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The yeast *UME6* gene product is required for transcriptional repression mediated by the *CAR1 URS1* repressor binding site

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ABSTRACT

***URS1* is known to be a repressor binding site in *Saccharomyces cerevisiae* that negatively regulates expression of many genes including *CAR1* (arginase), several required for sporulation, mating type switching, inositol metabolism, and oxidative carbon metabolism. In addition to the proteins previously shown to directly bind to the *URS1* site, we show here that the *UME6* gene product is required for *URS1* to mediate repression of gene expression in the absence of inducer. We also show that mutations in the *CAR80* (*CARGRI*) gene are allelic to those in *UME6*.**

INTRODUCTION

Expression of the arginase (*CAR1*) gene in *Saccharomyces cerevisiae* is regulated by the opposing actions of positive and negative regulators (1–6). The promoter of this gene, whose expression is induced by arginine contains four functional elements: two inducer-independent UASs, UAS_{C1} and UAS_{C2}, an inducer-dependent UAS, UAS_I, and the negatively acting *URS1* element (7). UAS_{C1} consists of multiple ABF1 and RAP1 binding sites (8, 9), while UAS_{C2} is composed of several RAP1 sites, a GCR1 site, and an as yet unidentified transcription factor recognition site (9). The UAS_I element contains three homologous sequences two of which are required for minimum activity and all three for full activity (7, 10). The *ARG80* (*ARGRI*), *ARG81* (*ARGRII*) and *ARG82* (*ARGRIII*) gene products were shown by Wiame to be required for induced production of arginase activity (11), but whether they are required for operation of UAS_{C1}, UAS_{C2}, or UAS_I has not yet been reported. It has been reported that the former two gene products bind to a large DNA fragment derived from the *CAR1* promoter region (12, 13). *URS1* has been shown to be the binding site for a repressor protein (14). In the absence of inducer, the negative action of proteins binding to *URS1* maintains expression at a low level, essentially neutralizing the transcriptional activation capabilities of the inducer-independent UAS_{C1} and UAS_{C2} elements (7). The appearance of arginine in the cell, either as a result of its addition to the culture medium or release from the cell vacuole in response to nitrogen starvation, permits the inducer-dependent UAS_I to operate. The combined action of the three UAS elements then overcomes the negative action mediated by the *URS1* site and the proteins associated with it (7).

The *cis*-acting *URS1* element was originally identified by locating the sequence lesion of a *cis*-dominant mutation (*CAR1-0*$^-$, 11) that resulted in inducer-independent expression of the *CAR1* gene, i.e. loss of inducibility (2, 3). Saturation mutagenesis demonstrated the *URS1* element consisted of a symmetrical 9 bp sequence, AGCCGCCGA, that bound a specific protein(s) (14). Recently, the protein binding to this sequence has been purified to homogeneity (15). Through studies in many laboratories, it became apparent that sequences similar to *URS1* were present in many genes including, but not limited to, several required for sporulation (16, 17), mating type specification (18), heat shock response (19), oxidative metabolism (20), and inositol metabolism (21). In a number of cases, it was shown that deletion of the *URS1*-homologous sequence resulted in significantly increased expression of those genes (7, 21).

The presence of a common *cis*-acting element, *URS1*, in many unrelated genes raised the possibility that analogously common *trans*-acting factors might be associated with it. In 1971, Wiame's laboratory (11) identified a mutated locus (*car80* [*cargRI*]) which generated a phenotype similar to one that might be expected of a *trans*-acting factor associated with the *URS1* site. The *car80* mutation, which is unlinked to *CAR1*, possessed the same phenotype as *CAR1-0*$^-$, but was recessive (2, 11). Genetic studies of sporulation and mating type specification have similarly resulted in identification of mutations that exhibit phenotypes potentially expected of negatively-acting regulators (22–25). Among them are mutations in the *SIN3* = *SDI1* = *UME4* = *RPD1* (23, 24), *UME1*, *UME2*, *UME3*, *UME5*, and *UME6* loci (22, 25).

The purpose of this work was to determine whether mutations that generated phenotypes expected of negative *trans*-acting factors affected the transcriptional repression function mediated by the *URS1* site. We demonstrate that mutation of the *UME6* locus results in loss of *URS1* function and that a *car80* mutation is allelic with one at *ume6*. *UME6* = *CAR80* does not, however, encode the *CAR1 URS1* binding factor.

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MATERIALS AND METHODS

Strains, media, and culture conditions

The yeast and bacterial strains used in this work are listed in Table 1. Yeast cultures for beta-galactosidase assay were cultured in YNB (Difco) minimal medium. Glutamate or arginine was provided as nitrogen source at a final concentration of 0.1%, and supplements were added as described (26, 27). Rich media for yeast and *E. coli* transformation were YPD and LB, respectively (2). Presporulation and sporulation media were used as described elsewhere (26, 27). Culture conditions for growth were described by Sumrada and Cooper (2).

Plasmid constructions

Standard cloning procedures were performed according to Maniatis et al. (28). *CAR1 UAS-lacZ* and *CYC1 UAS-CAR1 URS1-lacZ* fusion plasmids whose replication origins are *ARS1* have been described earlier (3, 7, 14). In order to make *ARS1-CENIV* versions of above plasmids, we constructed expression vectors containing *ARS1-CENIV* elements (plasmids pHP41 and pHP81) as follows. The *Nde*I-*Xmn*I fragments containing the *ARS1* and *CENIV* elements were isolated from plasmid YCp50. It was substituted for the *Eco*RI fragment containing *TRP1* and *ARS1* of plasmid pNG15 (7) to yield plasmid pHP41. One of the two *Nco*I sites of plasmid pHP41 (the one downstream of the *lacZ* gene) was destroyed with partial *Nco*I digestion followed by Klenow treatment and blunt-end ligation. The *Bam*HI-*Nco*I fragment, containing the *CYC1* promoter region with the *CYC1 UAS* elements, of plasmid pNG22 (7, 14) was then exchanged for the *Bam*HI-*Nco*I fragment containing *CYC1* promoter region devoid of the *UAS* elements of plasmid pHP41 to yield plasmid pHP81. *ARS1-CENIV* versions of *CAR1-lacZ* fusion plasmids were constructed by substituting the *Bam*HI-*Sma*I fragments of the *CAR1-lacZ* fusion plasmids, which have *ARS1* replication origin for the *Bam*HI-*Sma*I fragment of plasmid pHP41. In order to make *ARS1-CENIV* versions of *CYC1 UAS-CAR1 URS1-lacZ* fusion plasmids, *Bam*HI and *Nco*I sites were used with the same way as above.

Yeast and bacterial transformation

Yeast strains were transformed using lithium acetate by the method of Ito et al (29). *E. coli* strain HB101 was transformed using the Tschumper and Carbon modification (30) of Mandel and Higa method (31).

Beta-galactosidase assay

Beta-galactosidase activities of yeast transformants were determined using yeast cells whose optical density (A_{600}) is 0.6 to 0.7 (Gilford Response Spectrophotometer) by the method of Guarente and Mason (32). Activities were expressed in units defined by Miller (33), but were based on 10 mls of culture rather than 1ml. Since many of plasmids used in this work have *ARS1* origin, we took the same precautions described earlier (7, 34) to avoid potential problems that might result from varying plasmid copy number. In addition, we also used *ARS1-CENIV* versions of plasmids containing inserts identical to those used in the *ARS1* versions. Although activities supported by the *ARS1-CENIV* versions were much lower than those supported by *ARS1* versions, the patterns of activities were, with one exception that is subsequently discussed, the same irrespective of the plasmid replication system present.

Table 1 Strains Used

Strain	Genotype
S. cerevisiae	
Y271	MATa, his4-519, leu2-3, 112, lys2, trp1-1, ura3, can1-100
Y270	MATa, his4-519, leu2-3, 112, lys2, trp1-1, ura3, can1-100, ume6::LEU2
RSY280	MATα, his4-519, leu2-3, 112, lys2, trp1-1, ura3, can1-100, ume6::LEU2
0231a	MATa, car80(cargRI)
TCY1	MATα, lys2, ura3
TCY15	MATa, lys5, ura3
HPY12	ura3; derivative of 0231a
DY150	MATa, his3-11,15, leu2-3,112, ade2-1, trp1-1, ura3-52, can1-100
DY984	MATa, his3-11,15, leu2-3,112, ade2-1, trp1-1, ura3-52, can1-100, sin3Δ::ADE2
HPY61	(MATα, his4-519, leu2-3, 112, lys2, trp1-1, ura3, can1-100, ume6::LEU2 / MATa, lys5, ura3)
HPY71	(MATα, his4-519, leu2-3, 112, lys2, trp1-1, ura3, can1-100, ume6::LEU2 / MATa, car80, ura3)
E. coli	
HB101	hsdS20(r⁻, m⁻), recA13, supE44, proA2, rpsL20 (Sm^r)

Sporulation test

It was reported that homozygous *ume6* diploid strains were sporulation-defective (25). To ascertain whether the *car80* and *ume6* mutations would complement one another for this trait, sporulation frequency of a *car80,ume6* and various heterozygous diploid strains were determined. Cells of opposite mating type from freshly grown colonies were mixed on a YPD plate. After allowing mating to occur overnight at 30°C, the mating mixture was streaked onto a selective plate and incubated for 3 days at 30°C. Single colonies were isolated from these plates and tested for sporulation (26, 27). After these cells were grown on sporulation media for 3 days, asci and total cells were counted. Sporulation frequency (%) was calculated as the number of sporulated cells per the number of total cells × 100.

Electrophoretic Mobility Shift Assay (EMSA)

The methods used for cell growth, preparation of crude cell extracts, and reaction mixtures for the EMSAs were as described by Luche et al. (14). The DNA fragment used for this assay was the recently described *CAR1* probe covering positions −161 to −133 (15).

RESULTS

Requirement of *UME6* product for *CAR1 URS1* function

To ascertain whether or not the *UME6* gene product was required for repression of *CAR1* expression in the absence of inducer, we transformed wild-type and *ume6* disruption mutant strains (Y271 and Y270, respectively) with wild-type and mutant *CAR1-lacZ* fusion plasmids. As shown in Figure 2, a plasmid containing the entire wild-type upstream region of *CAR1* (pRS46) supported reporter gene expression possessing a three-fold response to addition of arginine. This response to inducer is significantly below the ten-fold observed in wild-type strains (RH218 or E1278b) we normally use to study *CAR1* expression (6, 7). It is, unfortunately, characteristic of the wild-type used in previous studies of *UME6* product function by the investigators who identified the locus and hence used in the present experiments (25). The poor induction response in wild-type strain Y271 and others of its genetic background probably derives from the fact that it contains a mutation in the *CAN1* gene, whose product is one component of the arginine permease. The *can1* mutation results in a limited rate of arginine entry into the cell. From

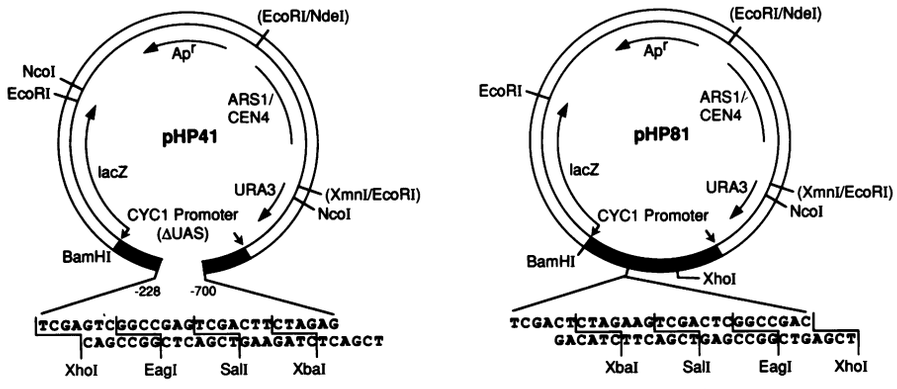

Figure 1. Expression vector plasmids (pHP41 and pHP81) used in this work. Plasmids pHP41 and pHP81 were constructed as described in Materials and Methods.

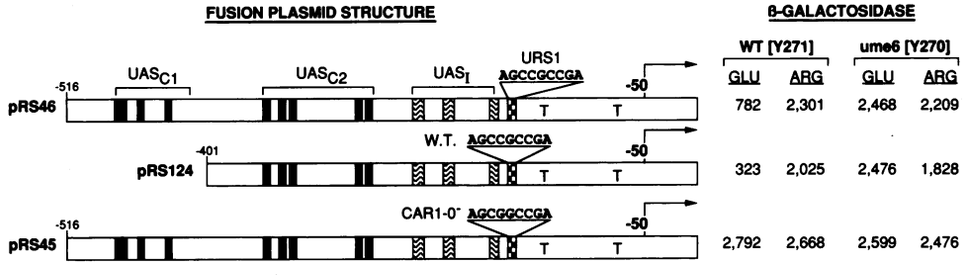

Figure 2. Beta-galactosidase production supported by plasmids containing *CAR1* upstream region in wild-type and *ume6* disruption mutant strains. Plasmids and areas designated have been described earlier (7). T's indicate the positions of TATA sequences. Numbers at the left of the plasmid inserts indicate the 5' termini of the *CAR1* upstream region in the *CAR1-lacZ* gene fusions relative to the translation start site. The arrow represents the start site and direction of *CAR1* transcription. GLU and ARG indicate the nitrogen sources used in the experiments, glutamate and arginine, respectively. Activities were expressed in Miller units (33) but were based on 10 ml of culture rather than 1 ml.

previously described experiments, we were aware that deleting *CAR1* UAS_{C1}, an inducer-independent *CAR1* UAS (plasmid pRS124) (7), would result in a more pronounced response to inducer by lowering the level of *CAR1* expression that occurred in the absence of inducer. This in turn would provide us a more sensitive assay of *CAR1 URS1* function in the genetic background used to generate the *ume6* disruptions. In agreement with this expectation, plasmid pRS124, which contains this deletion, supported a six-fold response to inducer in strain Y271 (Figure 2). Mutation of the *CAR1 URS1 cis*-acting element by a transversion mutation at position −153 (plasmid pRS45) provided a positive control to demonstrate how loss of *URS1* function effected *CAR1* expression in strain Y271, the isogenic parent of the *ume6* disruption mutant. As shown in Figure 2, a plasmid carrying this transversion mutation (plasmid pRS45) supported reporter gene expression in strain Y271 that was completely inducer-independent. When the above plasmids were used to transform a *ume6* disruption mutant strain (Y270), high level, inducer-independent reporter gene expression was observed with all of the plasmids (Figure 2). These data indicated that the *UME6* product was required to maintain *CAR1* expression at a low level in the absence of inducer, but did not identify the *cis*-acting element through which *UME6* product functioned. Two possibilities existed. *UME6* product might function at the level of the inducible *CAR1* UAS, UAS_I, and prevent its operation in the absence of arginine. In this case, loss of *UME6* product by gene disruption would be expected to permit UAS_I to activate transcription in the absence of inducer. Alternatively, *UME6* product might function in association with the *CAR1 URS1* element forming part of the complex repressing transcriptional activation by the *CAR1* UASs. In this case, loss of *UME6* product would be expected to result in loss of transcriptional repression mediated by *URS1*.

Our first attempt to distinguish these possibilities was made by determining the effects of *ume6* gene disruption on the abilities of plasmids containing only *CAR1* UAS_I and *URS1* to support inducible reporter gene expression (Figure 3). Plasmid pLK39, which contained wild-type alleles of both *CAR1* UAS_I and *URS1* was previously reported to support little B-galactosidase production even in a wild type strain (RH218) because the *URS1* element mediated far stronger negative regulation of transcription than the positive regulation mediated by UAS_I (7). As shown in Figure 3, little reporter gene product synthesis was supported by plasmid pLK39 in strain Y271 regardless of whether or not inducer was present. Similar results were observed whether the insert was carried on an *ARS1* (plasmid pLK39) or *ARS1-CENIV* vector (plasmid pHP43). The *ARS1* vector construction responded slightly more to inducer than did the *ARS1-CENIV* vector construction (Figure 3), but it is not known whether or not this difference is physiologically significant. As expected from previously reported results with our wild-type strain RH218 (7), removal of the *URS1* element from the insert of plasmid pLK39

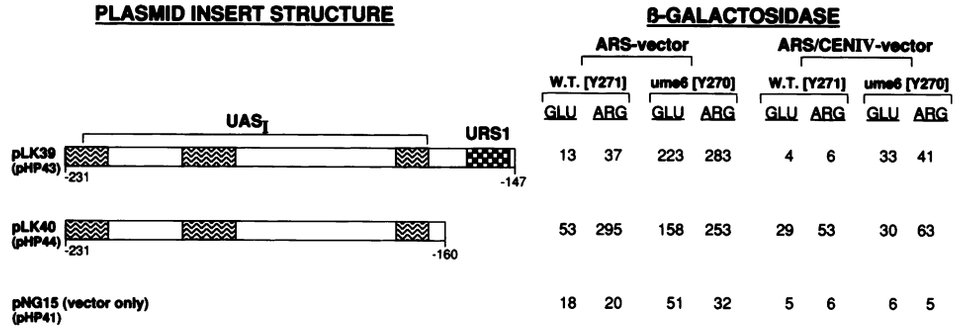

Figure 3. Beta-galactosidase production of wild-type and *ume6* mutant strains transformed with expression vector plasmids containing either the *CAR1 UAS$_I$* and *URS1*, or *CAR1 UAS$_I$* elements. Plasmid pLK39, pLK40 and pNG15 contain only an *ARS1* replication origin and have been described earlier (7). Plasmids pHP43 and pHP44 were constructed by substituting the *Sma*I-*Bam*H1 fragement (containing *CAR1 UAS$_I$-URS1* or *CAR1 UAS$_I$*) from pNG15-based plasmids pLK39 and pLK40 for the *Sma*I-*Bam*H1 fragement of plasmid pHP41 which has *ARS1* and *CENIV*. Throughout this work, plasmid numbers that appear within parentheses in the figures designate that these plasmids contain the *ARS1-CENIV* replication system. GLU and ARG indicate the nitrogen sources used in the experiments, glutamate and arginine, respectively. Activities were expressed in Miller units (33) but were based on 10 ml of culture rather than 1 ml.

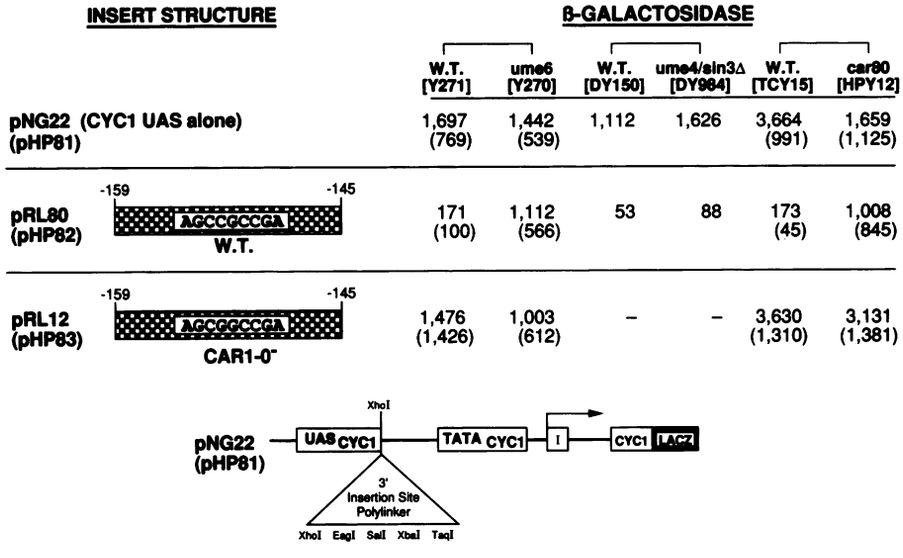

Figure 4. Reporter gene expression supported by the *CYC1 UAS* elements in the presence or absence of the *CAR1 URS1* element in wild-type and mutant strains. Pertinent structures of the parent expression vector plasmids, pNG22 and pHP81 are shown at the bottom of the figure. Sequences that were cloned into the 3' polylinker insertion site, and their *CAR1* coordinates are shown in the figure. Plasmids pRL80, pRL12 and pNG22 containing *ARS1* origin have been described earlier (14). Plasmids pHP82 and pHP83 were constructed by substituting the *Nco*I-*Bam*HI (containing the *CYC1 UAS*, and *CAR1 URS1* or *CAR1 URS1-0$^-$* elements) from pNG22-based plasmids for the *Nco*I-*Bam*HI fragement of plasmid pHP81 which has *ARS1* and *CENIV*. 0.1% arginine was used as nitrogen source. The strains used in each experiment are shown at the top of the figure. All experimental values enclosed within parentheses were derived from *ARS1-CENIV* plasmids. The numbers of these plasmids also appear in parentheses. Values obtained with *ARS1* plasmids are not enclosed within parentheses.

resulted in reacquisition of a response to inducer (Figure 3, plasmids pLK40 and pHP44 in strain Y271). As noted in Figure 2, the response to inducer was again modest (two to six-fold) in this strain. We noticed, however, that the response to inducer observed with the *ARS1* plasmid was again higher than that observed with the *ARS1-CENIV* plasmid just as observed with plasmids pLK39 and pHP43.

In *ume6* mutant strain Y270, plasmids pLK39 and pHP43 supported inducer-independent reporter gene expression. B-galactosidase production in the absence of arginine (GLU) was 17 and 8-fold higher, respectively, than seen in wild-type strain Y271. The *ume6* mutant transformed with plasmid pLK40 supported approximately the same levels of reporter gene expression in the presence of inducer as the wild-type. However, this plasmid in the *ume6* mutant supported three-fold more B-galactosidase production than wild-type when inducer was absent. The three-fold loss of inducer-dependence observed in a *ume6* disruption mutant transformed with the *ARS1*-containing plasmid (pLK40) was not observed when the *ARS1-CENIV* version (plasmid pHP44) was used to transform the same mutant. This loss of inducer response due to an elevated basal level was, however, observed when the *ARS1* vector control (plasmid pNG15) was used as the source of transforming DNA. Therefore, we do not consider these results physiologically significant. These observations suggested that, although disruption of the *UME6* gene had a small and questionable effect upon the inducer-

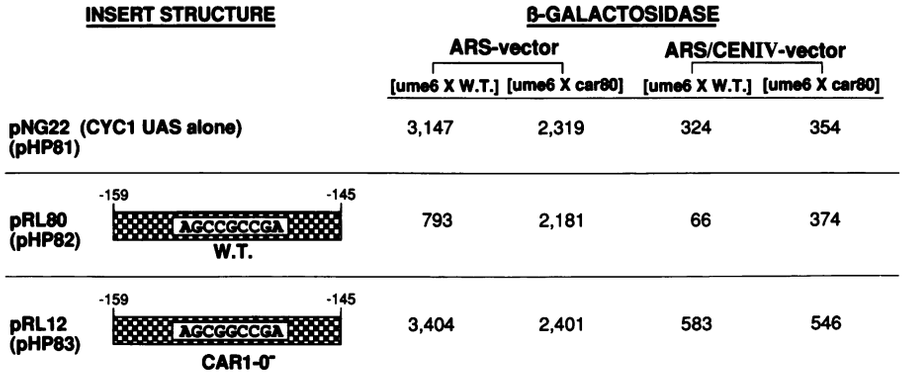

Figure 5. Reporter gene expression supported by the *CYC1 UAS* elements in the presence or absence of the wild type *CAR1* or *CAR1-0*$^-$ mutant *URS1* elements in diploid strains HPY61 and HPY71 constructed by crossing strains RSY280 to TCY15 and RSY280 to HPY12, respectively. Plasmids and nitrogen source used here are the same as those used in Fig. 4.

dependence of transcriptional activation mediated by *CAR1* UAS_I, the primary element through which *UME6* product functioned was *URS1*.

We more directly tested this suggestion by assaying *URS1* and *UME6* product function in the heterologous expression vector system originally used to define the *CAR1 URS1* element, i.e. the *CYC1-lacZ* fusion vector containing only the wild-type *URS1* element from the *CAR1* gene or a transversion mutant allele of it (*CAR1-0*$^-$) cloned 3′ to the *CYC1 UAS* elements (14). We used both of the previously described plasmids containing *ARS1* (plasmids pRL80 and pRL12) as well as identical versions containing *CENIV* (plasmids pHP82 and pHP83) in addition to *ARS1* to transform the wild-type and *ume6* disruption mutant strains. *CYC1 UAS*-mediated reporter gene expression was high in both wild type and *ume6* mutant strains carrying either *ARS1* or *ARS1-CENIV* plasmids (plasmid pNG22 and pHP81, Figure 4). When the wild type *URS1* fragment was cloned 3′ of the *CYC1 UAS* elements (plasmids pRL80 and pHP82) and these plasmids used to transform wild-type strain Y271, an eight to ten-fold decrease in *CYC1 UAS* activity was observed. In other words, *URS1* functioned normally in its negative control of the heterologous UAS and did so whether the plasmid carried an *ARS1* or *ARS1-CENIV* replication elements (plasmids pRL80 and pHP82). In the *ume6* mutant, on the other hand, no such decreases were observed (plasmids pRL80 and pHP82 in strain Y270, Figure 4). Similarly as expected, no down regulation of the heterologous UAS was observed when the *CAR1 URS1* transversion mutant (*CAR1-0*$^-$) was used in the control experiment (plasmids pRL12 and pHP83). When this experiment was repeated with a *sin3* (*ume4*) mutant only a modest effect on normal *URS1* operation was observed (Figure 4).

Requirement of *CAR80* (*CARGRI*) product for *CAR1 URS1* function

Wiame's laboratory isolated a mutant strain that produced arginase in an inducer-independent manner (11). The mutation in this strain (0231a) was in a locus designated *CAR80* (*CARGRI*) which was not linked to *CAR1* (11). We subsequently demonstrated that this strain contained steady state *CAR1* mRNA at fully induced levels even when inducer was absent (2). This was consistent with *CAR80* product exerting its regulation of arginase production at transcription (2). These observations prompted us to query whether or not *CAR80* was required for *URS1* function. This was done using the plasmids just described as the sources of DNA to transform wild type and *car80* mutant strains and testing their ability to support reporter gene expression. As shown in Figure 4, the *car80* mutation exhibited a phenotype that was very similar to that observed with the *ume6* disruption mutation. i.e. ability of the *CAR1 URS1* element to down regulate *CYC1 UAS*-mediated transcriptional activation was lost in the *car80* mutant strain.

Table 2 Complementation of ume6 Sporulation Defect by Wild Type and cargRI Mutations

MATα\MATa	W.T. (Y271)	ume6 (Y270)	W.T. (TCY15)	car80 (HPY12)
W.T. (TCY1)	36	38	40	36
ume6 (RSY280)	40	0	35	4

After diploid cells were grown on sporulation media for 3 days, sporulated cells were counted. Sporulation frequency (%) was calculated as the No. of sporulated cells per the No. of total cells x 100. Haploid strains used to construct the diploid strains are indicated in the table.

Assay of complementation between *car80* and *ume6* mutations

The similar phenotypes of the *car80* and *ume6* mutations prompted the question of whether or not they might be allelic. This information was particularly significant, because the *UME6* gene has been cloned and sequenced (25). The *ume6* disruption possessed two easily assayable characteristics: a decreased frequency of sporulation and loss of *CAR1 URS1* function. Therefore, we crossed wild-type and *car80* point mutant haploid strains to the *ume6* disruption mutant and sporulated the resulting diploids. As shown in Table 2, the wild type *CAR80* allele fully complemented the *ume6* disruption allele as far as the *ume6* sporulation phenotype was concerned. In contrast, the *car80* mutant allele was incapable of complemention, i.e. the *car80,ume6* diploid was sporulation deficient just as the *ume6* homozygous diploid. In a similar fashion, the wild-type *CAR80* allele effectively complemented the *ume6* mutation in the *URS1* functional assay described in Figure 4, whereas the *car80* mutation did not (Figure 5). The plasmids and experimental format used in this assay were identical to the experiment described in Figure 4; only the transformation recipient strains were different.

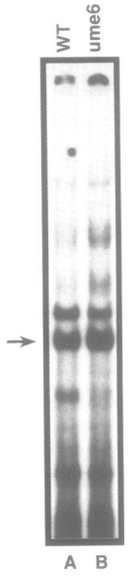

Figure 6. EMSA of protein extracts derived from wild type and *ume6* disruption mutant strains. The procedures used in this experiment are described in Methods. Thirty six micrograms of each extract were used. Reaction mixtures without protein extract did not contain any of the bands discussed in the text.

Does *UME6/CAR80* encode the *URS1* binding protein?

The requirement of *UME6* = *CAR80* product for *URS1* function raises the possibility that this locus might encode the *URS1* binding protein. We have recently purified this protein to homogeneity and found it to be heteromeric (15). To test the question of whether or not *UME6* = *CAR80* encodes one of the monomers of this heteromeric protein, we conducted EMSAs of a DNA fragment containing the *URS1* element using crude extracts derived from wild-type and the *ume6* disruption mutant strains. Extracts from both wild-type and *ume6* disruption mutant strains were capable of forming the same protein-DNA complex in EMSAs that was previously demonstrated to be the one to which the heteromeric *URS1* binding protein was bound (arrow, Figure 6). A complex below that of *URS1* and its heteromeric protein was observed to disappear in the *ume6* disruption mutant, but we do not, at present, have the reagents necessary to determine whether or not this higher mobility complex contains the *UME6* product. There was also a lower mobility complex observed in this experiment, but it was present regardless of whether wild type or mutant extract was used (Figure 6).

DISCUSSION

Data presented in this work demonstrate the *UME6* gene product, previously identified as being required for regulated expression of several sporulation-specific genes (25), is also required to maintain expression of the *CAR1* gene at a low level when inducer is absent. These observations support the idea that *UME6* is probably not a sporulation-specific regulatory gene, but most likely encodes a general transcription factor that participates in the negative transcriptional regulation mediated by the *URS1* binding site. If this conclusion is true, disruption of *UME6* will be expected to alter expression of many of the genes whose promoters contain sequences homologous to the *CAR1 URS1* element (14). Among these genes are those that participate in sporulation and mating type specification, genes encoding heat shock proteins, proteins required for oxidative metabolism, inositol metabolism, and glycolysis (16–21,35).

The above observations also indicate that *URS1*-mediated repression of *CAR1* transcription requires *trans*-acting elements in addition to the heteromeric protein that binds to the *URS1* site (15). The mechanisms involved in fulfilling these requirements, however, cannot be identified at present; several possibilities exist. The *UME6* product may form a protein-protein complex with the heteromeric *URS1* binding protein. Such a complex, if it exists, was not stable enough to be detected in our EMSAs of protein binding to *URS1* DNA. Another alternative, which is also untestable at the moment, is that *UME6* product may positively regulate functioning of the *URS1*-binding heteromer through a post-translational modification of the *URS1*-binding protein. A further possibility, which we do not favor, is that *UME6* product positively regulates expression of the genes encoding heteromeric *URS1* binding protein. If *UME6* product did so, we would have expected to see a loss of the *URS1*-heteromeric protein complex in the EMSA when the *ume6* disruption mutant extract was used for the source of protein. This was not observed experimentally.

Our results are most consistent with the suggestion that repression of *CAR1* transcriptional activation may be a more complicated process than steric hindrance such as might occur by binding a repressor protein to some operator sites in bacteria. The idea of a steric hindrance model generates the question of why *trans*-acting factors, in addition to the heteromeric protein which binds to the *URS1* site, are required for negative control. It might be suggested that the DNA-heteromeric protein complex is too small to accomplish the task. We do not favor this interpretation. We favor a model in which repression of transcriptional activation is more involved. If protein-protein interaction is important to *URS1*-mediated negative control of *CAR1* expression, transcriptional repression might occur because one or more proteins that bind to the heteromeric *URS1* binding protein also interact with some component of the UAS-associated proteins or components of the core transcriptional apparatus with which they interact. By this model, the heteromeric *URS1* binding protein carries specificity for the gene to be negatively regulated, while *UME6* product or another *trans*-acting factor carries specificity for the protein-protein interaction that occurs with the UAS or core transcriptional apparatus-associated proteins. This view of *URS1* binding protein function predicts that the *URS1* could be situated either 5′ or 3′ of the UAS sites. In most genes studied thus far, it is situated 3′ of the UAS. However, in the case of *GDH2* there is a *URS1* site situated 5′ of the UAS (36). Moreover, in our early characterization of the *URS1* site, we demonstrated that it would function, albeit less well, when placed over 400 bp upstream of the *CYC1 UAS* (Figure 5, ref. 6).

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