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Mutation in *Saccharomyces cerevisiae* Conferring Streptomycin and Cold Sensitivity by Affecting Ribosome Formation and Function

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A cold-sensitive, streptomycin-sensitive mutant of *Saccharomyces cerevisiae* accumulates a 28*S* ribonucleoprotein particle when grown at low temperature. This particle contains 17*S* ribosomal ribonculeic acid which is degraded when exposed to ribonuclease. The particle does not serve as a precursor to 60 and 40*S* ribosomal subunits nor is it turned over when growth is allowed to resume at the permissive temperature; rather it is only diluted by growth. That streptomycin sensitivity (allelic with cold sensitivity) is ribosomal is evidenced by the inhibition of protein synthesis in vitro by streptomycin and the binding of labeled streptomycin to the mutant but not the parental 40*S* ribosomal subunit.

Ribosome synthesis and function have been studied most extensively in bacteria (15). The results obtained from mutant analysis of antibiotic resistance and dependence, ribosomal ambiguity, and cold sensitivity have contributed significantly to our present understanding of the bacterial ribosome. A similar body of knowledge for eukaryotic ribosomes is lacking.

Investigations designed to determine the nucleo-cytoplasmic interactions involved in ribosome formation in eukaryotes are complicated by the difficulties of the genetics and culturing of these organisms. Studies on ribosome synthesis in HeLa cells (4, 22) indicate that there is active movement of ribosomal components from the nucleus to the cytoplasm and vice versa. These cells lack a tractable genetic system and are awkward to handle and grow; such difficulties limit the prospects of their playing a major role in systematic genetic studies to elucidate the mechanisms of formation and regulation of eukaryotic ribosomes. However, Warner (23) has compared the processes of ribosome synthesis in HeLa cells and in yeast and found them to be practically indistinguishable. These similarities, plus the well-defined genetic system and the adaptability to microbial methods of growth and manipulation, make yeasts a favorable system for study. Mutant analysis of ribosome biosynthesis by yeasts using antibiotic- and cold-sensitive mutants might be expected to produce important results.

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Although cycloheximide (3, 17) and blastocidin S (10) have been shown to inhibit the growth of *Saccharomyces cerevisiae* by inhibiting ribosome function, streptomycin is generally without effect on the cytoplasmic ribosomes of yeasts. However, two reports (1, 8) indicate that certain natural strains of *Saccharomyces cerevisiae* are sensitive to streptomycin. Similarly, it has been reported that a wild strain of *Endomyces magnusii* (a yeast-like fungus) is sensitive to streptomycin (200 μg/ml), chloramphenicol (200 μg/ml), and cycloheximide (30 μg/ml) in an in vitro protein synthesizing system (19).

Hartwell et al. (7) have isolated a large number of temperature-sensitive (restrictive temperature, 36 C) mutants which are deficient in ribosome synthesis at high temperature. Two of these mutants are also cold sensitive. Recent investigations of cold-sensitive bacterial mutants indicate that this class of mutants is a rich source of strains which are unable to assemble ribosomal subunits at low temperature (6, 20); also it has been shown that both cold sensitivity and altered antibiotic sensitivity can result from a single mutation in a gene encoding a single ribosomal protein (14).

Recently we isolated a streptomycin-sensitive mutant (AA-89) by selecting for increased sensitivity to phenotypic streptomycin suppression (1). This strain also proved to be cold sensitive. In this paper we describe some of the properties of this mutant. We show that its 40*S* ribosomes have gained the property of binding streptomycin, and that properties of streptomycin

and cold sensitivity are a consequence of a single or very closely linked mutations.

MATERIALS AND METHODS

Strains and culture techniques. The mutant, AA-89, which was isolated by antibiotic suppression as previously reported (1) was derived from 66A4-295 (*his1*, α) (Chris Korch, U. C. Davis) which was in turn derived from wild strain S288C (α) (R. K. Mortimer, U. C. Berkeley). Procedures for mating, sporulation, and ascus dissection were those of Mortimer and Johnson (12).

The growth media used were YEPD (1% yeast extract, 2% peptone, and 2% dextrose) and minimal (Difco yeast nitrogen base + 2% dextrose).

Preparation of cell lysates for sedimentation analysis. Exponentially growing cells were inoculated into 25 ml of YEPD medium at 30 C. After the culture reached an optimal density (OD) corresponding to 2×10^7 cells/ml, 100 μCi of [^{3}H]uracil or 2 μCi of [2-^{14}C]uracil was added. To label the ribonucleic acids (RNA) synthesized at 15 C, radioactive uracil was added at the time of shift from 30 to 15 C or 30 min later, depending on the experiment. Cells were harvested at times or OD indicated (see figure legends), washed twice with 0.01 M tris(hydroxymethyl)-aminomethane (Tris) buffer- 0.1 M NaCl- 0.1 mM $MgCl_2$ (pH 7.4) and suspended in this or other buffers as indicated. Cells were broken in an Eaton press (5) and debris was removed by three centrifugations at 10,000 $\times$ *g* for 20 min at 4 C, leaving a turbid supernatant fluid which contained the ribosomes. Extracts were used immediately or stored at −80 C until used.

Sucrose gradient sedimentation analysis. Linear sucrose gradients (5 to 20%) containing Tris buffer were made at 4 C with the gradient-mixing apparatus described by Rosenbloom and Schumaker (18) from sucrose solutions which were filtered through membrane filters (HA 0.45 μm pore size; Millipore Corp., Bedford, Mass.). Gradients were centrifuged, unless otherwise stated, in a Beckman model L2-50 ultracentrifuge by using an SW39 rotor at 4 C run at 37 K rpm for 200 min. Fractions (10 drops) were collected from the bottom of the gradient tube with a multispeed transmission pump (Harvard Apparatus Co., Dover, Mass.). An average of 30 to 32 fractions were collected per 5-ml gradient. Bray's solution (6 ml) was added to each vial which was then counted for 10 min in a Nuclear-Chicago, Mark II liquid scintillation spectrometer. The counts of ^{3}H and ^{14}C were corrected for channel overlap and were plotted by computer (U. C. Davis, Computer Center).

Sucrose density gradient analysis of RNA sedimentation patterns. Proteins associated with ribosomal RNA were released by adjusting the concentration of sodium dodecyl sulfate (SDS) of the suspending medium to 0.5%. Sucrose gradients (5 to 20%) were prepared as described above except that SDS buffer was substituted for Tris buffer and the gradients were prepared at room temperature. The gradients were centrifuged in the Beckman model L2-50 ultracentrifuge at 15 C and 37 K rpm for 330 min in an SW39 rotor. Fractions were collected as described for the Tris buffer gradients except that 20 drops were collected per fraction; 30 to 32 fractions were collected per 5-ml gradient.

Preparation of ribosomes and "pH 5 enzymes" for in vitro protein synthesis studies. The procedure used was that of Lamb et al. (11) with minor modifications. Cells were grown overnight in YEPD, washed twice with sterile water, broken (Eaton press) into Tris buffer (50 mM Tris, 5 mM $MgCl_2$, 10 mM KCl, and 1 mM 2-mercaptoethanol, pH 7.5), thawed, and centrifuged three times at 10,000 $\times$ *g* for 20 min at 4 C. The resulting supernatant fraction was collected and centrifuged in a type 50 rotor at 4 C and 41.5 K rpm in a Beckman model L ultracentrifuge for 90 min to pellet the ribosomes. The ribosomes were suspended in the same buffer for later use. The supernatant fraction was adjusted to pH 5 with acetic acid to precipitate the pH 5 enzymes. These enzymes were dissolved in the same buffer after centrifugation, precipitated at pH 5, and then dissolved in the same buffer (pH 5 enzymes).

Poly(U)-directed incorporation of [^{14}C]phenylalanine into acid-precipitable material. The complete reaction mixture (total volume 0.1 ml) contained: 0.2 mM guanosine triphosphate (GTP), 1 mM adenosine triphosphate (ATP), 5 mM phosphoenol pyruvate (PEP), 7.5 units of pyruvate kinase, 25 μg of poly(U), 0.1 μCi of [^{14}C]phenylalanine (325 μCi/μmol), 10 OD units (1.0 OD unit is that amount of RNA absorbing at 260 nm that gives an OD reading of 1) of ribosomes (approximately 1 mg), 1 mg of pH 5 enzymes, and various inhibitors at the concentrations indicated. Duplicate mixtures were incubated on a Dubnoff shaking water bath for 30 min at 30 C. Cold 7% trichloroacetic acid in 33% ethanol (3 ml) was added to stop the reaction. The tubes were heated to 85 to 90 C for 15 min and the precipitate was collected on a 25-mm (0.45 μm pore size) membrane filter (prewashed with 7% aqueous acid containing 100 μg of cold L-phenylalanine/ml), and then washed three times with 3-ml portions of 7% aqueous acid containing 100 μg of cold L-phenylalanine/ml. The filters were dried for 1 h at 60 C in glass vials, cooled, and suspended in 5 ml of toluene scintillation fluid (2-5-diphenyloxazole [PPO], 4.0 g, and 1-4-bis 2'(5'phenyloxazolyl) benzene [POPOP], 0.4 g, in 1.0 l toluene). Radioactivity (counts per minute) was measured in a Nuclear-Chicago Mark II liquid scintillation spectrometer.

Isolation of the 28S particle from AA-89. A culture of AA-89 was grown in YEPD to a density of 5×10^7 cells/ml and placed on a shaker at 15 C for 12 h. All subsequent procedures were done at 4 C or less. After harvesting, washing, and breaking the cells (Eaton press), 1.0 ml of extract (A_{260} at 100 units/ml) was layered on 34-ml sucrose gradients (5 to 20% sucrose in 0.01 M Tris, 0.1 M NaCl, 0.1 mM $MgCl_2$, pH 7.4) and centrifuged in a Beckman Ti 60 rotor for 70 min at 59 K rpm in a Beckman L-2-65B ultracentrifuge. Fractions from the gradient were collected in an ISCO (Instrumentation Specialities Co., Lincoln, Neb.) model UA-2 ultraviolet analyzer by pushing the gradient (top first) through a flow cell and recording the absorption at 254 nm. Fractions

containing the 28S ribonucleoprotein were pooled and samples were either layered on 5-ml (5 to 20%) sucrose gradients in Tris buffer or brought to 0.5% SDS and then layered on 5-ml (5 to 20%) sucrose gradients in SDS buffer. These gradients were centrifuged and collected as described above for each type of gradient, i.e., Tris or SDS.

Isolation of ribosomal subunits. Cells were grown overnight (14 to 18 h) in YEPD, washed twice with water, and broken (Eaton press) into a standard low Mg buffer (0.1 M Tris, 0.02 M KCl, 0.5 mM Mg acetate, pH 7.5). The lysate was thawed and centrifuged three times at 10,000 × g for 20 min at 4 C. The remaining supernatant liquid was centrifuged in a type 50 rotor for 90 min at 41.5 K rpm in a Beckman model L ultracentrifuge to pellet the ribosomes. The ribosomes were then suspended in a volume of standard low Mg buffer which would result in a final concentration of ~100 OD units/ml at 260 nm. The ribosome suspension was brought to a final concentration of 5 mM Mg acetate and 0.35 M KCl by adding an equal volume of 0.5 mM Mg acetate plus 0.68 M KCl (final concentration ~50 OD units/ml). This mixture was preincubated 3 to 5 min at room temperature, diluted 2.5 times, and 2.5 ml (50 OD units) was then layered on a 25-ml 10 to 30% linear sucrose gradient made in high KCl buffer (0.01 M Tris, 0.35 M KCl, 5 mM Mg acetate, 0.1 mM 2-mercaptoethanol, pH 7.5). The gradients were centrifuged in a Ti 60 rotor at 4 C and 59 K rpm for 70 min in a Beckman model L-2-65B ultracentrifuge. The gradient was collected by placing a 50-μliter disposable pipette through the gradient to the bottom of the tube and pumping with a polystallic pump (Buchler Instruments, Fort Lee, N.J.) through a flow cell in a Beckman Kintrac VII spectrophotometer which recorded the absorbance at 260 nm. The ribosomal peaks (60 and 40S) from mutant and wild-type gradients were collected and dialyzed in standard low Mg buffer for at least 3 h or overnight.

Protein synthesis in vitro by combinations of isolated subunits. The dilute nature of the dialyzed subunits required some modification of the standard assay. A total volume of 1 ml instead of 0.1 ml was used. The reaction mixture contained the same concentration of ingredients as the standard assay (see above) with the exception of ribosomes or subunits. Subunits were added in the ratio 2.5:1 OD units (260 nm) of 60 to 40S subunits, respectively. The final concentration was 3.5 OD units/ml as opposed to 10 OD units/ml for the standard assay. When 60S subunits were assayed alone, 2.5 OD units were added and for the 40S alone 1.0 OD unit was added.

Purification of ^{3}H-labeled streptomycin. Tritiated streptomycin sulfate (143 μCi/μg), made by exposing the streptomycin to 3 C of tritium gas for 2 weeks, was obtained from New England Nuclear Corp. The ^{3}H-labeled material was purified by the procedure described by Carter et al. (2). The final specific activity of the purified [^{3}H]streptomycin chloride was 7,000 counts per min per μg.

[^{3}H]streptomycin binding experiment. The isolated ribosomal subunits were frozen after dialysis and thawed at 4 C for use in this experiment. The procedure used was that described by Kaji and Tanaka (9) for [^{3}H]dihydrostreptomycin binding to *Escherichia coli* ribosomes, with a few modifications. The reaction mixture (0.5 ml) contained 20 μg of the subunit tested, 25 μg of poly(U), 175,000 counts/min of [^{3}H]streptomycin (25 μg), 0.01 M Tris, 0.01 M Mg acetate, and 0.02 M KCl, pH 7.5. The mixture was incubated for 30 min at 25 C and stopped by addition of 2 ml of cold buffer (0.01 M Tris, 0.01 M Mg acetate, 0.02 M KCl, pH 7.5) and filtered on membrane filters (0.45 μm pore size; Millipore Corp.) prewashed with buffer containing 100 μg of streptomycin/ml. The filters were washed four times with 2-ml portions of buffer, placed in glass scintillation vials, and dried for 1 h at 60 C. Toluene scintillation fluid (5 ml) was added to each vial and ^{3}H-counts were determined in a Nuclear-Chicago Mark II liquid scintillation spectrometer.

The procedure was further modified for [^{3}H]streptomycin binding with fractions collected from complete gradients. That is, instead of collecting fractions of subunit peaks, a gradient was broken into 15 20-drop fractions, and each dialyzed (4 h) fraction was tested for binding. In these samples the volume was 1.0 ml, the poly-U concentration was 250 μg/ml, and 350,000 counts/min of [^{3}H]streptomycin (50 μg) was added.

RESULTS

Effect of temperature and streptomycin on the growth rates of mutant (AA-89) and wild type (S288C). The mutant grows slightly more slowly than wild type at 30 C (1.9 times) but much more slowly at 15 C (7.1 times). Whereas wild type is capable of continued growth after a 3-h lag upon the addition of 10 mg of streptomycin/ml, the mutant is incapable of growth in the presence of this concentration of the drug (Table 1). Thus, the mutant strain (AA-89) is both cold-sensitive and streptomycin-sensitive.

Crosses. Tetrad analysis of 12 asci from a cross between AA-89 and XC500A resulted in 2:2 segregation of both streptomycin and cold sensitivity in all 12 parental ditype asci, indicating that the two phenotypic characters result from a single mutation or from two very closely linked mutations.

Ribosomal subunits. Wild type (S288C) was grown and labeled for 12 h in the presence of

TABLE 1. *Doubling times (in hours) of mutant (AA89) and wild type (S288C) at 15 and 30 C in YEPD medium in the presence of 10 mg of streptomycin/ml*

Mutant	Without streptomycin		Streptomycin (10 mg/ml)
	30 C	15 C	30 C
S288C	1.8	7.0	2.5
AA-89	3.4	50	∞

[^{3}H]uracil at 30 C for use as an internal marker; the mutant (AA-89) was grown for 12 h at 30 C; [^{14}C]uracil was added and the culture was shifted to 15 C for an additional 12 h. The addition of label before the culture actually reaches 15 C results in some incorporation of label into 60 and 40*S* subunits, but this does not occur if label is added 30 min after the shift to 15 C. Extracts were made and analyzed by zonal sedimentation (Fig. 1). The distribution of ribosomal RNA is drastically altered in AA-89 when grown at 15 C. Normal wild-type patterns result when AA-89 is grown at 30 C and when S288C is grown at 15 and 30 C, as has been previously reported (1).

Formation of a 28S particle at low temperature. Ribosome profiles of cells grown at 30 C and labeled at the time of shift to various temperatures are presented in Fig. 2. The results obtained with cultures grown at 25 C were identical to those from cultures grown at 30 C and are, therefore, omitted. Temperatures of 30 and 20 C do not prevent growth of the mutant, whereas temperatures of 15 and 10 C do. Material sedimenting at 28*S* is apparent in cultures labeled at 20, 15, and 10 C, and the relative amount of this material increases progressively as growth temperature is decreased. Ribosome profiles of wild type grown at these temperatures failed to show any accumulation of material at 28*S*.

Formation of the 28S particle as a function of time at 15 C. A culture of AA-89 labeled with [^{3}H]uracil at 30 C and one labeled with [^{14}C]uracil at 15 C for various times were analyzed to determine the sedimentation patterns of ribosomal subunits (Fig. 3) and the RNA species present (Fig. 4).

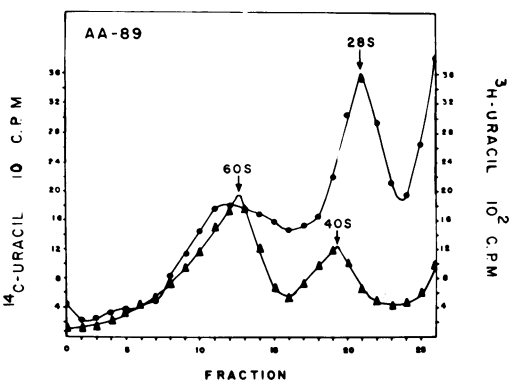

FIG. 1. *Ribosome subunit sedimentation patterns from a mixture of cell lysates of wild type (S288C) (▲) grown at 30 C for 12 h in the presence of [^{3}H]uracil and mutant (AA-89) (●) grown at 15 C for 12 h with [^{14}C]uracil.*

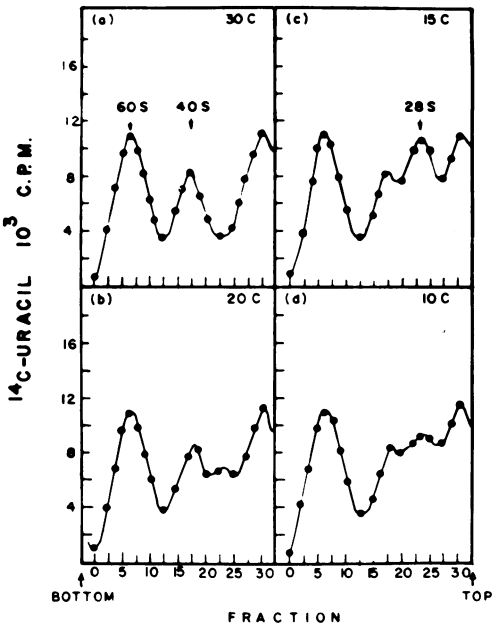

FIG. 2. *Formation of the 28S particle as a function of temperature. (a) AA-89 at 30 C; (b) AA-89 at 20 C; (c) AA-89 at 15 C; (d) AA-89 at 10 C. Cells were allowed to double at 30 and 20 C. Cells were grown for 12 h at 15 C (0.6 doublings). Cells were grown for 24 h at 10 C (0.3 doublings). Zone sedimentation analysis, done by using sucrose gradients, was performed on the cell extracts (Materials and Methods). No 28S peak is detectable in extracts of S288C grown at these temperatures.*

The [^{3}H]uracil-labeled material is, in effect, a zero time control and shows that no detectable 28*S* material is present prior to shift to 15 C. At 4 h after the shift, a distinct peak sedimenting at approximately 28*S* becomes apparent and increases in magnitude with time of incubation up to 12 h when further accumulation stops (a sample taken at 24 h was identical to the 12-h sample). The RNA species in the 28*S* peak sediments at 17*S* and hence is probably the same as that found in the normal 40*S* subunit. That the 17*S* RNA is, in fact, contained in the 28*S* particle can be deduced from its kinetics of formation. No relative increase in the 40*S* subunit occurs during this period (Fig. 3); on the other hand, the rate of formation of the 28*S* particle parallels that of the 17*S* RNA.

The particle contains RNA that sediments at 17S. As proof that 28*S* particles contain 17*S* RNA, fractions indicated by the shaded area (I) in Fig. 5a was pooled (as described in the Materials and Methods) and centrifuged in Tris-buffer gradients with (Fig. 5c) and without (Fig. 5b) 0.5% SDS. The isolated particle sedi-

ments at 28S (Fig. 5b) and the RNA from this particle cosediments with authentic 17S RNA from the 40S subunit (Fig. 5b).

Fate of the 28S particle during subsequent growth at 30 C. The fate of the 28S ribonucleoprotein particle which accumulates at low temperature was followed by labeling the culture with [^{14}C]uracil for 12 h at 15 C, washing away the label, and suspending the cells in YEPD plus 50 μg of cold uracil/ml at 30 C. Samples were taken at the times indicated and were analyzed in Tris-buffer sucrose gradients (Fig. 6). The 28S particle appears to be stable and does not move into the 40S region during the first doubling (OD 420 nm) (4 to 7 h); it is lost only through dilution by growth.

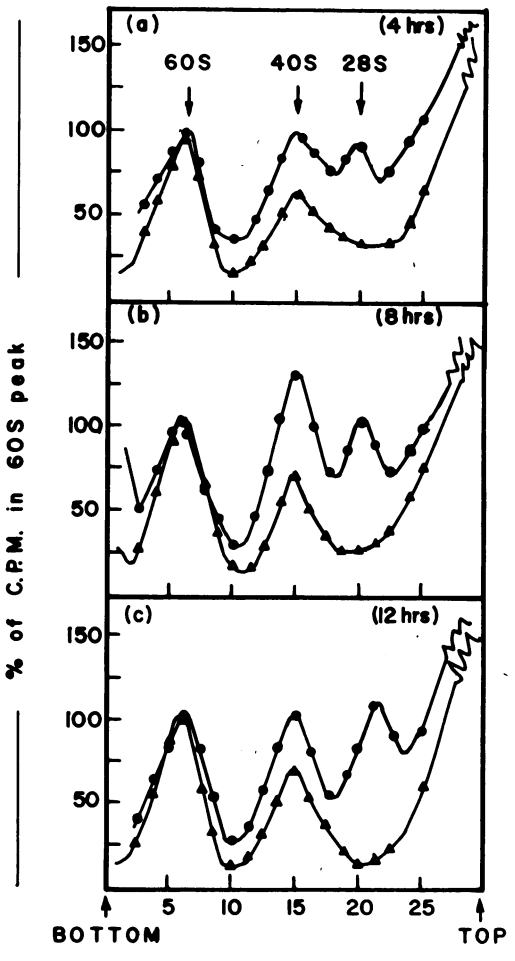

FIG. 3. *Formation of the 28S particle as a function of time at 15 C. The 60S peaks were adjusted to 100% and the counts in the other peaks were plotted as percent of that in the 60S peak. Symbols: ▲, AA-89 grown at 30 C in the presence of [^{3}H]uracil; ●, AA-89 grown at 15 C in presence of [^{14}C]uracil. Tris-buffer 5-ml (5 to 20%) sucrose gradients were layered with 0.1 ml of mixed lysate and centrifuged 200 min at 37K rpm in an SW39 rotor at 4 C.*

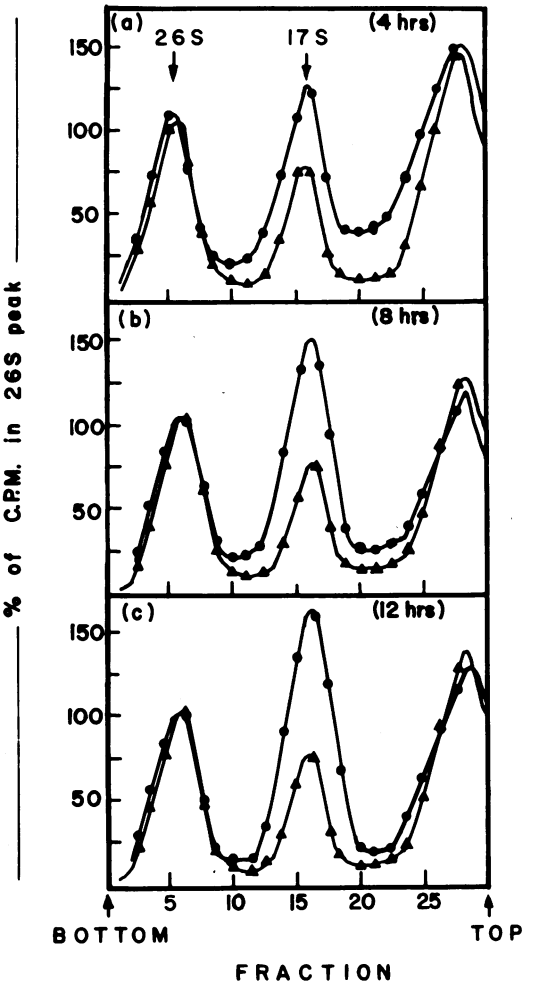

FIG. 4. *Formation of 17S RNA as a function of time at 15 C. The same samples were used as in Fig. 3 except lysates were brought to 0.5% SDS and 0.1 ml of lysate was layered on SDS-buffer 5-ml sucrose gradients (5 to 20%) and centrifuged at 37K rpm for 330 min at 15 C in an SW39 rotor. The 26S peaks were adjusted to 100% and the counts in the other peaks were plotted as percent of the counts in the 26S peak. Symbols: ▲, AA-89, grown in 30 C in the presence of [^{3}H]uracil; ●, AA-89 grown at 15 C in the presence of [^{14}C]uracil.*

Stability of the 28S particle. The particle was found to be degraded when an extract from the mutant grown at 15 C was incubated for 30 min at 4 C or when treated with 2 μg of RNase/ml (Fig. 7). The incubation for 30 min at 4 C was effective only if the cells were broken; intact

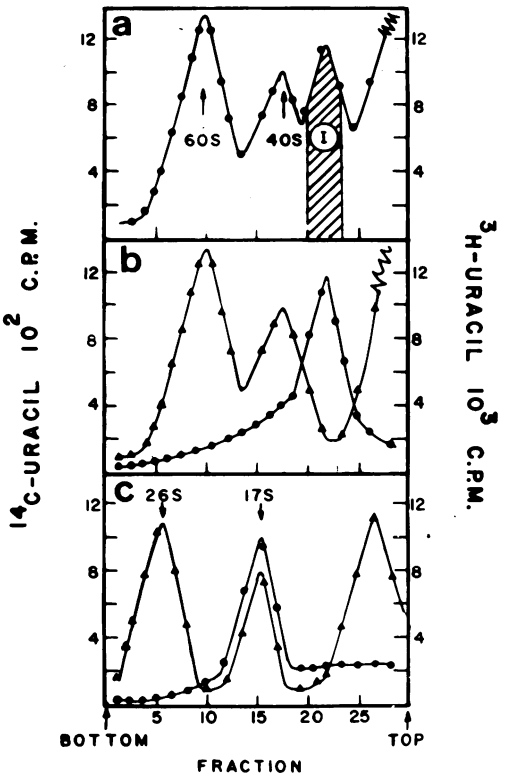

FIG. 5. *Isolation of the 28S peak. (a) Tris-buffer 5 to 20% sucrose gradient of lysate of AA-89 grown 12 h at 15 C. Cross-hatched region was isolated by collection fractions and re-run in the same system (b). (c) Same as b except the lysate was adjusted to 0.5% SDS and layered on a SDS-buffer 5-ml 5 to 20% sucrose gradient. Symbol: ●, AA-89 labeled with [^{3}H]uracil at 30 C.*

cells incubated for 30 min at 4 C prior to breaking contain a normal complement of the 28*S* ribonucleoprotein. The cells were always broken in the Eaton press which was cooled in dry ice. The organism and lysate were frozen at all times during the breaking process. The frozen lysates were thawed and immediately layered on the gradients and centrifuged.

In vitro incorporation of [^{14}C]phenylalanine into poly(U)-directed protein synthesis by ribosomes from AA-89 and S288C. The results of a typical incorporation experiment are summarized in Table 2. Blastocidin S and cycloheximide, known inhibitors of yeast ribosome function, inhibit protein synthesis by both AA-89 and S288C ribosomes. Streptomycin and neomycin, however, inhibit only the AA-89 ribosomes and not those from the wild type, S288C.

To determine which ribosomal subunits were responsible for these differences, the 40 and 60*S* ribosomes from strain AA-89 and S288C were dialyzed, mixed in various combinations, and assayed for their ability to promote protein synthesis in the presence and absence of neomycin (Table 3). The combination of 40*S* subunits from AA-89 and 60*S* subunits from S288C was inhibited by 100 μg of neomycin/ml, whereas 60*S* from AA-89 and 40*S* from S288C were not. Complete ribosomes from AA-89 were sensitive to neomycin, whereas complete ribosomes from S288C were resistant. Thus neomycin sensitivity resides in the 40*S* subunit.

Binding of streptomycin to ribosomal subunits from AA-89 and S288C. Results of experiments to determine the binding of [^{3}H]streptomycin to the isolated ribosomal subunits clearly establish that 40*S* subunits from the mutant gained the property of binding streptomycin.

No significant binding of [^{3}H]streptomycin to either of the subunits (60 or 40*S*) from S288C (Fig. 8) was observed; there was no binding of the [^{3}H]streptomycin to the 60*S* subunit of AA-89; however, a significant amount of the [^{3}H]streptomycin was bound to the 40*S* subunit of the mutant, AA-89 (Fig. 8a).

DISCUSSION

A high proportion of cold-sensitive mutants of bacteria which are unable to grow on complex media at low temperature have been shown to have mutations in genes that affect ribosome synthesis (6, 20). Further, many of these mutations are located on the chromosome near the streptomycin- and spectinomycin-resistance loci, loci which are known to encode ribosomal proteins (6, 20). Here we show that similar classes of cold-sensitive mutants exist in yeast. The mutant strain AA-89 lost the ability to grow at low temperature and became sensitive to streptomycin, and it accumulated altered ribosome-like particles.

The mutant (AA-89) was isolated on the basis of its increased sensitivity to streptomycin (1) but it proved also to be cold-sensitive (incapable of sustained growth at 15 C). The two properties of antibiotic and cold sensitivity are apparently the consequence of a single genetic lesion as indicated by tetrad analysis (13) of 12 asci from a cross between AA-89 and a haploid wild strain, XC500A.

Previous studies with bacteria have shown (14, 20) that cold sensitivity and altered antibiotic sensitivity are usually associated with the loss of ability to synthesize ribosomes at low temperature. This generalization seems also to apply to yeasts. At low temperature AA-89

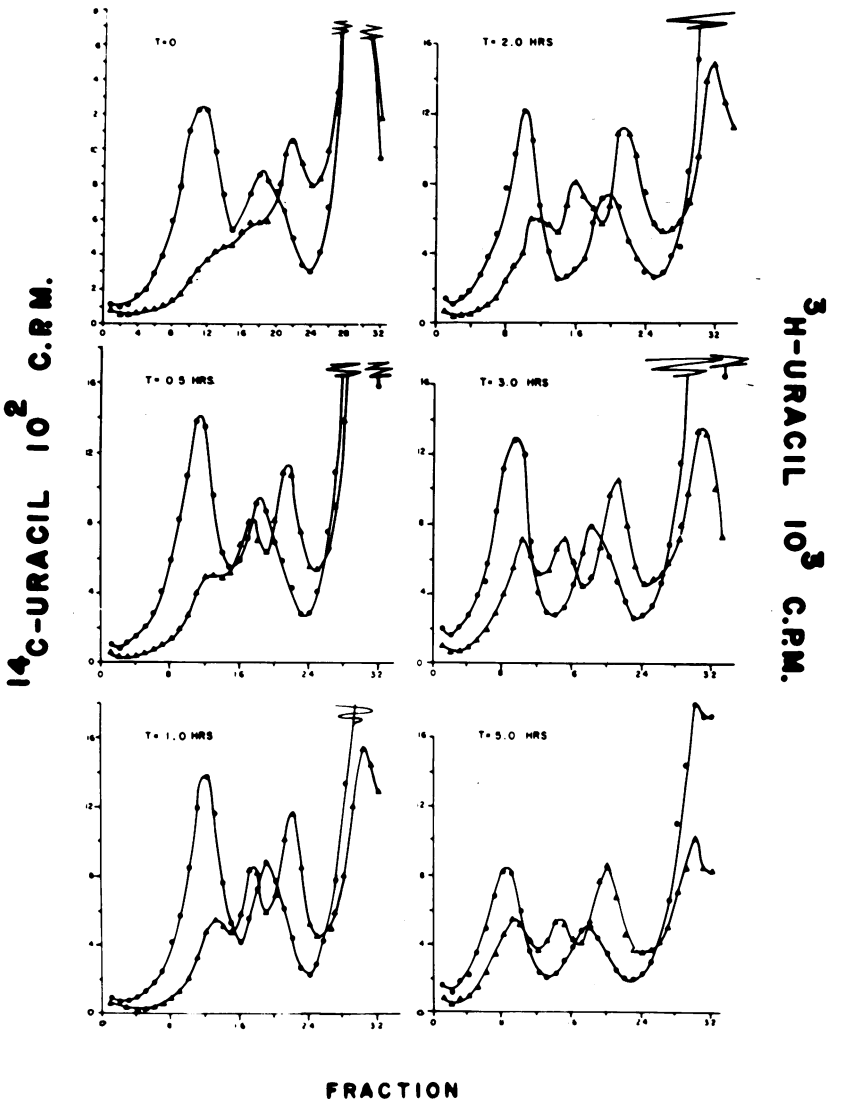

FIG. 6. *Shift from 15 C to 30 C of AA-89 labeled with [^{14}C]uracil at 15 C (▲). Cells were harvested, washed, and suspended in YEPD + 50 μg of cold uracil/ml at the time of shift. AA-89 was also grown at 30 C in the presence of [^{3}H]uracil (●). Cell lysates from both were mixed and 0.1 ml was layered on 5-ml Tris-buffer sucrose gradients (5 to 20%) and spun at 37K rpm for 200 min in an SW39 rotor at 4 C.*

accumulates, during a transient period of growth after shift to 30 C, a 28*S* ribonucleoprotein particle which seems to be an abortive precursor of the normal 40*S* ribosome. Like the normal 40*S* ribosome particle, it contains 17*S* RNA (Fig. 4 and 5) but it fails to mature into a 40*S* subunit when cells containing the 28*S* particle are allowed to continue growth at the permissive temperature, 30 C.

The presence of the 28*S* ribonucleoprotein particle is associated with inhibition of growth. As it accumulates with time at low temperature, growth rate progressively slows, and both its accumulation and inhibition of growth increase as temperature is decreased (Fig. 2 and 3). Thus, the particle might well be an inhibitor of protein synthesis as has been shown for preribosomal particles in bacteria (21).

In contrast to some cold-sensitive mutants of bacteria, AA-89 does not synthesize normal ribosomal subunits at the restrictive temperature. If the culture is completely equilibrated to

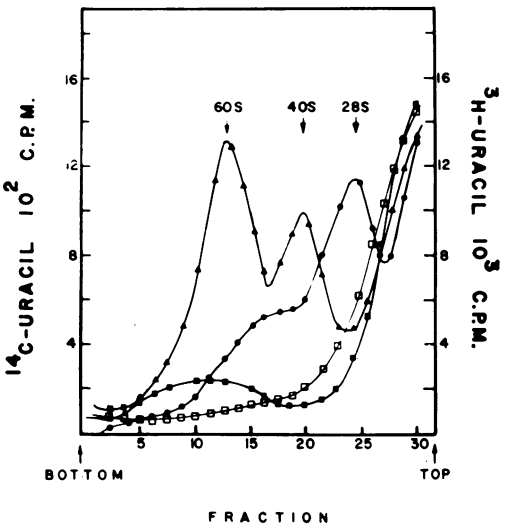

FIG. 7. *Incubation at 4 C and RNase treatment of the 28S particle. AA-89 was grown and labeled with [^{14}C]uracil at 15 C and (○) incubated for 30 min at 4 C, broken and run on a Tris-buffer sucrose gradient; (□) cells were broken and treated with 2 mg of RNase/ml and layered on sucrose gradient; (■) cells were broken, incubated for 30 min at 30 C, and then layered on the 5 to 20% sucrose gradient. (▲) AA-89 was grown at 30 C.*

15 C before radioactive uracil is added, little radioactivity can be detected in the 40 or 60*S* ribosomes (Fig. 6a). Similarly, some cold-sensitive mutants of bacteria lose the ability to assemble both 30 and 50*S* subunits.

As is the case with preribosomal particles of bacteria, the 28*S* particle produced by AA-89 at low temperature is very sensitive to nuclease attack. Treatment with 2 μg of RNase/ml or merely holding a lysate for 30 min at 4 C results in complete degradation of the particle. But the particle is quite stable in the cell; it is not degraded in nongrowing cells at low temperature nor during the recovery of cells returned to permissive temperature. These results suggest that the particle and RNase are in some way effectively separated in the intact cell.

The streptomycin and neomycin sensitivity of the mutant is most probably ribosomal. Inhibition of protein synthesizing systems from both the mutant and wild type are similarly sensitive to the known ribosomal inhibitors blastocidin S and cycloheximide, but only the mutant is sensitive to streptomycin and neomycin (Table 2) and this sensitivity of isolated ribosomal subunits and 80*S* ribosomes apparently resides in the 40*S* subunit (Table 3). Hybrid 80*S* ribosomes, formed by mixing 40*S* subunits from AA-89 and 60*S* subunits from S288C, are sensitive to neomycin, whereas no such inhibition occurs when 40*S* subunits from S288C and 60*S* subunits from AA-89 were used. The lower sensitivity of hybrid ribosomes (Table 3) to

TABLE 2. *Poly(U)-directed in vitro incorporation of [^{14}C]phenylalanine by isolated ribosomes from S288C and AA-89 in the presence and absence of various inhibitors*

Complete reaction mixture[a] + 80*S* ribosomes of strain listed	Inhibitor	[^{14}C] phenylalanine (counts/min)[a]	% Of total counts/min	% Inhibition
AA-89		386	100	
AA-89	Blastocidin S[b]	173	44.8	55.2
AA-89	Cycloheximide[b]	59	15.3	84.7
AA-89	Streptomycin[b]	61	15.8	84.2
AA-89	Neomycin[c]	58	15	85
S288C		467	100	
S288C	Blastocidin S[b]	47	10.1	89.9
S288C	Cycloheximide[b]	83	17.8	82.2
S288C	Streptomycin[b]	425	91.5	8.8
S288C	Neomycin[c]	536	115	

[a] Complete reaction mixture described in Materials and Methods. Background of t = 0 control + trichloroacetic acid subtracted.
[b] 100 μg/ml.
[c] 10 μg/ml.

TABLE 3. *Poly(U)-directed in vitro incorporation of [^{14}C]phenylalanine by mixed ribosome subunits from S288C and AA-89 in the presence and absence of neomycin*

40*S*[a]	60*S*[a]	80*S*[a]	Neomycin (μg/ml)	Counts/min	% Of total	% Inhibition
		AA-89	0	1,723	100	
		AA-89	100	292	17	83
		S288C	0	1,057	100	
		S288C	100	991	93.7	6.3
AA-89				268	18.9	
	AA-89			606	42.5	
AA-89	AA-89			1,419	100	
S288C				459	37.5	
	S288C			688	55.9	
S288C	S288C			1,230	100	
AA-89	S288C			1,158	100	
AA-89	S288C		100	713	61.5	38.5
S288C	AA-89			1,240	100	
S288C	AA-89		100	1,437	115	

[a] Complete reaction mixture described in Materials and Methods, plus ribosomes or ribosome subunits indicated. The subunits were mixed in a 2.5:1 ratio of 60*S*:40*S* based on OD_{260}.

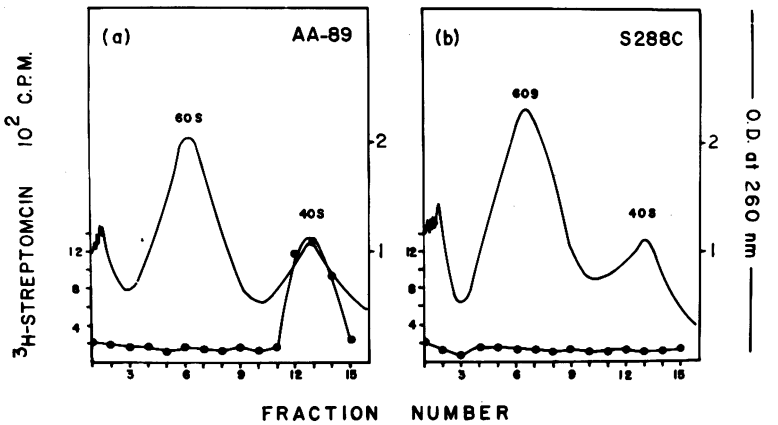

FIG. 8. *Fractions of complete sucrose gradients (Materials and Methods) of S288C and AA-89 were collected and monitored at 260 nm (–). The effluent from the flow cell was collected in a 20-drop fraction, dialyzed, and tested for binding with [^{3}H]streptomycin (●).*

inhibition by antibiotics is somewhat perplexing but may be explained by S288C 40*S* subunit contamination of the isolated S288C 60*S* fraction. This suspicion is strengthened by the high basal level of protein synthesis catalyzed by this fraction (55.9%).

The studies on in vitro protein synthesis suggest that the 40*S* subunit of AA-89 determines the mutant's sensitivity to streptomycin but these results are not conclusive. Binding studies with *Escherichia coli* ribosomal subunits (9) and with reconstituted subunits (16) have established that P-10 protein of the 30*S* (smaller) subunit is responsible for binding streptomycin. Our in vitro protein synthesis results suggest that the inhibitory effect of streptomycin on incorporation of label into protein may be due to the binding of streptomycin to the 40*S* (smaller) ribosomal subunit. This suggestion is consistent with subsequent experiments (Fig. 8).

We suspect that a single mutational event in a gene encoding a protein of the 40*S* subunit is responsible for the duo phenotype of cold and antibiotic sensitivity.

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LITERATURE CITED

1. Bayliss, F. T., and R. T. Vinopal. 1971. Selection of ribosomal mutations by antibiotic suppression in yeast. Science **174**:1339–1341.
2. Carter, H. E., R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell, and W. A. Strong. 1945. Isolation and purification of streptomycin. J. Biol. Chem. **160**:337–342.
3. 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.
4. Darnell, J. E. 1968. Ribonucleic acids from animal cells. Bacteriol. Rev. **32**:262–290.
5. Eaton, N. R. 1962. New press for disruption of microorganisms. J. Bacteriol. **83**:1359–1360.
6. Guthrie, C., H. Nashimoto, and M. Nomura. 1969. Structure and function of *E. coli* ribosomes. VII. Cold-sensitive mutants defective in ribosome assembly. Proc. Nat. Acad. Sci. U.S.A. **63**:384–391.
7. Hartwell, L., C. McLaughlin, and J. R. Warner. 1970. Identification of ten genes that control ribosome formation in yeast. Mol. Gen. Genet. **109**:42–56.
8. Ierusalimskii, N. D., L. A. Shevchenko, and E. V. Grishankova. 1963. Changes in some physiological requirements of yeast as a result of adaptation to streptomycin. Mikrobiologiya **32**:13–16.
9. Kaji, H., and Y. Tanaka. 1968. Binding of dihydrostreptomycin to ribosomal subunits. J. Mol. Biol. **32**:221–230.
10. Kijima, H., and K. Imahori. 1968. Site of action of blasticidin S in cell free system of microorganisms. Sci. Pap. Coll. Gen. Educ., Univ. Tokyo **18**:173–190.
11. Lamb, A. J., G. D. Clark-Walker, and A. W. Linnane. 1968. The biogenesis of mitochondria. 4. The differentiation of mictochondrial and cytoplasmic protein synthesizing systems *in vitro* by antibiotics. Biochim. Biophys. Acta **145**:664–670.
12. Mortimer, R. K., and J. R. Johnston. 1959. Life span of individual yeast cells. Nature (London) **183**:1751–1752.
13. Mortimer, R. K., and D. C. Hawthorne. 1969. Yeast genetics, p. 385–460. *In* A. H. Rose and J. S. Harrison (ed.), The yeast, vol. 1. Academic Press Inc., New York.
14. Nashimoto, H., and M. Nomura. 1970. Structure and function of bacterial ribosomes. XI. Dependence of 50S ribosomal assembly on simultaneous assembly of 30S subunits. Proc. Nat. Acad. Sci. U.S.A. **67**:1440–1447.
15. Nomura, M. 1970. Bacterial ribosome. Bacteriol. Rev. **34**:228–277.
16. Ozaki, M., S. Mizushima, and M. Nomura. 1969. Identi fication and functional characterization of the protein controlled by the streptomycin-resistant locus in *E. coli* Nature (London) **222**:333–339.

17. Rao, S. S., and A. P. Grollman. 1967. Cycloheximide resistance in yeast: a property of the 60S ribosomal subunit. Biochem. Biophys. Res. Commun. **29**:696-704.
18. Rosenbloom, J., and V. N. Schumaker. 1963. Analytical ultracentrifugation of T4r bacteriophage DNA in preformed sucrose density gradients. Biochemistry **2**:1206-1211.
19. Shevchenko, L. A., and I. I. Filippovich. 1965. Action of antagonists of protein synthesis on the ribosomal apparatus of yeast cells. Dokl. Akad. Nauk SSSR. **161**: 1461-1464.
20. Tai, P. C., D. P. Kessler, and J. L. Ingraham. 1969. Cold-sensitive mutations in *Salmonella typhimurium* which affect ribosome synthesis. J. Bacteriol. **97**:1298-1304.
21. Tai, P. C., and J. L. Ingraham. 1971. A ribosome-like particle accumulated at low temperature by a cold-sensitive mutant of *Salmonella typhimurium*. Biochem. Biophys. Acta **232**:151-166.
22. Warner, J. R. 1966. Ribosome formation in the Hela cells in the absence of protein synthesis. J. Mol. Biol. **19**:373-382.
23. Warner, J. R. 1971. The assembly of ribosomes in yeast. J. Biol. Chem. **246**:447-454.