Combinatorial Regulation of the *Saccharomyces cerevisiae* *CAR1* (Arginase) Promoter in Response to Multiple Environmental Signals†

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Received 6 May 1996/Returned for modification 17 June 1996/Accepted 16 July 1996

***CAR1* (arginase) gene expression responds to multiple environmental signals; expression is induced in response to the intracellular accumulation of arginine and repressed when readily transported and catabolized nitrogen sources are available in the environment. Up to 14 *cis*-acting sites and 9 *trans*-acting factors have been implicated in regulated *CAR1* transcription. In all but one case, the sites are redundant. To test whether these sites actually participate in *CAR1* expression, each class of sites was inactivated by substitution mutations that retained the native spacing of the *CAR1* *cis*-acting elements. Three types of sites function independently of the nitrogen source: two clusters of Abf1p- and Rap1p-binding sites, and a GC-rich sequence. Two different sets of nitrogen source-dependent sites are also required: the first consists of two GATAA-containing *UAS*_{NTR} sites that mediate nitrogen catabolite repression-sensitive transcription, and the second is arginine dependent and consists of three *UAS*_I elements that activate transcription only when arginine is present. A single *URS1* site mediates repression of *CAR1* arginine-independent upstream activator site (*UAS*) activity in the absence of arginine and the presence of a poor nitrogen source (a condition under which the inducer-independent Gln3p can function in association with the *UAS*_{NTR} sites). When arginine is present, the combined activity of the *UAS* elements overcomes the negative effects mediated by *URS1*. Mutation of the classes of sites either singly or in combination markedly alters *CAR1* promoter operation and control, supporting the idea that they function synergistically to regulate expression of the gene.**

One of the more complex and well-studied nitrogen catabolic genes in *Saccharomyces cerevisiae* is *CAR1*, which encodes arginase (5, 18, 21, 25–27, 29, 31–34, 39, 40, 43–46, 50–52, 56–63, 66–68, 76–79). Arginase production is induced by arginine (the native inducer [82]) and is repressed when cells are growing on a preferred nitrogen source such as ammonia (5, 18, 60–63, 83, 84). The latter response was subsequently designated nitrogen catabolite repression (NCR) (18, 19, 83, 84). Early genetic analysis of arginine-dependent *CAR1* expression was conducted by Grenson, Wiame, and their students, who isolated seven types of mutants which have played central roles in our understanding of *CAR1* regulation (2, 18, 32, 39, 83, 84). Mutation of the *ARG81/ARGR1*, *ARG82/ARGR2*, and *ARG83/ARGR3* loci results in the loss of inducible arginase production and the ability to effectively use arginine as nitrogen source (2, 18, 32, 39, 83, 84). Reversion of *arg81* and *arg82* mutants generated five additional mutant classes, all of which produced arginase with various degrees of inducer independence (18, 26, 30, 32, 83, 84). Mutations falling into three complementation groups, *CAR80/CARGRI/UME6*, *CAR81/CARGRII*, and *CAR82/CARGRIII*, were recessive, suggesting that they affected genes encoding *trans*-acting factors (18, 26, 30, 83, 84). The remaining two classes of mutations were dom-

inant and were linked to the *CAR1* structural gene (26, 28, 32, 33, 35, 40, 83). One of these *cis*-dominant classes was derived from Ty insertions in the *CAR1* upstream region and has not figured prominently in subsequent molecular studies (28, 30, 40, 77, 83). The second class was a *cis*-dominant mutation, *CAR1-0*[–], that possessed a phenotype similar to that of *car80* mutations (30, 77–79, 83).

Isolation of the wild-type *CAR1* gene and the *cis*-dominant *CAR1-0*[–] mutant allele on recombinant plasmids permitted comparison of their 5'-flanking regions, with the finding that *CAR1-0*[–] differed from the wild type by only one nucleotide, a C-to-G transversion at position –153 (40, 77, 79). Deletion of 13 bp surrounding the *CAR1-0*[–] mutation in a *CAR1-lacZ* fusion plasmid generated the same phenotype as the point mutation, i.e., high-level β-galactosidase production whether the nitrogen source was glutamate (a noninducing nitrogen source) or arginine, indicating that a negatively acting promoter element (designated *URS1*) had been mutated (79). The *URS1* element, consisting of the 9-bp sequence 5'-AGCCGC CGA-3' (52, 77–79), was subsequently found upstream of many yeast genes (52) and shown to bind Rpap/Bufp in vitro (50, 51). Car80p was also shown to be required for *URS1*-mediated transcriptional repression (66) and to bind to the element as well (1, 76). *URS1*, situated upstream of *IME2*, was shown to be a bifunctional site; it participates in repression of *IME2* expression during vegetative cell growth and is required for transcriptional activation of *IME2* during sporulation (5, 6). A similar observation was made for the *SPO13* gene (75, 76).

Dissection of the *CAR1* promoter, by deletion analysis and determination of binding and expression characteristics supported by short DNA fragments, defined four *CAR1* upstream regions capable of transcriptional regulation, *UAS*_{CI}, *UAS*_{CH}, *UAS*_I, and *URS1* (43–46, 52, 77–79, 81). The arbitrarily defined *UAS*_{CI} and *UAS*_{CH} regions supported constitutive reporter

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† Dedicated to the memory of Marcelle Grenson, who departed from this world on 15 February 1996. Her perceptive view of nitrogen regulation, especially that associated with arginine, glutamate, ammonia, and γ-aminobutyrate metabolism, and her ingenious mutant selection techniques resulted in identification of genetic loci and ideas which are the foundation of many current studies, including this one. The field of nitrogen regulation in *S. cerevisiae* is poorer because of the loss of this important contributor.

gene expression when cloned into a heterologous expression vector (43, 44, 46). Genetic and in vitro biochemical studies demonstrated the UAS_C regions potentially to contain two Abf1p-binding sites (44) and five Rap1p-binding sites (46). However, the degrees to which individual sites were required for the DNA fragments containing them to support high-level β -galactosidase production when cloned into a *CYC1-lacZ* expression vector varied (44, 46). The UAS_I region was shown to consist of three tandem sequences required for arginine-inducible reporter gene expression (43, 45, 81). The ability of the three UAS_I elements to support inducer-dependent reporter gene expression varied, with the 5'- and 3'-most elements being the least and most effective, respectively (81). Although at least two of the *CAR1* UAS_I elements are required for induced transcription, they are not required to be situated in a specific orientation relative to one another or to the TATA element (45, 81). Moreover, a single UAS_I element derived from *ARG5.6* is similarly capable of transcription, further demonstrating the functional independence of the elements (45). Even though several studies have investigated the precise UAS_I element sequence, a single broadly accepted sequence in agreement with all published data has not yet emerged (25, 29, 34, 43, 45, 58). The homologous sequences we originally identified as arginine-responsive are shown in Table 2 (43, 45). Among specific transcription factors binding to UAS_I sequences are the Arg80, Arg81, and Mcm1 proteins (33, 34, 45, 57–59, 67, 68); binding has also been reported to require arginine (58).

A working model integrating the operation of the above classes of *CAR1* *cis*-acting elements was proposed at the time the biochemical lesion of *CAR1-0⁻* was discovered (78, 79). According to this model, the strong negatively acting *URS1* site mediates repression of transcriptional activation supported by constitutively operating upstream activator sequences ($UASs$) holding expression at a low, basal level in the absence of inducer (79). When arginine is present, the inducer-responsive UAS_I elements become functional and, together with the constitutive UAS_{CI} and UAS_{CII} elements, act synergistically to overcome the repressive effects of *URS1*, resulting in high-level *CAR1* expression (79). To avoid confusion, it should be emphasized that *URS1*-mediated transcriptional repression and nitrogen catabolite repression are not mechanistically related functions.

The earliest report that arginase production was influenced by the quality of nitrogen source available was Middlehoven's demonstration that it was ammonia repressible (60–63). This physiological response, now designated NCR, allows *S. cerevisiae* to prioritize its use of nitrogen sources (13, 18, 19, 22, 83, 84). NCR occurs at the level of transcriptional activation of genes encoding the permease and catabolic enzyme systems needed to degrade poor nitrogen sources (e.g., arginine, allantoin, proline, and γ -aminobutyrate). When readily used nitrogen sources (e.g., asparagine, glutamine, or ammonia in some strains) are available, NCR-sensitive genes are expressed only at low levels (18, 19). Upon depletion of these repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes dramatically increases (18, 19). Initial work with the *DAL5* gene demonstrated that two dodecanucleotide UAS_{NTR} elements, containing the core sequence GATAA, are required to mediate NCR-sensitive transcriptional activation; one GATAA-containing element is not active (13, 19, 20, 22, 70, 71). Gln3p, containing a zinc finger motif homologous to those of the GATA family of DNA-binding proteins (20, 27a, 64a, 65), binds to UAS_{NTR} elements (4, 27, 64a) and is a strong transcriptional activator when tethered by LexA upstream of a promoter devoid of UAS elements (27). Gln3p is also required

TABLE 1. Strains used in this work

Strain	Genotype
<i>S. cerevisiae</i>	
RH218.....	$MATa$ <i>trp1 CUP1 gal2 SUC2</i> Mal^-
TCY1.....	$MAT\alpha$ <i>lys2 ura3</i>
RR91.....	$MAT\alpha$ <i>ura3 lys2 gln3::hisG</i>
RJ71.....	$MAT\alpha$ <i>lys2 ura3 gat1Δ::hisG URA3 hisG</i>
RJ72.....	$MAT\alpha$ <i>lys2 ura3 gln3Δ::hisG gat1Δ::hisG URA3 hisG</i>
JCY125.....	$MAT\alpha$ <i>lys2 ura3 ure2Δ::URA3</i>
<i>E. coli</i> DH5 α	F' <i>endA1 hsdS20</i> (r_K^- m_K^+) <i>supE44 thi-1 recA1 gyrA</i> (Nal^+) <i>relA1 Δ(lacZYA-argF)U169</i> [ϕ 80d <i>lac</i> Δ (<i>lacZ</i>) M15]

for maximal levels of induced *CAR1* expression (20). Mutations at the *URE2* locus result in loss of NCR sensitivity, and Ure2p has been suggested to be a negative regulator of Gln3p function; the mechanism through which this occurs, however, is not known (15, 19, 23, 24). A second GATA family protein, Gat1p, has also recently been shown to be required for NCR-sensitive gene expression (16, 17, 74b); whether Gat1p is required for *CAR1* expression is not known.

The above information suggested that the multiple responses of *CAR1* expression to environmental signals might involve as many as 14 positive and negative *cis*-acting sites working in combination. However, all of the data upon which this suggestion was based were derived predominantly from genetic and in vitro biochemical studies with synthetic fragments of *CAR1* DNA. Few if any data exist to evaluate whether the in vitro results are physiologically significant. Therefore, we had two objectives: (i) to determine whether the 14 putative *cis*-acting sites actually participate in regulated *CAR1* expression and (ii) to test the proposed model of *CAR1* transcriptional regulation (79), by determining whether the UAS_{CI} and UAS_{CII} elements were required for regulated *CAR1* expression. The results answered both questions positively, although whether all of the Rap1p sites were individually required could not be ascertained. This work also demonstrates requirements for a novel GC-rich *cis*-acting element and the Gat1p for maximum *CAR1* expression.

MATERIALS AND METHODS

Strains used in this work. *S. cerevisiae* and *Escherichia coli* strains used in this work are listed in Table 1.

Plasmid constructions. Plasmid pS3 (Fig. 1) is identical to plasmid pNG15, except that it contained a different polylinker cloned into the *XhoI* site. Plasmid pNG6, the precursor of plasmid pNG15, contains a *XhoI* site but does not contain a polylinker. Plasmid pS3 was constructed by cloning oligonucleotide BS52/53 (Fig. 1) into the *XhoI* site of plasmid pNG6. Plasmid pS87 was constructed by cloning the 2.9-kb *SacI-HindIII* fragment from plasmid pRS46 (78) into the vector backbone of plasmid pHP41 digested with *SacI* and *HindIII* (66). This vector (designated plasmid pS84) was digested with *HindIII*, and the ends were blunted by treatment with the Klenow fragment of DNA polymerase I. The *TRP1* marker was obtained by digesting plasmid pRS314 (73) with *DrdI* and isolating the 1.3-kb fragment. The ends of this fragment were blunted by treatment with T4 DNA polymerase (New England BioLabs), and it was ligated into plasmid pS84 to create plasmid pS87 (Fig. 1). Fusion vectors (see Fig. 3, 5, and 7) were created by substitution of the wild-type *Bam*HI fragment of plasmid pS87, containing the entire *CAR1* upstream region, with various mutant upstream regions (see below).

Promoter mutations. Mutations in the *CAR1* promoter (see Table 2) were created by three different methods of mutagenesis. Plasmids pS100B, pS95FB, pS88, and pS96B were generated with the pALTER site-directed mutagenesis system from Promega. Oligonucleotides with the mutations specified in Fig. 2 were synthesized and used for in vitro DNA synthesis reactions which yielded plasmids containing the desired mutation(s). Throughout this work, all DNA initially synthesized in vitro was sequenced to verify its structure. The mutant fragments were then cloned into the expression vector plasmid pS87 (Fig. 1).

Plasmid pS88 contains a single base pair alteration at position -252, which

FIG. 1. Plasmids used in this work. Heterologous expression vector pS3 was constructed as described in Materials and Methods. The *CAR1-lacZ* fusion vector, pS87, contained destroyed *HindIII* sites, which are indicated by parentheses. Mutant *car1* promoters replaced the wild type by substitution of the *Bam*HI fragment in plasmid pS87.

generated a *NheI* restriction enzyme site. This was done to facilitate the cloning of oligonucleotides necessary to construct plasmids pS94B, pS108B, pS109B, pS113B, pS116B, pS118B, and pS119B. Except for plasmid pS94B, β -galactosidase values obtained with these plasmids should be compared with those obtained with plasmid pS88 as the "wild-type" parent plasmid. Mutations contained in each of these promoter fragments are depicted in Fig. 2.

The final method of creating mutations involved using the mutagenic primer employed to make plasmid pS100B as a PCR primer with various mutagenized templates to generate *urs1* double mutants (49). The other PCR primer we used was a sequencing primer complementary to the polylinker cloning site common to each mutant template. The heat-stable DNA polymerase used in these reactions was Pwo polymerase from Boehringer Mannheim. Reaction conditions were standard and were as specified by the manufacturer (49). Plasmids pS104B, pS105FB, pS106B, pS110B, and pS111B were created in this manner. Plasmids pS112B and pS115B were generated by PCR methods with a mutant primer to the *UAS_{NTR}* site of the *CAR1* promoter and a *UAS_I* mutant template. All fragments of DNA which were generated by PCR were sequenced before being assayed in plasmid pS87. Mutations present in these promoter fragments are listed in Fig. 2. Every promoter fragment used in this study contained the same number of bases as the wild-type *CAR1* promoter; i.e., there was no deletion or addition of bases, and only base substitutions were used.

Transformation and β -galactosidase assays. Transformation of *S. cerevisiae* RH218 has been described previously (77). All β -galactosidase assays in this work were performed with transformants of strain RH218. Transformants were plated on yeast nitrogen base (YNB; Difco) (0.17%) plates supplemented with 2% glucose, 0.5% Casamino Acids, and 0.5% ammonium sulfate. Isolated transformant colonies were then patched onto plates containing the same medium and grown for exactly 2 days before inoculation into the liquid culture medium used for determination of β -galactosidase activity (37, 64). The same transformant colony patch was used for inoculation into medium containing each of the different nitrogen sources. This medium contained 2% glucose, 0.17% YNB, and

TABLE 2. *cis*-acting element homologous sequences upstream of *CAR1*

Element	Coordinates	Sequence ^a
<i>URS1</i>	–156 to –148	AGCCGCCGA
Consensus		AGCCGCCGA
<i>UAS_{I-A}</i>	–244 to –222	GTCTCTAGCTCTTGCCCTTCGCA
<i>UAS_{I-B}</i>	–187 to –209	ATGCGCTGTTGATTGCCATTAGC
<i>UAS_{I-C}</i>	–182 to –160	GCTCGCTGAATTTTCACTTAGC
Crabeel et al. (25)		CCNYTAANGGCARNNNANAGCGA
GC rich	–281 to –266	AGCCGAGACGCCGCGA
<i>RAP1-C</i>	–486 to –474	AAAACAAACAACA
<i>RAP1-D</i>	–446 to –458	GCACCCGTAGTTC
<i>RAP1-E</i>	–333 to –321	CCGCCCTTTGATT
<i>RAP1-F</i>	–321 to –309	TGCTCCTTCATT
<i>RAP1-G</i>	–295 to –283	TATCCCTTACAAG
Consensus		RTCCACCCANCMCC
<i>ABF1-A</i>	–474 to –460	ATCACATCATACGGA
<i>ABF1-B</i>	–336 to –350	ATCGCGGGCGACGGT
Consensus		RTCRYNNNNACG
<i>UAS_{NTR}</i>	–416 to –427	TTTATGATAAAC
	–404 to –393	ACACTGATAAAC
Consensus		TTNCTGATAAGG

^a Underlined bases indicate those which were mutated (see Fig. 2).

0.1% of the indicated nitrogen source. These cultures were grown for 16 to 24 h to a cell density (A_{600}) of 0.45 to 0.85 as measured on the Spectronic Genesys 5 spectrophotometer. These A_{600} values correspond to cells in the logarithmic growth phase. After the desired cell density was reached, duplicate 25-ml samples were transferred from each culture to tubes containing 0.5 ml of 1% cycloheximide and placed on ice. Even though most of the plasmids used in this work contain the stable *CEN/ARS*-based origin of replication, transformants were never frozen or stored; the cultures were assayed immediately. After incubation on ice, cells were collected by centrifugation and resuspended in 1.0 ml of Z buffer (37). The cells were permeabilized by addition of 50 μ l of chloroform and 0.1% sodium dodecyl sulfate. The cell suspensions were then vortexed at high speed for 20 s. The β -galactosidase assay was performed by the method of Guarente and Mason (37), and the units are those used by Miller (64). Each β -galactosidase activity reported in this work is the mean value from two independent transformants; i.e., four enzyme assays (duplicate samples from each transformant) were performed for each value reported. Data in Table 3 were collected over a 6-month period from 32 independent transformants containing either plasmid pS87 or pS100B. They provide a statistically significant assessment of the variation we observed over the entire range of β -galactosidase values reported. The percent variation observed was greatest for the values obtained when asparagine was used as nitrogen source. The most important characteristic of the experimental data obtained were the patterns of activity in response to either various environmental conditions or mutations observed; they were absolutely invariant from experiment to experiment. Activities for Fig. 3, 5, and 7 were determined in sets; the data in each figure derive from a single transformation with the set of plasmids shown in that figure as the sources of DNA. It is for this reason that results are more appropriately compared within each figure rather than between them.

Miscellaneous methods. Northern (RNA) blotting was performed as described by Coffman et al. (15). The DNA fragment used as a probe in the Northern blot analysis was the 750-bp *Bgl*II fragment from plasmid pTC42 containing the *CAR1* gene (77). Radioactive labeling of this fragment was performed by the random-primed method previously described (15). Equal loading of each lane was verified with the labeled histone H4 gene as a control (15).

The sequences of oligonucleotides cloned into plasmid pS3 to yield plasmids pS15, pS18, pS19, pS20, pS21, and pS22 appear in Fig. 2, and the plasmids were constructed by previously described procedures, with the exception that the oligonucleotides were cloned into the *Asp*718 and *Xho*I sites of plasmid pS3 (52). Oligonucleotide sequences in Fig. 2 are presented as single strands for the sake of brevity. Each one also contained 4-bp extensions (*Asp*718 site on the 5' end and *Xho*I on the 3' end) to permit cloning. Cloning of the various mutant promoters into plasmid pS87 followed standard cloning procedures.

A	pS86	{-156}AGCgGCCGA{-148}
	pS88	{-259}CGGCTAGcGCGAA{-247}
	pS94B	{-234}CTTGgtaccCGCAAAGCACCGTGCTGCTAcgttacATCAACAGCGCATCGCCGCTCGTGAATTggatagTAG{-161}
	pS95FB	{-429}ATGcTcgaCATAAACTTAGATATCAACACTaAcAAA{-394}
	pS96B	{-282}AAGaattcACattataAAA{-264}
	pS100B	{-157}TAGaattCGA{-148}
	pS108B	{-259}CGGCTAGcGCGAA{-247} {-489}ACActtAgActagtaATgACATCATagGGATtACTAgctctaagAAT{-443}
	pS109B	{-259}CGGCTAGcGCGAA{-247} {-352}GCACCCcTCGCCCGCcATCCcttagacTaGtaagCTCCTCCATTACAGTTTTTTTCctcgagTgcatAaAAG{-280}
	DOUBLE MUTANTS (urs1)	
	pS104B	{-234}CTTGgtaccCGCAAAGCACCGTGCTGCTAcgttacATCAACAGCGCATCGCCGCTCGTGAATTggatagTAG{-161} {-157}TAGaattCGA{-148}
	pS105FB	{-429}ATGcTcgaCATAAACTTAGATATCAACACTaAcAAA{-394} {-157}TAGaattCGA{-148}
	pS106	{-282}AAGaattcACattataAAA{-264} {-157}TAGaattCGA{-148}
	pS110B	{-259}CGGCTAGcGCGAA{-247} {-489}ACActtAgActagtaATgACATCATagGGATtACTAgctctaagAAT{-443} {-157}TAGaattCGA{-148}
	pS111B	{-259}CGGCTAGcGCGAA{-247} {-352}GCACCCcTCGCCCGCcATCCcttagacTaGtaagCTCCTCCATTACAGTTTTTTTCctcgagTgcatAaAAG{-280} {-157}TAGaattCGA{-148}
	MULTIPLE MUTANTS	
	pS112B	{-429}ATGcTcgaCATAAACTTAGATATCAACACTaAcAAA{-394} {-234}CTTGgtaccCGCAAAGCACCGTGCTGCTAcgttacATCAACAGCGCATCGCCGCTCGTGAATTggatagTAG{-161}
	pS115B	{-429}ATGcTcgaCATAAACTTAGATATCAACACTaAcAAA{-394} {-234}CTTGgtaccCGCAAAGCACCGTGCTGCTAcgttacATCAACAGCGCATCGCCGCTCGTGAATTggatagTAG{-161} {-157}TAGaattCGA{-148}
	pS113B	{-259}CGGCTAGcGCGAA{-247} {-489}ACActtAgActagtaATgACATCATagGGATtACTAgctctaagAAT{-443} {-352}GCACCCcTCGCCCGCcATCCcttagacTaGtaagCTCCTCCATTACAGTTTTTTTCctcgagTgcatAaAAG{-280}
	pS116B	{-259}CGGCTAGcGCGAA{-247} {-489}ACActtAgActagtaATgACATCATagGGATtACTAgctctaagAAT{-443} {-352}GCACCCcTCGCCCGCcATCCcttagacTaGtaagCTCCTCCATTACAGTTTTTTTCctcgagTgcatAaAAG{-280} {-157}TAGaattCGA{-148}
	pS118B	{-259}CGGCTAGcGCGAA{-247} {-489}ACActtAgActagtaATgACATCATagGGATtACTAgctctaagAAT{-443} {-352}GCACCCcTCGCCCGCcATCCcttagacTaGtaagCTCCTCCATTACAGTTTTTTTCctcgagTgcatAaAAG{-280} {-282}AAGaattcACattataAAA{-264}
	pS119B	{-259}CGGCTAGcGCGAA{-247} {-489}ACActtAgActagtaATgACATCATagGGATtACTAgctctaagAAT{-443} {-352}GCACCCcTCGCCCGCcATCCcttagacTaGtaagCTCCTCCATTACAGTTTTTTTCctcgagTgcatAaAAG{-280} {-282}AAGaattcACattataAAA{-264} {-157}TAGaattCGA{-148}
B	pS3 OLIGONUCLEOTIDE INSERTS	
	pS15	{-303}TTTTTTCCTATCCCTTACAAGAAGCCGAGACGCCCGGAAAATATCGGCTAGTCCGA{-248}
	pS18	{-287}ACAAGAAGCCGAGACGCCCGGAAAATATCGG{-257}
	pS19	{-287}cacctcAGCCGAGACGCCCGGAAAATATCGG{-257}
	pS20	{-287}ACAAGataatctGACGCCCGGAAAATATCGG{-257}
	pS21	{-287}ACAAGAAGCCGAGACTaatatAAAATATCGG{-257}
	pS22	{-287}ACAAGAAGCCGAGACGCCCGGAAAacggatG{-257}

FIG. 2. (A) Mutant sequences contained in each promoter. The number of each *CARI-lacZ* fusion plasmid is indicated in boldface type. Mutated sequences are indicated by lowercase letters. Numbers flanking each sequence denote the nucleotide positions relative to ATG. (B) Oligonucleotides used for the construction of heterologous expression vectors. Only the top strand of each fragment is indicated. Lowercase letters denote mutant bases. Each oligonucleotide contained 4-bp extensions on either end, *Asp*718 on the 5' end and *Xho*I on the 3' end, to permit cloning of the fragments into plasmid pS3.

RESULTS

Strategy to test the *UAS-URS* balance control hypothesis of *CARI* regulation. The earliest studies correlating the regulation of *CARI* expression with its promoter sequence suggested that the promoter consisted of positive and negative *cis*-acting

elements and their cognate *trans*-acting factors operating in combination to achieve the observed in vivo responses to various growth conditions. The essence of the proposal was that the functions of proteins binding to positive and negative *cis*-acting elements antagonize one another's operation and that specific environmental conditions, operating through these

TABLE 3. Variation in β -galactosidase assay results^a

Plasmid	No. of independent transformants evaluated	Nitrogen source	β -Galactosidase activity
pS87	16	Asparagine	27 $\pm$ 3
pS87	16	Proline	47 $\pm$ 5
pS100B	14	Asparagine	392 $\pm$ 65
pS100B	13	Proline	1,216 $\pm$ 125
pS87	15	Arginine	1,491 $\pm$ 99
pS100B	10	Arginine	3,083 $\pm$ 175

^a Data presented in this table include all values falling in the range of ± 1 standard deviation of the arithmetic mean for the entire data set. Plasmids pS100B and pS87 were analyzed 16 and 20 times, respectively (32 and 40 assays), for each culture condition used. This method of analysis was chosen because some experimental values were clearly outliers relative to the bulk of the data, and it provided an objective criterion for identifying the aberrant observations and eliminating them from consideration.

transcription factors, modulate this balance toward increased or decreased *CAR1* expression (43, 79). For example, in the absence of inducer, the proposal postulates the strength of positive- versus negative-element transcriptional effects to be such that *CAR1* expression occurs only at a low, basal level. When inducer is present, the combined action of the functioning positive elements is able to overcome *URS1*-mediated repression such that *CAR1* expression occurs at high level. This model was derived almost exclusively from in vitro promoter dissection studies in which specific transcription factors were shown to bind small fragments of the *CAR1* promoter that mediated activation or repression of reporter gene expression when cloned into heterologous expression vectors (43–46, 50, 52, 57, 58, 81). With 14 potential sites identified upstream of *CAR1* (Table 2), our objective was to test the physiological significance of this hypothesis of *CAR1* regulation.

A single negative site (*URS1*) was found upstream of *CAR1*, and for this reason it was the only promoter site to be identified by classical genetic methods (77, 78, 83). The remaining sites, situated in arbitrarily designated regions, *UAS_{CI}*, *UAS_{CH}*, and *UAS_I*, are present in multiple copies. The presence of multiple sites influenced the strategy by which we tested their contribution to promoter activity. Since relative in vitro contributions of individual sites within a class had already been compared and reported (43–46, 52, 58, 81), we tested the significance of the hypothesis by mutating each class of sites and measuring the effects of the mutations on the operation of an otherwise native *CAR1* promoter. Multiple point mutations, previously shown to inactivate totally each of the particular elements (Fig. 2) (44–46, 52, 81), were used in each case so that the spacing of the putative elements relative to one another and to the TATA box was not disturbed. To test the antagonistic aspect of the proposed model, the effects of each of the mutations were assayed in the presence and absence of a functional *CAR1 URS1* element.

To facilitate the construction of mutant promoters, a single restriction site was introduced upstream of the translational start. This was accomplished by a single T-to-C base change at position –252 (plasmid pS88). Since this single base change rendered the plasmid mutant at that position, we compared *lacZ* expression values obtained with plasmid pS88 with those obtained with the unaltered, wild-type plasmid pS87 (Fig. 3). The observed differences were small, thereby permitting us to use the engineered “wild type” as the parent for some of the subsequent plasmids. The difference observed, however, is probably real, since a related observation was made during deletion analysis of *CAR1* promoter fragments (43). The point

mutation we introduced into the sequence may have slightly damaged the *UAS_{I-A}* site. For this reason, both plasmids pS87 and pS88 were frequently included as controls in our experiments.

Mutation of inducer-responsive *CAR1 UAS_I* elements. The inducer responsiveness of *CAR1* expression requires three homologous *cis*-acting sites designated *UAS_I* (Table 2) (25, 29, 43, 45, 58, 81). Studies in three laboratories have demonstrated these sites to be of increasing strength from 5' to 3' and to bind several proteins including those encoded by *MCMI*, *ARG81*, and *ARG82* (3, 25, 29, 31, 33, 34, 45, 56–58, 67, 68, 81). To assess the contribution of these sites to overall promoter function, all three were mutated in a *CEN*-based plasmid containing the entire *CAR1* upstream region fused to the *E. coli lacZ* gene. The wild-type parent plasmid, pS87, supported highly inducible *lacZ* expression (Fig. 3). Basal-level expression was NCR sensitive (compare the values obtained with proline and asparagine), even though the absolute values were low. Mutation of the negatively acting *URS1* site (Table 2) resulted in 20- and 30-fold increases in *lacZ* expression observed in cells growing with asparagine or proline, respectively, as the sole nitrogen source (Fig. 3, plasmid pS100B). The NCR sensitivity, however, was maintained; i.e., the values with proline were about 2.5-fold higher than those with asparagine as the nitrogen source. Note also that β -galactosidase production increased approximately twofold (in arginine) in plasmid pS100B compared with the wild type (plasmid pS87), an observation consistent with the negative function of *URS1*. When the three *UAS_I* elements were mutated (plasmid pS94B), arginine responsiveness was lost. Not surprisingly, the ratios of β -galactosidase levels supported by plasmids pS87 and pS94B in cells with arginine as the nitrogen source were similar to those of arginase levels originally reported for wild-type versus *arg81* mutant cells growing with ammonia plus arginine (25, 76). Mutation of both the *UAS_I* and *URS1* elements (Fig. 3, plasmid pS104B) resulted in restoration of *lacZ* expression that was about 20 to 60% of fully induced wild-type levels (compare plasmids pS87 and pS104B). If β -galactosidase levels are compared for plasmids pS104B and pS100B (i.e., in a *urs1* mutant background), mutation of *UAS_I* resulted in 1.4- and 1.3-fold decreases with asparagine or proline as the nitrogen source but a 6.3-fold decrease with arginine. Together, these results supported the conclusions that (i) *UAS_I* elements are responsible for inducer responsiveness of the wild-type gene and (ii) a protein(s) putatively bound to *URS1* was responsible for repressing the nitrogen source-independent function of transcription factors operating through the *CAR1 UAS* elements remaining in the *uas_I urs1* double mutant, thus maintaining a low basal level of expression in the absence of inducer.

Mutation of NCR-sensitive *UAS_{NTR}* elements. Work initiated with *DAL5* (13, 70, 71) and subsequently carried out with most identified nitrogen-catabolic genes has demonstrated that *UAS_{NTR}* elements, containing the sequence GATAA at their cores, can serve as Gln3p-binding sites and are responsible for NCR-sensitive gene expression (27, 71). The newly discovered Gat1p also mediates NCR-sensitive transcription through some *UAS_{NTR}* elements (16, 17, 74b). *CAR1* contains two GATAA sites at positions –425 to –421 and –399 to –395 relative to the translational start site (Table 2; see Fig. 3, 5, and 7). As shown in Fig. 4, *CAR1* basal expression, (steady-state mRNA production; proline was the nitrogen source for this experiment) is NCR sensitive (Fig. 4b, lanes A and B), requires Gln3 and Gat1 proteins for maximum expression (Fig. 4a, lanes A and B and lanes A and D), and becomes largely NCR insensitive in a *ure2* null mutant (Fig. 4b, lanes B and D). In other words, *CAR1* expression exhibits characteristics typical

FIG. 3. Effect of mutations in the *UAS_I* and *UAS_{NTR}* elements of the full-length *CAR1* promoter. Mutated sites are denoted as white boxes; the exact sequences of the mutations are given in Fig. 2. All other sequences remain wild type except for the *URS1* site, which may or may not be mutated. The color key for the sites is as follows: red, *URS1*; blue, *UAS_I*; black, GC rich; yellow, Rap1p; purple, Abf1; green, *UAS_{NTR}*. As closely as possible, each schematic promoter is drawn to scale. Coordinates are relative to the translation start site. The repressive nitrogen source asparagine (ASN), the "derepressive" nitrogen source proline (PRO), and the inducing nitrogen source arginine (ARG) were provided at concentrations of 0.1%. Transformation, growth, and assay of the fusion plasmids in strain RH218 are described in Materials and Methods. Values provided are the mean of two independent transformants. NCR-sensitive expression is expected to be high with a nonrepressive nitrogen source (proline) and low with a repressive nitrogen source (asparagine) (70).

of a gene with moderate NCR sensitivity, confirming the conclusion of our earlier studies (21, 77–79). To assess the contribution of the two GATAA sequences to *CAR1* expression supported by an intact promoter, both sequences were mutated in plasmid pS95FB (Fig. 3). The loss of these two elements did not adversely affect basal-level expression with asparagine as the nitrogen source, a growth condition under which *UAS_{NTR}* elements, and hence presumably Gln3p, do not support transcription (13, 71), but decreased the proline (a derepressive nitrogen source in which Gln3p functions) and fully induced levels by about half compared with values observed with wild-type plasmid pS87. When *URS1* was mutated along with *UAS_{NTR}* (plasmid pS105FB), uninduced, basal-level expression (that observed with proline or asparagine) was 7- to 11-fold greater than wild-type expression (plasmid pS87) but was no longer two- to threefold decreased by the repressive nitrogen source, asparagine (compare asparagine and proline values for plasmid pS105FB). Two- to threefold is a magnitude similar to that estimated in the Northern blot experiment discussed above (Fig. 4b, compare lanes A and B). Although fully induced expression was not as high in plasmid pS105FB as in plasmid pS100B, it was more arginine responsive (compare the arginine and proline values for the two plasmids). In this regard, it is important to recall that transcriptional activation supported by *UAS_{NTR}* is constitutive with proline as the nitrogen source. This accounts for the high proline value and hence the low arginine responsiveness obtained with plasmid pS100B (sevenfold induction comparing asparagine and arginine values and 2.7-fold induction comparing proline and arginine values).

Next, we mutated both the *UAS_I* and *UAS_{NTR}* elements in the presence and absence of a mutated *URS1* element. With a wild-type *URS1*, the double mutant exhibited basal-level expression regardless of the nitrogen source used; both inducibility and NCR sensitivity were lost (Fig. 3, plasmid pS112B). The *uas_I uas_{NTR} urs1* triple mutant, however, remained capable of significant reporter gene expression that was independent of the nitrogen source (plasmid pS115B). This result was another indication that additional *UAS* elements were present, as suggested previously from *in vitro* studies (43, 78, 79).

Mutation of *RAP1* and *ABF1* sites upstream of *CAR1*. Work from several laboratories has demonstrated that Rap1 and Abf1 proteins are *UAS* transcription factors that bind to *UAS* elements upstream of many genes (8–12, 38, 47, 48, 53, 54, 72). Moreover, they are often situated and function together (10, 14). *ABF1* and *RAP1* *UAS* elements mediate transcriptional activation independent of the nitrogen source; hence, they operate constitutively with respect to the experimental conditions used in this work. Although operation of the Abf1p is affected by the carbon source (74) and Rap1p binding is influenced by the degree to which Rap1p is phosphorylated (41, 80), these types of regulation were not investigated in our studies. The *CAR1* *UAS_{CI}* and *UAS_{CH}* regions contain five sequences more or less homologous to those binding Rap1p and two homologous to those binding Abf1p (Fig. 5; Table 2). Analysis of small *CAR1* DNA fragments demonstrated that these *CAR1* sequences bound Rap1 and Abf1 proteins *in vitro* and supported reporter gene expression when cloned into a heterologous expression vector (44, 46). To assess whether the Rap1p and Abf1p elements contributed *in vivo* to the consti-

tutive *lacZ* expression observed with plasmid pS88 in Fig. 5, the putative *CAR1* Rap1p and Abf1p sites were mutated (Fig. 2). As shown with plasmids pS88 and pS108B, mutation of the 5' cluster of putative Abf1p- and Rap1p-binding sites (those in region *UAS_{CI}*) reduced *lacZ* expression observed with all three nitrogen sources by three- to fivefold. A maximum reduction of twofold, depending on the nitrogen source, was observed upon mutating the 3' cluster of putative Rap1p- and Abf1p-binding sites (those in region *UAS_{CH}*) (Fig. 5, compare the data obtained with plasmids pS88 and pS109B). One of the Rap1p site-homologous sequences in the *UAS_{CH}* region was not mutated because previous studies demonstrated that its homology was so limited that it did not significantly contribute to the overall UAS capability of a *UAS_{CH}* region DNA fragment (46). Mutation of the *UAS_{CI}* and *UAS_{CH}* region Rap1p and Abf1p sites in a *urs1* mutant background again demonstrated the contribution of these sites to overall *CAR1* promoter strength. Mutation of the *UAS_{CI}* or *UAS_{CH}* elements decreased β -galactosidase production by one-third with asparagine or arginine as the nitrogen source and three- to fourfold with proline (Fig. 5, compare plasmids pS100B, pS110B, and pS111B).

Mutation of the GC-rich sequences upstream of *CAR1*. During analysis of Bui1-3p/Rap1p binding to *URS1*-homologous sequences, we reported protein binding to *CAR1* GC-rich sequences (Table 2) (Fig. 5 and 6 of reference 51). Additionally, a DNA fragment containing these sequences mediated constitutive reporter gene transcription when cloned into a heterologous expression vector (Fig. 7 of reference 51). There were also three other GC-rich sequences between positions -376 and -316; however, we have found that they do not support significant UAS activity, and hence they were not studied further (74a). Our first experiment with the *UAS_{CH}* GC-rich sequences demonstrated that both of them (Table 2) were required for reporter gene transcription in a heterologous expression vector system. A DNA fragment containing *CAR1* sequences -287 to -257 was used as the parent fragment for this experiment (Fig. 2 and 6) and was similar to one shown earlier to support UAS activity (plasmid pLK76 [-304 to -251] in reference 43, and plasmid pS15 in this work). This DNA fragment supported 331 U of β -galactosidase activity compared with 8 U obtained with the expression vector alone (Fig. 6, plasmids pS18 and pS3). Mutation of 6 nucleotides immediately 5' of the first GC-rich sequence, AGCCGA, and immediately 3' of the second GC-rich sequence, GCCGCG, did not significantly affect transcription supported by the mutant DNA fragments compared with the parental wild type (plasmids pS18, pS19, and pS22). In contrast, mutation of the AGCCGA sequence decreased reporter gene expression to 44 U (plasmid pS20). Similarly, mutation of the second GC-rich sequence, GCCGCG, decreased *lacZ* expression to 13 U (plasmid pS21).

To assess the contribution of the GC-rich sequences to native *CAR1* expression derived from a full promoter, they were mutated (Fig. 5, plasmids pS96B and pS106B). Regardless of the *URS1* element allele used, loss of the GC-rich sequences decreased *lacZ* expression by one-third when arginine was provided as the nitrogen source (induced condition). Mutation of the GC-rich sequences also resulted in a two- to sevenfold decrease with asparagine as the nitrogen source (Fig. 5, compare pS87 with pS96B and pS100B with pS106B). The observed reporter gene expression, however, remained as NCR sensitive as in the wild type (compare proline and asparagine values). These data indicate that the GC-rich sequences contribute to the overall strength of the *CAR1* promoter, especially when the arginine-dependent *UAS_I* element is inactive (noninducing growth conditions). They also suggest that the

FIG. 4. Northern blot analysis of *CAR1* expression under different physiological conditions. (a) Effect of *gln3Δ* (strain RR91) and *gat1Δ* (strain RJ71) single mutations or a *gln3Δ gat1Δ* (strain RJY725) double mutation on the steady-state levels of *CAR1* mRNA when cells were grown with 0.1% proline (PRO) as the nitrogen source and compared with wild-type (WT) strain TCY1. (b) Effect of *ure2* gene deletion on steady-state levels of *CAR1* mRNA produced with different nitrogen sources. Wild-type (WT) strain TCY1 and *ure2Δ* strain JCY125 were grown under nitrogen-repressive conditions with glutamine (GLN) as the nitrogen source or in the poor nitrogen source proline (PRO). Nitrogen sources were provided at concentrations of 0.1%.

GC-rich sequences do not directly participate in the NCR-responsive regulation of *CAR1*.

Mutation of multiple *CAR1* elements mediating constitutive *UAS* activity. Since early studies demonstrated *CAR1* to be inducible (5, 30, 40, 43, 53–56, 77–79, 82, 83), it was reasonable to determine if regulation of the gene was mediated exclusively through the inducer-dependent *UAS_I* element. Although proposing a single inducer-dependent UAS would adequately explain induction and low, basal-level *CAR1* expression in the absence of an inducer, it fails to explain the *urs1* point (*CAR1-0⁻*) and deletion mutant phenotypes, which point to a more complex operation (30, 78, 79, 83). Therefore, it was important to know whether the elements mediating arginine-independent *CAR1* expression were vital to its regulation, as predicted by the positive-negative balance hypothesis (43, 78, 79). To determine whether *CAR1* could operate effectively without the constitutive elements, we mutated all of them in wild-type and *urs1* mutant backgrounds (Fig. 7). Mutation of both the 5' and 3' clusters of putative Rap1p- and Abf1p-binding sites in a wild-type *URS1* genetic background decreased both the basal and induced levels by 2.5- to 5-fold (Fig. 7, plasmids pS88 and pS113B). Similar results were obtained in the *urs1* mutant background (two- to threefold decrease) except with proline as the nitrogen source, when the decrease was eightfold (Fig. 7, compare plasmids pS100B and pS116B).

The last set of multiple mutants analyzed was those in which

FIG. 5. Effect of mutations in the *CAR1* Abf1p, Rap1, and GC-rich UAS elements. Mutated sites are denoted by white boxes, and the exact sequences of the mutations are given in Fig. 2. All other sequences remain wild type, and their identities, by color, are given in the legend to Fig. 3. All other conditions were as described in the legend to Fig. 3. The vertical red line indicates the position of the engineered *NheI* restriction site.

all known UAS elements mediating constitutive reporter gene activation (putative Rap1p- and Abf1p-binding sites and GC-rich sequences) were mutated. As shown in Fig. 7, inactivation of all constitutively functioning sites resulted in only a very weak arginine-inducible promoter (compare plasmids pS88 and pS118B). The fully induced value for β -galactosidase production supported by plasmid pS118B was 30-fold less than that for the wild type (plasmid pS88) under the same growth

FIG. 6. Reporter gene (*lacZ*) expression supported by DNA fragments derived from the *UAS*_{C2} region of the *CAR1* promoter. The nitrogen source for this experiment was arginine, provided at a final concentration of 0.1%. The DNA fragments were cloned into the *Asp718-XhoI* sites of plasmid pS3. These plasmids were used to transform strain RH218. Transformants were assayed as described in Materials and Methods. The coordinates are given with respect to the translation start site. Mutant bases are indicated by lowercase letters. The β -galactosidase (β -gal) activity is given on the right.

FIG. 7. Analysis of multiple mutations in *CAR1* Abf1p, Rap1p, and GC-rich UAS elements. Mutated sites are denoted by white boxes, and the exact sequences of the mutations are given in Fig. 2. All other conditions are as in Fig. 3. The vertical red line indicates the position of the engineered *NheI* restriction site.

conditions (Fig. 7). When the repressive effects mediated by *URS1* were relieved by mutation (plasmid pS119B), arginine inducibility increased 6-fold but was still 10-fold below that observed with plasmid pS100B. In fact, the pattern of activities supported by this mutant plasmid was not unlike that supported by a synthetic DNA fragment containing only the three *UAS_I* elements cloned into a heterologous expression vector (Fig. 6) (43). Together, these data argue that the three arginine-dependent *UAS_I* elements contribute only weakly to the fully induced level of activation observed with a wild-type promoter. The observation that *lacZ* expression did not increase in proline-containing medium over the values observed in asparagine-containing medium with plasmids pS113B, pS116B, pS118B, and pS119B, all of which contain wild-type *UAS_{NTR}* elements, argues that combinatorial operation of the putative Abf1p- and Rap1p-binding sites is also required for *CAR1* NCR-sensitive expression which occurs through the *UAS_{NTR}* elements. The combined operation of *UAS_{NTR}* and nonrelated UAS elements has been observed previously (69).

Mutation of other promoter element-homologous sites upstream of *CAR1*. Comparison of the *CAR1* upstream region with documented UAS elements revealed three additional sequences that were homologous to the Hap2, Hap3, Hap4, Hap5 (positions -372 to -380), T₄C (positions -263 to -271), and STRE elements (positions -144 to -148), respectively (6, 36, 42, 55). We mutated each of them in turn and assessed their effects upon β -galactosidase production. None of the mutations generated a detectable phenotype (data not shown). It may be important, however, that no assays were performed with diploid cells.

DISCUSSION

Work from several laboratories has demonstrated the existence of multiple sequences homologous to transcription factor-binding sites in the 5' region of *CAR1* (1, 27, 34, 44–46, 50–52, 57, 58, 76). In most cases, these factors have been shown

to bind to wild-type but not mutant alleles of these sequences (1, 27, 34, 44–46, 50–52, 57, 58, 76). Moreover, the putative UAS elements support reporter gene expression in heterologous expression vectors (43–46, 58). This work extends our view of the *CAR1* promoter by demonstrating that these putative UAS elements indeed participate in and are required for native wild-type *CAR1* expression and regulation. The remarkable feature of the *CAR1* promoter is the number of functional sites it contains. If one eliminates the weakest Rap1p site, *CAR1* still possesses 11 to 13 functional *cis*-acting elements (Fig. 8); there is some uncertainty about the precise number of Rap1p sites that actually function in vivo. The redundancy of *CAR1* UAS elements accounts both for the failure to isolate *cis*-acting mutants of them and for the stepwise loss of activity observed in nested 5' deletions of *CAR1* upstream sequences (43). In fact, if entire classes of elements had not been deleted in this work, their contributions to *CAR1* expression would not have been possible to demonstrate convincingly. It is also important to emphasize the utility of inactivating the *URS1* element in order to visualize some of the mutant phenotypes. The potential masking effect of *URS1* was recognized when it was first identified as a *cis*-acting element and its effect on induced activation was supported by an isolated *UAS_I* element (Fig. 6 in reference 43).

CAR1 is an example of a number of genes that respond to multiple environmental stimuli; this results in flexible expression levels, each suited to the particular condition(s) encountered by the cell (6, 7, 9, 11, 14, 18, 19, 47, 48, 55, 60–63, 75, 83). The expression levels appear to be mediated by multiple *cis*-acting elements and *trans*-acting factors, individual classes of which each respond to single environmental determinants functioning in combination to accomplish the appropriate level of *CAR1* expression. Transcription factors putatively bound in vivo to the various *cis*-acting control elements are presumably responsible for the layered and synergistic regulation of *CAR1* promoter operation. The earliest studies of arginase production demonstrated it to be inducible and NCR responsive (18,

FIG. 8. Structure and regulation of the *CAR1* promoter in *S. cerevisiae*. Color coding is as described in the legend to Fig. 3.

60–63, 84). As shown in this work, both NCR-sensitive and arginine-dependent UAS elements are required for previously observed nitrogen source effects on *CAR1* expression, thus explaining these early results.

The only *cis*-acting mutation, other than Ty insertions, to be isolated upstream of *CAR1* by classical genetic methods was the *CAR1-0⁻* mutation from Wiame's laboratory (30, 32, 35, 40, 77–79, 83). The phenotype generated by that point mutation in the middle of the *CAR1* *URS1* element was the key to unraveling the complex structure of the promoter of this gene (43, 52, 77–79). The revealing characteristic was that the mutant produced high-level arginase activity with glutamate as the nitrogen source, i.e., under noninducing conditions (78). This and the demonstration that the *CAR1-0⁻* point mutation destroyed a negatively acting element (i.e., point and deletion mutations yielded the same phenotype) suggested that *CAR1* expression consisted of strong arginine-independent and arginine-dependent UAS elements and a *URS1* negatively acting element. The actions mediated by all of these elements acting in various combinations were responsible for observed *in vivo* *CAR1* regulation.

These observations generated our working model of how *CAR1* regulation might be accomplished (43, 78, 79). This model can now be refined (Fig. 8). Rap1p, Abf1p, Gln3p, and putatively Gat1p sites, along with the GC-rich sequences, are responsible for high-level constitutive *CAR1* expression when cells are provided with a poor nitrogen source such as proline or, in some strains, glutamate (43). The function of these strong activators is prevented by the action of a protein(s), e.g., Car80p and perhaps Rpap, bound to the *URS1* element. In other words, negative actions of the *URS1* proteins balance positive actions of the above transcription factors (43, 78, 79). When arginine becomes available within the cell, transcriptional activation mediated by the arginine-dependent *UAS_I* elements cooperates with the arginine-independent elements mentioned above. In this way, the negative action of *URS1* is overcome and elevated expression ensues. Participation of *UAS_{NTR}* elements in *CAR1* expression accounts in part both for the moderate NCR sensitivity of the gene, its requirement for Gln3p and Gat1p for maximum expression, and the inability of arginine-mediated induction to completely overcome NCR (16, 17, 74b). Without *UAS_{NTR}* participation, the remaining UAS elements are insufficient to overcome *URS1* and hence yield a gene with only 50% of its fully induced expression (compare plasmids pS87 and pS95FB).

The three other classes of NCR-insensitive, constitutively functioning UAS elements that contribute, along with *UAS_{NTR}*, to *CAR1* expression also explain why the NCR sensitivity of *CAR1* is moderate rather than strong as observed for the allantoin, proline, or γ -aminobutyrate utilization pathway genes (15–19). The *UAS_{NTR}* elements upstream of *CAR1* demonstrated here provide a second means of regulating arginase production mediated by the quality of the nitrogen supply. The first is by inducer exclusion resulting from the NCR-sensitive expression of the amino acid permeases *CAN1* and *GAP1* (21, 30).

An additional piece of useful information is presented in this work. If the effects of mutating the *UAS_{NTR}*, Rap1p, and Abf1p elements are compared, it is apparent that the three sites do not function in a totally independent fashion. This is best observed by comparing data obtained with plasmids pS100B and pS116B in Fig. 7. *lacZ* expression supported by plasmid pS100B with asparagine and proline as nitrogen sources exhibits expected NCR sensitivity; the proline value is two to three times higher than the asparagine value. With plasmid pS116B, NCR sensitivity is lost; i.e., the proline and asparagine values are the same, even though the *UAS_{NTR}* elements are not mutated. This characteristic is also observed with mutations that eliminate one or the other of the Rap1p-Abf1p site clusters in a *urs1* mutant background. The proline values are decreased to a greater degree by the Rap1p-Abf1p site mutations than are the asparagine values.

Measurements made in both wild-type *URS1* and *urs1* mutant backgrounds yielded an observation that may be useful in studies of the mechanism through which *URS1* mediates repression of transcriptional activation. Regardless of the array of sites involved, *URS1* mediates strong repression; i.e., it does not exhibit detectable specificity in its repressive action. This correlation points toward the *URS1*-related transcription factors operating at the level of the general transcriptional machinery more than at the level of interaction with the *CAR1* transcriptional activation factors. The operation of so many *trans*-acting factors to support *CAR1* transcription will also no doubt ultimately influence our view of the interactions of the general transcriptional machinery with UAS-associated transcription factors. The pertinent question is how many of the possible 11 to 13 *trans*-acting factors that seem to function upstream of *CAR1* can simultaneously interact and function in conjunction with that machinery.

ACKNOWLEDGMENTS

We thank members of the UT Yeast Group who read the manuscript and offered suggestions for its improvement. Oligonucleotides used in these studies were prepared by the UT Molecular Resource Center.

This work was supported by Public Health Service grant GM-35642 awarded to T.G.C. by the National Institute of General Medical Sciences.

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