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A *cis*-Acting Element Present in Multiple Genes Serves as a Repressor Protein Binding Site for the Yeast *CAR1* Gene

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Induction of the arginase (*CAR1*) gene expression in *Saccharomyces cerevisiae* has previously been shown to require participation of a *cis*-dominantly regulated upstream repression sequence (URS). Deletion of this element results in high-level expression of the *CAR1* gene without inducer. To determine the structure of the *CAR1* URS element, we performed a saturation mutagenesis. Results of the mutagenic analysis indicated that the *CAR1* URS was a 9-base-pair palindromic sequence, 5′-AGCCGCCGA-3′. A DNA fragment containing this sequence was shown to bind one or more proteins by a gel shift assay. DNA fragments containing point mutations that completely eliminated URS function were not effective competitors in this assay, whereas those which supported URS function were effective competitors. Sequences in the 5′-flanking regions of 14 other genes were found to be homologous to the *CAR1* URS. These sequences were shown to support varying degrees of URS function in the expression vector assay, to bind protein as demonstrated by the gel shift assay, and to compete with a DNA fragment containing the *CAR1* URS for protein binding. These results indicate that the *CAR1* URS element possesses the characteristics of a repressor binding site. Further, they are consistent with the suggestion that sites homologous to the *CAR1* URS may be situated in the 5′-flanking regions of multiple unrelated yeast genes. The widespread occurrence of this element raises the possibility that it is the target site for one or more negatively acting general transcription factors.

Levels of arginase, the product of the *CAR1* gene in *Saccharomyces cerevisiae*, respond to both induction and nitrogen catabolite repression (5–8). In the presence of inducer (arginine or its analog, homoarginine), arginase is produced at high levels (5–8). In the absence of inducer, only low, basal levels of arginase production occur. By using the cloned *CAR1* gene as a probe, induction of *CAR1* was shown to be accompanied by a large increase in steady state *CAR1*-specific mRNA (12). This observation, and those indicating that sequences required for induction were situated outside of the transcribed region, led to the suggestion that induction was transcriptionally regulated (13–17; L. Kovari, I. Kovari, and T. G. Cooper, unpublished data).

Wiame isolated the first mutant with a defect in this induction process; the mutation was designated *CAR1-o*$^-$ (20). The mutant strain produced arginase at high levels in the absence of inducer. The *CAR1-o*$^-$ mutation was tightly linked to the *CAR1* gene and exhibited a *cis*-dominant phenotype (20). Isolation and sequence analysis of the mutant *CAR1-o*$^-$ allele demonstrated that it differed from the wild type by only a single base, a C-to-G transversion at position −153 (14). Deletion of a 13-base-pair (bp) fragment containing position −153 from the upstream region of the *CAR1* gene resulted in the same phenotype, *CAR1* expression in the absence of inducer (14–16). These two results suggested that nucleotide 153 was an important part of a negatively acting element that was responsible in some way for *CAR1* induction. This suggestion was supported by cloning a DNA fragment covering nucleotides −158 to −146 of *CAR1* 3′ to the *CYC1* upstream activation sequence (UAS) and finding that this insertion inhibited operation of the *CYC1* UAS (16). When a similar DNA fragment containing the C-to-G transversion at position −153 was used in place of the wild-type fragment, no inhibition was observed.

The information discussed above led to the hypothesis that a negatively acting site or upstream repression sequence (URS) is situated between positions −158 and −146. The purpose of this work was to define the boundaries and required internal positions of the putative URS site and to ascertain whether protein binding to the site could be demonstrated. The results reported here suggest that the *CAR1* URS consists of a 9-bp palindromic sequence, 5′-AGCCGC CGA-3′, which was observed to bind one or more proteins as assayed by a gel retardation assay. Related sequences found in 14 other genes were shown to function similarly in a heterologous expression vector system and to compete effectively with the *CAR1* URS for protein binding.

(Preliminary reports of this work have already appeared [R. Luche and T. G. Cooper, Abstr. Annu. Meet. Yeast Genet. Mol. Biol., Am. Soc. Genet. 1989, p. 138A].)

MATERIALS AND METHODS

Yeast and bacterial strains. Throughout this work, *S. cerevisiae* RH218 (*MATa trp1 CUP1 gal2 SUC2* Mal$^-$) was used as the transformation recipient. *Escherichia coli* HB101 (*hsdR hsdM recA13 supE44 lacZ24 leuB proA2 thi-1* Sm^r) was used for plasmid propagation and maintenance.

Growth media and transformation. Before transformation, strain RH218 was grown in YEPD medium (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 2% glucose) supplemented with 0.004% tryptophan (10). After transformation, cultures were grown on YNB plates (0.17% yeast nitrogen base, 0.5% Casamino Acids [Difco], 2% glucose, 2% agar). Top agar for the YNB plates contained 0.17% yeast nitrogen base, 0.5% Casamino Acids, 2% glucose, 0.1% ammonium sulfate, 22% sorbitol, and 3%

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TABLE 1. Plasmids used

Plasmid	Gene	Synthetic Fragment
pRLM1	CAR1	GGCCGGTAGCCGCCGAGGG CCATCGGCGGCTCCCAGCT
pRL57	CAR1	GGCCGCCTCGGCGGCTACG CGGAGCCGCCGATGCAGCT
pRL59	ENO1	GGCCGTCCTAGCCACCTCAAGG CAGGATCGGTGGAGTTCCAGCT
pRL62	CTT1	GGCCGCTCTGGCTGCAGGCTAG CGAGACCGACGTCCGATCAGCT
pRL64	CYB2	GGCCGCAAGAACCGCCAAGAACG CGTTCTTGGCGGTTCTTGCAGCT
pRL65	HSE2	GGCCGTCCATCGGCGGCAAAAG CAGGTAGCCGCCGTTTTCAGCT
pRL66	GAL-UASg	GGCCGTAGAAGCCGCCGAGCGG CATCTTCGGCGGCTCGCCAGCT
pRL67	CTA1	GGCCGAATTAGCCGCGCAAGTTG CTTAATCGGCGCGTTCAACAGCT
pRL68	MES1	GGCCGTGTTAGCCGCCGAAACG CACAATCGGCGGCTTTGCAGCT
pRL70	HSF1	GGCCGTTGGAGCCGCCAAAAAAG CAACCTCGGCGGTTTTTTCAGCT
pRL71	TOP1	GGCCGCTCTAGCCGCCGACGACG CGAGATCGGCGGCTGCTGCAGCT
pRL73	CAR2	GGCCGGCTAGCCGCCGACGCCG CCGATCGGCGGCTGCGGCAGCT
pRL74	G3PD4	GGCCGTATCCCTGCGCGGCTAAG CATAGGGACGCGCCGATTCAGCT
pRL75	PYK1	GGCCGAAATAGCCGCCATGACCG CTTTATCGGCGGTACTGGCAGCT
pRL77	ILV2	GGCCGCCTAGCCGCCGGAGCCG CGGATCGGCGGCCTCGGCAGCT
pRL78	ARG4	GGCCGTCTTGTGGTGGTTACTCG CAGAACACCACCAATGAGCAGCT

Noble agar. Cultures used for β-galactosidase assay were grown in a medium containing 0.17% yeast nitrogen base with 2% glucose and 0.1% asparagine (final concentrations). Strain HB101 was grown in LB medium (0.5% yeast extract, 0.5% $NaCl_2$, 1% NZ-amine, 50 mg of thymine per liter, 5 mg of thiamine per liter) or, for plasmid amplification, in M-9 medium (0.7% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% $NaCl_2$, 1% NH_4Cl_2, 1.2 mM $MgSO_4$, 5 mg of thiamine per liter, 50 mg of thymine per liter, 0.4% $CaCl_2$, 0.5% Casamino Acids, 2% glucose) for plasmid amplification. Transformed cultures of strain HB101 were grown in the presence of 200 mg of ampicillin (Sigma grade) per liter.

Oligonucleotide synthesis. Oligonucleotides were synthesized with a model 380B DNA synthesizer (Applied Biosystems, Foster City, Calif.). Oligonucleotides were purified by gel electrophoresis.

Plasmid construction. The vectors used were plasmids pNG17 and pNG22. These plasmids differ only by the presence (plasmid pNG22) or absence (plasmid pNG17) of the *Xho*I fragment containing the *CYC1* UAS. Plasmids pRL1 to -78 were constructed by using synthetic oligonucleotides (Table 1) ligated into the *Eag*I and *Sal*I sites of either plasmid pNG17 or plasmid pNG22. Verification of all plasmid insert structures, including sites used for cloning purposes, was obtained by dideoxy sequencing (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio) (18).

β-Galactosidase Assay. β-Galactosidase activities in yeast transformants were determined by the method of Guarente and Mason (3), using cultures grown in YNB-asparagine medium. Units are those of Miller (9), but for 25 ml of cells rather than 1 ml. Since all of the plasmids used in this work contained an autonomously replicating sequence, we took precautions to avoid problems that might result from varying copy number. All of the plasmids used in a given figure or table were transformed into the same sample of host cells. Random transformants were used as soon as they were large enough to serve as inocula. These inocula were grown up and assayed immediately; transformants were never subcultured or stored. Each experiment was repeated at least twice. All assays were performed in duplicate. The absolute values obtained from the assays varied somewhat, but the patterns of activity, including the subtle ones, observed from one construction to another were invariant. The data from repeated experiments generally varied less than 15%. Data from duplicate assays generally varied less than 5%. Occasionally, spurious values were obtained that differed by more than 30% from results of repeated experiments; in these cases, the spurious values could not be repeated and were ignored. The plasmids in Fig. 3 and Table 2 were divided into groups of manageable size and assayed by the methods described above. The data derived from these groups of plasmids were then normalized against that obtained with plasmid pNG22, which was included as a control with all groups of plasmids assayed.

Preparation of yeast protein extracts for DNA-binding assays. The methods used for preparation of protein extracts were those of the laboratory of L. Guarente (personal communication). Cultures of strain RH218 (200 ml) were grown to a cell density of A_{600} = 1.0 in liquid YEPD medium (Response spectrophotometer; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The cells were harvested by centrifugation and suspended in 400 μl of extraction buffer containing 200 mM Tris (pH 8.0), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% (vol/vol) glycerol, 7 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The cells were broken by vortexing with two-thirds the extract volume of glass beads eight times at 1 min each time, with 1 min of cooling on ice in between. After standing for 30 min in ice, the glass beads were removed by centrifugation (5 min at full speed in an Eppendorf centrifuge). The supernatant solution was then clarified by centrifugation at 4°C for 1 h in an Eppendorf centrifuge operating at full speed. After ammonium sulfate precipitation (50% saturation achieved by adding an equivalent volume of saturated solution to the soluble supernatant), the pellet was suspended in 300 μl of buffer containing 20 mM HEPES, 5 mM EDTA (pH 8.0), 7 mM β-mercaptoethanol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The resulting protein concentration (approximately 25 mg/ml) was determined by the method of Bradford (1).

Protein-DNA binding. Protein-DNA binding was carried out in a total volume of 20 μl. The reaction mixture contained (at final concentrations) 4 mM $MgCl_2$, 40 mM NaCl, and 4 mM Tris (pH 8.0), 60 to 100 ng of a γ-^{32}P-end-labeled DNA fragment, a 100- to 200-fold excess (8 to 12 μg) of heavily sonicated calf thymus DNA, and 1 μl of protein extract prepared as described above. The binding mixture was incubated at room temperature for 20 min and then loaded onto a 5%, preelectrophoresed polyacrylamide gel. The gel was prepared and run in 1× TBE for approximately 3.5 h at 10 V/cm at room temperature. Variation in the time

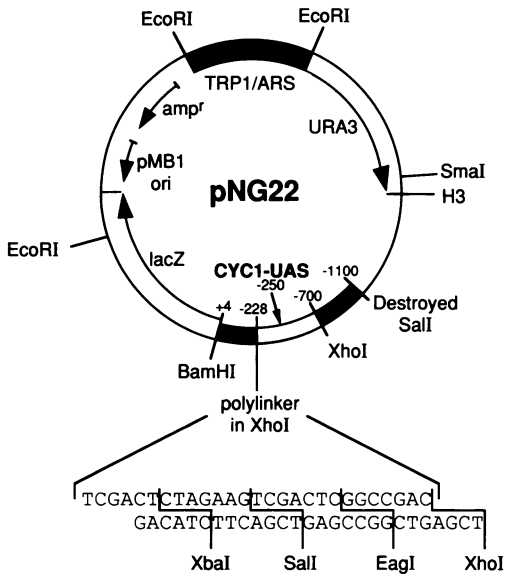


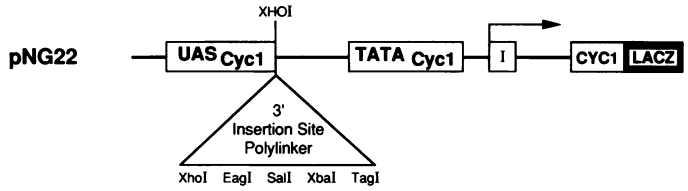
FIG. 1. Expression vector plasmid used in this work. This plasmid was derived from plasmids pGS13 and pHY100 (21).

of electrophoresis accounts for the different mobilities observed in Fig. 8. After electrophoresis, the gels were dried onto 3MM paper (Whatman, Inc., Clifton, N.J.) and used to expose radiographic film.

RESULTS

Localization of the *CAR1* URS. Previously reported experiments localized the *CAR1* URS to a DNA fragment covering positions −158 to −146 of the *CAR1* 5′-flanking region (16). In that study, we used a *CYC1-lacZ*-containing vector to assay URS function. Cloning a URS-containing fragment into either the *Xho*I site or a short polylinker engineered into the *Xho*I site 3′ of the *CYC1* UAS resulted in marked loss of β-galactosidase production (16). We used the same assay system for URS function throughout this work (vector in Fig. 1). On the basis of earlier results (16), we decided to use position −158 as the 5′ terminus of the DNA fragment to be mutagenized. However, these studies did not identify the 3′ terminus of the URS element. To generate this information, we constructed and analyzed four deletion plasmids (Fig. 2). The vector without a synthetic DNA fragment in the polylinker site supported 20 U of β-galactosidase production. When a DNA fragment containing positions −164 to −133 (plasmid pRL58) was cloned into the polylinker, we observed a 12-fold inhibition of *CYC1* UAS function (Fig. 2). The next DNA fragment, covering positions −159 to −144 (plasmid pRL1), supported similar inhibition as did one covering positions −159 to −146 (plasmid pRL2) (Fig. 2). Deletion of the next 3 bp (plasmid pRL3 covering positions −159 to −149) resulted in partial loss of the ability of the DNA fragment to mediate repression of *CYC1* UAS function. Finally, a DNA fragment spanning positions −159 to −152 was largely inactive (plasmid pRL4; Fig. 2). Using these data as a guide, we chose a DNA fragment (plasmid pRLM1) covering positions −158 to −145 for detailed genetic analysis. (This fragment is slightly larger than necessary for URS function.) This was done so that alterations flanking the putative element could be used as negative controls; it was our expectation that mutating these bases would have no effect on URS-mediated function.

Saturation mutagenesis of the *CAR1* URS. To identify the



Plasmid	Inserted Sequences	β-Galactosidase Activity
pNG22	None	207
pRL58	-164 GGCCGTTAGCGGTAGCCGCCGAGGGGTCTAAAGAGTAG -133 CAATCGCCATCGGCGGCTCCCCAGATTTCTCATCAGCT	17
pRL1	-159 GGCCGGTAGCCGCCGAGGGG -144 CCATCGGCGGCTCCCCAGCT	14
pRL2	-159 GGCCGGTAGCCGCCGAGG -146 CCATCGGCGGCTCCAGCT	11
pRL3	-159 GGCCGGTAGCCGCCGG -149 CCATCGGCGGCCAGCT	50
pRL4	-159 GGCCGGTAGCCGG -152 CCATCGGCCAGCT	156

FIG. 2. 3′ deletion analysis of a DNA fragment containing the *CAR1* URS element. The synthetic oligonucleotides indicated were cloned into plasmid pNG22. The resulting plasmids were then used to transform yeast strain RH218. The transformants were grown and assayed for β-galactosidase activity as described in Materials and Methods.

Base Substitutions	-158 5'-G	T	A	G	C	C	G	C	C	G	A	G	G-3'
	Wild-Type URS1 CAR1 Sequence												
T	13	13	15	**121**	13	**148**	140	200	22	30	56	10	20
C	**13**	26	123	48	13	13	279	13	13	**13**	120	13	12
A	16	20	13	36	106	126	**309**	80	**104**	22	13	**16**	16
G	13	26	104	13	73	2500	13	135	23	13	48	13	13

FIG. 3. Effect of point mutations on the function of the *CAR1* URS. Double-stranded synthetic oligonucleotides were prepared in which the sequence contained in plasmid pRLM1 was altered one base at a time to the three alternative bases at each position. The DNA fragments were cloned into plasmid pNG22, and the resulting DNA was used to transform yeast strain RH218. Transformants were grown in minimal glucose-asparagine medium and assayed for β-galactosidase activity as described in Materials and Methods. All values reported were normalized to the value observed for parental plasmid, pNG22 (200 U). The sequence across the top is the wild-type sequence. Substitutions appear at the left. The values shown were those observed for plasmids that possessed the indicated substitution at the indicated position. Values presented in boldface type were derived from plasmids that were also analyzed by gel shift analysis.

specific bases required for *CAR1* URS function, we synthesized a set of double-stranded DNA fragments covering positions −158 to −146 and altered one base at a time. Each base was mutated sequentially to the remaining three possible nucleotides. The 52 DNA fragments were individually cloned into the polylinker site of plasmid pNG22. After verification of each construction by DNA sequence analysis, the plasmids were used to transform wild-type yeast strain RH218. The transformants were grown in minimal glucose-asparagine medium and assayed for β-galactosidase activity. Mutation of bases at positions −158, −157, −149, −147, and −146 had a minimal effect on the ability of the DNA fragment to serve as a URS regardless of the substitution made (Fig. 3). Full URS function yielded 13 U of β-galactosidase activity, compared with 200 U when the URS element was deleted. Mutation of the remaining positions had a marked effect on the ability of the DNA fragment to serve as a URS. Substitution of cytosine or guanine for the normally occurring adenine at position −156 resulted in approximately 50% loss of URS function. However, substituting thymine for adenine had no effect. This was the first of several instances in which a mutant fragment with a less conservative substitution (a transversion) was functional, whereas one with the conservative substitution (a transition) was not. These mutations identified the 5′ extremity of the URS element. Mutation of the normally occurring adenine at position −148 to thymine or guanine at this position had a modest but demonstrable effect on URS activity. However, mutation to a cytosine resulted in 60% loss of function. These mutations identified the 3′ extremity of the URS element. Substitution of cytosine or adenine for the normally occurring guanine at position −155 resulted in only modest loss of URS function (36 to 48 U, compared with 13 U for the wild type). However, substituting thymine resulted in a 50% loss of function. A pyrimidine nucleotide was the only requirement at position −154; both cytosine and thymine at this position supported full URS function. Purine nucleotide substitutions again resulted in an approximately 50% loss of function. In contrast, position −153 displayed high specificity; any mutation at this position had high impact on the ability of the DNA fragment to serve as a URS. In fact, a C-to-G transversion at this position resulted not only in loss of URS function but in a 10-fold increase in β-galactosidase production. This high level of *lacZ* expression was also observed when the DNA fragment containing the C-to-G transversion at position −153 was cloned into an expression vector that did not contain the *CYC1* UAS (R. Luche and T. G. Cooper, unpublished data). We therefore concluded that β-galactosidase production supported by the mutant DNA fragment derived from artifactual generation of a UAS by the C-to-G transversion. A similar conclusion was reported earlier for analysis of the *CAR1-o*$^-$ mutation (16). Increased β-galactosidase production was shown to result from generation of a UAS by this transversion (16). Similar high specificity was observed for the guanine at position −152. Any mutation here resulted in major loss of URS function. At position −151 only an adenine could be substituted, and then only with 40% loss of URS function. The other substitutions resulted in even greater losses of function. At position −150, any nucleotide except adenine generated a wild-type phenotype.

The sequence identified by this mutant analysis as required for inhibition of the heterologous *CYC1* UAS was 5′-AGCCGCCGA-3′, which is a perfect palindrome. The symmetry of the sequence can also be recognized in a roughly symmetrical response of each position to mutation. For example, the two cytosines at positions −153 and −151 were highly specific. The two cytosines flanking them, positions −154 and −150, exhibited much lower specificity, with an adenine substitution generating the strongest mutant phenotype. A crudely similar argument can be made for mutation of the remaining positions in the sequence. Whether this symmetry of response to mutation of the element has any significance was not ascertained.

Protein binding a DNA fragment containing the *CAR1* URS. The genetic analysis presented above was based on an indirect assay of *CAR1* URS function, i.e., its ability to inhibit *CYC1* UAS function when cloned downstream of it. If the sequence designated *CAR1* URS is a repressor binding site, it would be expected to bind one or more proteins. This was tested by incubating a radioactive 19-bp DNA fragment, covering positions −158 to −146, in the presence or absence of a crude yeast cell extract to see whether its mobility was altered by components of the extract. In the absence of cell extract, the radioactive DNA migrated to its expected position at the bottom of the gel (Fig. 4, lane A). When yeast cell extract was added to the radioactive DNA before gel electrophoresis, a new species was observed that migrated much more slowly than expected of the DNA fragment alone (lane

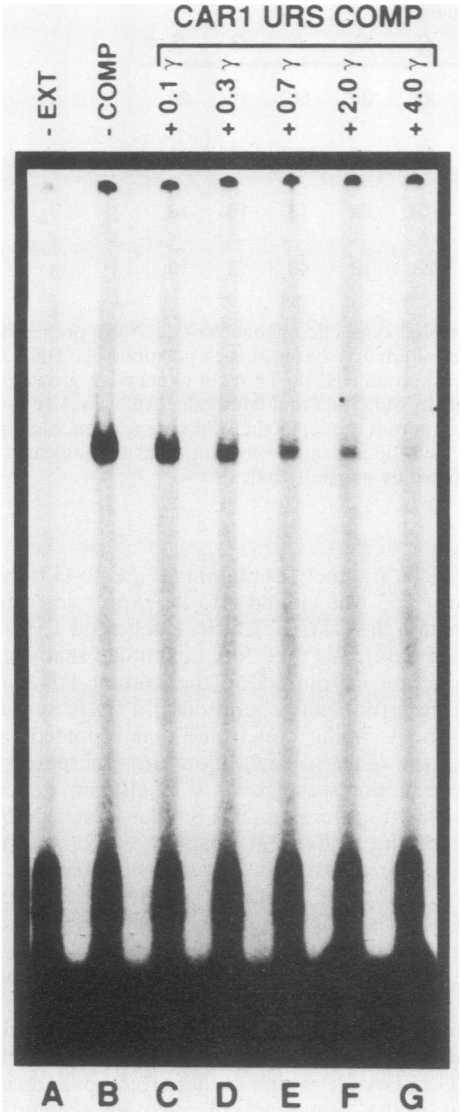


FIG. 4. Protein binding to a DNA fragment containing the *CAR1* URS element. A ^{32}P-labeled 19-bp oligonucleotide (100 ng) covering positions −158 to −146 and flanked by *Eag*I and *Sal*I restriction sites was used as a probe. Cell extract was omitted from the reaction mixture resolved in lane A. Competitor DNA was omitted the reaction mixture resolved in lane B. The reaction mixtures resolved in lanes C to G contained increasing amounts of the unlabeled 19-bp oligonucleotide as specific competitor. All reaction mixtures (see Materials and Methods) contained a 100-fold excess calf thymus DNA as nonspecific competitor. Complexes were resolved on a 5% nondenaturing acrylamide gel at 200 V for approximately 3.5 h as described in Materials and Methods.

B). This species could be abolished by increasing amounts of a nonradioactive DNA fragment containing the *CAR1* URS (lanes C to G). This experiment was repeated with a DNA fragment convering positions −164 to −133. The results were the same; i.e., nonradioactive competitor DNA effectively abolished the radioactive species (data not shown). This result was consistent with the suggestion that one or more proteins bound to a DNA fragment covering positions −158 to −146 of the *CAR1* 5′-flanking region.

Protein binding to a URS-containing fragment (Fig. 4) prompted the question of whether binding could be corre-

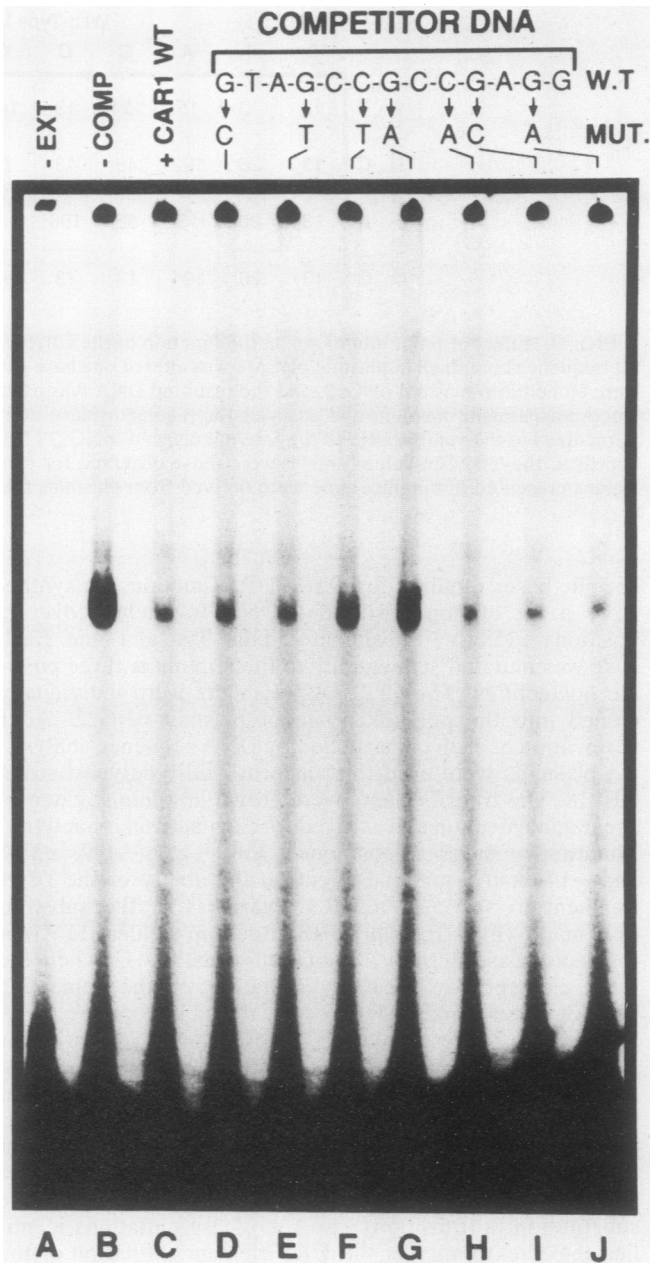


FIG. 5. Effects of point mutations on the ability of a DNA fragment containing the *CAR1* URS to form protein-DNA complexes. A ^{32}P-labeled (prepared by using the polynucleotide kinase reaction) 38-bp oligonucleotide (100 ng per reaction mixture) covering positions −164 to −133 of the *CAR1* 5′-flanking region and flanked by *Eag*I and *Sal*I restriction sites was used as a probe. Binding of the radioactive DNA was competed for by approximately a 20-fold molar excess (1 μg per reaction mixture) of an unlabeled DNA fragment that contained point mutations in the *CAR1* URS (lanes D to J). Detailed reaction conditions are described in Materials and Methods. Lanes: A, results obtained with a reaction mixture that did not contain cell extract; B, results obtained for the labeled *CAR1* URS-containing fragment in the absence of competitor; C, results obtained when approximately a 20-fold molar excess of an unlabeled 19-bp oligonucleotide covering positions −158 to −146 of the *CAR1* upstream region was included in the reaction mixture; D to J, results obtained with unlabeled 22- to 23-bp oligonucleotides containing point mutations at positions indicated at the top. These results correspond to the data presented in boldface type in Fig. 3.

TABLE 2. Inhibition of UAS function mediated by yeast gene promoter fragments with homology to the *CAR1* URS[a]

Plasmid	Gene	Sequence with Putative Homology to the CAR1 URS	ß-Galactosidase Activity
pRL2	CAR1	-158 5'- CGGT **AGCCGCCGA** GGTC -3' -146	13
pRL68	MES1	-248 TGTT **AGCCGCCGA** AACG -232	12
pRL65	HSE2	-174 CTTT **TGCCGCCGA** TGGA -190	13
pRL71	TOP1	-451 CTCT **AGCCGCCGA** CGAC -435	13
pRL75	PYK1	-864 AAAT **AGCCGCCAT** GACC -848	13
pRL67	CTA1	-247 AATT **AGCCGCGCA** AGTT -231	28
pRL74	G3PDH	-586 ACTT **AGCCGCGCA** GGGATA -602	28
pRL73	CAR2	-180 GGCT **AGCCGCCGA** CGCC -164	35
pRL77	ILV2	-484 GCCT **AGCCGCCGG** AGCC -468	46
pRL64	CYB2	-169 CAAG **AACCGCCAA** GAAC -153	47
pRL78	ARG4	-201 GAGT **AACCACCAC** AAGA -217	69
pRL59	ENO1	-218 TCCT **AGCCACCTC** AAGG -164	74
pRL62	CTT1	-222 GACT **AGCCTGCAG** CCAGAG -236	107
pRL66	GAL-UASg	-233 TAGA **AGCCGCCGA** GCGG -217	112
pRL70	HSF1	-615 TTGG **AGCCGCCAA** AAAA -599	112
pNG22		—	200

[a] Yeast promoter fragments with homology to the *CAR1* URS were cloned into the polylinker of expression vector plasmid pNG22. The resulting plasmids were transformed into yeast strain RH218. Transformants were assayed for β-galactosidase activity as described in Materials and Methods. The levels of activity observed in two different experiments were normalized to the value observed with the parental plasmid, pNG22 (200 U). The core sequences of these promoter fragments are aligned to the 9-bp core of the *CAR1* URS. Letters in small print represent linker bases. Sequences present within the genome in reverse orientation relative to that presented in the table are indicated by reversing the coordinates; i.e., the fragment appears in the table with the 3' terminus at the left.

lated with the ability of the DNA fragment to function. We made this determination by using seven of the mutant DNA fragments that had been cloned into the *CYC1-lacZ* vector as competitor DNAs in a competition experiment similar to the one shown in Fig. 4. The mutant DNA fragments tested were those that supported β-galactosidase activities in Fig. 3 (indicated by boldface values); i.e., they possessed mutations at positions −158, −155, −153, −152, −150, −149, and −147, respectively. Five of the seven mutant fragments competed as effectively as wild-type DNA (Fig. 5; compare lanes D, E, and H to J with lanes B and C). Abilities of the other two mutant fragments (those with mutations at positions −153 and −152) to effectively compete with the wild-type DNA fragment were markedly decreased even though they were present at approximately a 20-fold molar excess relative to the wild-type fragment (Fig. 5; compare lanes F and G with lanes B and C). This result suggested that for mutations with the strongest phenotypes in the indirect expression assay, there was a demonstrable correlation with their loss of ability to act as successful competitors. We interpreted this result as being consistent with the suggestion that these two mutant fragments were less well able to bind the protein(s) responsible for shifting the mobility of the radioactive DNA species.

Appearance of sequences with homology to the *CAR1* URS in multiple yeast genes. When the *CAR1* URS was first identified, a search of genomic sequence data banks revealed that sequences with homology to *CAR1* URS were present in the 5'-flanking regions of multiple other genes as well (16). The mere presence of homology between these short DNA sequences and the CAR1 URS does not address the question of whether they are functional. Therefore, we tested the ability of these DNA fragments, derived from the promoter regions of other yeast genes, to serve as URS elements in the *CYC1-lacZ* expression assay system used for our mutational analysis. Of the 14 sequences assayed, four were found to be just as effective as the *CAR1* URS at inhibiting *CYC1* UAS-mediated transcriptional activation (plasmids pRL68, pRL65, pRL71, and pRL75; Table 2). The remaining 10 sequences were effective in the range of 50 to 90%. To ascertain whether these 14 DNA fragments were also capable of binding protein, each was radioactively labeled by

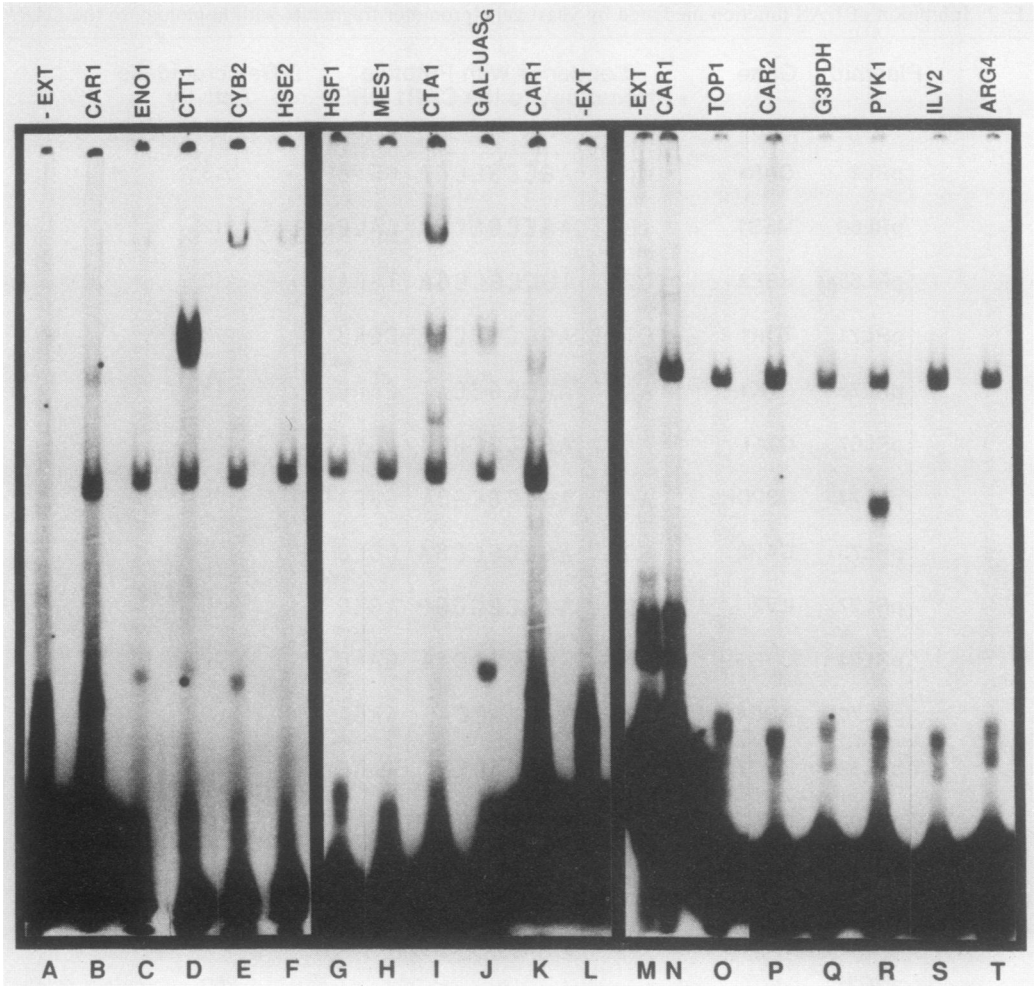


FIG. 6. Retardation of DNA fragments derived from the 5'-flanking regions of other yeast genes and possessing homology with the *CAR1* URS. The three panels represent data obtained from three different acrylamide gels that were used for the analysis. Specific structures of the labeled DNA fragments may be found in Tables 1 and 2; they were all of similar length (22 or 23 bp) and were flanked by the same restriction sites. The fragments used were those cloned into the plasmids analyzed in Table 2. The genes from which the labeled DNA fragments were derived appear above the lanes. Lanes D, E, I, J, K, and R contained additional complexes (see text). The 38-bp DNA fragment containing the *CAR1* URS was included in all three panels to facilitate comparison. Reaction conditions are described in Materials and Methods.

means of the polynucleotide kinase reaction and incubated with a crude yeast extract before electrophoresis. All 14 DNA fragments exhibited one or more retarded species, and all 14 possessed a species with a mobility similar to that observed with the *CAR1* URS (Fig. 6, lanes B, K, and N). Multiple retarded species were noted with several of the DNA probes (lanes D, E, I, J, K, and R). These species were not observed if a blunt-ended DNA probe was used in place of one with 5' extensions (data not shown). The species observed with the *CAR1* probe and species with the same mobility observed with other DNA probes were not affected by removing 5' extensions from the probes. All 14 DNA fragments were also found to be effective competitors of a radioactive DNA fragment containing the *CAR1* URS for protein binding (Fig. 7).

The competition experiments just described used a 14- to 22-fold molar excess of competitor DNA and thus might be subject to some ambiguity. Therefore, we carried out a more thorough set of competition experiments. DNA fragments, containing homology to the sequence 5'-AGCCGCCGA-3', from either the *CTT1* or *CYB2* gene were effective competitors of the *CAR1* URS (species A; Fig. 8A and B). Species B was not observed with a blunt-ended DNA probe (data not shown). Similarly, DNA fragments derived from the *CTT1* promoter and *CAR1* URS effectively competed with a labeled fragment from the *CYB2* promoter (species A; Fig. 8C and D), and DNA fragments from the *CYB2* promoter and *CAR1* URS were effective competitors of a DNA fragment from the *CTT1* promoter (species A; Fig. 8E and F). A similar set of competition experiments were performed with DNA fragments derived from the *CAR1* and *CTA1* genes (data not shown). Again, the DNA fragments competed with one another as described above.

DISCUSSION

Data presented in this work demonstrated that a DNA fragment covering positions −158 to −146 upstream of the *CAR1* coding region exhibits two characteristics expected of a repressor binding site. First, the DNA fragment was shown to mediate inhibition of the operation of a heterologous UAS

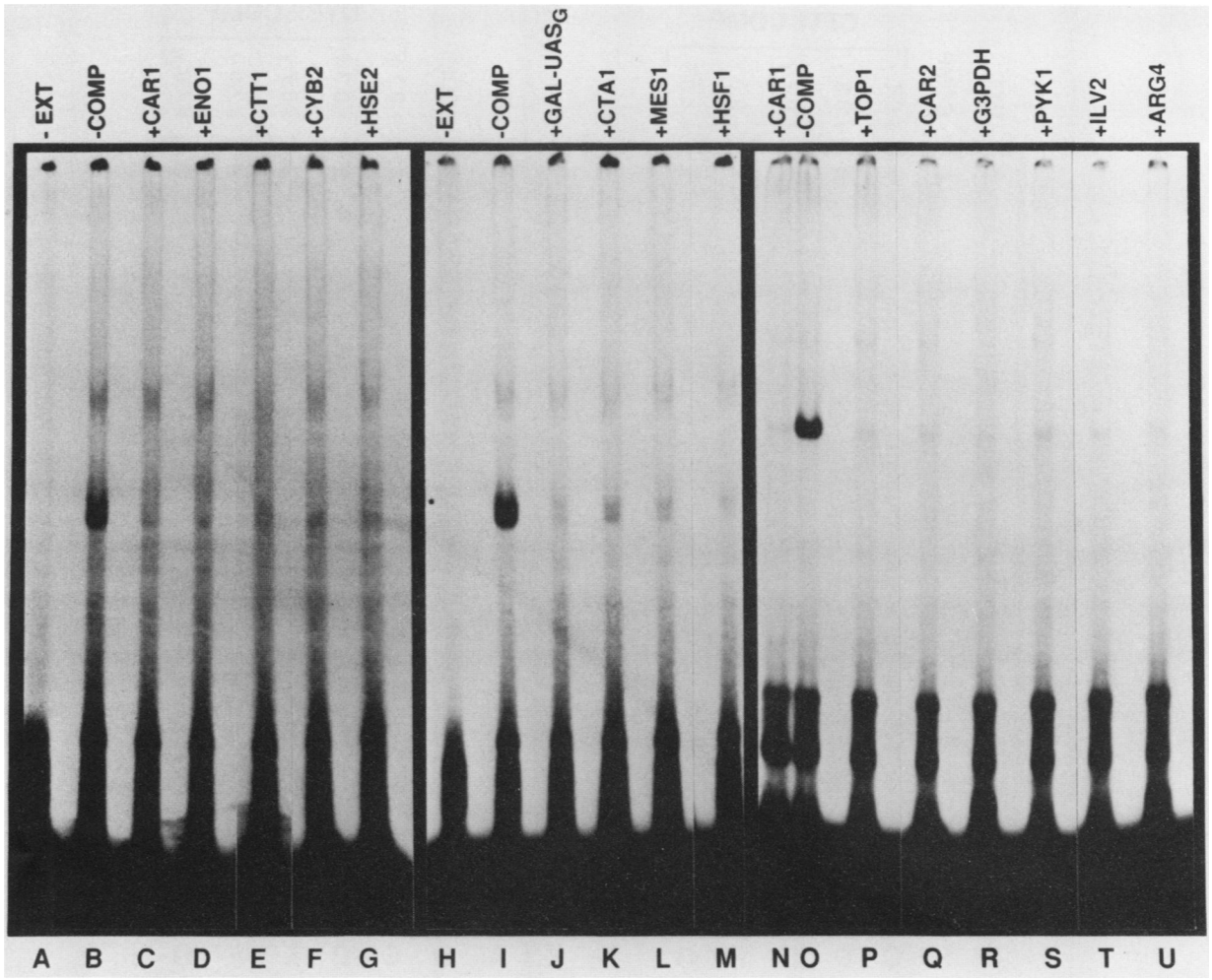


FIG. 7. Competition analysis using a labeled DNA fragment containing the *CAR1* URS competed with DNA fragments containing homology to the *CAR1* URS and derived from other yeast genes. In all three panels, 60 ng of a 32-bp ^{32}P-labeled oligonucleotide covering positions −164 to −133 of the *CAR1* 5′-flanking region was used as a probe. A 165-fold excess (by weight) of calf thymus DNA and 22-fold molar excess of competitor DNA were added to each reaction mixture; accuracy of the values cited is limited by the accuracy of the spectrophotometric assays of DNA concentrations. The reaction mixtures resolved in lanes A and H contained with no cell protein. The reaction mixtures resolved in lanes B, I, and O did not contain competitor DNA. All other lanes contained unlabeled competitor DNA. The identities of the competitor DNA fragments are indicated above the lanes. Their structures are as indicated in Table 2.

element. Inhibition of homologous UAS elements has also been demonstrated (L. Kovari and T. G. Cooper, unpublished data). Second, the DNA fragment was shown to bind one or more proteins, as demonstrated by gel retardation assays. Protein binding was competed for by wild-type DNA fragments containing positions −158 to −146 of *CAR1*, but this ability was lost in two *CAR1* mutant DNA fragments. These fragments were also shown to be incapable of mediating inhibition of *CYC1* UAS function.

The sequence identified to be required for *CAR1* URS function is a palindrome, 5′-AGCCGCCGA-3′. This finding contrasts with the more frequent observation that symmetrical *cis*-acting sites are found to be invertedly repeated sequences rather than palindromes. The consensus *GCN4* protein binding site, for example, was shown by saturation mutagenesis to be 5′-ATGACTCAT-3′ (4). The symmetrical *CAR1* URS primary structure seems to parallel a crude symmetry in the response of bases on each end of the palindromic sequence to mutation. First, only the center three bases of the palindromic structure were found to be absolutely required for URS-mediated inhibition of UAS function. This finding correlates with the observation that of all mutant DNA fragments tested in the competition gel shift assay, only those fragments with mutations at center positions −153 and −152 lost their ability to serve as effective competitors. Second, base substitutions at analogous positions flanking the core of the palindromic sequence exhibited similar phenotypes (compare mutations at positions −154 and −150). Third, there appeared to be unexpectedly high flexibility in the sequence that could serve as a URS even though the site was only 9 bp long. With exception of the three center bases, every possible nucleotide substitution yielded a mutant fragment that supported at least 40% of the URS function. In other words, mutating the nucleotides on either side of the core of the regulatory site resulted in weak phenotypes, whereas mutating the core itself resulted in a strong phenotype in the expression vector assay. These observations correlate with the findings that DNA fragments

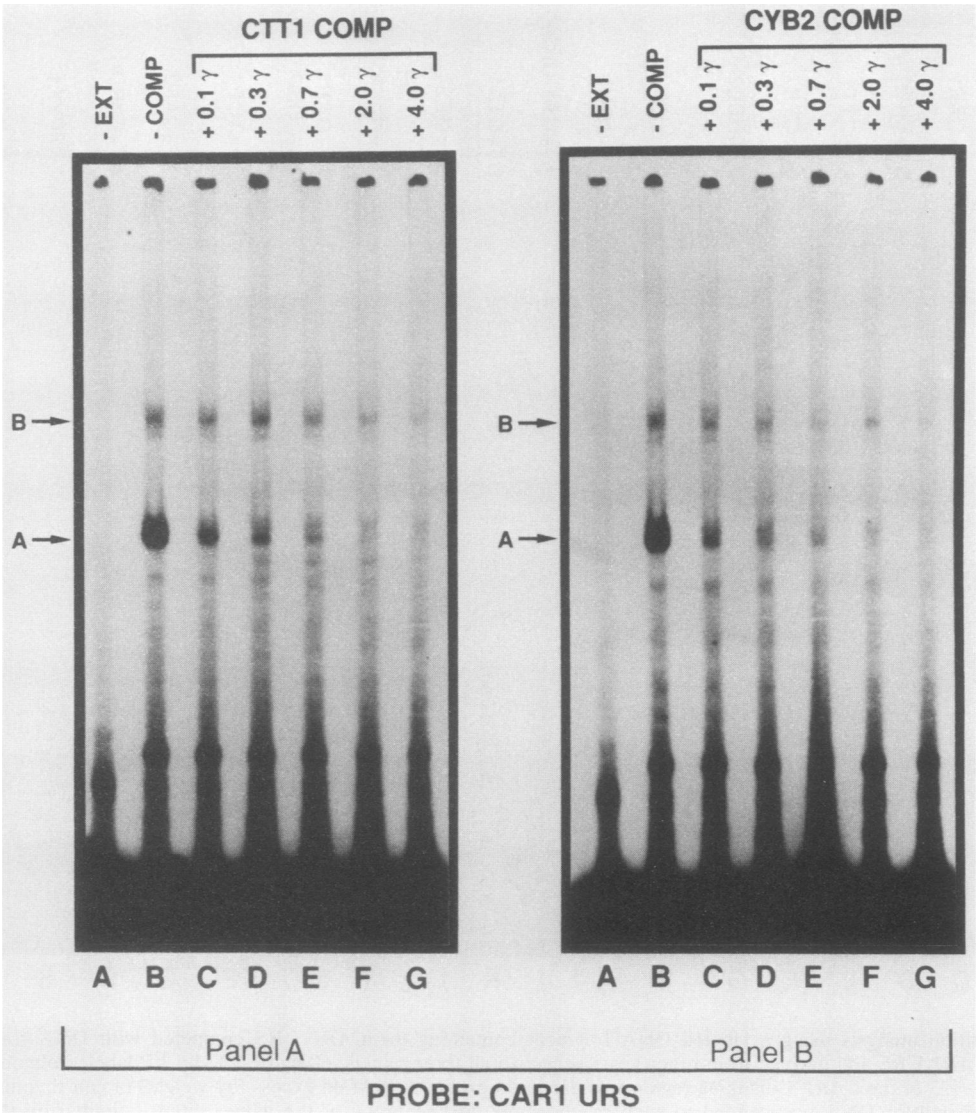


FIG. 8. Competition between DNA fragments derived from the *CAR1*, *CTT1*, and *CYB2* genes for protein binding. All lanes in panels A and B contained 60 ng of a 32-bp ^{32}P-labeled oligonucleotide containing the *CAR1* URS; the oligonucleotide covered positions −168 to −133 of the *CAR1* upstream region. The reaction mixtures resolved in lane A of each panel were devoid of cell extract. The reaction mixtures resolved in lane B of each panel contained cell extract but no competitor DNA. The reaction mixtures resolved in lanes C to G of panel A contained increasing amounts of unlabeled *CTT1* 17-mer (the DNA fragment cloned into plasmid pRL62) as competitor. The reaction mixtures resolved in lanes C to G of panel B contained increasing amounts of unlabeled *CYB2* 23-mer (the DNA fragment cloned into plasmid pRL64) as competitor. Panels C and D contained 60 ng of a 23-bp ^{32}P-labeled oligonucleotide covering positions −169 to −153 of the *CYB2* gene (the DNA fragment cloned into plasmid pRL64). The format of the experiment was as described for panels A and B. Unlabeled competitor DNAs used in panels C and D were the *CTT1* 17-mer (DNA fragment cloned into plasmid pRL62) and the *CAR1* 13-mer (DNA fragment cloned into plasmid pRL57). Panels E and F contained 60 ng of a 17-bp ^{32}P-labeled oligonucleotide covering positions −222 to −236 of the *CTT1* gene (the DNA fragment cloned into plasmid pRL62). The format of the experiment was as described for panels A and B. Unlabeled competitor DNAs used in panels E and F were the *CYB2* 23-mer (DNA fragment cloned into plasmid pRL64) and the *CAR1* 19-mer (DNA fragment cloned into plasmid pRL57). Retarded species indicated as A was the only one that remained when blunt-ended DNA probes were used; the species designated C and the rapidly migrating species near the bottom of the gel were not observed with a blunt-ended probe. Therefore, it was the only one considered to represent physiologically significant complex formation. All species designated A possessed the same mobility when resolved in adjacent lanes. This was not true for species designated B.

with mutations in positions flanking the center three bases of the palindrome retained their ability to be good competitors of the wild-type fragment for protein binding. Our genetic results are, in formal terms, consistent with the expectations of mutating a functionally duplicated sequence on either side of the core. On the other hand, our results contrast markedly with the report by Hill et al. that almost all single-base-pair mutations within the 9-bp *GCN4* protein binding site significantly reduced the ability of the site to function (4). These contrasts may reflect different overall motifs of protein binding at the *CAR1* URS and *GCN4* sites.

Multiple, unrelated yeast genes were found to possess sequences with homology to the *CAR1* URS in their 5′-flanking regions (16). DNA fragments containing these sequences were demonstrated to possess two characteristics expected of a repressor binding site as described above. In

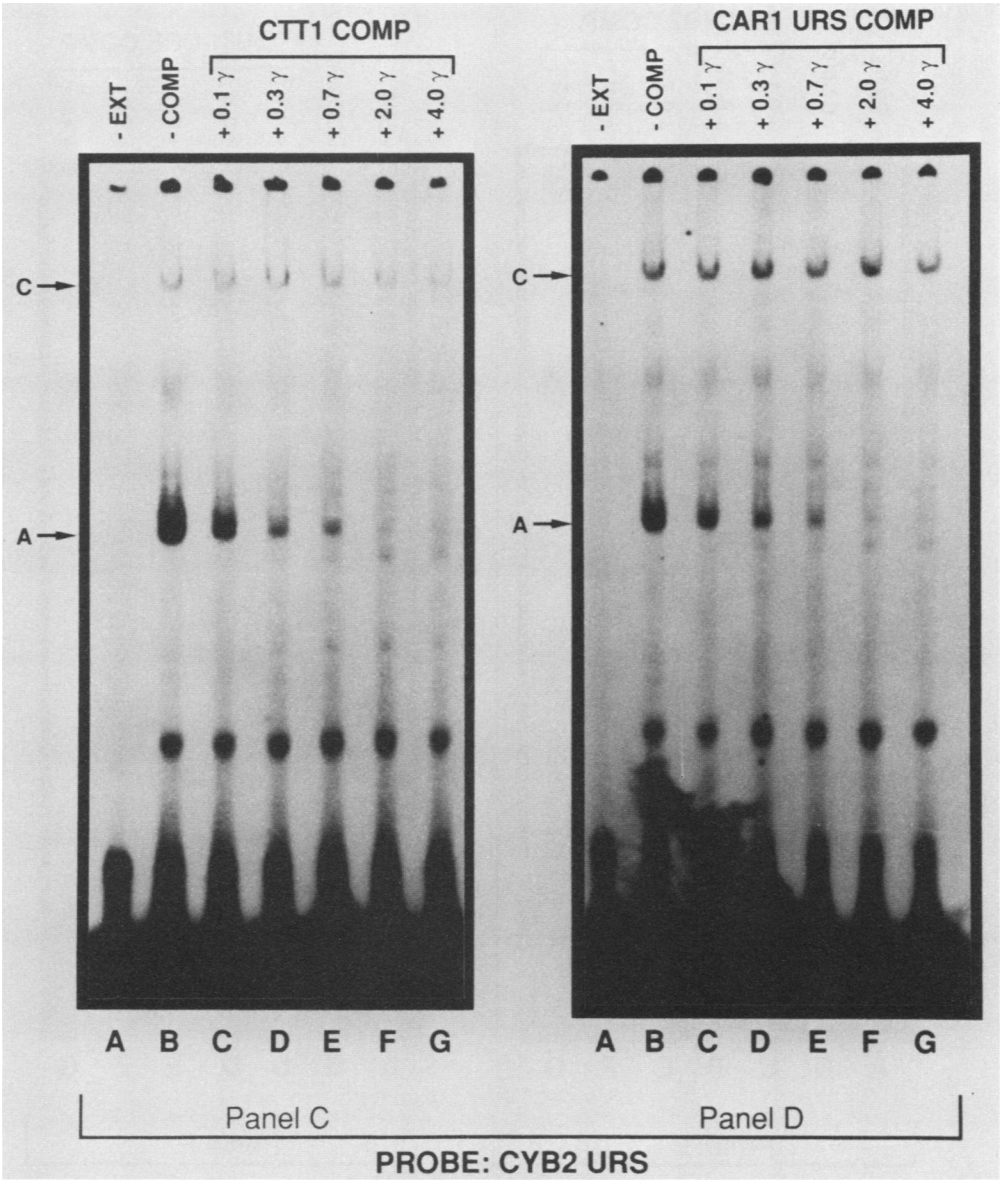


FIG. 8—*Continued.*

particular, they were successful competitors of protein binding to the *CAR1* −158 to −146 DNA fragment. This observation raises the possibility that some or all of these sequences contain a target site for the same protein or family of proteins if such exists. What is not known for the other genes possessing a sequence homologous to the *CAR1* URS is whether the *CAR1* URS-like sequence functions in control of their expression. More work on each of the genes possessing sequences with homology to the *CAR1* URS will be required before that conclusion can be drawn. In this regard, it may be pertinent that in several instances, the homologous sequences have been deleted, and in each case there was a marked increase in gene expression (2, 11). These results are consistent with our interpretation.

Although genetic analysis of the *CAR1* URS permitted identification of sequences in other genes that potentially perform a function similar to that observed in *CAR1*, it also highlights the difficulties of attempting to identify potential *cis*-acting elements on the basis of their homology alone. The data we obtained argue that one cannot easily specify conservative substitutions. This was emphasized by the observation that a transversion mutation at position −156 supported significantly greater URS function than did the more conservative transition mutation. It may be difficult to recognize physiologically significant homologies when thymine is a permissible substitution for adenine but guanine is not. A second important outcome of the genetic analysis was that the completeness of the data set permitted us to see the limitation of a less complete analysis. If all bases are mutated to all possible alternatives, it is possible to obtain a reasonable idea of the length of the element and the nucleotides that are important for function. A less complete data set (for example, the construction and assay of only transversion mutations at each position of the sequence that we analyzed) would have resulted in missing the requirement of nucleotides at positions −156, −155, −150, and potentially −148

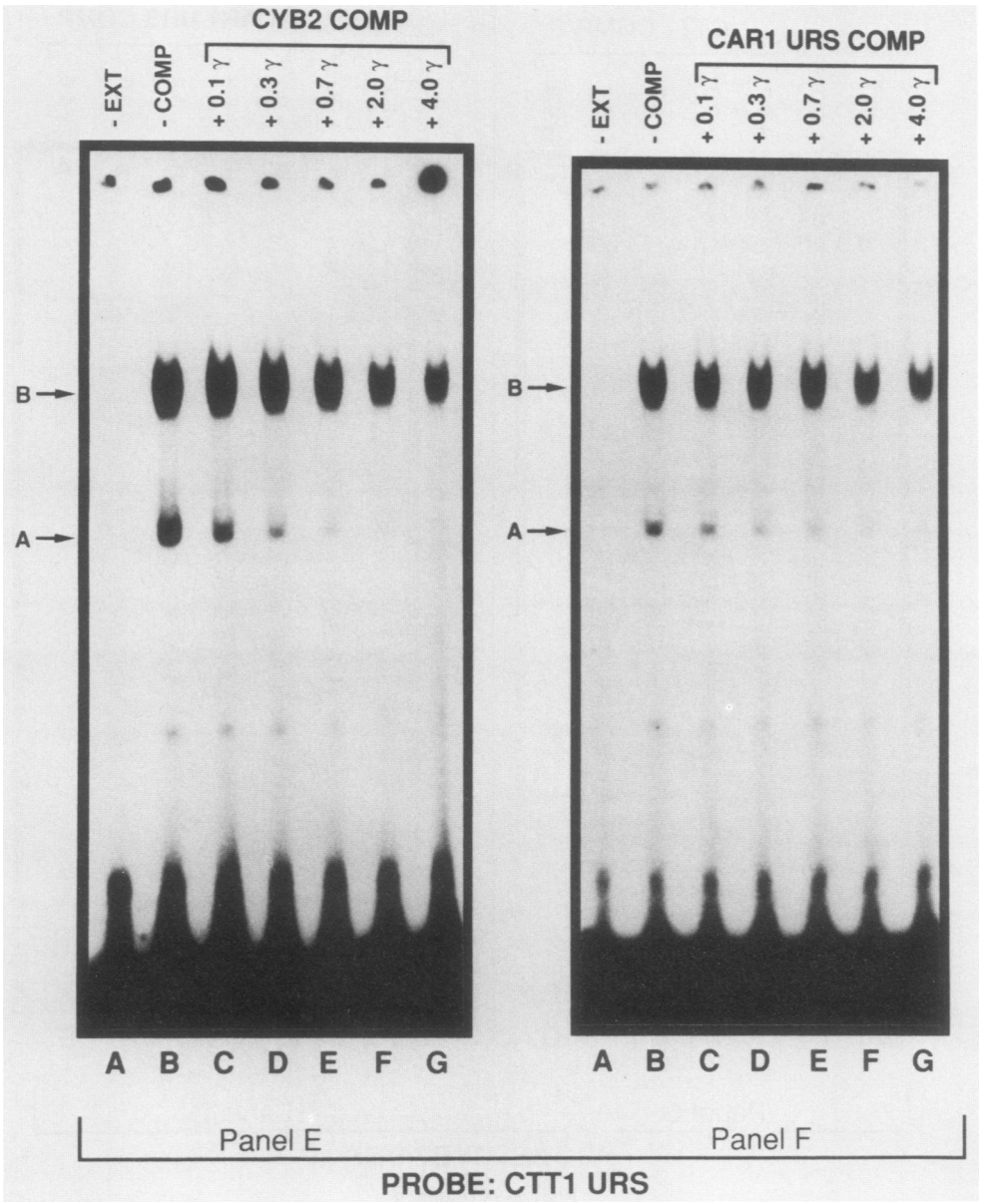


FIG. 8—*Continued.*

for URS function. Limitation of this genetic analysis, however, may be signaled by the result obtained with plasmid pRL66 (Table 2). That plasmid contained a perfect copy of the *CAR1* 5′-AGCCGCCGA-3′ sequence yet supported only 50% inhibition of *CYC1* UAS activity. In addition, plasmids pRL59 and pRL78 contain a centrally located adenine rather than guanine. According to the data in Fig. 3, these variants with this structure would not be expected to function; 65% inhibition of UAS function, however, was observed. The reason for this incongruence between expectation and observation may be trivial. For example, there may be other functions supported in part or wholly by this DNA fragment and the restriction joints of the polylinker. Alternatively, the incongruence may be substantive and indicate that the URS site is larger or more complex than our focused genetic analysis was able to visualize. These possibilities cannot be distinguished in the absence of further biochemical characterization of the DNA site and its cognate binding protein.

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