JOURNAL OF BACTERIOLOGY, Nov. 1973, p. 818-822
Vol. 116, No. 2
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Modification of Ribosomes in Cryptopleurine-Resistant Mutants of Yeast

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Received for publication 20 July 1973

Cryptopleurine-resistant mutants of *Saccharomyces cerevisiae* were isolated. A single, recessive nuclear gene, very closely linked to the mating locus (2.1 centimorgans), is responsible for resistance. Ribosomes from the mutants were found to be resistant to cryptopleurine when analyzed by poly(U)-directed polyphenylalanine synthesis. Analysis of the distribution of ribosomes between monosomes and polysomes in sensitive cells exposed to cryptopleurine suggests that some step is inhibited during the elongation phase of protein synthesis.

Cryptopleurine is a plant bark alkaloid (2, 5) that has been shown to inhibit protein synthesis by the cytoplasmic systems from a variety of eukaryotic cells (1, 3, 9, 10, 12). In general, the systems from both bacteria and mitochondria were less sensitive than those from eukaryotes (9). These properties suggested that cryptopleurine-resistant mutants of yeast might have an alteration in the protein synthetic apparatus and that the gene conferring resistance to the drug could therefore be used as a genetic marker for the affected element. Accordingly, we have isolated and have begun characterizing cryptopleurine-resistant mutants of *Saccharomyces cerevisiae*. The gene conferring resistance, *cry*, is very closely linked to the mating locus, and ribosomes isolated from the mutants are resistant, in vitro, to the inhibitor.

MATERIALS AND METHODS

Reagents. Deacylated yeast transfer ribonucleic acid (tRNA), polyuridylic acid(poly U), and GTP were obtained from Miles Laboratories, Inc. Liquifluor and ^{3}H-phenylalanine (6 Ci/mmol) were obtained from New England Nuclear Corp. Glusulase was a product of Endo Laboratories. Cryptopleurine was pruchased from Chemsea Manufacturing Pty., Ltd., New South Wales, Australia. Dextran T-500 and polyethylene glycol 6000 were obtained from Pharmacia Fine Chemicals Inc. and Baker Chemicals, respectively. Components for media were obtained from Difco.

Media. Cells used for in vivo labeling experiments were grown overnight in YM-1 medium (7) containing in grams per liter: yeast extract, 10; peptone, 10; adenine and uracil, 0.01 each; succinic acid, 10; and glucose, 20. The pH was adjusted to 5.8 with 10 N NaOH. YM-5 medium contained one-fifth the yeast extract and peptone. For all other experiments, cells were grown in YPD medium containing in grams per liter: peptone, 20; yeast extract, 10; and glucose, 20. Supplemented minimal medium contained per liter: 6.7 g of amino acid-free yeast nitrogen base, 20 g of glucose, and 20 to 40 mg of the appropriate supplements. When plate assays were performed, 20 g of agar per liter were also added. Presporulation medium contained in grams per liter: yeast extract, 8; peptone, 3; and glucose, 100. Sporulation medium contained 10 g of potassium acetate, 1 g of yeast extract, and 0.5 g of glucose per liter.

Strains. The strains used in these studies are listed in Table 1.

Isolation of mutants. Mutants of strain D587-4B were isolated following mutagenesis by ethylmethane sulfonate (EMS) (4). Cells from an overnight culture grown in YPD medium were washed with water by centrifugation and incubated for 50 min at 28 C in 0.1 M sodium phosphate (pH 8.0) containing 3% EMS. Mutagenesis was terminated by suspending the cells in a 0.5 volume of 5% $Na_2S_2O_3$ for 15 min. Survival was about 50%. The cells were washed, resuspended in water, and diluted 10× into YPD medium for expression overnight at 28 C. Approximately 10^8 cells from the expressed cultures were plated on YPD agar containing 1 μM cryptopleurine. A total of four mutants from independently expressed cultures were selected for further study.

Tetrad analysis. Matings were performed by mixing haploid cultures together on YPD agar and incubating overnight. Diploids were subsequently isolated by prototrophic selection (13). Single colonies were transferred to 10 ml of presporulation medium and were incubated at 28 C without shaking for 24 to 36 h until fermentation appeared to be maximal. The cells were washed, suspended in 5 ml of sporulation medium, and incubated with shaking for 3 to 5 days. Sporulation was usually 80 to 95%, and only cultures with a high degree of sporulation were used for

analysis. The sporulated cultures were stored at 4 C in 1 ml of sporulation medium. Tetrads were dissected, and the data were analyzed by standard procedures (11). All four spores germinated in about 60 to 65% of the asci dissected. Only data from such tetrads were analyzed.

In vivo protein synthesis. Overnight cultures of strain A364A in YM-1 medium were suspended in YM-5 medium to a density of 1.5×10^7 cells/ml. Reconstituted ^{14}C-protein hydrolysate (0.1 μCi/ml), and varying amounts of cryptopleurine were added to each culture. Samples (1.0 ml) were removed at various times, and trichloroacetic acid-precipitable radioactivity was determined.

Polysome analysis. Strain A364A was grown in YM-1 medium to a density of 2×10^7 cells/ml or less, washed once in distilled water by centrifugation, and suspended in 1 M sorbitol at a density of 2×10^8 cells/ml. Glusulase was added to give a concentration of 1%, and the resulting mixture was incubated for 1 h at 23 C. Spheroplasts were centrifuged and then resuspended in YM-5 medium plus 0.5 M $MgSO_4$ at a density of 1.5×10^7 cells/ml, incubated for 2 h at 23 C, and concentrated 10× by centrifugation. After incubation with 20 μCi of ^{14}C-protein hydrolysate per ml, the spheroplasts were centrifuged and suspended in lysing buffer containing 0.01 M tris(hydroxymethyl) aminomethane (Tris)-hydrochloride, 0.1 M NaCl, 0.03 M $MgCl_2$, and 20 μg of poly-L-ornithine per ml, pH 7.4. Sodium deoxycholate and Brij-58 were added to give final concentrations of 0.5% each (8). Lysates were layered on 10 to 40% sucrose gradients containing lysing buffer. Centrifugation was for 2 h at 27,000 rpm in the Spinco SW40 Ti rotor. Gradients were monitored at 260 nm and collected in 0.7-ml fractions. Trichloroacetic acid-precipitable radioactivity was determined after addition of 100 μg of bovine serum albumin to each fraction.

Preparation of ribosomes and elongation factors. Cells were grown in YPD medium at 28 C in a New Brunswick Microferm laboratory fermentor, harvested at mid-log phase by continuous flow centrifugation, and stored in liquid N_2. Ribosomes and elongation factors were prepared by a modification of the dextran-polyethylene glycol two-phase extraction procedure (6, 14). Cells in two volumes of buffer 1 (0.05 M Tris-acetate, pH 7.0; 0.05 M NH_4Cl; 0.012 M Mg Cl_2; and 0.001 M dithiothreitol (DTT)) were ruptured by passage through a French pressure cell at 12,000 lb/in^2. Debris was removed by centrifugation at 30,000 × *g* for 20 min. Solutions of 20% (wt/wt) Dextran T500 and 30% (wt/wt) polyethylene-glycol 6000 were added to give final concentrations of 1.4 and 6.4%, respectively. The resulting suspension was stirred for 10 min in ice, and the phases were separated by centrifugation at 1,500 × *g* for 10 min. Under these conditions, all of the ribosomes and the bulk of both elongation factor activity and aminoacyl tRNA synthetase activity was in the dextran (lower) phase.

The dextran phase was diluted with two volumes of buffer 2 (buffer 1 plus 0.5 M NH_4Cl), insoluble material was removed by centrifugation at 20,000 × *g* for 10 min, and ribosomes were pelleted by centrifugation at 105,000 × *g* for 3.5 h. The ribosomes were suspended in buffer 2 and centrifuged through 10% glycerol containing buffer 2 at 105,000 × *g* for 2.5 h. The final ribosomal pellet was suspended in buffer 1 containing 10% glycerol and stored in liquid N_2.

TABLE 1. *Strains used in these studies*

Strain	Genotype	Source or explanation
D587-4B	α *his1-1*	F. Sherman
D585-11C	a *lys1-1*	F. Sherman
X2928-3D	a *gal1 ade1 leu1 trp1 met14 his2*	R. Mortimer
A364a	a *ade1 ade2 ura1 tyr1 his7 lys2 gal1*	L. Hartwell
PS1-3C	a *lys1-1*	D587-4B × D585-11C
PS19-33D	a *lys1-1 cyh*	PS1-3C × D587-4B (*cyh*)
D587-4B (cyh)	α *his1-1 cyh*	A mutant of D587-4B, resistant to cyclohexamide with resistant ribosomes.
cry1-1 - cry1-4	α *his1-1 cry1*	Mutants of D587-4B resistant to cryptopleurine

The dextran-phase postribosomal supernatant fluid was dialyzed at 4 C against 0.05 M Tris-hydrochloride (pH 7.5), 0.05 M KCl, and 0.001 M DTT overnight, and was used as a source of elongation factors as well as aminoacyl-tRNA synthetases.

Preparation of ^{3}H-phe-tRNA. The reaction for charging tRNA contained (in 15 ml): 0.04 M Tris-hydrochloride (pH 8.0); 0.01 M magnesium acetate; 0.02 M DTT; 0.006 M NH_4Cl; 0.003 M ATP; 0.0002 M cold amino acids, except for phenylalanine; 940 *A* units of tRNA at 260 nm; 15 mg of dialyzed dextran-phase proteins; and 900 μCi of ^{3}H-*phe*. After 20 min at 30 C, the charged tRNA was isolated by two phenol extractions, passed over a Sephadex G-25 column in H_2O, and lyophilized. The degree of charging was 1.5 to 3%, based on total tRNA.

Polyphenylalanine synthesis. Reaction mixtures of 0.05 ml contained buffer 1, 0.02 M DTT, 8 μg of poly(U), 0.04 *A* units of ribosomes at 260 nm, 10 to 12 μg of dialyzed dextran-phase protein; 0.001 M guanosine 5'-triphosphate and 10 to 15 pmol of ^{3}H-*phe*-tRNA. After an appropriate incubation period, the reactions were terminated by addition of 0.5 ml of 10% trichloroacetic acid, heated at 90 C for 10 min, and filtered on GF-C glass fiber filters (Whatman). Radioactivity was determined by using Liquifluor scintillation fluid in a Beckman LS-100 scintillation spectrometer at about 30% efficiency.

RESULTS

Site of inhibition by cyrptopleurine in vivo. Protein synthesis is inhibited in a variety of eukaryotic cells by low concentrations of cryptopleurine (1, 3, 9). As shown in Fig. 1, protein synthesis in *S. cerevisiae* was strongly in-

hibited by 0.5 μM and completely abolished by 2 μM of cryptopleurine. Before beginning a study of mutants resistant to cryptopleurine, we wished to know precisely which stage during protein synthesis is inhibited in vivo. Such information could be valuable in the characterization of resistant mutants.

Analysis of the distribution of ribosomes between the monosome and polysome fractions in inhibited cells permits some conclusions to be drawn about the site of inhibition. This approach has been used extensively in yeast to answer such questions (8). A culture of spheroplasts was divided into two parts which were subsequently incubated with labeled amino acids, with and without cyrptopleurine. After 30 min, sucrose gradient analysis of the polysomes was carried out. Although protein synthesis was strongly inhibited by cryptopleurine, the polysome profile was essentially the same as the uninhibited control (Fig. 2). These data indicate that cryptopleurine inhibits some step in protein synthesis subsequent to initiation. Inhibition of polyphenylalanine synthesis by cryptopleurine (1, 9) is consistent with inhibition of a step required for elongation.

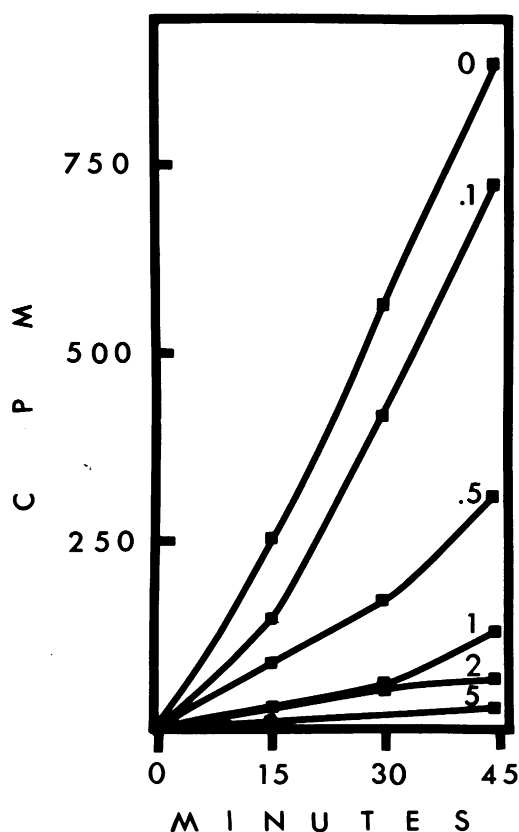

FIG. 1. *Effect of cryptopleurine on protein synthesis in intact yeast cells. Strain A364A was grown as described in Materials and Methods and suspended in YM-5 medium containing ^{14}C-protein hydrolysate and 0.1, 0.5, 1.0, 2.0, or 5.0 μM cryptopleurine. After the indicated time, 1-ml samples were removed for determination of trichloroacetic acid-precipitable radioactivity.*

Isolation of mutants resistant to cryptopleurine and mapping of the cry gene. Because cryptopleurine very likely inhibits some reaction of the elongation phase of translation, mutants resistant to inhibition would be expected to have an alteration in some element of the translation apparatus involved in elongation. Accordingly, we isolated and characterized such mutants. After ethyl methane sulfonate mutagenesis, four independently derived mutants of strain D587-4B were obtained which were resistant to up to 10 μM of cryptopleurine in YPD plates.

Preliminary characterization of four independently isolated mutants showed that a single, recessive nuclear gene is responsible for resistance. In the course of routine genetic analysis, we found linkage between the gene for cryptopleurine resistance (*cry* locus) and the mating locus. In Table 2, the various crosses performed are listed with the ascus-type ratios obtained between *cry* and the mating locus. No nonparental ditype asci were obtained with any mutant, showing that the resistance gene in each of the four mutants is linked to the mating locus. The second division segregation frequency for *cry* was determined in cross 2 to be 44%. In the two tetratype asci obtained in cross 2, *cry* had the first division pattern, suggesting that *cry* is between the centromere and *a*/α. Of the 188 asci, 4.2% were tetratype indicating a map location for *cry* 2.1 centimorgans from the mating locus on the centromere side.

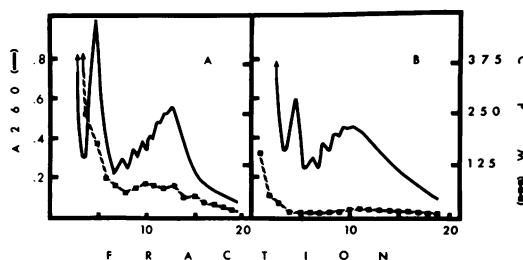

FIG. 2. *Effect of 30 min of incubation with 5 μM cryptopleurine on the polyribosome profile. Spheroplasts of strain A364A were incubated with or without 5 μM cryptopleurine in the presence of ^{14}C-protein hydrolysate for 30 min. The spheroplast lysates were analyzed by sucrose gradient centrifugation. A, Control; B, cryptopleurine treated.*

TABLE 2. *Ascus-type ratios between cryptopleurine resistance and the mating locus*

Cross	PD	NPD	TT[a]
(1) *cry1-1* × PS1-3C	27	0	1
(2) *cry1-1* × X2928-3D	46	0	2
(3) *cry1-1* × PS19-33D	61	0	2
(4) *cry1-2* × PS1-3C	26	0	1
(5) *cry1-3* × PS1-3C	14	0	1
(6) *cry1-4* × PS1-3C	6	0	1

[a] PD, NPD, and TT refer to parental ditype, nonparental ditype, and tetratype asci, respectively.

Cross 3 was performed to test whether *cry* is linked to the cycloheximide resistance locus. Strain PS1933D contains a cycloheximide resistance gene which produces ribosomes resistant to cycloheximide (unpublished data). The ascus type ratios between *cry* and *cyh* were 11 parental ditype:10 nonparental ditype, and 43 tetratype asci, showing that the two genes are not linked. Furthermore, neither gene appears to be pleiotropic, because interpretation of the tetrad data was unambiguous.

Analysis of ribosomes from the mutants. To determine how the *cry* mutation produces resistance to cryptopleurine, ribosomes and factors were isolated from the parent D587-4B, as well as from the mutant *cry1-1*. The ribosomes were washed twice with 0.5 M NH_4Cl and were free of detectable elongation-factor activity. A comparison of the inhibition of polyphenylalanine synthesis at different cryptopleurine concentrations, with ribosomes from both the parent and the mutant, is shown in Fig. 3. Higher concentrations of cryptopleurine were required for inhibition when ribosomes from the mutant were used. Similar cryptopleurine concentration curves with the two ribosomes were done with factors from the mutant. As shown in Table 3, the concentration of inhibitor required for 50% inhibition was about 100× higher when the ribosomes from *cry1-1* were used, regardless of the source of factors. Similarly, ribosomes from the parent were sensitive even in the presence of factors from the mutant. Ribosomes from another mutant, *cry1-2*, were also examined and had essentially the same properties as those from *cry1-1*. We conclude that the *cry* gene confers resistance to cryptopleurine by virtue of a ribosomal alteration.

DISCUSSION

Cryptopleurine is an inhibitor of eukaryotic protein synthesis, which appears to act during the elongation phase in vivo as well as in vitro. The exact step affected by cryptopleurine has yet to be determined. Previously reported results indicated that cryptopleurine may interfere with the peptidyl transfer reaction (12). However, a relatively high concentration was only partially inhibitory. This result must be reconciled with a report that a chemically similar compound, tylocrebrine, appeared to interfere with translocation and not peptidyl transfer (10). We have undertaken studies to determine the site of inhibition of polyphenylalanine synthesis with yeast ribosomes.

Yeast mutants resistant to cryptopleurine have a single, altered nuclear gene 2.1 centimorgans from the mating locus. Ribosomes isolated from resistant mutants were shown to be resistant, in vitro, to cryptopleurine. Preliminary

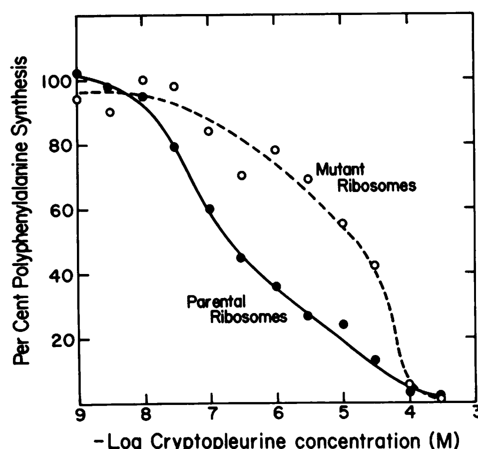

FIG. 3. *Inhibition of polyphenylalanine synthesis by cryptopleurine with ribosomes from strains D587-4B and cry1-1. Polyphenylalanine synthesis was carried out for 10 min with ribosomes from either the parent or the mutant in the presence of increasing concentrations of cryptopleurine. Elongation factors from the parent were used. Incorporation of phenylalanine in the absence of cryptopleurine was 2.7 pmol with the parental ribosomes and 2.9 pmol with the mutant ribosomes.*

TABLE 3. *Inhibition of polyphenylalanine synthesis by cryptopleurine*

Source of ribosomes	Source of factors	Concn of cryptopleurine giving 50% inhibition[a]
D587-4B	D587-4B	1.8×10^{-7} M[b]
D587-4B	*cry1-1*	3.0×10^{-7} M[c]
cry1-1	D587-4B	1.8×10^{-5} M[d]
cry1-1	*cry1-1*	2.2×10^{-5} M[e]

[a] Polyphenylalanine synthesis was carried out as described in Materials and Methods.

[b-e] For polyphenylalanine synthesis 100% values were 2.7, 3.5, 2.9, and 3.2 pmoles, respectively.

experiments to localize the resistance on one or the other subunit have been hampered by the fact that 40S subunits from the mutant are less active than those from the parent. It therefore appears that ribosomes resistant to cryptopleurine may have altered 40S subunits. Studies are currently in progress to correlate such ribosomal alterations with resistance to cryptopleurine.

While these studies were in progress, we learned that similar results have been obtained by A. Jiminez, P. Grant, and J. Davies (personal communication).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA-12565 (L. S.) and CA-10628 (C. M.) from the National Cancer Institute. L. S. is a recipient of a Faculty Research Associate grant from the American Cancer Society.

We thank F. Sherman and R. Mortimer for supplying strains. Special thanks are extended to F. Sherman, N. Eaton, and B. Dorfman for their helpful advice on tetrad analysis.

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