other words, GLN3 and DAL80 probably both bind GATAA-containing elements, albeit likely with different association constants. On the basis of the presumption that GLN3 also binds to elements containing GATAA sequences, we suggest that DAL80 and GLN3 proteins at times compete for the same binding site and that some UAS_{NTR} elements serve as both upstream activation elements and repression elements. A corollary of this suggestion is that the degree of transcriptional activation observed with promoters containing such UAS_{NTR} elements will be a function of the overall competition of the DAL80 and GLN3 proteins for these shared sites.

Structural characteristics for DAL80 binding are quite distinct from those of the metazoan GATA family of DNA-binding proteins. The latter protein family possesses two conserved zinc finger motifs (41), whereas DAL80 possesses only one. The C-terminal metazoan zinc finger motif possesses specificity for GATA binding; the other stabilizes the DNA-protein interaction. There is no evidence that the metazoan proteins form homodimers, though these proteins interact with other transcription factors (17). DAL80 protein operation, on the other hand, probably does involve dimerization. The DAL80 protein is also much smaller than the metazoan proteins and GLN3. This size difference may have important mechanistic implications. For example, if DAL80

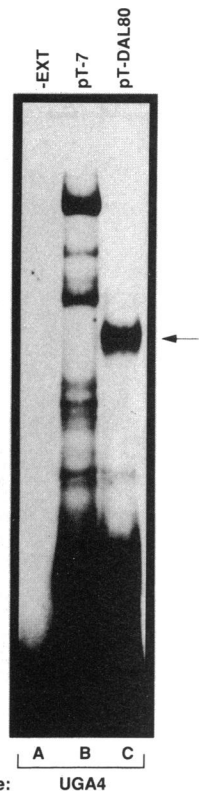

FIG. 8. DAL80 protein binding to a DNA fragment derived from the *UGA4* gene. Reaction conditions are as described in the legend to Fig. 2, right panel. Each of the complexes present in lane B is also seen in lane C when an overexposure of the film is viewed. In such overexposures, however, the DAL80-specific complex present in lane C (arrow) is not observed in lane B.

represses gene expression exclusively through competition with the GLN3 protein for some DNA binding sites, domains required to interact with components of the core transcription complex would be unnecessary.

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