The Transcriptional Activators BAS1, BAS2, and ABF1 Bind Positive Regulatory Sites as the Critical Elements for Adenine Regulation of ADE5,7

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Adenine repression of the purine nucleotide biosynthetic genes in Saccharomyces cerevisiae involves down-regulation of the activator protein BAS1 or BAS2 by an unknown mechanism. To determine the minimal cis-acting requirements for adenine regulation, hybrid promoter constructs were made between ADE5,7 promoter fragments and a CYC1-lacZ reporter. A 139-nucleotide fragment containing two BAS1 binding sites was sufficient to confer adenine regulation on the CYC1-lacZ reporter. Analysis of deletion and substitution mutations led to the conclusion that the proximal BAS1 binding site is both necessary and sufficient for regulation, whereas the distal site augments the function of the proximal site. By performing saturation mutagenesis, we found two essential regions that flank the proximal site. An ABF1 consensus sequence is within one of these regions, and mutations that impaired in vitro ABF1 binding impaired promoter activity in vivo. A second region is AT-rich and appears to bind BAS2. No substitution mutations led to high level constitutive promoter activity as would be expected from removal of an upstream repression sequence. Our results indicate that ABF1, BAS1, and BAS2 are required for ADE5,7 promoter function and that adenine repression most likely involves activator modification or a negative regulator that does not itself bind DNA.

The de novo synthesis of purine nucleotides requires 10 enzymatic steps to form the first purine nucleotide, inosine monophosphate. IMP is converted to either AMP or GMP in two steps. The products of at least 13 genes are required for this synthesis, since mutations in any one of these lead to an adenine requirement (1). Most of these genes encode the biosynthetic enzymes that participate in specific steps of the pathway (ADE1, ADE2, ADE4, ADE5,7, ADE6, ADE8, ADE12, ADE13), others encode enzymes required to produce additional substrates necessary to complete the pathway (ADE3, ASP5), and the function of one gene product is unknown (ADE9). Strains with mutations in two additional genes, BAS1 and BAS2, have a partial adenine requirement (2). In these latter mutants, expression of the biosynthetic enzymes is low, indicating a positive regulatory role for these gene products (3, 4).

Expression of the adenine biosynthetic genes is repressed when cells are grown in the presence of adenine (3–6). This adenine-mediated repression occurs at the transcriptional level (7). ADE gene transcription is unregulated in bas1 or bas2 mutant strains (3). The expression of BAS1 and BAS2 is not regulated by adenine levels (8, 9), however, indicating that adenine repression occurs by down-regulating the activator functions of the BAS1 or BAS2 proteins.

BAS1 and BAS2 were identified as transcriptional activator proteins required for the basal expression of the HIS4 gene (2). BAS1 binds to DNA using an amino-terminal myb motif (8). This tryptophan-rich motif is repeated three times in the BAS1 protein (8, 10). BAS1 binds to two sites in the HIS4 promoter (8) and to two sites in each of the promoters for the ADE2 and ADE5,7 genes (3). The consensus sequence derived from these six BAS1 binding sites is TGACTC. Interestingly, this hexanucleotide sequence forms the core of the binding site for GCN4 protein as well, although flanking nucleotides differently affect the binding affinity of these two proteins (10, 11). Whereas GCN4 has a preference for the sequence RRTGACT-CATTT (R represents A or G; Ref. 11), none of the known BAS1 sites or any predicted sites in other ADE gene promoters have either an A nucleotide at the 3' base following the conserved hexanucleotide core or any other sequence conservation.

BAS2 binds to DNA via an amino-terminal homeodomain that is closely related to the engrailed protein of Drosophila (8). The BAS2 binding site at HIS4 has been mapped to an A + T-rich repeat, TTAA (8). The results of electrophoretic mobility shift assays suggested that BAS2 binds to the ADE2 and ADE5,7 promoters; however, no specific binding sites were identified (3). In addition to participating with BAS1 in transcriptional activation of HIS4 and the ADE genes, BAS2 (also known as PHO2 and GRF10) stimulates transcription of PHO5 (12) and HO (13). High level transcription of HO requires BAS2 in conjunction with SWI5 (13, 14). Binding of BAS2 and SW15 to the HO promoter is cooperative in vitro (13, 15). BAS2 also interacts with PHO4 to induce PHO5 transcription under phosphate starvation conditions (16). Binding of PHO4 is BAS2-dependent and restricted to derepressing conditions as shown by in vivo footprinting experiments (17). There is evidence that BAS2 and PHO4 physically interact at the promoter and that this interaction is regulated by phosphate (18).

Because expression of the genes that are activated by BAS1 and BAS2 is lower when cells are grown in adenine excess, adenine antagonizes the activation function of one or both of these proteins. Excess adenine could inhibit their ability to interact with one another, to bind to DNA, or to interact with components of the transcriptional machinery. Down-regulation of BAS protein function could also occur by covalent modification or through binding of a negative regulator, analogous to binding of the repressor GAL80 to the activator GAL4 (12). Alternatively, a DNA binding repressor might interact with negative control sites in the promoter, as described for the MIG1-TUP1-SSN6 complex that mediates the carbon catabo-

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Adenine Regulation by BAS1, BAS2, and ABF1 at the UAS<sub>ADE5,7</sub>

Table I

Oligonucleotides

Sequences of the oligonucleotides are listed from their 5′ end. Comments indicate what the oligonucleotide was used for. Underlined positions indicate restriction enzyme sites used for cloning, as indicated in the text. seq., sequencing primer; mut., mutant.

| Name   | Comments     | Sequence                                                                 |
|--------|--------------|--------------------------------------------------------------------------|
| RO-26  | CYC1 seq.    | 5′-GCCATATGATCATGATGC-3′                                                  |
| RO-27  | End −271     | 5′-CGTCCCGGTGAGTGGAGAAGC-3′                                               |
| RO-28  | −133         | 5′-CGTCTCGAGGTTCAAGGCCCATGC-3′                                            |
| RO-29  | −233         | 5′-CGTCTCGAGCATTTTTTTTTCTAGGTCAC-3′                                     |
| RO-30  | −180         | 5′-CGTCTCGAGGCTGTTATACAGGAGACG-3′                                        |
| RO-31  | Distal site  | 5′-CGTCTCGAGCATTTTTTTTTCTAGGTAATGC-3′                                    |
| RO-36  | Proximal site| 5′-CGTCTCGAGGCTTTATACAGGACG-3′                                           |
| RO-39  | −211         | 5′-CGTCTCGAGGGCAGGTGGATGTA-3′                                            |
| RO-40  | −183         | 5′-CGTCTCGAGGGGACCTGGGAC-3′                                              |
| RO-41  | −145         | 5′-CGTCTCGAGGACATAATCTTACGAC-3′                                          |
| RO-43  | −195         | 5′-CGTCTCGAGGACATTTTTTTCTAGGAAATGC-3′                                    |
| RO-53  | −211         | 5′-CGTAGATCTCCGCGGAGGCTGACG-3′                                           |
| RO-54  | Cluster 1    | 5′-CGTAGATCTCCGCGGAGGCTGACG-3′                                           |
| RO-55  | Cluster 2    | 5′-CGTAGATCTCGCAGGNNAGTTGAG-3′                                           |
| RO-56  | Cluster 3    | 5′-CGTAGATCTCGCAGGNNAGTTGAG-3′                                           |
| RO-57  | Cluster 4    | 5′-CGTAGATCTGGGNNAGTTGAG-3′                                              |
| RO-58  | Cluster 5    | 5′-CGTAGATCTGGGNNAGTTGAG-3′                                              |
| RO-59  | Cluster 6    | 5′-CGTAGATCTGGGNNAGTTGAG-3′                                              |
| RO-60  | Cluster 7    | 5′-CGTAGATCTGGGNNAGTTGAG-3′                                              |
| RO-61  | Cluster 8    | 5′-CGTAGATCTGGGNNAGTTGAG-3′                                              |
| RO-62  | Cluster 9    | 5′-CGTAGATCTGGGNNAGTTGAG-3′                                              |
| RO-63  | Cluster 10   | 5′-CGTAGATCTGGGNNAGTTGAG-3′                                              |
| RO-64  | Cluster 11   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-65  | Cluster 12   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-66  | Cluster 13   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-67  | Cluster 14   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-68  | Cluster 15   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-69  | Cluster 16   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-70  | Cluster 17   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-71  | Cluster 18   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-72  | Cluster 19   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-73  | Cluster 20   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-74  | Cluster 21   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-75  | Cluster 22   | 5′-CGTCTCGAGCAGATTTTTTTCTAGGAAATGC-3′                                   |
| RO-97  | Linker       | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-98  | Linker       | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-103 | BAS2         | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-104 | BAS2         | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-107 | BAS1         | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-108 | BAS1         | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-118 | ARS1         | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-119 | ARS1         | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-120 | ARS1 mut.    | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-121 | ARS1 mut.    | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| RO-126 | Vector       | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| FZP20  | III and IV   | 5′-GATCTGCTCGTTCCATGAG-3′                                                |
| FZP21  | III and IV   | 5′-GATCTGCTCGTTCCATGAG-3′                                                |

The abbreviations used are: UAS, upstream activation sequence; GST, glutathione S-transferase; bp, base pair; kb, kilobase pair; His<sub>6</sub>-, a six-histidine tag; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; SD, synthetic dextrase.

To define more clearly the cis- and trans-acting regulatory elements required for adenine regulation, we performed an extensive analysis of the ADE5,7 promoter. First, we identified the minimal sequences from the UAS<sub>ADE5,7</sub> sufficient to confer adenine-regulated activation (UAS<sub>ADE5,7</sub><sup>1</sup>) function to a heterologous CYC1-lacZ reporter lacking its native UAS<sub>CYC1</sub>. We then subjected the minimal UAS<sub>ADE5,7</sub> to extensive mutagenesis by making successive 3-nucleotide substitutions across this element to identify precisely the critical nucleotides contained therein. Our results indicate that of the two BAS1 binding sites at UAS<sub>ADE5,7</sub>, the gene-proximal copy is the more critical, being both necessary and sufficient for adenine-regulated promoter activity. Two additional regions were essential for UAS<sub>ADE5,7</sub> function. One region, located 5′ to the critical BAS1 site, is a binding site for ABF1. The other, located 3′ to the critical BAS1 site, is an extended A + T-rich element that appears to be a Bas2 binding site. We found that none of the nucleotide substitutions in the UAS<sub>ADE5,7</sub> led to constitutively derepressed expression, the phenotype expected from the loss of a repressor binding site. Thus, our results suggest that the repression of UAS<sub>ADE5,7</sub> function in adenine-replete cells involves a modification of one of the three transcriptional activator proteins, BAS1, BAS2, or ABF1, rather than binding of a repressor.

Experimental Procedures

Strains and Media—Saccharomyces cerevisiae strains AY854 (a GCN4 BAS1 BAS2 ura3–52), AY856 (a GCN4 bas1–2 BAS2 ura3–52), AY858 (a GCN4 BAS1 bas2–2 ura3–52), AY860 (a GCN4 bas1–2 bas2–2 ura3–52), AY967 (a gcn4 BAS1 BAS2 ura3–52), and AY962 (a gcn4 bas1–2 bas2–2 ura3–52) were provided by Kim Arndt (Cold Spring Harbor Laboratories). ElectroMax Escherichia coli strain DH10B (Life Technologies, Inc.) was used for transformation (19).

E. coli cells were grown in LB medium supplemented with 100 µg/ml ampicillin. S. cerevisiae cells were grown in synthetic dextrose (SD) medium (20) supplemented with 0.5 mM arginine and 0.5 mM histidine. Adenine was added to 0.15 mM in the cultures grown under repressing conditions.

Oligonucleotides and Plasmids—Table I lists the oligonucleotides that were used as polymerase chain reaction (PCR) and sequencing primers for cloning and as probes for DNA binding assays as described.
below. Deletion of an XhoI fragment that contained the UASocy, from plasmid pLG6992 (21) generated pLG6992ΔXhoI. Plasmid pCM81, a derivative of pLG6992ΔXhoI, contains an oligonucleotide that replaces the unique XhoI site with adjacent BglII and XhoI sites (22): Fragments of the ade5,7 promoter were generated by the PCR using plasmid pYEADES5,7 (5.5 kb) and the primers described in Table I, placed in the relevant figure and table legends. PCR reactions were performed using Taq polymerase (Perkin-Elmer) under the following conditions: 30 cycles of denaturing at 95 °C for 2 min, annealing at 55 °C for 1 min, and chain extension at 72 °C for 30 s. PCR fragments were digested with either XhoI or XhoI and BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and inserted into XhoI digested with BglII, separated from primers by electrophoresis, purified, and 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Adenine Regulation by BAS1, BAS2, and ABF1 at the UAS\textsubscript{ADE5,7}

Yeast strains were transformed with plasmid pR116 containing an ADE5,7-lacZ fusion on a YEB3 CEN plasmid and were grown in SD medium with and without adenine (see “Experimental Procedures”). The bas1-2 and bas2-2 deletion alleles have been described (2). Extracts prepared from each transformant were assayed for β-galactosidase activity by determining the smallest DNA fragment from adenine repression or whether repression involves a DNA binding site. However, whether repression is completely inhibited in adenine-replete cells, it was not known.

Identification of a Minimal ADE5,7 Promoter Fragment with UAS Function—The results in Table II suggest that the ability of BAS1 and BAS2 to activate ADE5,7 transcription is completely inhibited in adenine-replete cells. It was not known, however, whether ADE5,7 promoter activity is dependent on any additional positive regulators that might be subject to adenine repression or whether repression involves a DNA binding repressor. To address these issues, we set out to identify the cis-acting sequences required for adenine-regulated promoter activity by determining the smallest DNA fragment from ADE5,7 sufficient to confer adenine-repressible UAS function on a CYC1-lacZ reporter (21). Various fragments of the ADE5,7 promoter were generated by PCR and inserted into pUC18-lacZ upstream of the CYC1 transcription initiation sites where the UAS\textsubscript{CYC} normally resides (Fig. 1). These constructs were introduced into four different yeast strains carrying wild-type GCN4, BAS1, and BAS2, and β-galactosidase expression was assayed after growing transformants in minimal medium containing or lacking adenine. As expected, expression of β-galactosidase in transformants of each strain bearing the parental CYC1-lacZ construct lacking UAS\textsubscript{CYC} was very low (~10 units) and essentially unaffected by adenine supplementation or mutations in any of the regulatory genes (Table III, line 19). The ADE5,7 promoter fragments exhibited a wide range of expression from the CYC1 promoter in the wild-type strain, and in many cases promoter activity was repressed by adenine in the medium. As seen with the authentic ADE5,7 promoter (Table II), expression depended upon the wild-type alleles of BAS1 and BAS2 but not upon GCN4 (Table III). In addition to these adenine-regulated constructs, promoter fragments with 3' end points at positions 1 - 183 (Table III, lines 3 and 7) or at -160 (Table III, lines 2 and 10) exhibited significant activity that was independent of GCN4, BAS1, and BAS2 and was largely unaffected by adenine in the medium.

Fig. 1 presents the β-galactosidase activities of the constructs analyzed in Table III after subtracting the promoter activity that was independent of BAS1, BAS2, and GCN4 (see Fig. 1 legend). The constructs containing both the distal and proximal BAS1 binding sites (3) exhibited the highest expression under derepressing conditions (Fig. 1A, lines 1, 2, 4, 5, and 6). In addition, each of these constructs showed substantial repression by adenine, with repression ratios ranging from 8.4 to 44. One notable difference among this group of constructs was observed in the comparison between pR136 and pR148, where removal of the 12 nucleotides between -145 and -133 led to a roughly 5-fold increase in expression under both growth conditions (compare lines 4 and 5). We attribute this difference to the removal of a negative element that is either located between positions -133 and -145 or was formed by the novel junction between position -133 in the ADE5,7 fragment and the CYC1 promoter sequences. Because the magnitude of adenine repression was not diminished by removal of the -133 to -145 interval, no additional studies were carried out on these sequences. Truncating the ADE5,7 fragment from position -145 to -160 (compare lines 5 and 6) led to a decrease in UAS\textsubscript{ADE5,7} function by roughly a factor of 2, suggesting that a positive element resides within this interval. As shown below, the -145 to -160 interval contains a binding site for BAS2.

The two constructs lacking the gene-proximal BAS1 site but containing the distal site (Fig. 1A, lines 3 and 7) showed very low unregulated activity, suggesting that the proximal BAS1 site is critical for UAS\textsubscript{ADE5,7} function. In accord with this idea, three constructs containing the proximal site but lacking the distal BAS1 site (lines 8–10) showed substantial promoter activity that was adenine-repressible. As a group, these last three constructs showed weaker UAS\textsubscript{ADE5,7} function than the constructs containing both BAS1 sites, suggesting that the presence of two BAS1 sites confers greater UAS\textsubscript{ADE5,7} function than occurs with the proximal site alone. As above, removal of positions -160 to -145 from pR145 decreased UAS\textsubscript{ADE5,7} function, consistent with the presence of a positive element in this region.

The results obtained with constructs pR151, pR150, and pR149 (lines 11–13) revealed that the region adjacent to the proximal BAS1 site is not sufficient for high level UAS\textsubscript{ADE5,7} function. The very low expression shown by these constructs suggested that a critical positive element resides between positions -211 and -195. Although they exhibited very low promoter activity under derepressing conditions, it appears that all three constructs are adenine-repressible.

To confirm the different contributions of the two BAS1 binding sites to UAS\textsubscript{ADE5,7} function, we introduced point mutations into each BAS1 site in construct pR134, containing the ADE5,7 promoter fragment from position -233 to -160. A mutation that alters BAS1 binding site from TGAGTC to TGAATTC was introduced into one or both of the BAS1 sites in pR134. An equivalent substitution was shown to impair in vitro binding of BAS1 to its sites in the ADE2 promoter (3). The mutation in the distal BAS1 binding site reduced expression by a factor of about 4 but did not completely eliminate adenine regulation (Fig. 1B, pR137). In contrast, a mutation in the proximal site virtually eliminated both promoter activity and adenine regulation (pR138). The doubly mutated construct pR140 also showed very low expression, similar to pR138. Comparable results were obtained when these point mutations were introduced singly into pR136, which contains the ADE5,7 fragment extending from position -133 to -233 (Fig. 1B, lines 5–7). These findings agree with the results of the deletion analysis in Fig. 1A in showing that the two BAS1 sites are not equivalent; the proximal site is essential for promoter activity and adenine repression, whereas the distal site only augments UAS\textsubscript{ADE5,7} function.

Identification of Multiple Positive Regulatory Elements in UAS\textsubscript{ADE5,7}—Given the absence in the literature of defined...
Adenine Regulation by BAS1, BAS2, and ABF1 at the UAS\textsubscript{ADE5,7} binding sites for BAS2 at \textit{ADE5,7} and \textit{ADE2} and our identification of a positive element located between the two BAS1 binding sites, we decided to map all the DNA elements in the UAS\textsubscript{ADE5,7} critical for promoter function and adenine regulation by saturation mutagenesis. To accomplish this goal, we introduced random substitutions into the 22 successive groups of three nucleotides that span the 67-nucleotide \textit{ADE5,7} fragment in pR145. This construct was chosen because it contains the smallest fragment that conferred high level promoter activation in pR145. This construct was chosen because it contains the smallest fragment that conferred high level promoter activity that is not attributed to the three transcriptional activators GCN4, BAS1, or BAS2 by subtracting the activity found in strain AY862 (Δ\textit{ADE5,7}) from the wild-type construct. It can be seen that mutations in three of the four regions severely impaired expression under derepressing conditions (boxed sequences I–III), whereas mutations in the fourth region decreased expression by only about 40–50%. Mutations outside of these four regions had little (<25%) or no effect on expression. Region I is defined by mutations in clusters numbered 2–6, spanning positions 208 to 219 (Fig. 2), that lowered expression under derepressing conditions from 120 units to 6–16 units. These were the only clustered substitutions mutations that also substantially decreased expression under repressing conditions, lowering it from 10 units to 3–5 units (Table IV, lines 3–19). Thus, most of the mutations in region I reduced the magnitude of adenine repression but did not abolish it, decreasing the repression ratio from ~7 (measured for the parental construct) to values of 2–4-fold (Table IV). Region I is coincident with the interval between –211 and –195 whose deletion led to a dramatic reduction in UAS\textsubscript{ADE5,7} function. Thus, the results of deletion and substitution mutations agree in suggesting that region I stimulates UAS\textsubscript{ADE5,7} function by roughly an order of magnitude, without being absolutely required for regulation by adenine.

The sequence in region I conforms exactly to the consensus binding site for ABF1 (29) (5’-CGTNNNNNRYGAY-3’ in which R represents any purine, Y represents any pyrimidine, and N represents any nucleotide (30), and the data in Table IV are consistent with the idea that region I functions as an ABF1 binding site. Mutations in clusters 2–3 and 5–6 alter the conserved positions in this bipartite consensus sequence and have a large effect on expression, whereas mutations in cluster 4 alter the nonconserved positions in the consensus sequence (NNR) and have a relatively weak effect on promoter activity (Table IV). Region II is defined by mutations in clusters 10–13, spanning positions –184 to –173 (Fig. 2), of which the most severe decreased promoter function under derepressing conditions by

### Fig. 1. Deletion and substitution mutations defining the UAS\textsubscript{ADE5,7}

The top section shows a schematic of the 139-nucleotide fragment from the \textit{ADE5,7} promoter with nucleotide positions numbered relative to the initiation codon. Hatched and solid boxes represent the distal and proximal BAS1 binding sites, respectively, that contain the core sequence TGACTC (3). The distal site is located between –217 and –212, and the proximal site is located between –184 and –179. A, constructs used in the deletion analysis. PCR fragments with 5’ end points at positions –271, –233, –211, or –195 and 3’ end points at –183, –160, –145, or –133 were generated using oligonucleotides listed in Table I and are indicated by the lines shown below the schematic. These fragments were inserted in place of UAS\textsubscript{Cyc7} in plasmid pLG669Z\textsubscript{XhoI}, and the resulting constructs were introduced into yeast strain AY854 (wild type). β-Galactosidase activities in whole cell extracts were determined after growing the transformants under derepressing (–Ade) or repressing conditions (+Ade) and are listed in Table III. These results have been corrected for UAS activity that is not attributed to the three transcriptional activators GCN4, BAS1, or BAS2 by subtracting the activity found in strain AY862 (\textit{gcn4 bas1 bas2}) and are shown to the right of each construct. In cases where the corrected value was a negative number, it is enclosed by parentheses. NA, not applicable. B, constructs used to analyze mutations in the BAS1 binding site. A point mutation from TGACTC to TGAATTC in the core binding site. Mutations in clusters 10–13, spanning positions –184 to –173 (Fig. 2), of which the most severe decreased promoter function under derepressing conditions
Table III

Analysis of UAS_{ADE5,7} function conferred on a CYC1-lacZ fusion by different fragments from the ADE5,7 promoter

Plasmids containing either no insert (line 19, vector pLG6992\AA hol) or containing various fragments from the ADE5,7 promoter (lines 1–18, plasmids pR133 to pR152) were introduced into strains AY54 (GCN4 BAS1 BAS2), AY860 (GCN4 bas1–2 bas2–2), and AY962 (gen4 bas1–2 bas2–2), and the transformants were assayed for β-galactosidase activities after growth under repressing (+ Ade) and derepressing (− Ade) conditions, as described in Table II. Values shown are averages of the results obtained from 2–4 cultures assayed in triplicate, and the S.D. values are less than 30%. The nucleotide positions of the ends of the ADE5,7 fragments that are inserted at the XhoI site of pLG6992\AA hol are listed in the column labeled “ADE5,7 fragment.”

| Plasmid | ADE5,7 fragment | GCN4 BAS1 BAS2 | GCN4 bas1 bas2 | gen4 BAS1 BAS2 | gen4 bas1 bas2 |
|---------|-----------------|----------------|----------------|----------------|----------------|
|         |                 | − Ade + Ade    | − Ade + Ade    | − Ade + Ade    | − Ade + Ade    |
| 1. pR133 | −271 to −133    | 410 25         | 8.9 14         | 200 24         | 8.1 16         |
| 2. pR135 | −271 to −160    | 560 110        | 47 62          | 390 61         | 30 47          |
| 3. pR142 | −271 to −183    | 110 110        | 140 150        | 100 100        | 94 92          |
| 4. pR136 | −233 to −133    | 290 15         | 8.9 12         | 240 13         | 6.3 7.4        |
| 5. pR148 | −233 to −145    | 990 60         | 30 35          | 670 42         | 21 18          |
| 6. pR134 | −233 to −160    | 440 69         | 43 54          | 220 47         | 26 28          |
| 7. pR143 | −233 to −183    | 96 100         | 120 130        | 100 98         | 88 85          |
| 8. pR146 | −211 to −133    | 42 8.5         | 7.5 9.2        | 28 5.8         | 5.0 5.8        |
| 9. pR145 | −211 to −145    | 200 27         | 24 26          | 160 24         | 20 21          |
| 10. pR144 | −211 to −160    | 94 48          | 46 54          | 80 40          | 34 38          |
| 11. pR151 | −195 to −133    | 4.3 1.8        | 2.3 2.6        | 1.4 1.6        | 1.8 1.9        |
| 12. pR150 | −195 to −145    | 19 5.4         | 7.6 6.4        | 16 5.0         | 5.9 6.7        |
| 13. pR149 | −195 to −160    | 13 6.0         | 10 8.5         | 9.2 4.7        | 6.5 6.1        |
| 14. pR137 | −233 to −160    | 110 49         | 45 56          | 84 38          | 22 21          |
| 15. pR138 | −233 to −160    | 35 35          | 43 51          | 32 31          | 26 25          |
| 16. pR140 | −233 to −160    | 27 31          | 37 39          | 29 30          | 24 23          |
| 17. pR139 | −233 to −133    | 64 11          | 7.4 7.4        | 45 8.6         | <4 4<        |
| 18. pR152 | −233 to −133    | 5.4 6.1        | 10 10          | 5.7 5.6        | 7.7 8.7        |
| 19. vector none | 10 10 | 14 14 | 5.3 5.9 | 9.7 11 |

about 10-fold. There was little change from the wild-type level under repressing conditions (Table IV, lines 26–36). A complete loss of adenine regulation distinguishes the mutations in this region from mutations in the other three regions. The first two clusters in region II, TGA and CTC, alter the core sequence of the proximal BAS1 binding site described previously at ADE5,7 (3) and identified above as being critical for UAS_{ADE5,7} function. As expected, all six substitutions in these two clusters essentially abolished expression and adenine repression. Mutations affecting the next two clusters in region II were also very deleterious, including the substitution of TCA for GTG at cluster 12 and GGA for TCC at cluster 13 (Fig. 2 and lines 34 and 35 in Table IV). We propose that all of the mutations in the 12-nucleotide sequence comprising region II impair UAS_{ADE5,7} function by reducing BAS1 binding to the ADE5,7 promoter.

Region III consists of clusters 15 and 16, spanning −169 to −164 (Fig. 2). The most severe mutations in this region decreased expression from 120 units to 13–40 units under derepressing conditions but had little or no effect under repressing conditions (Table IV, lines 39–40, 42, and 43). The sequence and location of this region, TAATAA, resembles the binding site described for BAS2 at HIS4 (8). The sequence of region IV comprising clusters 18–20, CAATAATGA, is related to that of region III except that two of the three TAA repeats are degenerate. Mutations in region IV have the weakest effects, decreasing expression under derepressing conditions by 50% or less (Table IV, lines 47–52). On the basis of their sequence and juxtaposition 3′ to the BAS1 site, we conclude provisionally that regions III and IV function as strong and weak binding sites, respectively, for BAS2.

None of the clustered substitutions in the UAS_{ADE5,7} led to high, unregulated promoter activity. The greatest level of expression under adenine-repressing conditions was observed for mutations in clusters 19 and 21 (Table IV, lines 50–51 and 53–56). The −2-fold increase above the repressed values elicited by these mutations clearly does not account for the 6.7-fold repression ratio seen with the parental construct. This result is noteworthy because it suggests that adenine repression does not involve binding of a repressor protein to an upstream repression sequence. If such a repressor existed, its binding site would have to coincide with one of the four positive control sites we identified in UAS_{ADE5,7} where, as shown below, ABF1, BAS1, or BAS2 binds. Given that only mutations in the BAS1 site completely abolished adenine regulation, a hypothetical repressor would probably have to compete with BAS1 for binding to its site in region II of UAS_{ADE5,7}.

Region 1 in UAS_{ADE5,7} Functions as a Binding Site for ABF1—Results from the mutational studies described above suggest that region I of UAS_{ADE5,7} functions as a binding site for ABF1. ABF1 is an abundant, essential yeast protein first shown to bind DNA at autonomous replicating sequences (ARSs) involved in replication (31). Later, ABF1 was shown to interact with telomeres, the silent mating cassettes, and the promoters of various metabolic genes (29, 30, 32). To demonstrate that region I is a binding site for ABF1, we performed EMSAs to detect DNA binding. Whole cell extracts containing ABF1 were prepared from a gen4 bas1 bas2 yeast strain (AT862) and incubated with radioactively labeled oligonucleotide probes containing the ABF1 binding site from ARS1 or UAS_{ADE5,7}. As shown in Fig. 3A, the extract contains a binding activity for both the ARS1 (lane 2) and ADE5,7 (lane 6) oligonucleotides. The addition of a 100-fold molar excess of unlabeled ARS1 oligonucleotides eliminated binding to the labeled ARS1 probe (lane 3) and to a large extent in the case of the ADE5,7 probe (lane 7). A 200-fold molar excess of unlabeled ARS1 oligonucleotide abolished binding to the ADE5,7 probe (data not shown). The tighter binding to ADE5,7 is consistent with the observation that the ABF1 binding site in the ADE5,7 fragment has greater homology to the derived consensus sequence for ABF1 binding than does the ARS1 sequence. The experiment was repeated using oligonucleotides containing a double point mutation in the ARS1 sequence (33) as the competitor DNA. This mutant oligonucleotide failed to compete with either of the labeled probes for protein binding (Fig. 3,
Adenine Regulation by BAS1, BAS2, and ABF1 at the UAS\textsubscript{ADE5,7}

Table IV
Effects of clustered substitutions on UAS\textsubscript{ADE5,7} function

Strain AY854 (GCN4, BAS1, and BAS2) was transformed with each of the constructs listed and assayed for β-galactosidase as described for Table II. Expression from each construct was assayed in duplicate on at least two independent transformants, and the values shown have standard errors less than 30%.

### TABLE IV
Effects of clustered substitutions on UAS\textsubscript{ADE5,7} function

| Plasmid number | Cluster number | Wild-type sequence | Mutant sequence | Number of changes | β-Galactosidase activity | Derepression ratio |
|----------------|----------------|--------------------|----------------|------------------|-------------------------|-------------------|
|                |                |                    | NA             | NA               |                         |                   |
| Empty vector   | NA             | NA                 | NA             | 6.4              | 6.0                     | 1.1               |
| Wild type      | NA             | NA                 | NA             | 120              | 18                      | 6.7               |
| 1. 134-1       | 1              | GCC                | GAG            | 3                | 79                       | 4.9               |
| 2. 134-2       |                |                    |                | 82               | 15                       | 5.5               |
| 3. 135-1       | 2              | CCG                | AAG            | 2                | 7.4                      | 2.3               |
| 4. 28-10       |                |                    |                | 7.0              | 3.2                      | 2.2               |
| 5. 135-5       |                |                    |                | 1                | 16                       | 4.9               |
| 6. 135-2       |                |                    |                | 2                | 6.3                      | 3.3               |
| 7. 29-1        | 3              | TCG                | GTG            | 2                | 8.6                      | 3.1               |
| 8. 29-7        |                |                    | AGG            | 2                | 9.8                      | 3.1               |
| 9. 136-2       |                |                    | GAT            | 3                | 5.9                      | 2.8               |
| 10. 137-1      | 4              | GTA                | TGG            | 3                | 60                       | 4.5               |
| 11. 137-2      |                |                    | GCG            | 2                | 20                       | 2.6               |
| 12. 138-1      | 5              | GTG                | AAA            | 3                | 18                       | 3.4               |
| 13. 138-4      |                |                    | ACC            | 3                | 14                       | 2.8               |
| 14. 31-2       |                |                    | CCG            | 2                | 9.6                      | 3.0               |
| 15. 138-3      |                |                    | GAG            | 2                | 74                       | 3.7               |
| 16. 138-5      |                |                    | TCA            | 3                | 12                       | 4.2               |
| 17. 139-1      | 6              | ACA                | AGG            | 3                | 14                       | 4.2               |
| 18. 139-3      |                |                    | AAC            | 2                | 11                       | 3.6               |
| 19. 139-4      |                |                    | TCT            | 2                | 14                       | 4.4               |
| 20. 140-2      | 7              | AGT                | GAC            | 3                | 120                      | 20                |
| 21. 141-1      |                |                    | GCC            | 2                | 120                      | 18                |
| 22. 141-4      |                |                    | AAA            | 3                | 120                      | 14                |
| 23. 142-2      | 9              | GAC                | CTA            | 3                | 40                       | 12                |
| 24. 142-3      |                |                    | GGT            | 2                | 240                      | 24                |
| 25. 142-1      |                |                    | GCC            | 3                | 210                      | 32                |
| 26. 143-3      | 10             | TGA                | AAG            | 3                | 7.8                      | 6.4               |
| 27. 143-2      |                |                    | GGA            | 1                | 16                       | 14                |
| 28. 143-4      |                |                    | CCG            | 2                | 14                       | 13                |
| 29. 144-2      |                |                    | CTC            | 2                | 7.7                      | 7.3               |
| 30. 144-1a     |                |                    | TAT            | 3                | 6.5                      | 7.0               |
| 31. 144-1c     |                |                    | TTG            | 2                | 10                       | 10                |
| 32. 145-1      | 12             | GTG                | ACC            | 3                | 37                       | 37                |
| 33. 145-2      |                |                    | TCC            | 2                | 48                       | 14                |
| 34. 145-3      |                |                    | TCA            | 3                | 7.8                      | 6.7               |
| 35. 146-2      | 13             | TCC                | GGA            | 3                | 12                       | 11                |
| 36. 146-1      | 4              | GCC                | CCG            | 1                | 120                      | 16                |
| 37. 147-1      | 14             | TGG                | GTC            | 3                | 78                       | 15                |
| 38. 147-3      |                |                    | GTC            | 3                | 120                      | 16                |
| 39. 148-1      | 15             | TAA                | ATC            | 3                | 25                       | 13                |
| 40. 148-3      |                |                    | TCA            | 1                | 35                       | 19                |
| 41. 148-2b     |                |                    | TTA            | 1                | 59                       | 13                |
| 42. 148-5      |                |                    | ATG            | 3                | 40                       | 18                |
| 43. 149-2      | 16             | TAA                | CCA            | 2                | 13                       | 6.5               |
| 44. 150-1      | 17             | CAG                | CTA            | 2                | 84                       | 11                |
| 45. 150-2      |                |                    | AAT            | 2                | 130                      | 17                |
| 46. 150-4      |                |                    | ACA            | 3                | 120                      | 24                |
| 47. 151-1      | 18             | CAA                | GAC            | 2                | 79                       | 16                |
| 48. 151-2      |                |                    | CTC            | 2                | 94                       | 17                |
| 49. 151-3      |                |                    | TCA            | 2                | 52                       | 11                |
| 50. 152-1      | 19             | TAA                | CCG            | 3                | 64                       | 33                |
| 51. 152-2      |                |                    | GAG            | 2                | 100                      | 42                |
| 52. 153-2      | 20             | TGA                | CCA            | 2                | 61                       | 17                |
| 53. 155-2      | 21             | ATA                | CTT            | 2                | 150                      | 32                |
| 54. 155-3      |                |                    | ACC            | 2                | 100                      | 24                |
| 55. 155-12     |                |                    | TTG            | 2                | 110                      | 32                |
| 56. 155-14     |                |                    | TGA            | 2                | 130                      | 30                |
| 57. 154-1      | 22             | TGCC               | ACAC           | 4                | 120                      | 17                |
| 58. 154-3      |                |                    | ACGA           | 4                | 220                      | 21                |
| 59. 154-4      |                |                    | CAAC           | 4                | 81                       | 12                |

lanes 4 and 8). These results strongly suggest that ABF1 can bind in vitro to ADE5,7 DNA.

To demonstrate that the bound protein is ABF1, antibodies against ABF1 (28) were added to the binding reactions containing yeast lysate and labeled ADE5,7 or ARS1 oligonucleotides. The addition of ABF1-antibody, but not preimmune serum,
produced a supershift of the DNA-protein complexes, whether the labeled probe contained ADE5,7 sequences (Fig. 3B, lanes 3–5) or ARS1 sequences (data not shown). These results prove that ABF1 binds to the ADE5,7 fragment under these in vitro conditions.

To demonstrate that the mutations in the presumed ABF1 binding site in the UASADE5,7 that impair promoter activation in vivo also reduce ABF1 binding in vitro, we performed EMSA using as probes either the wild-type UASADE5,7 fragment or mutant fragments bearing selected clustered substitutions described in Table IV. Fig. 3C shows that ABF1 binds with nearly equal efficiency to the wild-type ADE5,7 fragment (lanes 1 and 9), to a fragment bearing mutations with no effect on in vivo UASADE5,7 function (lane 5), and to fragments containing mutations that impair UASADE5,7 but lie outside of the presumed ABF1 binding site (lanes 6–8 and 14–16). In contrast, ABF1 did not bind to fragments containing mutations in the conserved positions of the consensus ABF1 binding site in UASADE5,7 (lanes 2, 4, 10, 12, and 13). Moreover, mutations that alter the central nonconserved positions of the consensus binding site had little or no affect on ABF1 binding (lanes 3 and 11), consistent with the known sequence requirements for ABF1 binding. We performed complementary experiments in which mutant and wild-type unlabeled fragments were compared for their ability to compete with labeled wild type ADE5,7 probe for binding of ABF1 (data not shown). These results established a perfect agreement between the effects of mutations in the ABF1 consensus binding site on ABF1 binding in vitro and UASADE5,7 function in vivo. We conclude that ABF1 binds to region I in vivo and is responsible for the positive role of this sequence in the UASADE5,7.

Region II in UASADE5,7 Functions as a 12-Base Pair Binding Site for BAS1—We proposed that mutations in region II, including the six nucleotides located 3’ to the TGACTC core, which are not conserved among different BAS1 binding sites, destroy UASADE5,7 function by interfering with binding of BAS1. To verify this, we investigated whether mutations in all four of the triplets composing region II interfere with BAS1 binding to the ADE5,7 fragment in vitro. We purified a GST-BAS1 fusion protein expressed in E. coli and used it in EMSA experiments with wild-type or mutant ADE5,7 probes. As shown in Fig. 4A, GST-BAS1 bound to the ADE5,7 fragment (lane 3), whereas GST alone did not (lane 2). Mutations in clusters 10–13 reduced or eliminated the ability of GST-BAS1 to bind to the ADE5,7 DNA (Fig. 4B, lanes 5–8), whereas mutations outside of region II had no effect on binding (lanes 2–4, 5, and 10). Mutations in the TGGTCC core sequence (lanes 5 and 6) and in the adjacent hexanucleotide sequence GTGTCC (lanes 7 and 8) reduced or abolished binding of GST-BAS1 to the ADE5,7 probe. Three additional mutations in the GTGTCC
sequence (constructs 26, 30, and 32 in Table IV) showed strongly diminished or no in vitro binding of GST-BAS1 (data not shown).

To confirm these results, we compared the ability of unlabeled mutant fragments present in 200-fold molar excess to compete with the labeled wild-type fragment for binding to GST-BAS1. These results, shown in Fig. 4C, completely mirrored those presented in Fig. 4B, in that only the mutations in clusters 10–13 of region II diminished the ability to compete with the wild-type ADE5,7 fragment for binding to GST-BAS1. Moreover, the mutations in the TGACTC core sequence (lanes 10 and 11) had a greater effect than did those in the adjacent hexanucleotide sequence (lanes 12 and 13). We conclude that region II comprises a 12-bp binding site for BAS1 and that all of the mutations in this region reduce UASADE5,7 function by decreasing BAS1 binding to the promoter.

Evidence That Regions III and IV in UASADE5,7 Function as BAS2 Binding Sites—Based on their A + T-rich sequence and location 3' to the BAS1 binding site, we suggested above that regions III and IV are binding sites for BAS2. To test this idea, we carried out EMSA experiments using a GST-BAS2 fusion protein expressed in E. coli to determine whether mutations in clusters 15 or 16 reduced binding of BAS2 to the ADE5,7 fragment. GST-BAS2 bound specifically to the wild-type ADE5,7 fragment (Fig. 4A, lane 4), whereas mutations in clusters 15 or 16 of region III significantly reduced binding of this interaction (Fig. 5A, lanes 1, 5, and 6). In an effort to verify these results, we carried out EMSA experiments comparing wild-type and mutant competitors using a purified polyhistidine-tagged form of BAS2 expressed in E. coli (13, 15). We saw small reductions in the ability of fragments containing mutations in clusters 15 or 16 or clusters 18 and 19 relative to the wild-type ADE5,7 fragment to compete with the labeled wild-type probe for (His)6-BAS2 binding (data not shown). We then combined these mutations in the same fragment and compared it with the wild-type fragment for ability to compete. As shown in Fig. 5B, we found that significantly greater amounts of the fragment containing mutations in clusters 15–19 were required to achieve the same degree of competition for (His)6-BAS2 binding to ADE5,7 DNA. These results support the idea that sequences in regions III and IV function as binding sites for BAS2. The fact that mutations in clusters 15 and 16 have only modest effects on BAS2 binding in vitro but substantially reduce UASADE5,7 function in vivo suggests that the in vitro binding reactions do not duplicate some important aspect of the in vivo interaction of BAS2 with the ADE5,7 promoter.

DISCUSSION

Evidence That Binding Sites for BAS1, BAS2, and ABF1 Are the Critical Elements of the UASADE5,7—Our results define the minimum promoter elements required for adenine-regulated transcription of ADE5,7 and provide a detailed picture of the spatial relationships among the DNA-binding proteins that interact with these sequences. These results provide the first complete description of a UAS from an adenine biosynthetic gene. Wild-type promoter activity and adenine repression require (i) two binding sites for BAS1 that are separated in the promoter by about 20 nucleotides, (ii) a single binding site for ABF1 located immediately 3' to the distal BAS1 site, and (iii) two A + T-rich elements located immediately 3' to the proximal BAS1 site to which monomers of BAS2 appear to bind, all within a region of only ~65 bp (Fig. 6). A 52-bp fragment containing a single binding site for each protein was sufficient to confer adenine-repressible activation, UASADE5,7 function, on the CYC1 basal promoter.

The conclusion that regions I and II function in UASADE5,7 as binding sites for ABF1 and BAS1, respectively, is based on the strong correlation between the effects of mutations in these sequences on protein binding in vitro and on UASADE5,7 function in vivo. The results of clustered substitution mutagenesis of region II provides in vivo evidence that BAS1 recognizes between 4 and 6 bp 3' to the highly conserved TGACTC core element present in all known BAS1 sites. Similarly, Havring et al. (10) showed that BAS1 recognizes the G residues on both strands of the sequence CGG located immediately 3' to the TGACTC core at the BAS1 binding site in the HIS4 promoter. This particular sequence is not conserved in either of the BAS1 binding sites at ADE5,7 or ADE2. Based on our findings, we suggest that sequence differences in the 4–6 bp on the 3' side of the TGACTC core element lead to differences in BAS1 binding affinity.

Our proposal that regions III and IV function as BAS2 binding sites is reasonable given that BAS2 is required for ADE5,7 promoter activation and that these are the only cis-acting elements in addition to the BAS1 and ABF1 sites that are critical for UASADE5,7 function in vivo. The BAS2 binding sites have

**Fig. 4. In vitro binding of a GST-BAS1 fusion protein to UASADE5,7**—A, binding of GST fusion proteins to a radiolabeled ADE5,7 probe (described in Fig. 3) was analyzed by EMSA. For lane 1, no extract was added; for lanes 2–4, 0.25 µg of purified GST fusion protein was added. In lanes 2–4, GST (lane 2), GST-BAS1 (lane 3), or GST-BAS2 (lane 4) was added to wild-type ADE5,7 DNA. **B,** binding of GST-BAS1 to wild-type and mutant fragments from ADE5,7. Labeled oligonucleotides identical to the ADE5,7 probe used above (lane 1) or containing point mutations in the clusters indicated along the top were employed in EMSAs as described above for A. The mutant fragments were the same as used in Fig. 3. The mutations in clusters 3 and 5 (numbers 7 and 13 in Table IV) led to reduced UASADE5,7 function and lie outside of the BAS1 binding site but within the ABF1 binding site. The mutation in cluster 7 (number 20 in Table IV) had no effect on UASADE5,7 function and lies outside of the binding site for BAS1. The mutations in clusters 10–13 alter the proximal TGACTC and the adjacent six nucleotides to the 3' side (numbers 27, 29, 34, and 35 in Table IV). The mutations in clusters 15 and 16 (numbers 39 and 43 in Table IV) decrease UASADE5,7 function and lie outside of the BAS1 binding site. **C,** competition by unlabeled ADE5,7 fragments for binding of GST-BAS1. EMSAs were performed as for A above using radiolabeled ADE5,7 DNA, GST-BAS1, and various unlabeled DNAs as competitors. For lane 1, no competitor was added; for lanes 2–10 competitors as indicated along the top of the figure were added a 1 in 200-fold molar excess. The mutants were the same as described for B.
not been identified in any other ADE gene promoter; however, the high A + T content of the region III and IV sequences and their location 3' of the proximal BAS1 binding site are both reminiscent of the juxtaposed BAS1 and BAS2 binding sites at *HIS4* (8). The identification of the BAS2 binding sites at *ADE5,7* is not definitive because mutations in regions III and IV that greatly impaired activation in vivo had relatively small effects on DNA binding in vitro, and it was necessary to combine multiple substitutions in these sequences before achieving a significant reduction in BAS2 binding. One possible explanation for these results is that purified BAS2 protein may not bind to *ADE5,7* DNA with high sequence specificity. Perhaps specific binding of BAS2 to regions III and IV is stabilized *in vivo* by its interaction with BAS1 bound at the adjacent sequences at positions −184 to −173 (Fig. 6). As discussed below, we recently obtained strong evidence for complex formation by BAS1 and BAS2 (9). Although no cooperativity was detected between BAS1 and BAS2 in binding to the *HIS4* promoter (8), the BAS2 site defined at *HIS4* (repeats of TTAA; Ref. 8) differs from that proposed here for *ADE5,7* (TAATAA and CAATAA). It is possible that BAS2 has a greater affinity for the *HIS4* sequences and does not require cooperativity with BAS1 to bind there with high specificity.

We recently obtained evidence for interactions between BAS1 and BAS2 by showing that a LexA-BAS1 fusion protein can recruit BAS2 to a promoter containing *lexA* operator se-
quences (9). This interaction at the lexA operator occurred even when the DNA binding activity of BAS2 was destroyed by mutations in its homeodomain. These mutations impaired activation of ADE5,7 transcription, however, indicating that the DNA binding activity of BAS2 was required for its binding to the native ADE5,7 promoter. To explain these observations, we propose that BAS1 cannot bind to the ADE5,7 promoter as effectively as LexA-BAS1 binds to the lexA operator and that an interaction between BAS1 and BAS2 is required for these proteins to bind with high affinity to their adjacent sites at ADE5,7.

We propose that the BAS2 binding sites at ADE5,7 are relatively weak and that cooperativity with BAS1 is required for stable binding of BAS2 to regions III and IV. Similarly, stable binding of BAS1 at the ADE5,7 proximal site depends on cooperativity with BAS2. In contrast, both proteins can interact more effectively with their respective binding sites at HIS4, and cooperativity is not required for promoter occupancy.

Possible Functions for ABF1 and the Distal BAS1 Binding Site in the ADE5,7 Promoter—Our results showed that the two BAS1 binding sites in the ADE5,7 promoter are not equivalent. Mutations in the distal site led to an approximately 6-fold loss in expression but no obvious impairment of adenine regulation. In contrast, the basal BAS1 binding site was absolutely required for promoter activity and regulation. Given that BAS1 and BAS2 must interact to form a potent activation complex (9), we propose that the juxtaposition of BAS2 binding sites on the 3' side of the proximal BAS1 site allows formation of the functional BAS1-BAS2 complex. The fact that the distal BAS1 site is flanked by an ABF1 site rather than a BAS2 binding site may explain the failure of the latter to support UAS228 to 145 function without the proximal site. In addition to ADE5,7, the genes ADE2, ADE4, ADE1, and ADE8 have two TGACTC core sequences that are potential BAS1 binding sites, and it was shown that BAS1 binds in vitro to both sites at ADE2. The proximal TGACTC sequence is more important for activation at ADE2 (3, 4, 6), whereas the distal site is more critical at ADE4 (34). It will be interesting to learn whether the more potent sites at these genes are uniquely flanked by BAS2 binding sites, in the manner we suggest for ADE5,7.

At ADE5,7, the two BAS1 binding sites are separated by 33 nucleotides, which is a 3.1 helical turns. The two TGACTC core sequences in the other ADE genes are also separated by an integral number of helical turns (with the exception of the ADE2 gene, which has the same number of helical turns). McBroom and Sadowski (35) have shown that ABF1 is able to bend DNA 120° around itself. If this occurred at the ADE5,7 promoter, then binding of ABF1 could bring the distal and proximal BAS1 sites into close proximity. Perhaps the distal BAS1 site has a higher affinity for BAS1, and the bend in the DNA introduced by ABF1 allows BAS1 bound at the distal site to stabilize binding of the BAS1-BAS2 complex to the proximal site. Alternatively, the bend in the DNA might allow BAS1 bound at the distal site to cooperate with the BAS1-BAS2 complex in promoting assembly of the transcription initiation complex, although it is incapable of carrying out this function alone.

Because the distal BAS1 site is less important than the ABF1 site for high level activation, ABF1 must perform a function distinct from promoting interactions between BAS1 molecules bound at the distal and proximal sites at ADE5,7. Perhaps ABF1 is required to mark the region as a promoter, and by bending the DNA keeps UAS228 to 145 free of nucleosomes. It has been proposed that Rap1 binding in the vicinity of BAS1 and BAS2 binding sites performs precisely this function in the HIS4 promoter (36). The proximity of the distal BAS1 site and the ABF1 site at ADE5,7 (Fig. 6) raises the possibility that binding of BAS1 and ABF1 is mutually exclusive. If so, ABF1 may be displaced by binding of BAS1 to the distal site in the course of assembling the transcription initiation complex. Additional in vivo studies using purified BAS1, BAS2, and ABF1 will be required to test this idea and to address the possibility that BAS1-BAS1, BAS1-BAS2, or BAS2-BAS2 interactions stabilize the binding of these proteins to UAS228 to 145.

The Mechanism of Adenine Repression of BAS1 and BAS2 Function—Our mutational analysis provided no evidence for a DNA binding repressor that would mediate adenine repression of ADE5,7 transcription. Mutations in the binding site for such a repressor would be expected to produce constitutively derepressed expression, whereas all of the mutations we generated led to reduced UAS228 to 145 function. While it is possible that a repressor protein binds to UAS228 to 145 with the same sequence requirements as ABF1, BAS1, or BAS2, this seems unlikely. Moreover, the fact that only mutations in the BAS1 site completely abolished adenine repression probably indicates that the hypothetical repressor would have to compete with BAS1 for binding to its proximal site. A simpler explanation for our findings is that adenine regulation occurs by covalent modification of BAS1 or BAS2, or the binding of a negative regulator to one of these two proteins, that inhibits their ability to interact with DNA or activate transcription. In addition, because BAS2 regulates expression of promoters unrelated to purine biosynthesis, it seems most likely that BAS1 is the target of this negative regulation. Our results using LexA-BAS1 fusions eliminated the possibility that the DNA binding domain of BAS1 is the target of adenine repression (9). Instead, we found that complex formation between BAS1 and BAS2 unmasks the latent activation function of BAS1 and is down-regulated by adenine. It is possible that the activation function of the BAS1-BAS2 complex is also negatively regulated by adenine. Several mechanisms can be proposed to explain how the ability of BAS1 to interact with BAS2 is negatively regulated, including phosphorylation, binding of a negative regulator, or prevention of its entry into the nucleus. Efforts are under way to distinguish between these possibilities and to identify other trans-acting factors that might be involved in this regulatory mechanism.
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REFERENCES

1. Jones, E. W., and Fink, G. R. (1982) in The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp. 181–209, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

2. Arndt, K. T., Styles, C., and Fink, G. R. (1987) Science 237, 874–880

3. Daignan-Fornier, B., and Fink, G. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6746–6750

4. Stotz, A., Muller, P. P., and Linder, P. (1993) Curr. Genet. 24, 472–480

5. Mantzala, P., and Zalkin, H. (1984) J. Biol. Chem. 259, 8478–8484

6. Gedvilaite, A., and Sasnauskas, K. (1994) Curr. Genetics 25, 475–479

7. Rolfes, R. J., and Hinnebusch, A. G. (1993) Mol. Cell. Biol. 13, 5099–5111

8. Høvring, P. I., Bostad, A., Ording, E., Myrset, A. H., and Gabrielsen, O. S. (1994) J. Biol. Chem. 269, 17663–17669

9. Huxley, C., Green, E. D., and Dunham, I. (1990) Trends Genet. 6, 236

10. Itu, H., Fukada, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168

11. Henikoff, S. (1986) J. Mol. Biol. 190, 519–528

12. Sherman, F. (1991) Methods Enzymol. 194, 3–21

13. Hanahan, D., Jessee, J., and Bloom, F. R. (1991) Methods Enzymol. 204, 63–113

14. Sherman, F. (1991) Methods Enzymol. 194, 3–21

15. Hanahan, D., Jessee, J., and Bloom, F. R. (1991) Methods Enzymol. 204, 63–113

16. Sherman, F. (1991) Methods Enzymol. 194, 3–21

17. Hanahan, D., Jessee, J., and Bloom, F. R. (1991) Methods Enzymol. 204, 63–113