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Cryptopleurine Resistance: Genetic Locus for a 40S Ribosomal Component in *Saccharomyces cerevisiae*

PAUL GRANT, LUCAS SÁNCHEZ, AND ANTONIO JIMÉNEZ

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706, and Instituto de Biologia Celular, Velazquez 144, Madrid 6, Spain

Received for publication 3 September 1974

Mutants resistant to the phenanthrene alkaloids tylophorine, tylocrebrine, and cryptopleurine have been isolated from the yeast *Saccharomyces cerevisiae*. A single recessive nuclear gene was responsible for resistance. This resistance locus, *cry*, was closely linked to the mating locus (2.2 centimorgans). The resistance was ribosomal and was due to an altered 40S ribosomal subunit. The mode of action of these drugs has been examined, and they appear to inhibit the translocation phase of protein synthesis.

The yeast *Saccharomyces cerevisiae* is a convenient eukaryotic organism for genetic analysis and biochemical studies. The protein synthetic system of *S. cerevisiae* has been the object of considerable study, but few mutants resistant to protein synthesis inhibitors have been well characterized.

Until recently, work with antibiotic-resistant mutants had been almost entirely limited to the glutarimide group of antibiotics, of which cycloheximide is an example (4, 11). These mutants have been shown to have an altered 60S ribosomal subunit. Mutants resistant to the 12,13-epoxytrichothecenes have recently been characterized (19). Such mutants also have an altered 60S ribosomal subunit but are not cross-resistant to cycloheximide. Skogerson, McLaughlin, and Wakatama (20) described the isolation of mutants resistant to the phenanthrene alkaloid, cryptopleurine, that have ribosomes resistant to cryptopleurine in vitro.

We have isolated spontaneous mutants of *S. cerevisiae* that are resistant to the phenanthrene alkaloids cryptopleurine, tylocrebrine, and tylophorine, but not cross-resistant to cycloheximide or trichodermin, and which have ribosomes that are resistant to the phenanthrene alkaloids in vitro. The resistance has been shown to be due to an altered 40S ribosomal subunit. The gene conferring resistance to the phenanthrene alkaloids, *cry*, has been found to lie close to the mating locus.

MATERIALS AND METHODS

Yeast transfer ribonucleic acid (tRNA) was obtained from General Biochemicals, Chagrin Falls, Ohio; polyuridylic acid [Poly(U)] from Miles Laboratories, Inc., Kankakee, Ill.; [U-^{14}C]phenylalanine from New England Nuclear, Boston, Mass.; [U-^{14}C]amino acid mixture from the Radiochemical Centre, Amersham, England; dithiothreitol, adenosine-5'-triphosphate, guanosine-5'-triphosphate, and creatine phosphokinase from Calbiochem, San Diego, Calif.; Glusulase from Endo Laboratories, Inc., Garden City, N.Y.; phosphocreatine from Sigma Chemical Co., St. Louis, Mo.; and 2-mercaptoethanol from Schwarz/Mann, Orangeburg, N.Y. Cryptopleurine was a gift from S. Földéak. Tylophorine and tylocrebrine were obtained from E. Gellert.

Media. YED medium contains 1% yeast extract (Difco), 2% peptone (Difco), and 2% glucose. YM-1 and YM-5 media are as described by Hartwell (8). YNB medium contains 0.7% yeast nitrogen base without amino acids (Difco) and 2% glucose, plus appropriate supplements. Presporulation medium was 10% glucose, 0.8% yeast extract, and 0.24% peptone. Sporulation medium was 0.1% yeast extract, 1% potassium acetate, and 0.05% glucose. All solid media were made by adding 2% agar to the above media.

Strains. The strains of *S. cerevisiae* used in this work are described in Table 1.

Isolation of cryptopleurine-resistant mutants. Resistant mutants were isolated by plating about 10^9 cells from stationary-phase cultures of *S. cerevisiae* on YED plates containing cryptopleurine (5 μg/ml). The plates were then incubated at 30 C. Eight spontaneous mutants were obtained, seven from strain A364A and one from strain Y166. Most of the reported work was done with the mutant isolated from strain Y166, which we have designated strain CRY6.

Tetrad analysis. Matings were performed by mixing haploid strains of opposite mating types on YED plates and incubating overnight at 30 C. Diploids were isolated by prototrophic selection on YNB plates containing the appropriate supplements. Single colonies were transferred to presporulation plates and incubated 2 days at 30 C. The cultures were then

transferred to sporulation plates and incubated 2 to 4 days at 30 C.

Tetrad analyses were performed using established techniques (14). Only complete tetrads were analyzed.

Effect of phenanthrene alkaloids on cell growth. Cell growth in YM-5 medium was followed at 600 nm in a Bausch and Lomb Spectronic 20. The minimal inhibitory concentration was defined as the lowest concentration of drug which did not allow a doubling of the optical density in 24 h after addition of the drug.

Ribosome and ribosomal subunit preparation. Ribosomes and subunits were prepared using a modification of the procedure of van der Zeijst, Kool, and Bloemers (22). Their procedure called for the preparation of spheroplasts before treating the culture with sodium azide. We found that incubation with sodium azide could be done on whole cells, thus eliminating the necessity for making spheroplasts, simplifying the procedure, and making it more amenable to large-scale ribosomal subunit preparations.

The yeast cultures were grown in YED medium at 30 C to a cell density of approximately 3×10^7 cells/ml. Sodium azide was then added to a final concentration of 1 mM, and incubation continued for 15 to 20 min at 30 C. The cultures were then quick chilled, harvested, washed with water, and resuspended in 50 mM tris(hydroxymethyl)aminomethane(Tris)hydrochloride buffer, pH 7.4, 10 mM $MgCl_2$, 88 mM KCl, and 10 mM 2-mercaptoethanol (Buffer 1) at a concentration of 0.5 to 1.0 g of cells/ml. All further operations were at 0 to 4 C unless otherwise indicated.

The cell suspension was passed through a French pressure cell (Aminco) one or two times at 18,000 lb/in^2. The cellular debris and unbroken cells were centrifuged at 30,000 $\times$ *g* for 5 min, and the supernatant liquid was decanted; the pellet was washed by resuspension in Buffer 1 and again centrifuged at 30,000 $\times$ *g* for 5 min. The pellet was discarded, and the supernatant fractions were pooled and recentrifuged at 30,000 $\times$ *g* for 30 min. The pellet was discarded, and the supernatant liquid was centrifuged a second time at 30,000 $\times$ *g* for 30 min. The supernatant liquid was decanted, and the pellet was discarded.

The supernatant liquid was then centrifuged at 160,000 $\times$ *g* in a Ti50 rotor for 90 min. The upper two-thirds of the supernatant liquid from this spin was saved for the preparation of supernatant factors. The ribosomal pellet was rinsed with 50 mM Tris-hydrochloride, pH 7.4, 12.5 mM $MgCl_2$, and 80 mM KCl, and 1 mM dithiothreitol (Buffer 2) and resuspended in the same buffer. The ribosome suspension was then centrifuged at 30,000 $\times$ *g* for 20 min. The supernatant fraction was saved and any pellet was discarded. Ribosomes were stored in Buffer 2 at −70 C.

Ribosomal subunits were prepared by diluting 100 optical density units at 260 nm (OD_{260}) of 80S ribosomes to 0.75 ml in Buffer 2. Then 0.25 ml of 2 M KCl was added to bring the final KCl concentration to 0.5 M. The ribosomal sample (100 OD_{260} units in 1 ml) was layered on a 38-ml, 10 to 30% (vol/vol) glycerol gradient containing 50 mM Tris-hydrochloride, pH 7.4, 5 mM $MgCl_2$, 0.5 M KCl, and 10 mM 2-mercaptoethanol. Gradients were centrifuged at 95,000 $\times$ *g* for 6.5 h at 15 C in an SW27 rotor.

TABLE 1. *Strains used in these studies*

Strain	Genotype
Y166	*α his4 trp5 MAL1*
CRY6	*α his4 trp5 MAL1* cryptopleurine resistant (a spontaneous mutant from strain Y166)
2181-1A	a *ade1 trp1 his2 gal1*
1/5	a *ade2 his4 cyh2*
A364A	a *ade1 ade2 ura1 tyr1 his7 lys2 gal1*
CRY4	a *ade1 ade2 ura1 tyr1 his7 lys2 gal1* cryptopleurine resistant (a spontaneous mutant from strain A364A)
SR_{17}	*α his4 trp5 MAL1 cyh2*

The gradients were analyzed by passage through an Isco density gradient fractionator, model D/UA-2. Fractions of approximately 1.1 ml were collected. The fractions containing the 40S subunits were pooled, as were those containing the 60S subunits, and centrifuged 9 to 10 h at 160,000 $\times$ *g* in a Ti50 rotor. The subunit pellets were resuspended in Buffer 2 and stored at −70 C.

Preparation of supernatant factors. The supernatant fraction from the first 160,000 $\times$ *g* centrifugation (the first ribosomal pelleting) was made up to 70% ammonium sulfate at 0 C. The precipitate was collected by centrifugation at 20,000 $\times$ *g* for 10 min, resuspended in a small amount (about one-tenth to one-fifth of the original volume) of 20 mM Tris-hydrochloride, pH 7.4 and 1 mM dithiothreitol, and dialyzed overnight against two changes of the same buffer. The supernatant preparation was then centrifuged at 20,000 $\times$ *g* for 20 min to remove any remaining precipitate. Any pellet was discarded, and the supernatant preparation was frozen and stored in small portions in liquid nitrogen. Occasionally a somewhat purer preparation was made by taking the fraction precipitating between 40 and 70% ammonium sulfate instead of the 0 to 70% fraction.

Preparation of polyribosomes for endogenous messenger RNA-directed peptide synthesis. Cultures were grown to about 10^7 cells/ml in YM-1 medium. The cells were centrifuged, washed twice with water, and resuspended in 1 M sorbitol. Spheroplasts were prepared by adding Glusulase to a final concentration of 1% (vol/vol) and incubating at 23 C for 1 h with gentle shaking. The spheroplast preparation was then poured into YM-5 medium containing 1 M sorbitol and incubated at 30 C with gentle shaking until the spheroplasts were actively synthesizing protein (1 to 2 h). The culture was then poured over frozen, crushed 1 M sorbitol, and spheroplasts were collected by centrifugation for 15 min at 9,000 $\times$ *g* at 0 to 4 C.

Lysis was achieved by resuspending the spheroplasts in a small volume of Buffer 2, adding 0.1

volume of 5% sodium deoxycholate and 0.15 volume of 5% Brij-58. Lysis was assisted by three strokes in a Potter-Elvehjem homogenizer. Cellular debris was removed by centrifugation at 10,000 × *g* for 10 min. Polyribosomes were then centrifuged at 160,000 × *g* for 90 min. Fifty to 60% of the ribosomes were found in polyribosomes.

Polyribosome analyses. Spheroplasts were prepared as described for the preparation of polyribosomes for endogenous messenger RNA-directed peptide synthesis. The spheroplasts were incubated in YM-5 medium containing 1 M sorbitol for 2 h at 30 C. Cryptopleurine was added to a portion of the culture (equivalent to 10 ml of the original culture) at a concentration of 130 μM, and incubation of both treated and untreated portions was continued at 30 C for 60 min. Cycloheximide was then added to both portions of the culture to a final concentration of 1 mM. The cultures were placed on ice for 5 min. The spheroplasts were collected by centrifugation at 2,500 × *g* for 5 min at 0 to 4 C. Lysis was achieved by resuspending the spheroplasts in a small amount of 10 mM Tris-hydrochloride, pH 7.4, 100 mM NaCl, 30 mM $MgCl_2$ (Lysis buffer) and adding one-tenth volume of 5% sodium deoxycholate, waiting 5 min, adding one-fifth volume of 5% Brij-58, and waiting 5 min. The entire sample was then layered on a 10 to 30% sucrose gradient containing lysis buffer. The gradients were centrifuged at 114,000 × *g* for 35 min in an SW50.1 rotor and analyzed by passage through an Isco density gradient fractionator, model D/UA-2.

Poly(U)-directed polyphenylalanine synthesis on 80S ribosomes. The reaction mixture for poly(U)-directed polyphenylalanine synthesis on 80*S* ribosomes contained, in 50 μliters: 50 mM Tris-hydrochloride, pH 7.7, 12.5 mM magnesium acetate, 80 mM KCl, 4 mM creatine phosphate, 40 μg of creatine phosphokinase per ml, 250 μg of yeast tRNA per ml, 1 mM dithiothreitol, 2 mM adenosine 5'-triphosphate, 0.1 mM quanosine 5'-triphosphate, 60 mCi of [^{14}C]phenylalanine per ml, 300 μg of poly(U) per ml, 16 absorbancy units (260 nm) of ribosomes per ml, and about 2 mg of supernatant enzymes per ml. Incubations were carried out at 30 C for 45 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The sample was heated at 90 C for 10 min, cooled, collected on glass-fiber filters (Whatman GF/C), washed with 5% trichloroacetic acid, and dried. The filters were counted in a toluene-based liquid scintillation solution.

Endogenous messenger RNA-directed protein synthesis. The system was the same as that for polyphenylalanine synthesis on 80*S* ribosomes, except all amino acids were present at 4 μM, poly(U) was omitted, and labeled phenylalanine was replaced by ^{14}C-reconstituted protein hydrolysate. The quantities of polyribosomes in the reaction mixtures were 5.3 and 3.9 absorbancy units at 260 nm/ml of Y166 and CRY6 polysomes, respectively. Reactions were incubated at 30 C for 60 min. The samples were prepared for liquid scintillation counting as described for polyphenylalanine synthesis on 80*S* ribosomes.

Poly(U)-directed polyphenylalanine synthesis on ribosomal subunits. Polyphenylalanine synthesis on ribosomal subunits was measured in a system described elsewhere (19) except that 0.25 OD_{260} units of 40*S* subunits and 0.5 OD_{260} units of 60*S* subunits were used per reaction. Incubations were for 10 min at 30 C. Reactions were terminated by adding 1 ml of 10% trichloroacetic acid. The samples were prepared for liquid scintillation counting as described for polyphenylalanine synthesis on 80*S* ribosomes.

RESULTS

The effect of cryptopleurine on growth of S. cerevisiae. The effect of cryptopleurine on cell growth in YM-5 medium is shown in Fig. 1. Concentrations of drug as low as 0.67 μM completely inhibited the growth of strain Y166. Similar levels of inhibition in the mutant strain CRY6 were only achieved with 130 μM cryptopleurine. However, the mutant was not completely resistant, as shown by the inhibition of growth by drug concentrations in the 3-μM range.

Minimal inhibitory concentrations for the three phenanthrene alkaloids are given in Table 2. At pH 5.8, the usual pH of YM-5 medium, tylocrebrine and tylophorine showed little activity. At pH 7.0, however, the minimal inhibi-

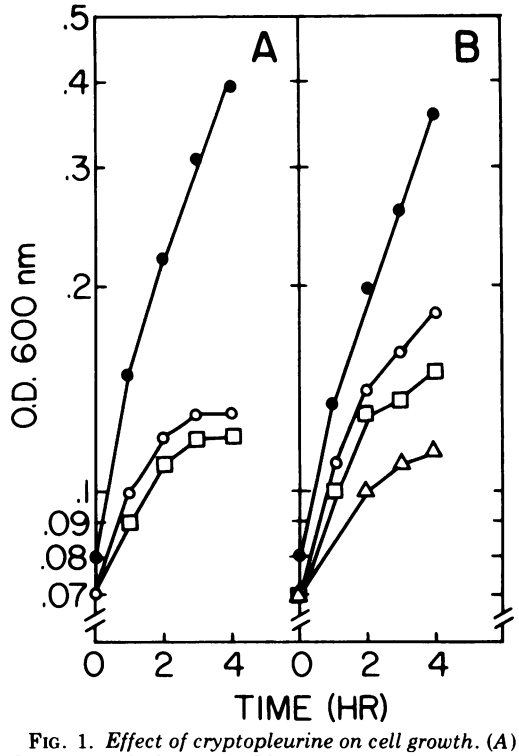

FIG. 1. *Effect of cryptopleurine on cell growth. (A) Y166, control (●), 0.67 μM (○), and 1.3 μM cryptopleurine (□). (B) CRY6, control (●), 2.7 μM (○), 13 μM (□), and 52 μM cryptopleurine (△).*

TABLE 2. *In vivo minimal inhibitory concentrations*

Drug	Minimal inhibitory concn (μM)[a]			
	Y166		CRY6	
	pH 5.8	pH 7.0	pH 5.8	pH 7.0
Cryptopleurine	0.65		130	
Tylocrebrine	260	2.0	1300	130
Tylophorine	260	2.0	1300	130

[a] Minimal inhibitory concentration is defined in Materials and Methods.

tory concentrations for these two drugs were similar to that for cryptopleurine. This possibly indicates some form of a permeability barrier to the charged forms of these two alkaloids at the lower pH.

Cryptopleurine-resistant mutants showed no cross-resistance to either cycloheximide or trichodermin in vivo, indicating that cryptopleurine probably has a mode of action different from that of either of these drugs. Conversely, mutants of *S. cerevisiae* resistant to cycloheximide or trichodermin were sensitive to cryptopleurine and related compounds.

Effect of cryptopleurine on polyribosome profiles. The addition of cryptopleurine to a spheroplast culture resulted in polyribosome profiles indistinguishable from the control (Fig. 2). Cryptopleurine strongly inhibited protein synthesis under these conditions but did not cause a breakdown or a decrease in the number of polyribosomes. Polyribosomes appeared to be "frozen" by cryptopleurine, indicating that inhibition by the alkaloid occurs in the elongation or termination phase of protein synthesis.

Effect of the phenanthrene alkaloids on in vitro protein synthesis. Experiments in an in vitro poly(U)-directed polyphenylalanine synthesizing system using heterologous supernatant and ribosomal fractions from strains Y166 and CRY6 demonstrated that the resistance in strain CRY6 was ribosomal (data not shown). Therefore, wild-type supernatant factors were used in all in vitro experiments shown.

The effect of the three phenanthrene alkaloids on polyphenylalanine synthesis on 80S ribosomes from strains Y166 and CRY6 can be seen in Table 3. The resistant mutant was resistant to all three of the alkaloids. However, from the data on the sensitive strain Y166, it appeared that, in vitro, tylocrebrine and tylophorine were not as potent inhibitors as cryptopleurine. Neither strain Y166 nor strain CRY6 showed any significant resistance to cycloheximide. The mutation to cryptopleurine resistance did not impart cross-resistance to cycloheximide, confirming the in vivo experiments showing no cross-resistance to cycloheximide.

Endogenous protein synthesis in an in vitro system containing yeast polyribosomes confirmed these observations. Low concentrations of the drugs (0.67 to 13 μM) drastically inhibited peptide synthesis when polyribosomes from strain Y166 were used (Fig. 3). Much higher concentrations of the drugs were required to inhibit peptide synthesis on polyribosomes from the resistant strain, CRY6.

Experiments were then done to determine which subunit of the ribosome was responsible for the resistance to cryptopleurine. Table 4

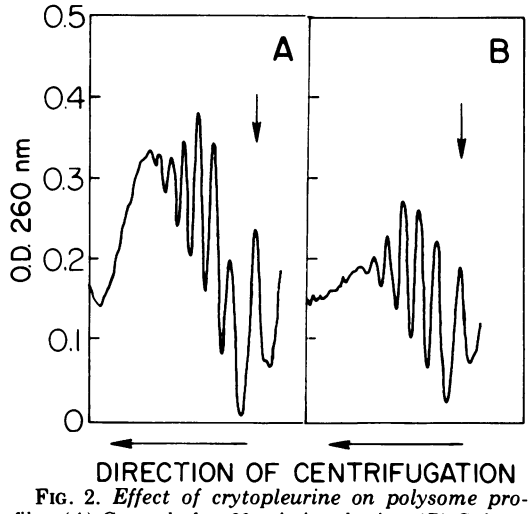

FIG. 2. *Effect of crytopleurine on polysome profiles. (A) Control after 60-min incubation. (B) Culture 60 min after addition of cryptopleurine (130 μM). The arrow indicates the position of 80S ribosomes.*

TABLE 3. *Effect of the phenanthrene alkaloids on poly(U)-directed polyphenylalanine synthesis on 80S ribosomes*[a]

Drug	Concn (μM)	Inhibition (%)	
		Y166	CRY6
Cryptopleurine	1.3	40	8
	7.8	52	20
	13	58	26
Tylocrebrine	2.6	11	4
	13	23	9
	52	44	20
Tylophorine	2.6	15	6
	13	34	15
	52	37	21
Cycloheximide	7.1	56	73
	18	66	86

[a] Conditions for assays are given in Materials and Methods.

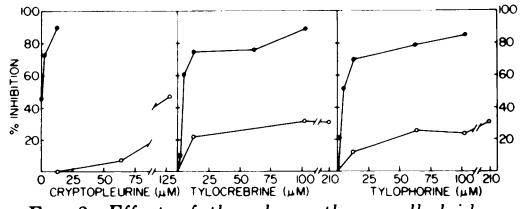

FIG. 3. *Effect of the phenanthrene alkaloids on endogenous mRNA-directed peptide synthesis. Symbols: Y166 (●); CRY6 (○).*

TABLE 4. *Effect of cryptopleurine on poly(U)-directed polyphenylalanine synthesis on ribosomal subunits*

Source of subunit		Conc of drug (μM)	Inhibition (%)	
40S	60S		Expt I[a]	Expt II[b]
Y166	Y166	13	47	36
Y166	CRY6	13	41	47
CRY6	Y166	13	12	0
CRY6	CRY6	13	9	6

[a] One hundred percent values for experiment I were 39, 28.6, 15.6, and 12.7 pmol of phenylalanine incorporated, respectively.

[b] One hundred percent values for experiment II were 13.2, 11.9, 8.8, and 9.4 pmol of phenylalanine incorporated, respectively.

shows the results of two experiments using various combinations of subunits of strains Y166 and CRY6 in an in vitro poly(U)-directed polyphenylalanine synthesizing system. Only those combinations containing 40*S* subunits from the resistant mutant, CRY6, were resistant to cryptopleurine, indicating that strain CRY6 contains an altered 40*S* ribosomal subunit which confers resistance to the phenanthrene alkaloids. The 40*S* subunits from cryptopleurine-resistant strains of *S. cerevisiae* were found to be less stable than those of wild type; this has been noted by Skogerson et al. (20).

Genetic analysis of cryptopleurine resistance. Genetic analysis of the cryptopleurine-resistant mutant, CRY6, indicates that a single, recessive, nuclear gene was responsible for cryptopleurine resistance. During genetic analysis the gene for resistance to cryptopleurine was found to be closely linked to the mating locus. Table 5 shows the ascus-type ratios obtained between the resistance locus, *cry*, and the mating locus in several crosses. Analysis of the tetratype ascus in the cross of strains CRY6 and 2181-1A gave a first division pattern with *trp1*, *cry* and the mating locus that indicated that *cry* is between the centromere and the mating locus. Comparing the first division segregation patterns of *trp5*, *cry* and the mating locus in the tetratype asci in the cross of strains CRY6 and 1/5 gave similar results. Combining the data from all the crosses places *cry* 2.2 centimorgans from the mating type locus on the centromere side.

Preliminary work with strain CRY4, a cryptopleurine-resistant mutant derived from a different strain, A364A, which also has resistant ribosomes, indicated that its lesion was allelic with that in strain CRY6 (Table 5).

The cross with strain 1/5 was done to test whether *cry* was linked to *cyh2*, a locus that determines cycloheximide resistance and gives 60*S* ribosomal subunits resistant to this drug (4, 11). The ascus type ratio was 6 parental ditype:8 nonparental ditype:27 tetratype, indicating no linkage between the two genes. A cross between strains CRY4 and SR_{17}, a strain containing the same *cyh2* mutation as strain 1/5, behaved in the same way: 3 parental ditype:7 nonparental ditype:18 tetratype.

Similar results have been reported by Skogerson, McLaughlin, and Wakatama (20) for the cryptopleurine-resistant mutants they isolated. It would appear that we are examining the same locus, although appropriate crosses have not been done to show that their mutations are allelic with ours.

DISCUSSION

The phenanthrene alkaloids, cryptopleurine, tylophorine, and tylocrebrine, have been shown to inhibit protein synthesis in a variety of eukaryotic systems including yeast (1, 6, 9, 10, 20). These drugs appear to have a primary effect on protein synthesis, although they also inhibit RNA and deoxyribonucleic acid synthesis to some extent (6, 10). Cryptopleurine has been shown to inhibit processing of the 37*S* ribosomal RNA precursor (12). Blockage of 37*S* ribosomal RNA precursor processing has also been observed with the protein synthesis inhibitors cycloheximide and anisomycin (12, 21).

In yeast spheroplasts (Fig. 2; 20) and rabbit reticulocyte lysates (10) the phenanthrene alka-

TABLE 5. *Ascus type ratios between cryptopleurine resistance and the mating locus*

Cross	Ascus ratios[a]		
	PD	NPD	T
CRY6 × 2181-1A	25	0	1
CRY6 × 1/5	58	0	3
CRY4 × SR_{17}	25	0	1

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

loids prevent the breakdown of polyribosomes. In rabbit reticulocyte lysates, it has been shown that tylocrebrine prevents the release of nascent peptide chains (10). This behavior suggests that these drugs inhibit a step in peptide chain elongation or termination. The fact that peptide synthesis is inhibited in an in vitro system using yeast polyribosomes (Fig. 3) indicates that inhibition probably occurs at a step in elongation, since normal initiation and termination do not occur in such a system. The effectiveness of the phenanthrene alkaloids in inhibiting in vitro poly(U)-directed polyphenylalanine synthesizing systems (Tables 3 and 4; 1, 20) also points to peptide chain elongation as the step in protein synthesis which is sensitive to these alkaloids. Huang and Grollman (10), however, reported that tylocrebrine does not inhibit poly(U)-directed polyphenylalanine synthesis on rabbit reticulocyte ribosomes. The reason for this difference in behavior between rabbit reticulocyte ribosomes and yeast or Ehrlich ascites tumor cell ribosomes is not known. Tylophorine and tylocrebrine do not affect peptidyl transferase as measured by the "fragment reaction" (1) or by release of nascent peptide chains from ribosomes (10). However, Pestka et al. (18) have reported a partial inhibition (40%) of peptidyl transferase activity at high levels of cryptopleurine.

Huang and Grollman (10) reported that tylocrebrine does not affect guanosine triphosphatase activity in a rabbit reticulocyte lysate system. However, tylocrebrine stimulates the binding of deacylated tRNA to ribosomes in the rabbit reticulocyte system (10).

Since peptide bond formation seems to be unaffected by the phenanthrene alkaloids, it would seem that they affect that portion of peptide chain elongation known as translocation, i.e., the relative movement of the ribosome along the messenger RNA.

Huang and Grollman (10) found that tylocrebrine inhibits translocation as measured in a two-step assay system that requires movement of peptidyl-tRNA from the aminoacyl-tRNA accepting site on the ribosome to the peptidyl-tRNA site and subsequent reaction with labeled aminoacyl-tRNA. However, puromycin releases 90% of the nascent peptide chains in vivo even in the presence of tylocrebrine (10). If translocation were completely inhibited by tylocrebrine, peptidyl-tRNA should accumulate in the aminoacyl-tRNA accepting site and be unable to react with puromycin. Stimulation by tylocrebrine of the binding of deacylated tRNA to the ribosome may suggest that these drugs somehow prevent the release of the deacylated tRNA from the ribosome after reaction of the peptidyl-tRNA with aminoacylated tRNA. Such a blockage would prevent further relative movement of the ribosome along the messenger RNA.

Battaner and Vazquez (1) reported that, when subunits from yeast ribosomes were pretreated with cryptopleurine or tylocrebrine and used in an in vitro poly(U)-directed polyphenylalanine synthesizing system, only the activity of the 60*S* subunit was affected. However, mixed ribosomal subunits from a cryptopleurine-resistant mutant, CRY6, and the sensitive parent, Y166, indicate that the alteration that produces resistance to these drugs is in the 40*S* subunit (Table 4). These latter results suggest that the site of action of these phenanthrene alkaloids is the 40*S* and not the 60*S* subunit.

Inhibition of the translocation step in protein synthesis by a drug acting on the smaller ribosomal subunit is unexpected; however, a parellel example has recently been reported by Burns and Cundliffe (3). They reported that spectinomycin inhibits translocation on *E. coli* ribosomes. It is known that resistance to spectinomycin can arise as the result of a point mutation in a protein from the 30*S* ribosomal subunit of *E. coli* (2).

Genetic analysis of the resistant mutant CRY6 indicates that resistance to the phenanthrene alkaloids is due to a single altered nuclear gene. Resistance to the phenanthrene alkaloids is recessive to sensitivity. The gene determining resistance, *cry*, lies approximately 2.2 centimorgans nearer the centromere of chromosome III than the mating locus. Skogerson, McLaughlin, and Wakatama (20) have reported cryptopleurine-resistant mutants lying at the same place. This mutation, *cry*, yields ribosomes that are resistant to cryptopleurine in vitro as a result of an altered 40*S* ribosomal subunit. The locus for cryptopleurine resistance provides the first genetic marker for the 40*S* ribosomal subunit in *S. cerevisiae*, or in any eukaryote.

The gene determining cryptopleurine resistance, *cry*, is not linked to *cyh2* or to a mutation conferring resistance to trichodermin (19). Both *cyh2* and the mutation to trichodermin resistance have been shown to produce altered 60*S* ribosomal subunits (4, 11, 19). Whereas there is presently no evidence indicating that these mutations affect ribosomal proteins and not ribosomal RNA or modifying enzymes, it is interesting to note that the known loci for ribosomal function in *S. cerevisiae* are not in a single cluster. The genetic loci *cry* and *cyh2*, trichodermin resistance, and the majority (60 to

70%) of the structural genes for ribosomal RNA (7, 13, 17) all lie on different chromosomes (15). The 140 ribosomal RNA cistrons do appear to exist in clusters of 10 to 32 cistrons, and three to ten of these clusters appear to lie on chromosome I of *S. cerevisiae* (5, 7, 13, 17). It appears that the organization of ribosomal structural genes in *S. cerevisiae* may prove to be different from the ribosomal gene cluster found in prokaryotes like *E. coli* (16).

While this work was in progress, it was learned that similar results had been obtained by K. Johnson and L. Skogerson (personal communication). We thank Dr. Skogerson for the opportunity to share data.

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LITERATURE CITED

1. Battaner, E., and D. Vazquez. 1971. Inhibitors of protein synthesis by ribosomes of the 80*S* type. Biochim. Biophys. Acta **254**:316-330.
2. Bollen, A., J. Davies, M. Ozaki, and S. Mizushima. 1969. Ribosomal protein conferring sensitivity to the antibiotic spectinomycin in *Escherichia coli*. Science **165**:85-86.
3. Burns, D. J. W., and E. Cundliffe. 1973. Bacterial protein synthesis: a novel system for studying antibiotic action *in vivo*. Eur. J. Biochem. **37**:570-574.
4. Cooper, D., D. V. Banthorpe, and D. Wilke. 1967. Modified ribosomes conferring resistance to cycloheximide in mutants of *Saccharomyces cerevisiae*. J. Mol. Biol. **26**:347-350.
5. Cramer, J. H., M. M. Bhargava, and H. O. Halvorson. 1972. Isolation and characterization of γDNA of *Saccharomyces cerevisiae*. J. Mol. Biol. **71**:11-20.
6. Donaldson, G. R., M. R. Atkinson, and A. W. Murray. 1968. Inhibition of protein synthesis in Ehrlich ascites-tumor cells by the phenanthrene alkaloids tylophorine, tylocrebrine, and cryptopleurine. Biochem. Biophys. Res. Commun. **31**:104-109.
7. Finkelstein, D. B., J. Blamire, and J. Marmur. 1972. Location of ribosomal RNA cistrons in yeast. Nature N. Biol. **240**:279-281.
8. Hartwell, L. 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. **93**:1662-1670.
9. Haslam, J. M., P. J. Davey, and A. W. Linnane. 1968. Differentiation *in vitro* by phenanthrene alkaloids of yeast mitochondrial protein synthesis from ribosomal systems of both yeast and bacteria. Biochem. Biophys. Res. Commun. **33**:368-373.
10. Huang, M. T., and A. P. Grollman. 1972. Mode of action of tylocrebrine: effects on protein and nucleic acid synthesis. Mol. Pharmacol. **8**:538-550.
11. Jimenez, A., B. Littlewood, and J. E. Davies. 1972. Inhibition of protein synthesis in yeast, p. 292-306. *In* E. Muñoz, F. Garcia-Ferrândiz, and D. Vazquez (ed.), Molecular mechanism of antibiotic action on protein biosynthesis and membranes. Elsevier, Amsterdam.
12. Jimenez, A., D. J. Tipper, and J. Davies. 1973. Mode of action of thiolutin, an inhibitor of macromolecular synthesis in *Saccharomyces cerevisiae*. Antimicrob. Ag. Chemother. **3**:729-738.
13. Kaback, D. B., M. M. Bhargava, and H. O. Halvorsen. 1973. Location and arrangement of genes coding for ribosomal RNA in *Saccharomyces cerevisiae*. J. Mol. Biol. **79**:735-739.
14. Mortimer, R. K., and D. C. Hawthorne. 1966. Genetic mapping on *Saccharomyces*. Genetics **53**:165-173.
15. Mortimer, R. K., and D. C. Hawthorne. 1973. Genetic mapping on *Saccharomyces*. IV. Mapping of temperature-sensitive genes and use of disomic strains on localizing genes. Genetics **74**:33-54.
16. Nomura, M., and F. Engbaek. 1972. Expression of ribosomal protein genes as analyzed by bacteriophage Mu-induced mutations. Proc. Nat. Acad. Sci. U.S.A. **69**:1526-1530.
17. Øyen, T. B. 1973. Chromosome I as a possible site for some rRNA cistrons in *Saccharomyces cerevisiae*. FEBS Lett. **30**:53-56.
18. Pestka, S., H. Rosenfeld, R. Harris, and H. Hintikka. 1972. Studies on transfer ribonucleic acid-ribosome complexes. XXI. Effect of antibiotics on peptidyl-puromycin synthesis by mammalian polyribosomes. J. Biol. Chem. **247**:6895-6900.
19. Schindler, D., P. Grant, and J. Davies. 1974. Trichodermin resistance, a mutation affecting eukaryotic ribosomes. Nature (London) **248**:535-536.
20. Skogerson, L., C. McLaughlin, and E. Wakatama. 1973. Modification of ribosomes in cryptopleurine-resistant mutants of yeast. J. Bacteriol. **116**:818-822.
21. Udem, S. A., and J. R. Warner. 1972. Ribosomal RNA synthesis in *Saccharomyces cerevisiae*. J. Mol. Biol. **65**:227-242.
22. Van der Zeijst, B. A. M., A. J. Kool, and H. P. J. Bloemers. 1972. Isolation of active ribosomal subunits from yeast. Eur. J. Biochem. **30**:15-25.