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Isolation and Characterization of Mutants that Produce the Allantoin-Degrading Enzymes Constitutively in *Saccharomyces cerevisiae*

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Degradation of allantoin, allantoate, or urea by *Saccharomyces cerevisiae* requires the participation of four enzymes and four transport systems. Production of the four enzymes and one of the active transport systems is inducible; allophanate, the last intermediate of the pathway, functions as the inducer. The involvement of allophanate in the expression of five distinct genes suggested that they might be regulated by a common element. This suggestion is now supported by the isolation of a new class of mutants (*dal80*). Strains possessing lesions in the *DAL80* locus produce the five inducible activities at high, constitutive levels. Comparable constitutive levels of activity were also observed in doubly mutant strains (*dur1 dal80*) which are unable to synthesize allophanate. This, with the observation that arginase activity remained at its uninduced, basal level in strains mutated at the *DAL80* locus, eliminates internal induction as the basis for constitutive enzyme synthesis. Mutations in *dal80* are recessive to wild-type alleles. The *DAL80* locus has been located and is not linked to any of the structural genes of the allantoin pathway. Synthesis of the five enzymes produced constitutively in *dal80-1*-containing mutants remains normally sensitive to nitrogen repression even though the *dal80-1* mutation is present. From these observations we conclude that production of the allantoin-degrading enzymes is regulated by the *DAL80* gene product and that induction and repression of enzyme synthesis can be cleanly separated mutationally.

The highly developed genetics of *Saccharomyces cerevisiae* make it a particularly advantageous eucaryote for studies of gene expression. The unique assets of the genetic approach are identification, by mutagenesis, of previously unknown regulatory elements and a formal description of their function even before either the elements themselves or biochemical assays for them have become available. These studies require, however, a model set of highly regulated genes. In this report they are the genes of the allantoin degradative pathway (Fig. 1). Our past work has shown that five enzymatic activities and four transport systems are required for the degradation of allantoin and its metabolites (6–8, 10, 12, 17, 21, 22, 24–28). Mutants have been isolated that each lack one of these functions with the exception of ureidoglycollate hydrolase (*DAL3*), which is currently under study (8, 17, 22, 26). Genetic characterization of these strains has thus far demonstrated the existence of two distinct genes, each encoding a pathway enzyme, and a third locus (*DUR1,2*) responsible for production of a bifunctional protein catalyzing the last two enzymatic steps of the pathway (6, 9; R. Sumrada and T. Cooper, J. Biol. Chem., in press). Four additional loci encode the transport functions associated with allantoin degradation (8, 22, 24). The chromosomal locations of all but one of these loci have been determined.

Earlier studies revealed that all of the enzymes and the urea active transport system are inducible (10, 12, 29). Allophanate, the last pathway intermediate, was identified as the native inducer; oxalurate was also found to serve as an equally efficient non-metabolizable inducer (20). Indirect studies with conditionally lethal mutants and drugs suggested that induction and repression of allantoin degradative enzyme synthesis was regulated at the level of transcription (1–3, 5, 11, 14–16). More recent studies, using recombinant plasmids harboring allantoin pathway genes, support this suggestion (7; T. G. Cooper, F. S. Genbauffe, and H.-S. Yoo, submitted for publication).

These past studies generate the following hypothesis. If allophanate induces expression of five distinct genes situated on several different chromosomes, it perhaps does so through the

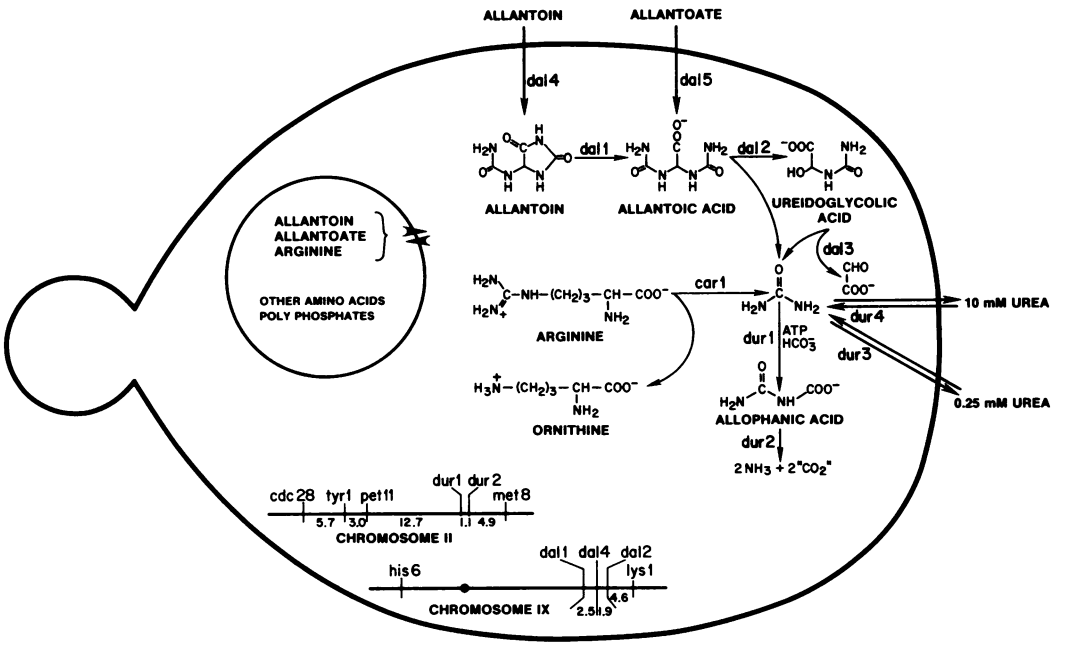

FIG. 1. Reactions of the allantoin pathway and the chromosomal locations of some of the structural genes associated with the pathway. The genetic loci are indicated above the reaction that is catalyzed by their products. The designations are: allantoinase, *dal1*; allantoicase, *dal2*; ureidoglycollate hydrolase, *dal3*; allantoin permease, *dal4*; allantoate permease, *dal5*; urea carboxylase, *dur1*; allophanate hydrolase, *dur2*; urea (low K_m) active transport, *dur3*; urea (high K_m)-facilitated diffusion, *dur4*; and arginase, *car1*.

mediation of one or more pathway-specific, regulatory protein elements. This report presents genetic evidence demonstrating the existence and characteristics of one such element. Preliminary reports of this work have been presented (G. Chisholm and T. G. Cooper, Abstr. 10th International Conference on Yeast Genetics and Molecular Biology, 1980, abstr. no. 224, p. 88; G. Chisholm and T. G. Cooper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, H51, p. 122).

MATERIALS AND METHODS

Strains and culture conditions. The strains used in the work are described in Table 1. All of the strains were derived from the Σ1278b genetic background. The minimal medium used throughout this work was that of Wickerham (31). Nitrogen sources were provided at 0.1% final concentration, and glucose was provided as the sole carbon source at a final concentration of 0.6%. The cultures were incubated at 30°C, and cell density was followed turbidimetrically by using a Klett-Summerson colorimeter equipped with a no. 54 band pass filter (500 to 570 nm); 100 Klett units was approximately equivalent to 30×10^6 to 40×10^6 cells per ml of culture.

Enzyme assays. Allophanate hydrolase and urea amido-lyase activities were assayed in cell extracts or nystatin-permeabilized cells by the method of Whitney and Cooper (28). Cell extracts were prepared by the method of Cooper et al. (8). Allantoinase, allantoicase, and ureidoglycollate hydrolase activities were measured by the colorimetric methods of Cooper et al. (8). Arginase activity was determined by using the proce-

TABLE 1. Strains used

Strain no.	Genotype
GC210[a]	α *lys2* (wild type)
GC213[b]	a *lys5* (wild type)
GC235	α *lys2 dur1*
GC299	α *lys2 dur1 dal80-1 amr1*
M1079	α *lys2* / a *lys5*
M1080	α *lys2 dal80-1* / a *lys5*
M1081	α *lys2 dal80-1* / a *lys5 dal80-1*
M1098	α *lys2 dur1-214* / a *lys5 dur1-214*
M1099	α *lys2 dur1-214 dal80-1* / a *lys5 dur1-214*
M1100	α *lys2 dur1-214 dal80-1* / a *lys5 dur1-214 dal80-1*
M1102	α *lys2 dal2-229* / a *lys5 dal2-229*
M1104	α *lys2 dal2-229 dur5-1* / a *lys5 dal2-229 dur5-1*

[a] Isogenic derivative of Σ1278b.
[b] Isogenic derivative of strain 12079d.

dure of Bossinger and Cooper (4); urea active transport was assayed by the procedure of Cooper and Sumrada (12). Allantoate transport was measured by the procedure of Turoscy and Cooper (24). The plate assay procedure used for measuring allophanate hydrolase activity in mutants and in tetrads was a modification of the procedure of Tabor et al. (23).

Preparation of [^{35}S]methionine-labeled urea amidolyase protein. Cell cultures were grown overnight to a cell density of 25 Klett units in an appropriate medium supplemented with 20 μg of tyrosine per ml. [^{35}S]methionine (1,060 Ci/mmol) was added at a final concentration of 2 μCi/ml to 20-ml samples of each test culture. The cells were then allowed to grow to a density of 80 Klett units. Cell extracts were prepared by the method of Cooper et al. (8). Radioactive urea amido-lyase protein was then isolated with the ligand immunoprecipitation method of Sumrada and Cooper (in press). The immunoprecipitated protein was suspended in 2× sample buffer and loaded onto sodium dodecyl sulfate-polyacrylamide gels for electrophoresis.

Gel electrophoresis. Radioactively labeled proteins were separated by electrophoresis through a 7.5% sodium dodecyl sulfate-polyacrylamide gel (acrylamide–bis-acrylamide, 74:1) at 10 mA of constant current. The buffer system used was that of O'Farrell with modifications as reported earlier (Sumrada and Cooper, in press). The proteins were stained with 0.25% Coomassie blue R250 (Bio-Rad). Autoradiography of the dried gels was performed with Kodak SB-5 film at −20°C for 48 to 96 h.

RESULTS

Isolation of constitutive mutants. The identification of allophanate as inducer of five separate enzymes prompted us to hypothesize that a pleiotropic regulatory element might be associated with this process. To search for the genetic locus encoding such an element, one must predict the phenotype generated by a mutant allele. Therefore, we arbitrarily assumed that the element acted in a negative manner and that mutating the locus would result in constitutive production of the allantoin-degrading enzymes. We also assumed that the mutant allele would markedly overproduce the allantoin enzymes. To identify the desired mutant we began with a strain that grew poorly on allantoin due to a lesion in the *dur1* locus. This strain (GC235) was spread on minimal medium containing allantoin as the sole nitrogen source and irradiated with UV light until 90% of the cells had been killed. Mutants that could now readily utilize allantoin were selected and then screened for constitutively produced urea amido-lyase activity by using a modification of the method of Tabor et al. (23). Of the 3,000 strains screened in this manner, 22 independent mutants were found to possess high levels of urea amido-lyase activity even when grown in the absence of inducer. One isolate (GC299) was mated with a wild-type strain and sporulated, and the meiotic products were assayed for urea amido-lyase in the absence of inducer (Fig. 2). Under the conditions of this assay a wild-type strain grown in the absence of inducer will produce a light spot on the autoradiogram, whereas a fully induced wild-type strain will produce a dark spot. Since the assay depicted in Fig. 2 was performed in the absence of inducer, the presence of dark spots on the autoradiogram indicates constitutive levels of urea amido-lyase activity. In all cases distinct 2 dark:2 light segregation was observed, thereby indicating that the phenotype was behaving as though derived from a single genetic locus. The slight variations seen in wild-type ascospores were due to small variations in the number of cells assayed and the presence of varying auxotrophies that were also included in the cross. Several other mutants were analyzed in this manner. In each case the results were identical to those just described. Complementation analysis of the 22 mutant alleles demonstrated that all of them were members of the same complementation group; i.e., no complementation was observed. The mutant locus was designated *dal80* and was found not linked to any of the allantoin pathway structural genes (data not shown).

Biochemical characterization of strains with mutations in the *DAL80* locus. A qualitative assessment of urea amido-lyase protein levels was made under various growth conditions by

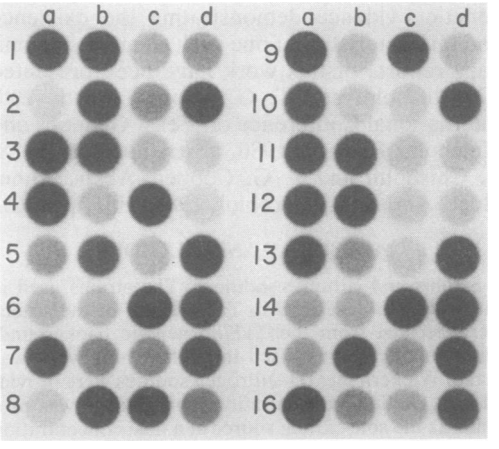

FIG. 2. Segregation of the *dal80-1* allele. Asci generated from a diploid strain (M1080) heterozygous at the *DAL80* locus were assayed with the procedures described in the text. Numbers 1 to 16 identify the various asci analyzed; letters a through d designate the four individual ascospores. Depicted in the figure is an autoradiograph of the assay filter. Dark spots indicate the presence of enzyme activity and hence a *dal80-1* allele, whereas pale spots are indicative of the wild-type allele.

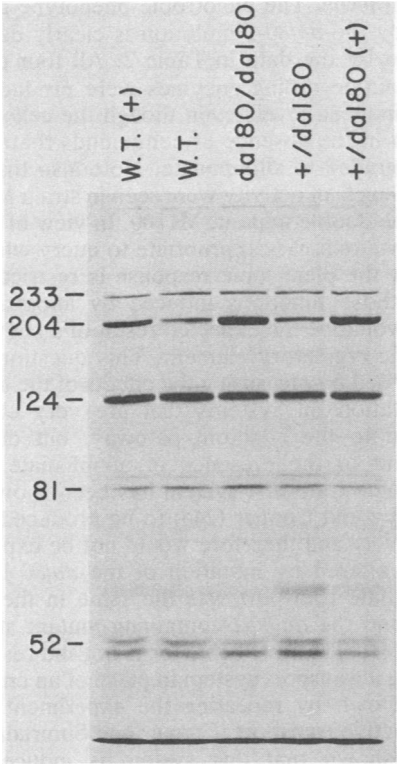

FIG. 3. Levels of urea amido-lyase protein (204 kilodaltons) in wild-type and mutant strains of *S. cerevisiae*. This figure depicts an autoradiograph of a sodium dodecyl sulfate-polyacrylamide gel of separated, radioactive proteins derived from cells grown under different conditions of induction and labeled as described in the text. Oxalurate (0.5 mM) was added to some of the cultures 5 min before the addition of the radioactive methionine, to serve as inducer. Immunoprecipitates were prepared as described in the text and subjected to sodium dodecyl sulfate gel electrophoresis. The molecular sizes indicated in the figure are in kilodaltons. W.T. (lanes 1 and 2), strain M1079; *dal80/dal80* (lane 3), strain M1081; *+/dal80* (lanes 4 and 5), strain M1080. The presence of oxalurate is designated by +.

using the immunoprecipitation method of Sumrada and Cooper (in press). As shown in Fig. 3 (lane 1), a 204-kilodalton species, previously shown to be urea amido-lyase (Sumrada and Cooper, in press), was observed when wild-type cells were grown in the presence of inducer. This species was markedly lowered in cells grown in the absence of inducer (Fig. 3, lane 2). In contrast, a strain harboring a lesion in the *dal80* locus produced large quantities of urea amido-lyase even when grown in inducer-free medium (lane 3). Constitutive overproduction of urea amido-lyase, so apparent in the diploid strain homozygous for the *dal80-1* mutation (lane 3), was lost in the heterozygote (lane 4), thereby demonstrating the recessiveness of *dal80* mutations to the wild-type allele. High-level induction of the heterozygote was still possible, however, as shown in lane 5. Figure 4 depicts a more quantitative measurement of

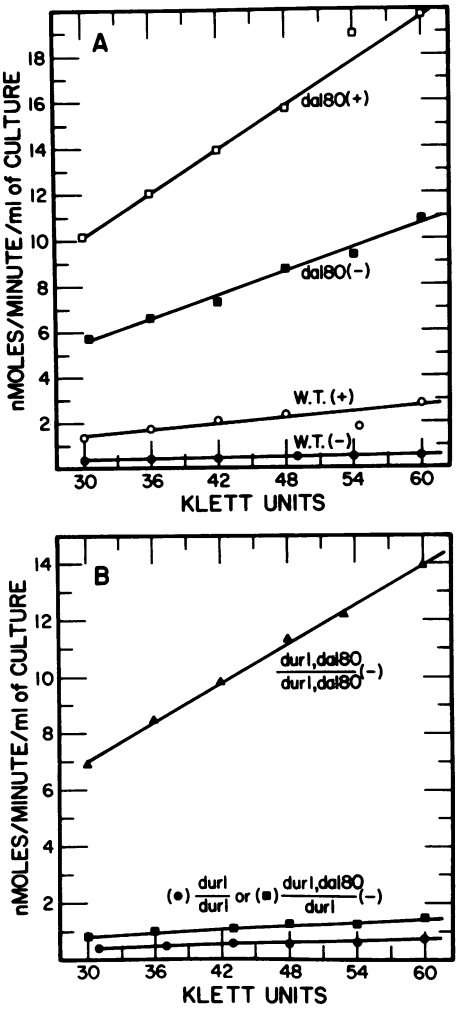

FIG. 4. Differential rate of allophanate hydrolase synthesis in wild-type and mutant strains of *S. cerevisiae*. All cultures were grown overnight in minimal proline medium. Some of them were provided with 0.25 mM oxalurate (+), and others were not (−). At a cell density of about 30 Klett units, samples of each culture were removed for assay as described in the text. Thereafter, sampling was continued at the cell densities indicated for approximately one generation. Strains used were: (A) M1079 (○,●) and M1081 (□,■); (B) M1098 (●), M1099 (■), and M1100 (▲). The significant portion of each strain's genotype has been used to label the curve generated by using that strain.

TABLE 2. Activities of the allantoin-degrading enzymes observed in wild type and mutant strains[a]

Enzyme assayed	Activity observed (nmol/min per mg of protein)		
	M1079 (wild type)	M1081 (*dal80*)	M1100 (*dur1 dal80*)
Allantoinase	60.2	160.6	143.6
Allantoicase	12.5	56.2	53.6
Ureidoglycollate hydrolase	26.7	238.6	232.2
Urea amido-lyase	0.05	1.6	—
Allophanate hydrolase	9.1	188.4	154.8

[a] Cells were grown to a cell density of 60 Klett units in glucose-proline minimal medium in the absence of inducer. Cell extracts were prepared and assays were performed as described in the text. —, Value not determined.

enzyme activity. Strain M1081, growing in inducer-free medium, produced approximately fourfold more enzyme than the fully induced, wild-type strain (Fig. 4A). It should also be pointed out that this mutant retains a residual capacity for induction; a value of slightly less than twofold was observed in this experiment. This result demonstrates that a wild-type yeast cell is capable of producing much more enzyme activity than is seen under normal growth conditions.

One trivial mechanism which would explain the constitutive production of the allantoin-degrading enzymes is internal induction. It was previously demonstrated that mutants with lesions in the *dur2* locus produce the allantoin pathway enzymes constitutively due to a build-up of allophanate, which can neither be degraded nor excreted (10, 29; Cooper, unpublished data) in these strains. The finding of Zacharski and Cooper (32) that wild-type cells normally contain large quantities of sequestered allantoin and arginine raised the possibility that the constitutivity seen in strains with mutated forms of the *dal80* locus is derived from release and metabolism of these reserve nitrogen sources. We evaluated this possibility by measuring the levels of the allantoin pathway enzymes in a doubly mutant strain (M1100; *dur1 dal80*). Strains with defects in the *dur1* locus produce inactive urea carboxylase and are hence unable to synthesize allophanate regardless of the starting material. Constitutive levels of allophanate hydrolase were observed in the double mutant (Fig. 4B). These data argue against the proposal that internal induction is the basis for constitutive enzyme synthesis in *dal80-1*-containing mutants.

Specificity of the element encoded by the *DAL80* locus. The pleiotropic phenotype generated by the *dal80-1* mutation is clearly demonstrated by the data in Table 2. All four of the allantoin-degrading enzymes were produced at fully induced levels even though the cells were grown in the absence of compounds that could be degraded to allophanate. Note also that the same levels of activity were seen in strain M1081 and the double mutant, M1100. In view of these observations, it is appropriate to query whether or not the pleiotropic response is restricted to only those functions induced by allophanate. This would be the expected result of a pathway-specific regulatory element. This question was addressed by measuring the effects of the *dal80-1* mutation on systems that are very closely related to the allantoin pathway, but do not respond to the presence of allophanate. The allantoate transport system has been shown by Turoscy and Cooper (24a) to be produced constitutively and therefore would not be expected to be altered by mutation of the *dal80* locus. Allantoate transport was the same in the wild type and the *dal80-1*-containing mutant strains (Fig. 5B). That this behavior is not the result of testing a transport system in place of an enzyme was shown by repeating the experiment with urea active transport. Cooper and Sumrada (12) have shown that this system is induced by allophanate. High constitutive levels of urea active transport were found in strain M1100 even though they were unable to synthesize allophanate (Fig. 5A).

Finally, we determined the inducibility of arginase in strains carrying the *dal80-1* mutation. Whitney and Magasanik (30) demonstrated earlier that arginine itself is the native inducer of arginase activity. Arginase activity remained at its uninduced, basal level in both wild-type and mutant strains (Fig. 6).

Nitrogen catabolite repression in strains carrying the *dal80-1* mutation. Bossinger et al. (5) have previously shown that the allantoin degradative enzymes are subject to repression when cells are provided with readily used nitrogen sources such as asparagine or glutamine. It was, therefore, of interest to ascertain whether or not strains mutated in the *dal80* locus were sensitive to repression. This we did by growing strain M1081 in the presence of various nitrogen sources and thereafter immunoprecipitating urea amido-lyase protein from crude cell extracts of these cells. Large amounts of urea amido-lyase were found in extracts derived from cells provided with proline or glutamate, two rather poor, nonrepressing nitrogen sources (Fig. 7). Alternatively, when highly repressive nitrogen sources such as asparagine, glutamine, or ammonia were provided, only very small quantities of urea amido-lyase were seen.

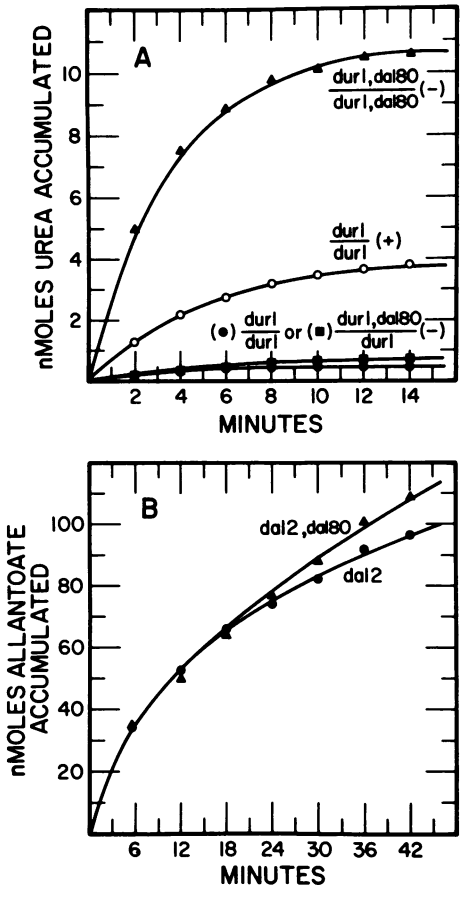

FIG. 5. Active transport of urea (A) and allantoate (B) in wild-type and constitutive mutant strains of *S. cerevisiae*. (A) Strains M1098, M1099, and M1100 were grown overnight to a cell density of 45 Klett units in minimal proline medium. Some of the cultures were provided with 0.25 mM oxalurate (+). Transport assays were performed as described in the text. (B) Strains were grown as described for (A), and the cells were assayed for allantoate transport. All data were normalized on the basis of cell number as determined with a Coulter Counter.

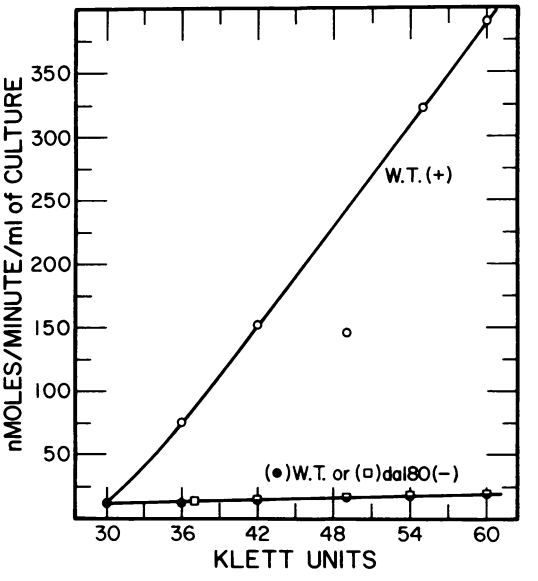

FIG. 6. Differential rate of arginase production in wild-type and mutant strains of *S. cerevisiae*. Both strains were grown overnight in proline minimal medium. Arginine (0.5 mM) was added as inducer where indicated (+). Samples were then removed as described in the legend to Fig. 4 and assayed for arginase activity as described in the text. The pertinent portion of the mutant strain's genotype has been used to label the curve obtained with that strain.

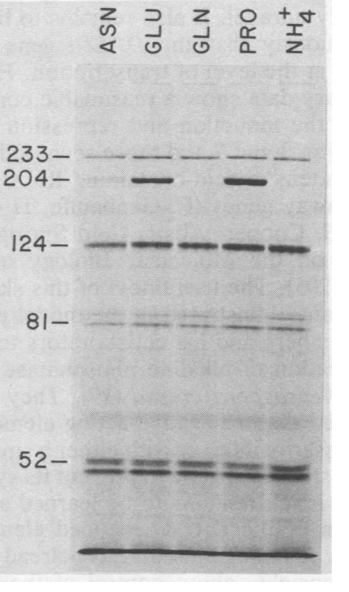

FIG. 7. Repression of urea amido-lyase production in a constitutive mutant strain (M1081) of *S. cerevisiae*. This figure depicts an autoradiograph similar to that shown in Fig. 2. In this case, however, different nitrogen sources were provided. The nitrogen sources used were: ASN, asparagine; GLU, glutamate; GLN, glutamine; PRO, proline; and NH_4^+, ammonia.

DISCUSSION

We have demonstrated the existence of a genetic locus encoding an element that pleiotropically regulates production of the allantoin-degrading enzymes in a seemingly negative manner. The regulatory action of this element is exquisitely specific, modulating production of only those functions induced by allophanate. Even other pathway activities that do not respond to allophanate, such as allantoate transport or arginase, are immune to control by the *DAL80*-encoded regulatory element. Recessiveness of mutations in the *dal80* locus argues

that control is exerted by a diffusible molecule. This conclusion is also supported by the pleiotropic phenotype of strains carrying mutations in the *dal80* locus and the fact that it is unlinked to any of the genes whose expression it regulates.

dal80-1-containing mutants possess two intriguing characteristics. They markedly overproduce urea amido-lyase and retain a residual level of inducibility (five alleles of five tested exhibited overproduction of the enzyme, and two alleles of two tested exhibited the residual inducibility). It will be of future interest to identify the molecular mechanisms underlying these two phenotypic characteristics. One possible explanation for the latter characteristic is that the element's function has not been totally destroyed by the mutation. Such an explanation, however, must be viewed with some skepticism, because we don't know whether or not the *DAL80*-encoded element interacts directly with allophanate or with the genes whose expression it regulates. There is more that prompts us to resist the temptation of prematurely proposing molecular models to explain the action of this element. Not only are we presently unaware of its associations with other important molecules in the control system, but we have also found several other classes of mutants that are specifically and pleiotropically altered in control of the allantoin-degrading enzymes (24a). Until the cast of players is complete, the story of their interactions will be difficult if not impossible to rigorously unravel. It also remains to be shown unambiguously that the *DAL80* gene product operates at the level of transcription. However, preliminary data show a reasonable congruence between the induction and repression patterns seen in Fig. 3 and 7 and those seen on hybridizing polyadenylic acid-containing RNA to allantoin pathway genes (F. Genbauffe, H.-S. Yoo, and T. G. Cooper, Abstr. Cold Spring Harbor Meeting on the Molecular Biology of Yeast, 1981, p. 205). The usefulness of this skepticism was elegantly illustrated in the model proposed by Metzenberg and his collaborators to explain the regulation of alkaline phosphatase production in *Neurospora crassa* (19). They cleverly recognized that a negative-acting element need not necessarily interact with either a small molecule or a structural gene to prevent its synthesis.

While much remains to be learned about the operation of the *DAL80*-encoded element, the *dal80-1*-containing mutants have already provided new insights about control of the allantoin degradative pathway. The strikingly high levels of urea amido-lyase in these mutants compared to those seen in fully induced wild-type cells again point to the importance of this enzyme in control of the pathway. Not only is it the multifunctional protein that both synthesizes and degrades the inducer of the pathway, it is also the limiting step in the pathway on a turnover number basis. The data presented in this report indicate that the enzyme is produced at only a small fraction (ca. 15 to 20%) of full capacity. The reason for such tight regulation is not clear.

The data depicted in Fig. 7 also bear on a hypothesis previously proposed by Lemoine et al. (18). These investigators claimed that induction and repression of the allantoin system are regulated in common, a phenomenon they designated as synergy. A similar model was proposed for control of the arginine-degrading enzymes arginase and ornithine transaminase (13). The data presented in this report argue against the validity of their hypothesis, because production of urea amido-lyase protein remained fully repressible by preferred nitrogen sources even in mutants harboring the *dal80-1* mutation. This observation is more consistent with the suggestion that induction and repression of the allantoin functions are mediated by separate elements which may operate by different mechanisms.

Finally, the above data generate a hypothesis. If regulatory elements exist that pleiotropically modulate expression of five distinct genes, it is to be expected that each of the five genes possesses a target site that is recognized by those elements. In other words, each gene should possess a homologous sequence or structure that interacts with the controlling element or elements. This is not to imply that *DAL80* encodes the specific protein that interacts with the structural gene control site. The isolation and characteristics of the constitutive mutants only point with certainty to the common regulation of the five genes. If such homologous sequences or structures exist, they may be visible when regulatory sequences adjacent to each of the five genes are compared. These studies are currently in progress.

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