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Ribosomal Suppressors and Antisuppressors in *Podospora anserina*: Resistance to Cycloheximide

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Informational suppressors and antisuppressors have been previously isolated in *Podospora anserina*, and a range of exclusively genetic arguments have led to the assumption that they correspond to ribosomal mutations. An in vivo and in vitro comparison of the effect of the ribosomal inhibitor cycloheximide on wild-type and mutant strains described in this paper confirms the ribosomal hypothesis for at least some mutants. Indeed, the four mutants in the *AS*3 gene were cycloheximide resistant, and their ribosomes were found to be resistant when analyzed by polyuridyl-directed polyphenylalanine synthesis. On the other hand, ribosomes from two *su*1 mutants were hypersensitive to the drug.

Genetic analysis of ribosomes in eukaryotes is still at an early stage (9, 17). The lack of screening procedures for the isolation of ribosomal mutations is a major problem. Selection for cold-sensitive mutants (2, 21, 26) and for resistance to protein synthesis inhibitors has proved a useful technique for selecting ribosomal mutants in several organisms. The ribosomal nature of cycloheximide (CHX)- (3, 8, 10, 16, 22), cryptopleurine- (7, 23), and trichodermin- (20) resistant mutants has been demonstrated in numerous studies. However, these selection procedures remain limited.

The ascomycete *Podospora anserina* is a convenient eukaryotic organism for genetic analysis and biochemical studies. In this organism, suppressors and antisuppressors were screened and described by Picard-Bennoun (14, 15). The suppressors were not gene specific and are therefore presumed to be informational suppressors. Because of their suppression pattern (they suppress nonsense, missense, and frameshift mutants), their weak dominance, and their pleiotropy, most of them are assumed to be mutations affecting ribosomal proteins. In such mutants, analogous to *ram* mutants of *Escherichia coli* (19), the misreading level would be enhanced. Five loci were defined: *su*1, *su*2, *su*3, *su*5, and *su*7. Some alleles (strong suppressors) are more efficient than others (weak suppressors). The antisuppressors (five loci: *AS*1, *AS*2, *AS*3, *AS*4, and *AS*5) were screened as mutations displaying an antagonistic action with respect to the suppressors. They would be similar to restrictive ribosomal mutants of *E. coli* (4 to 6). Such mutants are of major interest in the study of ribosomal control of translational fidelity in eukaryotes. Therefore, in spite of the strength of the genetic arguments, the ribosomal hypothesis must be ascertained by in vitro experiments. A first step in this direction is presented in this paper.

Ribosomes are very integrated structures. Even slight modifications of a ribosomal protein may change the structure and function of the organelle. A preliminary study (J. Cohen, personal communication) suggested that some of the *su* and *AS* mutants of *Podospora* display a level of resistance to CHX, trichodermin, and/or aminoglycosides different from that of the wild type. Therefore, we have undertaken a systematic study of the resistance of all the mutants to these antibiotics both in vivo and in vitro. In this paper, results concerning CHX are reported.

CHX (see reference 13 for review) acts at the ribosomal level by inhibiting chain initiation as well as polypeptide chain elongation (11, 13). The resistance is a property associated with the 60S ribosomal subunit (24).

By analogy with *E. coli* mutants (12, 28), it is possible that the proteins coded by *su* and *AS* genes in *Podospora* belong to the 40S subunit and yet affect the 60S subunit function. It is well known that a mutation in one subunit can change the response to an antibiotic that exerts its primary effect through the other ribosomal subunit (1).

MATERIALS AND METHODS

The main properties of and culture techniques for *P. anserina* were first described by Rizet and Engel-

man (18) and were last reviewed by Esser (Neurospora Newslett. 15:27, 1969.

Strains. The origin and phenotypes of all the strains used in this study have been described (14, 15).

The most important characteristics of the mutants displaying a level of resistance to CHX different from that of the wild type are presented in Table 1.

Resistance levels in vivo. Resistance levels to CHX of the different strains were determined on solid synthetic medium (M2) after growth at 27°C. Stock solutions of CHX were sterile filtered, and appropriate amounts were added to molten agar before pouring. Growth was always measured as the diameter of the thallus. Two techniques were used as follows.

(i) CHX concentration curves. After 2 days at 27°C, the percentage of growth was determined by comparing the growth on M2 with increasing concentrations of antibiotic with that on M2. Diameters of 10 thalli were measured for each strain at each CHX concentration.

(ii) Growth curves at a definite concentration. *Podospora* is a filamentous fungus that forms a relatively flat, circular mycelium growing only at the periphery. Therefore, the growth curve, measured by the increase in diameter, is linear. The slope of this line (millimeters of growth per day) gives the growth rate. The diameters of five thalli were measured each day during at least 6 days on M2 medium and on M2 plus CH_A. The ratio of the two rates, expressed as a percentage, yields a measure of the resistance level of a given strain.

Preparation of ribosomes and high-speed supernatant. This was done according to Begueret and Perrot (M. Perrot, thèse de 3è cycle, University of Bordeaux, Bordeaux, France, 1975). Roux flasks were inoculated with mycelia fragmented and disrupted in a Waring blender for 30 s. Cultures were grown at 27°C for 3 days in the dark and were harvested by filtration through cheesecloth. The mycelia were washed with distilled water and buffer A [0.44 M sucrose-50 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.6)-10 mM magnesium acetate-25 mM KCl-5 mM 2-mercaptoethanol]. They were squeezed dry and stored at −80°C. Mycelia were disintegrated in a grinding mill with acid-washed sea sand. When all hyphae were disrupted (by observation with a light microscope), 1 volume of buffer A was added. The resulting smooth paste was centrifuged in an SS34 rotor in a Sorvall RC2B centrifuge at 10,000 × *g* for 10 min to sediment cellular debris and sand. The supernatant S10-1 was saved, and the pellet was washed once more with buffer A and respun as above to obtain S10-2. S10-1 was used to obtain ribosomes and high-speed supernatant, whereas S10-2 (more diluted) was used only for ribosomes. Mitochondrial fragments and cellular debris were spun down by centrifugation at 25,000 × *g* for 20 min. The post-mitochondrial supernatants were centrifuged for 2 h in a 40 rotor at 140,000 × *g* in a Beckman Spinco model L ultracentrifuge. The pellets were taken up in buffer B (buffer A without sucrose). Their dispersion was made easier by gentle use of a Potter homogenizer. After a clarifying spin for 10 min at 10,000 × *g*, the solution was placed onto a 50% sucrose cushion made in buffer A and centrifuged at 100,000 × *g* for 15 h in the same rotor. Ribosomes passed the cushion and sedimented at the bottom of the tube. The pellet consisted of a clear, colorless precipitate covered by an opaque yellow layer, which was flushed out without disturbing the clear pellet. This layer contained ribosomes bound to the membranes. The pellet was resuspended in buffer B and stored at −80°C for several months

TABLE 1. *General characteristics of the mutants used*[a]

Strain	Mutagen	Suppressor or antisuppressor	% Germination	% Growth/ WT growth[b]	Female fertility	Cold or heat sensitivity	CHX		Ribosomal activity[c]
							In vivo	In vitro	
WT			100	100	+++				1
*su*1-1	NG	Weak suppressor	100	110	+++				1
*su*1-5	UV	Weak suppressor	100	100	+++			S	2.8
*su*1-25	NG	Strong suppressor	30	50	−	ts	R	S	1
*su*1-26	NG	Strong suppressor	90	50	−	ts	R		1.5
*su*1-51	ICR	Strong suppressor	65	80	+				0.2
*AS*3-1	NG	Antisuppressor	20	90	+	cs	R	NT	NT
*AS*3-2	EMS	Antisuppressor	20	85	+	cs	R	R	0.6
*AS*3-3	EMS	Antisuppressor	20	85	+	cs	R	R	0.5
*AS*3-4	NG	Antisuppressor	20	90	+	cs	R	NT	NT

[a] See references 14 and 15 for more information. Symbols and abbreviations are as follows: WT, wild type; NG, nitrosoguanidine; UV, ultraviolet light; ICR, ICR-170; EMS, ethylmethane sulfonate; +++, good fertility; + poor fertility; −, no fructification; ts, heat sensitive; cs, cold sensitive; R, more resistant than wild type; S, more sensitive than wild type; NT, not tested.

[b] Growth was measured as diameter of the thallus after 2 days at 27°C.

[c] Efficiency of incorporation by mutant ribosomes is compared with that of wild-type ribosomes for a 60-min incubation. The ratios are a mean of values measured in four experiments for *su*1-5, two for *su*1-25, eight for *su*1-26, two for *su*1-51, and seven for *AS*3-3.

without loss of activity. The concentration of ribosomes was estimated by ultraviolet light absorption at 260 nm.

After ultracentrifugation of S10-1, the upper two-thirds of the supernatant (S140) was saved. This was dialyzed overnight against buffer A and stored in small portions at −80°C. The S140 contains aminoacyl-transfer ribonucleic acid (tRNA) synthetases and factors necessary for the polyuridyl [poly(U)]-directed phenylalanine polymerization. Wild-type supernatants were used in all in vitro experiments in order to normalize the system.

Cell-free system: poly(U)-directed phenylalanine polymerization. The reaction mixture contained the following components in 125 μl: 8.125 μmol of tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.8; 3.44 μmol of KCl; 1.625 μmol of magnesium acetate; 2 μmol of 2-mercaptoethanol; 0.75 μmol of phosphoenolpyruvate; 0.25 μmol of adenosine 5'-triphosphate; 0.063 μmol of guanosine 5'-triphosphate; 150 μg of tRNA from yeast; 3.2 U of pyruvate kinase; 31.25 μg of poly(U); 12.5 μl of S140; and 2.5 optical density units (at 260 nm) of ribosomes.

The reaction was started by adding 12.5 μl of [^{14}C]phenylalanine (0.125 μCi; 436 mCi/mmol) and carried out at 27°C for 60 min unless otherwise indicated. At the end of the incubation, 125 μl of 10% trichloroacetic acid containing 4 g of phenylalanine per liter was added to the mixture. The tubes were put on ice for 10 min to allow complete precipitation and then heated for 20 min at 90°C. After 10 min on ice, the hot, acid-insoluble material was collected on glass-fiber filters (Whatman GF/C) and washed three times with 5% trichloroacetic acid containing 2 g of phenylalanine per liter and three times with 96% alcohol. The filters were then dried and transferred to vials containing scintillation fluid [3.92 g of 2,5-diphenyloxazole (PPO) and 80 mg of *p*-bis-(*p*-methylstyryl)benzene per liter of toluene]. Counting was done in an Intertechnique liquid scintillation counter with an efficiency of 80%. In each experiment a filter reference was made by adding trichloroacetic acid before [^{14}C]phenylalanine.

Inhibition of polyphenylalanine synthesis by CHX. The effect of CHX on in vitro protein synthesis was determined by two procedures: (i) CHX concentration curves and (ii) kinetics of [^{14}C]phenylalanine incorporation with and without CHX. In the first method, polyphenylalanine synthesis was carried out for 60 min in the presence of increasing concentrations of CHX. The inhibition is expressed as the percentage of [^{14}C]phenylalanine incorporated with and without CHX. In the second method, 125-μl portions, taken at various time intervals in a tube containing the reaction mixture necessary for all samples, were precipitated with trichloroacetic acid. The percentage of incorporation was determined by the incorporation rate ratios with and without CHX. The incorporation rates, given by the slope of a straight line, were calculated by the method of linear adjustment.

We observed that the rate of phenylalanine incorporation by ribosomes of a given strain varied from one experiment to another. These variations could be due to the quality of ribosome or supernatant extraction (supernatant activity decreases with conservation time) or to variation in the reaction mixtures. For this reason, wild-type and mutant ribosomes were always compared within the same experiment.

Source of reagents. CHX, pyruvate kinase, phosphoenolpyruvate, adenosine 5'-triphosphate, and guanosine 5'-triphosphate were purchased from Sigma Chemical Co., St. Louis, Mo.; poly(U) and tRNA were obtained from Miles Laboratories, Elkhart, Ind.; [^{14}C]phenylalanine was from CEA, Saclay, France; and Omnifluor was from New England Nuclear Corp., Boston, Mass.

RESULTS

In vivo analysis of *su* and *AS* strains. A preliminary analysis of all known suppressor and antisuppressor mutants at 10 loci was carried out: 60 mutants in the *su*1 locus, 14 in *su*2, 4 in *su*3, 1 in *su*5, 2 in *su*7, 4 in *AS*1, 2 in *AS*2, 4 in *AS*3, 8 in *AS*4, and 1 in *AS*5. Three implants of each mutant strain were tested on M2 plus 2 μg of CHX per ml, a concentration yielding 50% growth inhibition of the wild-type strain. Mutants differing even slightly from wild type were retained and crossed to wild type to check that antisuppression (or suppression) and drug resistance (or sensitivity) cosegregated. Three strains displaying hypersensitivity to CHX were discarded because this phenotype was controlled by another mutation. The other strains were further analyzed according to the procedure described in Materials and Methods.

The CHX concentration curves identified six strains displaying higher resistance to CHX than wild type. After 48 h of growth in the presence of 10 μg of CHX per ml, the inhibition was 100% for the wild type, 40% for the four *AS*3 mutants, and 53% for the *su*1-25 and *su*1-26 strains. With 20 μg of CHX per ml, growth of the two *su*1 mutants was not totally inhibited. The *AS*3 mutants still grew on drug concentrations higher than 40 μg/ml. This resistance level can be compared with that of the *cyh* 1-1 mutant, the ribosomes of which are resistant to CHX in vitro (M. Perrot, thèse de 3è cycle, 1975). CHX at 10 μg/ml caused only 25% growth inhibition for this mutant, and the growth was not totally inhibited at concentrations as high as 100 μg/ml. The *cyh*1 gene is not linked to any of the *su* and *AS* genes (M. Picard-Bennoun, personal communication).

Growth curves produced some supplementary data. Resistance was manifested at two levels: growth yield and the initial lag time necessary before growth began (Fig. 1). The values obtained for the *su*1-25, *su*1-26, *AS*3-2,

and wild-type strains are shown in Table 2. Results for the *su1-5* mutant studied in vitro were included. It can be seen that this mutant did not differ from wild type in vivo.

Analysis of ribosomes from the *AS3* mutants. There was a parallel between the resistance of *AS3* strains in vivo and the resistance of their ribosomes in a cell-free system. A comparison of the inhibition of polyphenylalanine synthesis at different CHX concentrations between ribosomes from the *AS3-3* and the wild-type strains is shown in Fig. 2A. Higher concentrations of CHX were required to obtain a given inhibition with mutant than with wild-type ri-

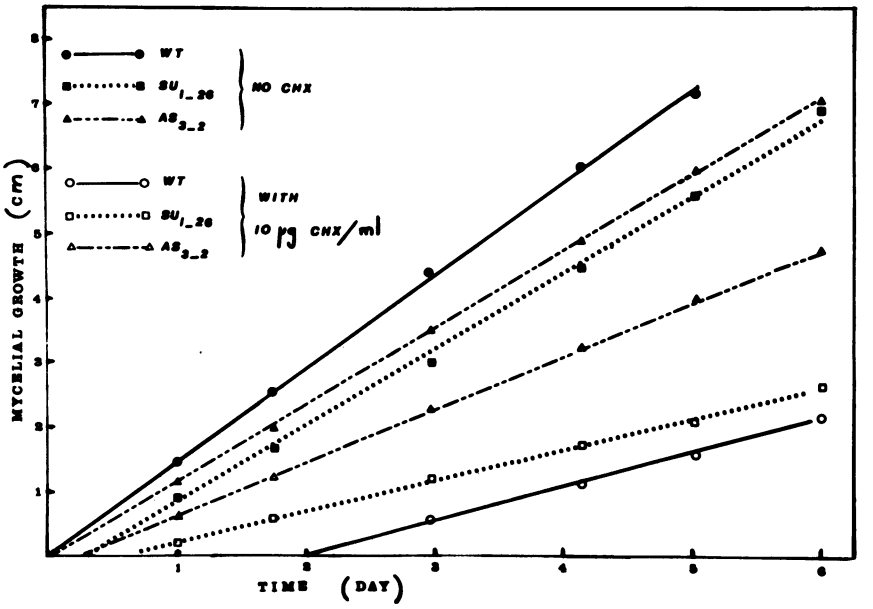

FIG. 1. *Comparison of the growth rates of wild-type (WT), sul-26, and AS3-3 strains measured on M2 and M2 plus 10 µg of CHX per ml as described in the text.*

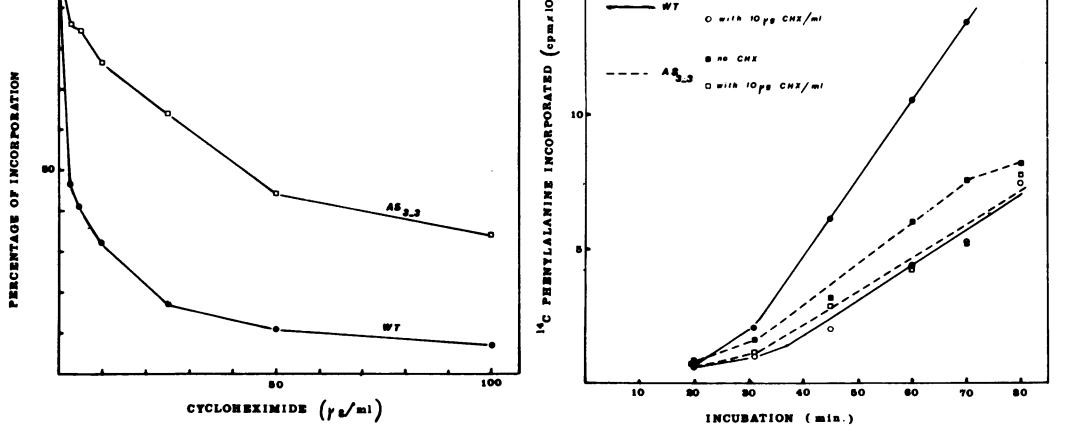

FIG. 2. *Influence of increasing concentrations of CHX on polyphenylalanine synthesis with wild-type (WT) and AS3-3 ribosomes. (A) Incorporation of [^{14}C]phenylalanine without CHX was (per unit of optical density [at 260 nm] of ribosomes): WT, 5.4 pmol; AS3-3, 2.2 pmol. (B) Kinetics of phenylalanine incorporation with WT and AS3-3 ribosomes without CHX and with 10 µg of CHX per ml. A rate of 10,000 cpm corresponded to 5.2 pmol of phenylalanine incorporated per optical density unit of ribosomes.*

TABLE 2. *Growth characteristics of four mutant strains compared with wild type*[a]

Strains	Characteristic											
	0[b]		2		4		10		20		40	
	Growth yield[c]	Growth lag[d]	Growth yield	Growth lag	Growth yield	Growth lag	Growth yield	Growth lag	Growth yield	Growth lag	Growth yield	Growth lag
Wild type	100	0	60	21	51	38	33	48	0		0	
*su*1-25	100	9	78	10	NT[e]	NT	37	12	30	18	24	30
*su*1-26	100	1	94	4	NT	NT	70	6	52	6	44	12
*AS*3-2	100	0	62	24	50	40	NT	NT	NT	NT	NT	NT

[a] As measured on M2 with increasing concentrations of CHX; the values were calculated from the growth curves as described in Materials and Methods.
[b] Micrograms of CHX per milliliter.
[c] Growth yield = [(growth rate on M2 + CHX)/(growth rate on M2)] × 100.
[d] Initial lag was measured on the graphs (in hours).
[e] NT, Not tested.

bosomes. Similar concentration curves were obtained in two other experiments, one using the same *AS*3-3 ribosomes and the other using ribosomes from the *AS*3-2 allele, when compared with wild-type ribosomes from different extractions. This result was confirmed by the kinetics data. (Fig. 2B). With 10 μg of CHX per ml, the incorporation rate was 82% of the control with mutant ribosomes and 45% with wild-type ribosomes. Approximate values were found in another experiment performed with ribosomes of a different *AS*3-3 strain.

Analysis of ribosomes from strains carrying an *su*1 mutation. *su*1-25 ribosomes were slightly hypersensitive to CHX, whereas there was no significative difference between *su*1-26 and wild-type ribosomes. The results of concentration curves were confirmed by kinetics at 10 μg of CHX per ml. In one such experiment, the incorporation rate was 63% of the control with wild-type ribosomes, 56% with *su*1-26 ribosomes, and 47% with *su*1-25 ribosomes. In another kinetics experiment, performed with ribosomes from other wild-type, *su*1-26, and *su*1-25 strains, the percentages of incorporation were, respectively, 77, 74, and 61%. By modifying the Mg^{2+} concentration, we hoped to test separately initiation and elongation, as is done for the reticulocyte system (11). The difference between *su*1-25 and wild-type ribosomes was increased at the high and low Mg^{2+} concentrations (Fig. 3). On the other hand, these results

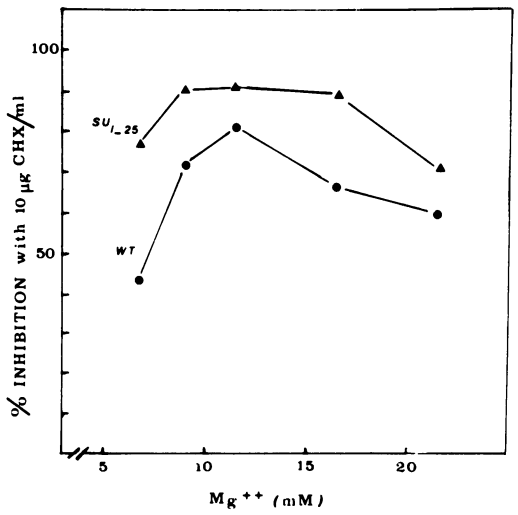

FIG. 3. *Effect of Mg^{2+} concentration on inhibition by CHX of polyphenylalanine synthesis with wild-type (WT) and su1-25 ribosomes. Standard assay conditions were modified to contain the indicated concentrations of Mg^{2+}.*

suggest that the *Podospora* and reticulocyte systems are different. The above results suggest that some mutations in the *su*1 gene could confer increased sensitivity to CHX. Therefore we tested ribosomes from strains displaying no difference from wild type in vivo (see Table 1 for *su*1-5). The *su*1-5 and *su*1-25 ribosomes behaved similarly. The hypersensitivity, although slight, was found again in four experiments performed with *su*1-5 and wild-type ribosomes from different strains and extractions. It was confirmed by kinetics at 5 and 10 μg of CHX per ml (Fig. 4). The incorporation rate was decreased more with *su*1-5 than with wild-type ribosomes: 51 and 62%, respectively, of the controls. The time lag before the polymerization reaction became linear was increased by CHX. The increase was greater with *su*1-5 than with wild-type ribosomes (11 min instead of 5).

Such hypersensitivity was not observed with either *su*1-51 or *su*1-1 ribosomes. Therefore, this characteristic seems to be a property of only some alleles of the *su*1 gene.

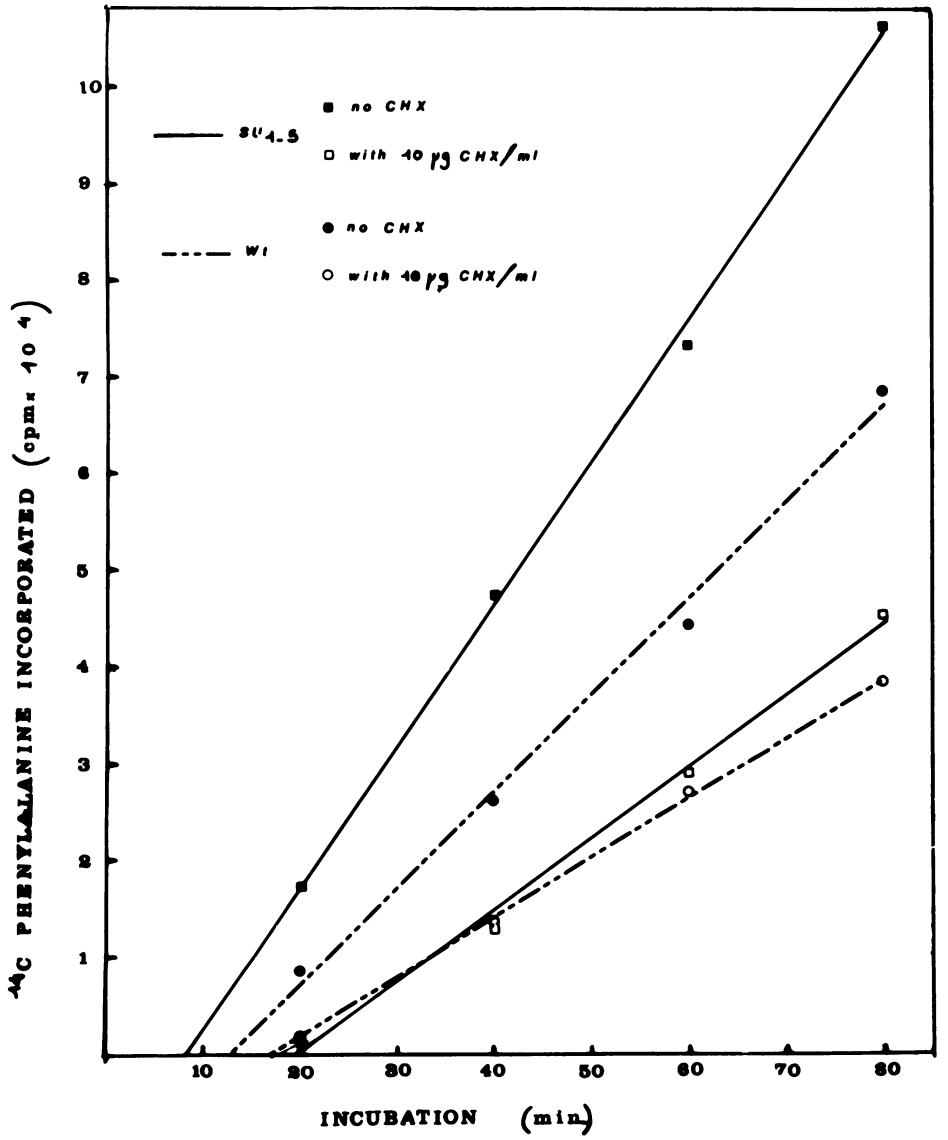

FIG. 4. *Kinetics of phenylalanine incorporation with wild-type (WT) and su1-5 ribosomes without CHX and with 10 μg of CHX per ml. For details, see text and Fig. 2B.*

Ribosome activity. There was a relationship between the genotype of the strains and the incorporation rates of phenylalanine by their ribosomes (Table 1, last column). Phenylalanine incorporation was less by a factor of two with *AS* than with wild-type ribosomes. The incorporation was often more efficient with *su* than with wild-type ribosomes.

DISCUSSION

In this paper, the in vivo and in vitro resistance to CHX of various suppressor and antisuppressor mutant strains of *Podospora* was analyzed. We found that several mutants display modified sensitivity to CHX, therefore confirming the hypothesis previously made that these mutations are expressed at the ribosomal level. It must be stressed that the differences between wild-type and mutant ribosomes were not drastic but were always reproducible. The difference observed in the incorporation rates of polyphenylalanine between *AS*, *su*, and wild-type ribosomes is an additional argument. *AS*3 and *su*1 strains are, therefore, mutated in genes coding for ribosomal components or modifying enzymes. Table 1 summarizes all the data concerning the nine mutants studied here. It shows in particular that most of the mutations are highly pleiotropic.

***AS*3 mutants.** Mutations in the *AS*3 gene lead to resistance to CHX in vivo as well as in vitro. A similar situation was observed in *Schizosaccharomyces pombe*, in which two CHX-resistant alleles of *cyh*1 have an antisuppressor effect (25). The resistance level of *AS*3 ribosomes seems low when it is compared with that of *cyh*1-1 ribosomes (M. Perrot, thèse de 3è cycle, 1975). In the same cell-free system, *cyh*1-1 ribosomes exhibit 91% incorporation of the control at 100 μg of CHX per ml. In this mutant, screened for its resistance to CHX, it is probably the binding site of the antibiotic that is altered. Indeed, resistance has been shown to be a property of the 60S subunit. *AS*3 mutants selected as antisuppressors probably act through another mechanism. The structural distortion due to the altered ribosomal component affects the CHX site. The *AS*3 gene could code for a protein of the small subunit because of its relationship to ribosomal ambiguity. However, modification of a 60S component leading to a more efficient termination of translation could also explain the properties of *AS*3 (15). By separating the ribosomal subunits of both *AS*3 and wild-type ribosomes and cross-reassociating them, we can attribute the modification conferring resistance to CHX to a particular subunit.

***su*1 mutants.** Although the *su*1-26 strains are resistant to CHX in vivo, their ribosomes do not differ from wild-type ribosomes in vitro. Such a situation has been found in *E. coli*: ribosomal mutants that are affected in protein L22 are resistant to erythromycin, but their ribosomes are not resistant to that drug in vitro (27). Ribosome-membrane interactions could explain these findings. Alternatively, the resistance could be related to a step in protein synthesis that cannot be tested in the cell-free system used, for instance the initiation level. A suitable cell-free system must be established to prove this assumption.

The *su*1-25 strain is resistant in vivo and hypersensitive in vitro; the above-mentioned hypothesis could account for this finding.

Isolated *su*1-5 ribosomes are hypersensitive in vitro, whereas there is no difference between this mutant and the wild-type strains in vivo. Several hypotheses for this finding can be advanced. The hypersensitivity may be too low to give a different phenotype at the cellular level. Alternatively, the hypersensitivity observed in vitro could be caused by use of the poly(U) system, but would not occur at the level of in vivo protein synthesis.

For these three *su*1 mutants, the relationship of the in vivo observations to the in vitro assays are unclear. However, it has been shown that ribosomes from *su*1-25 and *su*1-5 strains are modified.

In conclusion, the experiments presented here strongly suggest that *su*1 and *AS*3 genes code for ribosomal components (or, less likely, for modifying enzymes). Screening for informational antisuppressors and suppressors makes it possible to obtain ribosomal mutants other than those determining cold sensitivity and drug resistance in eukaryotes. It remains to be confirmed by in vitro experiments that these mutants are concerned with the control of translational fidelity. Further studies of these mutants should provide important information concerning this ribosomal function in eukaryotes.

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