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Neurospora crassa Cytoplasmic Ribosomes: Isolation and Characterization of a Cold-Sensitive Mutant Defective in Ribosome Biosynthesis

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Twenty-seven cold-sensitive mutants of *Neurospora crassa* were isolated by mutagenesis of wild-type conidia followed by filtration enrichment in complete medium at the nonpermissive temperature (10 C). Zone sedimentation analyses of cytoplasmic ribosomes isolated from the wild-type strain and from 14 of the mutant strains grown at 10 C indicate that one cold-sensitive mutant is defective in ribosome biosynthesis at that temperature: instead of the 2.3:1 mass ratio of 60*S*:37*S* ribosomal subunits characteristic of wild type, the mutant strain PJ30201 (called *crib-1* for cytoplasmic ribosome biosynthesis) exhibits a mass ratio of approximately 7.2:1. Ribosomal subunits synthesized by strain PJ30201 at 25 C are present in wild-type proportions. The cold-sensitive and ribosomal phenotypes segregate together in tetrads isolated from crosses between strain PJ30201 and the wild type indicating that a single nuclear gene mutation is probably responsible for both mutant phenotypes. The *crib-1* locus lies near the centromere in linkage group IV.

In bacteria, several different approaches have been directed at obtaining an understanding of ribosome structure, function, and assembly (for reviews, see references 6, 13, 16). Studies on the in vitro assembly of the 30*S* ribosomal subunit of *Escherichia coli* ribosomes from its component ribosomal ribonucleic acid and protein molecules indicated that a strongly temperature-dependent step is involved (21). On the assumption that in vivo ribosome assembly is also strongly temperature dependent, cold-sensitive mutants of bacteria have been isolated and a large proportion of them have been shown to have conditional blocks in ribosome assembly (9, 20).

In view of the success in bacteria of the genetic approach towards understanding ribosome structure and function, and on the assumption that the assembly of eukaryotic cytoplasmic ribosomes is also strongly temperature dependent, we hypothesized that selection for cold-sensitive mutants of *Neurospora crassa* would enrich for ribosome mutants in that organism also. Clearly, such mutants would be valuable for studying the structural and functional relationships of eukaryotic cytoplasmic ribosomes. Indeed, some cold-sensitive mutants of a related ascomycete, *Saccharomyces cerevisiae*, have been shown to be defective in ribosome formation at low temperatures (1, 2, 10).

This paper describes the methodology for the isolation and characterization of cold-sensitive, ribosome mutants of *N. crassa*.

MATERIALS AND METHODS

Culture techniques. The culture techniques and growth media used were essentially those of Beadle and Tatum (3) with the exception that Vogel minimal medium N (22), supplemented with 2% sucrose unless otherwise indicated, was substituted for Fries salts. The complete medium used was medium N supplemented with 1.0% sucrose, 0.25% Difco yeast extract, and 0.75% Difco malt extract. Where solid medium was required, 2.0% agar was added (3.0% in the case of growth tubes). All crosses were made on slants of Difco corn meal agar which were incubated at 25 C.

Strains. The wild-type strains of *Neurospora crassa*, used for crosses and for mutagenic treatment, were the St. Lawrence laboratory strains, *74 A* and *77 a*, further inbred in the laboratory of A. M. Srb (Cornell University) from whom they were obtained.

The standard mating-type testers used were the peach-fluffy strains, fl^p *A* and fl^p *a* (17). The strains used for mapping experiments were the following: alcoy (T[I; II]4637,*al-1;* T[IV; V]R2355, *cot-1;* T[III; VI]1,*ylo-1*) (17), *cot-1; inos*(C102[t]; 37401) (17), *arg-2*(33442), and *met-1*(38706). These strains were obtained from the Fungal Genetics Stock Center, California State University, Humboldt, Arcata, Calif.

Genetic mapping procedures. The technical methods for initial linkage group localization of cold-sensitive mutations were those described by Perkins et al. (17). These procedures employ the multiple

translocation linkage-tester strain, alcoy. Follow-up mapping crosses were analyzed using the methods described in Davis and de Serres (7).

Growth measurement. Comparison of mycelial growth rates was made using 500-mm-long growth tubes (18) which have an outside diameter of 13 mm. Each tube was half-filled with 18 ml of solid minimal medium, inoculated at one end with a small amount of an aqueous conidial suspension, and incubated at the required temperature. The lateral mycelial extension along the surface of the medium was marked at regular time intervals.

Mutagenic technique. Mutagenesis was performed on conidia harvested from 6.5-day-old cultures of wild type grown in 125-ml Delong flasks containing 30 ml of solid complete medium. The flasks were incubated at 30 C for the first 48 h and at 25 C for the remainder of the growth time (24). The mutagen was *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 15 μg/ml in water. Treatment was for 2.5 h at 25 C with shaking. Under these conditions 87% killing was obtained.

Selection for cold-sensitive mutants. Mutagen-treated conidia were inoculated to a final concentration of 1.5×10^6/ml in 1.0 liter of liquid complete medium in a 2.8-liter Fernbach flask, which was incubated with shaking at 10 C (the nonpermissive temperature). The filtration-enrichment procedure for mutant selection (23) was then employed.

Between 3 and 5 weeks after mutagenesis, samples of the remaining conidial suspension were spread on plates of solidified complete medium which were incubated at 10 C. After 72 h, the locations of the colonies which developed were marked and the plates were shifted to 25 C (the permissive temperature). New colonies which appeared at this temperature were isolated to slants of complete medium as the potential cold-sensitive mutant strains and were retested. After 5 days of incubation at 10 C, those strains which showed a markedly lower colony growth rate than that of the wild type were retained as the cold-sensitive mutants for further study.

Following the terminology of Bryant and Sypherd (5), Cld-S and Cld-R will be used to refer to the phenotypic growth responses of cold sensitivity and cold resistance (wild type), respectively.

Growth conditions for ribosomal isolation. Conidia from each strain were used to inoculate 125-ml Delong flasks, containing 30 ml of liquid minimal medium, which were shaken for 72 h at 25 C. The medium was decanted, and the mycelial growth from two flasks was blended for 15 s at high speed with 100 ml of fresh minimal medium in a sterile stainless-steel Eberbach semi-micro blender cup (4). The blended contents were transferred to a 300-ml Delong flask.

When the subsequent growth temperature was 25 C, [5-^{3}H]uridine (Schwarz-Mann, specific activity 8 Ci/mmol) was added immediately to a final concentration of 2 μCi/ml, and the flask was shaken for 24 h. When 10 C was the subsequent growth temperature, the flask was equilibrated to 10 C for 1 h with shaking, after which time [5-^{3}H]uridine was added to a final concentration of 2 μCi/ml, and the flask was shaken for 48 h.

Ribosome isolation. The procedures used for the isolation of cytoplasmic ribosomes from *N. crassa* were modified from those of Küntzel (11). All manipulations were carried out at 4 C. The mycelia from the flask were harvested on cheesecloth, washed with ice-cold water, and then washed with ice-cold buffer A [0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.8; 0.1 M NH_4Cl; 0.01 M $MgCl_2 \cdot 6H_2O$; 0.25 M sucrose]. Washed mycelia were homogenized with carborundum powder (grit no. 320) for 5 min in approximately 35 ml of buffer A, using a Ten-Broeck tissue grinder. The homogenate was centrifuged for 5 min at $3,020 \times g$ (5,000 rpm), and the supernatant liquid was then centrifuged for 20 min at $30,900 \times g$ (16,000 rpm). After removal of surface lipid material, the supernatant liquid was again centrifuged for 20 min at $30,900 \times g$. The resultant supernatant liquid was stirred for 5 min with 0.04 volumes of 20% aqueous Triton X-100, and the treated solution was centrifuged for 60 min at $218,000 \times g$ (50,000 rpm) in a Beckman model L ultracentrifuge, type 65 angle rotor. The resultant crude ribosomal pellet was washed several times with buffer B (0.01 M Tris-hydrochloride, pH 7.8; 0.1 M NH_4Cl; 0.01 M $MgCl_2 \cdot 6H_2O$), resuspended in 18 ml of buffer B by gentle homogenization, and centrifuged for 10 min at $12,000 \times g$ (10,000 rpm) to remove contaminating cellular debris. The supernatant liquid was centrifuged for 60 min at $218,000 \times g$ as before. The colorless ribosomal pellet (washed ribosomes) was resuspended in a small volume (0.5 to 1.0 ml) of buffer B and frozen at -20 C until required (routinely less than 7 days).

Ribosome analysis. The ribosomes of wild-type and Cld-S strains were examined under dissociating conditions by zone sedimentation at 4 C in linear sucrose gradients. Thawed ribosome samples were diluted with buffer B to a concentration of 100 absorbance units measured at 259 nm. Twelve-milliliter 5 to 20% (wt/vol) linear sucrose gradients were made up in buffer C (0.01 M Tris-hydrochloride, pH 7.8; 0.5 M KCl; 0.001 M $MgCl_2 \cdot 6H_2O$). Seven absorbance units at 259 nm of the ribosome suspension were layered onto the surface, and the gradients were centrifuged for 5.25 h at 36,000 rpm in a Beckman model L ultracentrifuge SW 36 rotor. This method is a modification of the high-salt method of ribosome dissociation described by Martin and Wool (14) and Falvey and Staehelin (8).

Following centrifugation, seven-drop fractions were collected from the bottom of the tube. Each fraction was mixed thoroughly with 4 ml of PCS solubilizer liquid scintillation cocktail (Amersham-Searle) in a Minivial and counted for 60 s in a Nuclear-Chicago model 6801 liquid scintillation counter.

RESULTS

Twenty-seven Cld-S mutant strains of *N. crassa* were obtained by the mutagenesis and selection procedure described above; all of them had extremely low growth rates at 10 C compared with that of the wild type. At 25 C the

growth rates of the mutant strains approached that of the wild type. Fourteen of the Cld-S strains were analyzed for possible ribosome aberrancies, as a result of which, one strain (PJ30201) was shown to be defective in the biosynthesis of cytoplasmic ribosomes at the restrictive temperature. In this paper, the term *crib* (cytoplasmic ribosome biosynthesis) will be used to denote a genotypic determinant for the biosynthesis of cytoplasmic ribosomes, and the ribosome mutation carried by the PJ30201 strain will be considered to lie in the *crib-1* locus.

Growth characteristics of strain PJ30201. In contrast to the wild type, strain PJ30201 exhibited a colonial growth habit on solidified complete medium at 10 C. Microscopic examination revealed some dichotomous branching of the hyphae, which were thinner than those of the wild type and which showed some tip lysis. At 25 C, the morphology of strain PJ30201 was wild type.

Figure 1 illustrates the growth characteristics of strain PJ30201 compared with that of the wild type, as measured in growth tubes incubated at 25 and 10 C. The PJ30201 strain exhibited an extreme Cld-S growth phenotype at 10 C, its growth rate being only 5.6% of the wild type rate at that temperature. At 25 C, the growth rate of the mutant strain was 79% that of the wild type. Calculations from the curves revealed that the wild type had a 3.9-times-greater linear growth rate at 25 C than at 10 C, whereas the factor for strain PJ30201 was 55. Maximal growth rates for the wild type under these conditions were 7.0 and 1.8 cm per day at 25 and 10 C, respectively.

Ribosome phenotype of strain PJ30201. The zone sedimentation profile of cytoplasmic ribosomal subunits isolated from the wild type cultured at 10 C is presented in Figure 2: the two subunits (called here 60*S* and 37*S* after Küntzel and Noll [12]) showed a mass ratio of 2.3:1 for the large (60*S*) relative to the small (37*S*) subunit.

At 10 C, the PJ30201 strain synthesized disproportional amounts of the two ribosomal subunits compared with the wild type, resulting in increased numbers of 60*S* subunits (Fig. 3). In fact, the mass ratio of 60*S*:37*S* subunits was about 7.2:1, almost triple the ratio characteristic of the wild type. At the permissive temperature (25 C), the PJ30201 strain synthesized ribosomal subunits in wild-type proportions (Fig. 4, mass ratio 2.3:1). Therefore, the evidence accumulated thus far indicates that the PJ30201 strain was defective in ribosome biosynthesis at 10 C, but not at 25 C.

Correlation of cold sensitivity with ribosome biosynthesis defect. To test more precisely the genetic basis of the ribosome phenotype of strain PJ30201, and whether or not it was directly associated with the Cld-S gene mutation, ordered tetrads were isolated from a cross of strain PJ30201 with the wild type. Cultures derived from four ascospores of one such tetrad (representing each of the four meiotic products) were examined for Cld-S and for

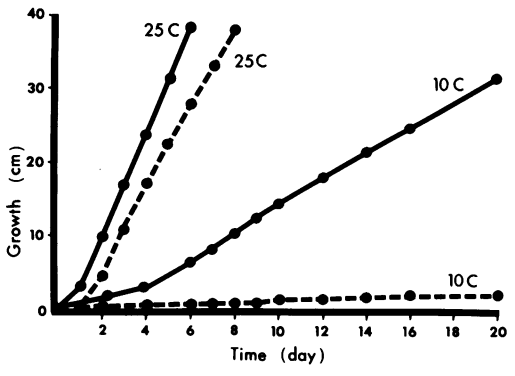

FIG. 1. *Comparison of the rates of lateral mycelial extension of the wild type and strain PJ30201 measured at 25 and 10 C in growth tubes as described. Symbols: ——, wild type; -----, PJ30201.*

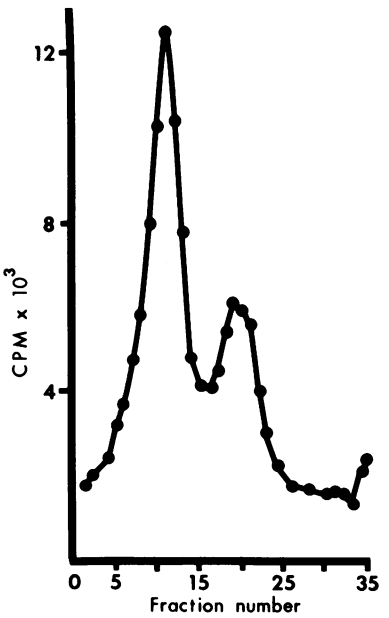

FIG. 2. *Zone sedimentation profile of ribosomal subunits of the wild type grown at 10 C. Conditions of growth and analysis were as described.*

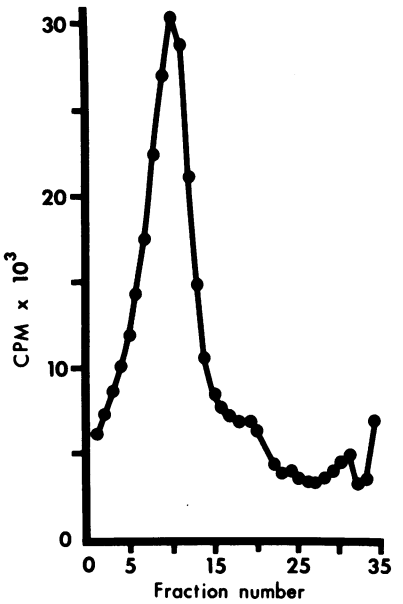

FIG. 3. *Zone sedimentation profile of ribosomal subunits of strain PJ30201 grown at 10 C. Conditions of growth and analysis were as described.*

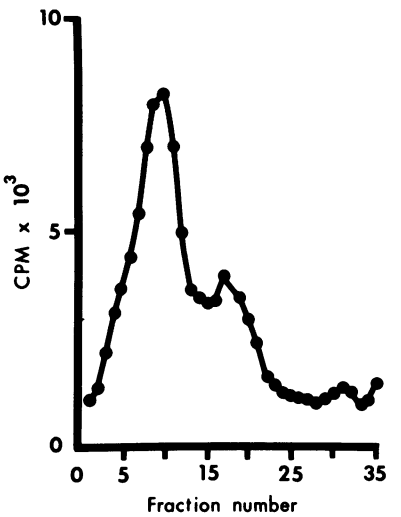

FIG. 4. *Zone sedimentation profile of ribosomal subunits of strain PJ30201 grown at 25 C. Conditions of growth and analysis were as described.*

ribosome phenotype. The growth rates of the four cultures as determined in growth tubes incubated at 10 C are presented in Fig. 5, and the zone sedimentation profiles of ribosomes isolated from the cultures grown at 10 C are shown in Fig. 6. The results show a 2:2 segregation of cold sensitivity and abnormal ribosomal subunits to cold resistance and wild-type ribosomal subunits, and thus it may be concluded that the two mutant phenotypes are probably the result of a single nuclear gene mutation.

Genetic analysis. Examination of five ordered tetrads isolated from a cross of strain PJ30201 with the wild type indicated 100% first-division segregation of Cld-S and Cld-R, and therefore the *crib-1* locus was probably located near a centromere. Analysis of random-spore progeny from crosses of strain PJ30201 with the *alcoy* strain and with strain *cot-1;inos* (these markers lie in linkage groups IV and V, respectively) showed that the *crib-1* locus was in linkage group IV.

A more precise map location for the *crib-1* locus was obtained by examining random-spore progeny of crosses of strain PJ30201 with a *met-1* strain and with an *arg-2* strain. (The auxotrophic markers lie in the right arm of linkage group IV.) The recombination frequency obtained between *crib-1* and *met-1* was 6.6%, and that between *crib-1* and *arg-2* was 17.0%. These recombination data, considered with the tetrad data and the known map locations of the two auxotrophic mutants, suggest that the *crib-1* locus lies between the centromere and the *met-1* locus in the right arm of linkage group IV, and the order of the loci is probably *centromere—crib-1—met-1—arg-2.*

DISCUSSION

The existing Cld-S mutations in bacteria that result in defective ribosome assembly fall into two general classes; namely, those affecting assembly of the 50*S* ribosomal subunit but not

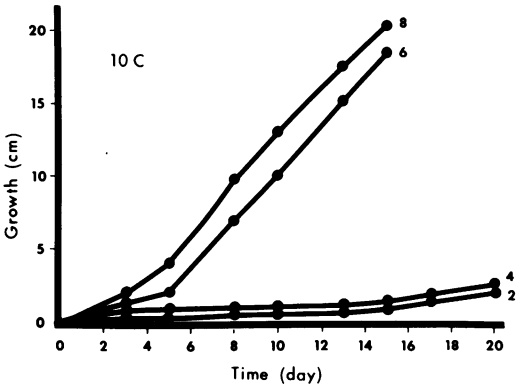

FIG. 5. *Rates of lateral mycelial extension at 10 C for cultures representing the four members of an ordered tetrad isolated from a cross between wild type and strain PJ30201. The numbers adjacent to the curves indicate the spore position within the ascus from which the culture tested was derived. Cultures derived from spores 2 and 4 are Cld-S, and cultures derived from spores 6 and 8 are Cld-R.*

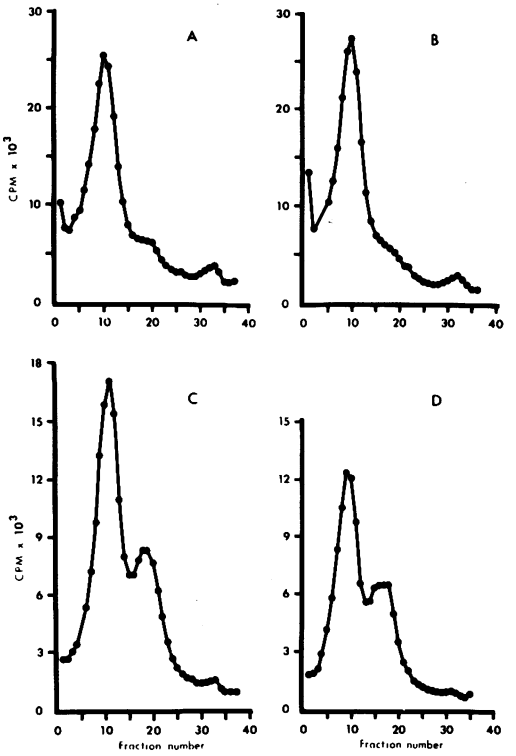

FIG. 6. *Zone sedimentation profiles of ribosomal subunits prepared from cultures derived from ascospores of an ordered tetrad isolated from a cross between wild type and strain PJ30201. The cultures were grown and labeled at 10 C as described. (A) Profile of a Cld-S segregant (ascospore 2). (B) Profile of a Cld-S segregant (ascospore 4). (C) Profile of a Cld-R segregant (ascospore 6). (D) Profile of a Cld-R segregant (ascospore 8).*

that of the 30S subunit, and those which affect the assembly of both subunits. In each case, few, if any, mature ribosomal subunits are produced and ribosomal precursor particles accumulate at the nonpermissive temperature. A priori there are at least two types of mutations that could result in defective ribosome assembly, for example: (i) mutations in genes coding for the structural components of the ribosome, either ribonucleic acid or protein; and (ii) mutations in genes coding for nonstructural ribosomal components, such as modifying enzymes necessary for correct ribosome synthesis and assembly. Most of the Cld-S, ribosome-assembly mutants isolated in *E. coli* and *Salmonella typhimurium* belong to the first class (9, 15, 19, 20). Recently, Bryant and Sypherd (5) reported the isolation of Cld-S ribosome-assembly mutants of *E. coli* which fall into the second category. That is, these authors propose that the mutants they have isolated may be defective in maturation factors necessary for the processing and assembly of ribosomal components into mature subunits.

In eukaryotes, Cld-S ribosomal mutants have been reported only in yeast (1, 2, 10). Hartwell et al. (10) have studied a number of temperature-sensitive mutants that are inhibited in ribosome synthesis at high temperature. Two of these mutants are concomitantly cold sensitive. The Cld-S ribosomal mutant studied by Bayliss and coworkers (1, 2) is also streptomycin sensitive and, when grown at low temperatures, accumulates a 28S ribonucleoprotein particle related to the small (40S) cytoplasmic ribosomal subunit.

The ribosome phenotype of the *N. crassa* mutant strain PJ30201 described here is different from any of the aforementioned Cld-S mutants; at 10 C, both 60S and 37S cytoplasmic ribosomal subunits are synthesized, but not in wild-type proportions. A priori this could be explained either in terms of an altered ribosomal structural component, or in terms of an altered extraribosomal component that is necessary for proper ribosome biogenesis or for the regulation of that process. These alternatives are currently being investigated.

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