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Synthesis and Turnover of Ribosomal Proteins in the Absence of 60S Subunit Assembly in *Saccharomyces cerevisiae*

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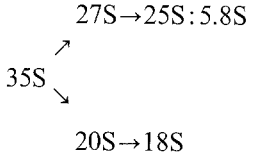
Summary. We have measured the synthesis and stability of ribosomal proteins in a temperature sensitive strain of yeast which at the restrictive temperature is specifically blocked in the processing of 27S ribosomal precursor RNA. We find that in the absence of 60S ribosomal subunit assembly, the synthesis of all the ribosomal proteins studied continued. However, the proteins of the 60S subunit fail to accumulate and are rapidly degraded.

Introduction

The biosynthesis of ribosomes is a complex process requiring the coordinate synthesis of several species of ribosomal RNA (rRNA) and over fifty different ribosomal proteins (Dennis and Nomura, 1975; Gorenstein and Warner, 1976). Three of the four species of rRNA arise from a series of endonucleic cleavages of a large precursor molecule. The steps in this maturation pathway have been described in yeast (Udem and Warner, 1972; Trapman and Planta, 1976):



Andrew et al. (1976) have described a temperature sensitive yeast strain, *ts* 351, which at the restrictive temperature, does not process the 27S percursor correctly. As a consequence the cells accumulate 40S ribosomal subunits, but fail to assemble 60S ribosomal subunits.

In this study, using methods permitting the identification of ribosomal proteins in whole cell extracts, we have asked: 1) whether the block in 27S RNA processing could be due to the lack of synthesis of the 60S proteins, and 2) whether ribosomal proteins made in the absence of 60S subunit assembly accumulate.

We find that at the restrictive temperature the synthesis of all the ribosomal proteins studied continued. However, the proteins of the 60S subunit fail to accumulate and are rapidly degraded.

Materials and Methods

Strain and Media

Mutant strain *ts* 351 is a derivative of A364A. It was originally isolated by Hartwell (1967) and was a kind gift from A. Hopper. Cells were grown on synthetic complete media (SC) containing, per liter, 10 g succinic acid, 6 g NaOH, 6.7 g yeast nitrogen base without amino acids (Difco), 20 g glucose, 20 mg each of adenine and uracil, 50 mg each of histidine, tyrosine and lysine.

Labeling and Extraction of RNA

Cells were converted to spheroplasts, labeled, and RNA was extracted as previously described (Udem and Warner, 1972). The RNA was analyzed on 2.75% polyacrylamide gels as described (Warner and Udem, 1972).

Labeling and Extraction of Proteins

Cells were grown at 23° in SC supplemented with 2 μCi/ml ^{14}C leucine to a density of 10^7 cells/ml. Cells were washed, converted to spheroplasts and incubated in SC containing 0.4 M $MgSO_4$ (SCM) for 90 min (Hutchinson and Hartwell, 1967). The protocol used for pulse labelling spheroplasts is given in the table and figure legends. Total protein was extracted by lysing spheroplasts in H_2O, followed by immediate addition of 2 volumes glacial acetic acid and 0.1 volume 1 M $MgCl_2$. RNA precipitates under these conditions and was removed by centrifugation at 25,000 × *g* for 15 min. Greater than 90% of the total cellular protein was thus solubilized. The proteins were dialyzed against several changes of 1% acetic acid and lyophilized.

Two Dimensional Gel Electrophoresis of Proteins

Total cell extracts were electrophoresed in two dimensional gels as previously described (Gorenstein and Warner, 1976). Electrophoresis in the first dimension was at pH 5 in a 4% acrylamide gel containing 8 M urea (Mets and Bogorad, 1974), and in the second dimension in a 17% acrylamide gel containing SDS (Maizel, 1971). Gels were stained with 0.2% Coomassie blue in 50% methanol and destained in 30% methanol. Ribosomal and non-ribosomal proteins were identified, excised from the gel and counted as previously described (Gorenstein and Warner, 1976).

Measurement of Ribosomal Protein Synthesis

The relative rate of protein synthesis, Ai, was calculated from the $^3H/^{14}C$ ratio found in each protein excised from the gel, and normalizing this ratio to the $^3H/^{14}C$ ratio in total protein:

$$Ai = \frac{(^3H/^{14}C);\ \text{th protein}}{(^3H/^{14}C)\ \text{total protein}}.$$

This method is valid for the measurement of the synthesis and accumulation of ribosomal proteins (Dennis, 1974) and is not dependent on the efficiency of protein extraction from the gel.

Results

Synthesis and Accumulation of rRNA in Mutant Cells

The synthesis of ribosomal RNA at the permissive and restrictive temperature was followed by pulsing the cells briefly with ^3H [CH_3] methionine. Total RNA was extracted and analyzed on polyacrylamide gels. Figure 1 A and B demonstrate the pattern of rRNA synthesis at 23° C and 36° C respectively. It can be seen that under both conditions the synthesis of all precursor species can be detected. However, Figure 1 B shows that although the transcription and processing of the 35S RNA are accelerated at the elevated temperature, as demonstrated by the appearance of mature 18S RNA, little or no 27S is converted to the mature species. In wild type cells (not shown) the processing of both 27S and 20S RNA are increased at the elevated temperature (Udem and Warner, 1972).

The apparent block in the processing of one of the precursors can be seen more clearly after a chase at the restrictive temperature. Figure 1 C shows that while the conversion of 20S RNA to the mature 18S species occurs normally, the 27S RNA disappears without the concomitant appearance of 25S RNA. Using the data from Figure 1 C and the ratio of methyl groups in 25S to 18S RNA (Klootwijk and Planta, 1973), we can calculate that after 90 min at the restrictive temperature, the efficiency of conversion of 27S to 25S RNA is less than 4%. These results, using ^3H [CH_3] methionine to specifically label rRNA, are in agreement with those of Andrew et al.

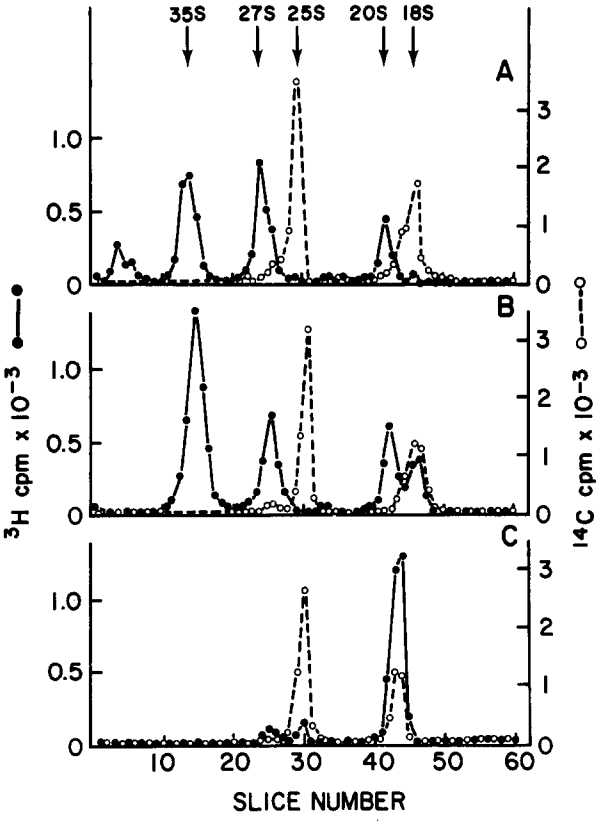


Fig. 1 A–C. Ribosomal RNA synthesis at the non-permissive temperature. A culture of *ts* 351 was grown in SC supplemented with 0.2 μCi/ml ^{14}C uracil for several generations. The cells were converted to spheroplasts and allowed to grow in SCM for 2 h. One aliquot was pulsed at 23° with 60 μCi/ml ^3H [CH_3]-methionine for 5 min (**A**). Another aliquot was shifted to 36° for 90 min and pulsed for 5 min with 60 μCi/ml ^3H [CH_3]-methionine (**B**) and chased for 10 min with 500 μg/ml unlabeled methionine (**C**). Metabolic activity was arrested by pouring the cultures into frozen 1 M sorbitol. RNA was extracted and electrophoresed as described in Methods. o---o ^{14}C labeled RNA; ●—● ^3H labeled RNA

(1976) using $^{32}PO_4$, and confirm their conclusion that at the restrictive temperature *ts* 351 is defective in the conversion of 27S to 25S RNA. The 27S RNA made under these conditions disappears, presumably due to rapid nuclease digestion (Fig. 1 C).

Synthesis of Ribosomal Proteins at the Restrictive Temperature

In order to determine whether the block in the maturation pathway of 27S RNA had an effect on the synthesis of ribosomal proteins, or was a result of the lack of synthesis of 60S ribosomal proteins, we determined the relative rates of synthesis, Ai, for a large number of 60S ribosomal protein, as well as a few 40S ribosomal proteins and some non-ribosomal proteins (designated by letters) as controls. The

Table 1. Synthesis of ribosomal and non-ribosomal proteins at permissive and restrictive temperatures

	Ai 23°	Ai 36°
60S Proteins		
1	0.90	0.70
2	1.44	0.84
6	1.60	0.89
8	1.24	0.74
11	1.24	1.20
15	1.00	0.56
16	1.40	1.30
18	1.30	1.00
22	1.00	0.70
23	1.31	1.20
24	1.20	1.10
25	1.20	1.10
27	1.70	0.67
28	1.20	0.56
29	1.00	0.89
33	1.10	0.60
38	1.44	1.30
39	1.40	1.30
44	0.90	0.51
47	1.20	0.79
48	1.60	1.20
49	1.50	1.10
57	1.16	1.16
58	0.86	0.59
62	1.10	0.70
64	1.20	0.89
65	1.75	0.53
40S Proteins		
5	1.60	1.90
13	1.24	1.10
21	1.10	0.96
36	1.20	1.30
63	1.31	1.10
Non-ribosomal proteins		
A	1.24	1.40
B	1.60	1.20
D	1.90	1.40

A culture of labeled spheroplasts as described in Methods was pulsed for 5 min with 100 μCi/ml ^{3}H leucine at 23°, and after one hour at 36°. Incorporation of labeled amino acid was arrested by pouring the cultures into frozen 1 M sorbitol. Total cell extracts were electrophoresed as described in Methods and the Ai for ribosomal and non-ribosomal proteins were determined

nomenclature used to identify the ribosomal proteins in 2D gels has been described previously (Gorenstein and Warner, 1976). Table 1 demonstrates that the synthesis of all 27 of the 60S subunit proteins which we examined continued at the restrictive temperature. Thus, the accurate processing of 27S ribosomal precursor RNA is not necessary for the continued synthesis of ribosomal proteins or their mRNAs, which we have shown to have a half life of 10 to 20 min at 36° (Warner and Gorenstein, 1977).

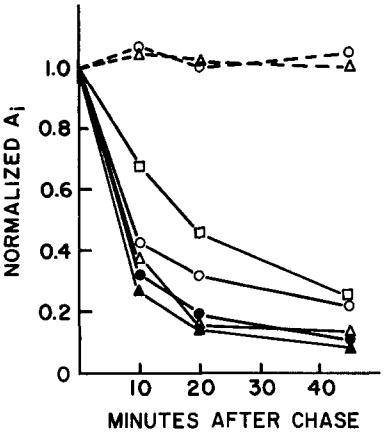


Fig. 2. Kinetics of ribosomal protein decay at the restrictive temperature. A culture of *ts* 351 was grown overnight in ^{14}C leucine at 23° as described in Methods. Spheroplasts were shifted to 36° for 1 h, pulsed for 5 min with 100 μCi/ml ^{3}H leucine and chased for various periods of time with 1 mg/ml unlabeled leucine. Aliquots were removed at the end of the pulse and chase periods. Total cell extracts were prepared and electrophoresed as described in Methods. The Ai values for a large number of ribosomal and non-ribosomal proteins were determined. Five representative proteins from the large subunit, one from the small subunit, and one non-ribosomal protein have been plotted. The Ai values for these proteins have been normalized to the first time point in order to facilitate comparisons. The solid lines represent 60S proteins: □ #24, ○ #15, △ #8, ● #38, ▲ #27. The dashed lines are control proteins: ○ #36 a 40S protein, △ A a non-ribosomal protein

Fate of Ribosomal Proteins Synthesized at the Restrictive Temperature

Studies in animal cells where actinomycin D may be used to inhibit the synthesis of rRNA have shown that in the absence of ribosome formation the synthesis of ribosomal proteins continues (Craig and Perry, 1971; Warner, 1977). However, these proteins fail to accumulate and are rapidly degrated (Tsurugi and Ogata, 1977; Warner, 1977). Figure 2 shows the kinetics of decay for 5 proteins of the large subunit, one 40S protein and one non-ribosomal protein. The 60S proteins appear to decay exponentially with half lives of 7 to 20 min, while control proteins are relatively stable. Table 2 demonstrates that a similar situation occurs for all 60S ribosomal proteins examined.

The last column in Table 2 presents the half lives of the 60S proteins made at the restrictive temperature. It can be seen that with the exception of protein #16, the decay observed is extremely rapid. In the

Table 2. Stability of ribosomal and non-ribosomal proteins at restrictive temperature

	Ai 5 min pulse	Ai 30 min chase	Half life in minutes $t^{1}/_{2}$
60S Proteins			
1	0.59	0.30	24
2	0.72	0.32	24
8	0.79	0.15	8
11	1.05	0.48	20
15	0.52	0.17	13
16	0.98	0.67	119
18	0.75	0.25	13
22	0.70	0.15	7
23	0.91	0.19	8
24	0.73	0.45	23
25	0.79	0.58	35
27	0.68	0.17	7
28	0.59	0.34	25
29	0.74	0.14	10
33	0.66	0.16	10
38	0.92	0.20	8
39	1.10	0.60	24
47	0.74	0.14	12
48	1.23	0.20	14
49	0.96	0.16	7
40S Proteins			
5	1.43	1.26	
13	0.95	0.83	
21	0.78	0.76	
36	0.99	0.91	
63	1.07	0.90	
Non-ribosomal proteins			
A	1.11	1.16	
B	1.56	1.50	
D	2.20	2.19	

A culture of labeled spheroplasts, as described in Methods, was maintained at 36° for 1 h. The cells were pulsed for 5 min with 100 μCi/ml ^{3}H leucine and chased with 1 mg/ml unlabeled leucine for 30 min. Aliquots were removed at the end of the pulse and chase periods. Total cell extracts were electrophoresed as described in Methods and the Ai for a large number of ribosomal and non-ribosomal proteins were determined. The values for the half lives of ribosomal proteins were obtained from the experiment described in Figure 2, using the 0, 10 and 20 min points. The $t^{1}/_{2}$ of each protein was calculated from the slope, fitted by least squares analysis, of a semi log plot

absence of subunit assembly, proteins #22, #27 and #49, with half lifes of 7 min, are among the most unstable proteins known (Goldberg and St. John, 1976). This rapid turnover is responsible for the Ai values of 60S proteins made at 36° being somewhat lower than the controls (Table 1), since considerable degradation of these proteins occurred during the labeling period. The contrast between the unstable 60S proteins and the stable 40S proteins is dramatic. Clearly, unless ribosomal proteins are part of a ribonucleoprotein particle which is correctly processed, they are subject to rapid degradation.

Discussion

The pattern of rRNA synthesis and maturation is similar in all eukaryotes (Hadjiolov and Nikolaev, 1976). In yeast the mature species are derived from a series of endonucleolytic cuts on a ribonucleoprotein particle containing a methylated 35S precursor molecule yielding particles containing 27S and 20S molecules. These are further trimmed to the 25S, 5.8S and 18S mature species found in 60S and 40S ribosomal subunits. We have corroborated the results of Andrew et al. (1976) by showing that at the restrictive temperature of 36°, the mutant strain *ts* 351 is unable to process the 27S precursor into the mature 25S species. The 27S RNA does not accumulate, and is rapidly degraded (Fig. 1C). In contrast, the synthesis of 18S RNA and 4S RNA, as well as total mRNA, continues at approximately normal levels (Andrew et al., 1976).

The absence of ribosomal proteins leads to impaired processing of ribosomal precursor RNA and ultimately to its degradation (Warner and Udem, 1972; Gorenstein and Warner, 1976). Furthermore, the synthesis of the various ribosomal proteins is coordinately regulated (Gorenstein and Warner, 1976). We asked therefore if the lesion in strain *ts* 351 could be associated with the regulation of the synthesis of the proteins of the large subunit. However, the data in Table 1 indicate not only that the synthesis of the 60S proteins continues, but also that at least 27 of them are synthesized in substantial amounts. Furthermore, we have observed no differences in comparing the electrophoretic migration of the 60S proteins of the wild type and mutant strains, suggesting, but not proving, that the *ts* gene does not specify one of these proteins. However, eight to ten of the 60S proteins are not resulved on our gels, for the most part because they are too acidic. It is possible that the synthesis or the structure of one of these proteins could be affected by the mutation. Since other causes of the mutant phenotype are also possible, further investigation is necessary.

The half life of mRNA for ribosomal proteins at 36° is 8–15 min (Gorenstein and Warner, 1976; Warner and Gorenstein, 1977). Since the cells used for the experiment of Table 1 had been at the restrictive temperature for sixty minutes before the pulse

labeling they must have been synthesizing the mRNA for the 60S proteins during the time preceding the pulse. We conclude that neither the total processing of ribosomal precursor RNA nor the synthesis of new ribosomes is necessary for the synthesis of mRNA for ribosomal proteins.

The proteins of the 60S subunit that are synthesized at the restrictive temperature undergo rapid decay (Fig. 2, Table 2). That this decay is a result of the lack of assembly of 60S subunits is apparent from a comparison with the proteins of the 40S subunit, which are quite stable. In HeLa cells, ribosomal proteins made in the absence of the transcription of ribosomal RNA are also very unstable (Warner, 1977). It appears to be characteristic of ribosomal proteins in eukaryotic cells that if they are not associated with ribosomal RNA, or properly assembled, they are degraded rapidly, usually with a half life of 5 to 10 percent of the generation time of the cell. In *E. coli* excess ribosomal proteins are also unstable but their decay is an order of magnitude slower (Dennis, 1974).

The half time of decay of the ribosomal proteins shown in Table 2 is quite reproducible. Some are clearly more stable than others. These differences do not appear to be correlated with the size or isoelectric point of the proteins. Protein #1 is at least two times the size of #8 but is more stable; #8 is at least twice the size of #39 but is less stable. All the proteins studied are highly basic, as shown by their rapid migration towards the cathode at pH 5. Thus, the generalization that small basic proteins are likely to be the most stable (Goldberg and St. John, 1976) does not hold in this case. It is worth noting that the rate of decay of the 60S proteins at the restrictive temperature is more than a hundred fold greater than the rate of turnover of total intracellular proteins in the parental strain, which Betz (1976) determined to be one percent per hour.

The question remains whether, on the one hand, the structure of free ribosomal proteins makes them unusually susceptible to the common cellular proteases (reviewed by Pringle, 1975), or, on the other hand, whether there may be a specific enzyme, perhaps located in the nucleolus, which degrades excess ribosomal proteins.

We have previously shown that ribosomal precursor RNA, synthesized in the absence of ribosomal proteins (Gorenstein and Warner, 1976), decays rapidly (Warner and Udem, 1972). Thus, it appears that yeast have developed housekeeping techniques which maintain a balance between ribosomal RNA and ribosomal proteins. Ribosomal components made in excess do not accumulate but are rapidly degraded.

The one clear exception to the general instability of the 60S proteins is #16. With a half life of nearly two hours, it is the most stable of the 60S proteins made in the absence of subunit assembly. This protein has been previously shown to exchange between ribosomes and cytosplasm (Zinker and Warner, 1976). Its stability may reflect the ability of this protein to exist in two states: associated with the ribosome where it is presumably protected, and free in the cytoplasm where it may be susceptible to proteolytic degradation.

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