Effects of Cycloheximide and 5-Fluorouracil on Formation of Low-Molecular-Weight Ribonucleic Acid in Yeast

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The effects of cycloheximide and 5-fluorouracil on the formation of low-molecular-weight ribonucleic acid (RNA) in yeast were investigated. Both compounds were found to affect the synthesis of low-molecular-weight RNA of ribosomal origin more than transfer RNA but less than the high-molecular-weight ribosomal RNA. 5-Fluorouracil-containing transfer RNA was separated from normal transfer RNA by chromatography on diethylaminoethyl cellulose at 80 °C in the presence of 7 M urea.

MATERIALS AND METHODS

Yeast strain used. The yeast strain used in this investigation was Saccharomyces carlsbergensis NCTC 74. Methods for the cultivation and the conversion of the cells into protoplasts have been described elsewhere (6).

Materials. FU was obtained from Calbiochem, Los Angeles, Calif. Cycloheximide was purchased from Sigma Chemicals Corp., St. Louis, Mo. Adenine-8-¹⁴C (specific activity, 55 mc/m mole), ¹⁴C-Methionine (specific activity, 52 mc/m mole), and carrier-free ³⁵S-labeled orthophosphate were products of Schwartz Bio Research, Inc., Orangeburg, N.Y.

Preparation of high-molecular-weight yeast RNA. Total high-molecular-weight yeast RNA was prepared as described previously (4). The fractionation of high-molecular-weight yeast RNA with phenol was carried out as described in another paper (11a).

Preparation of low-molecular-weight RNA. Low-molecular-weight RNA was prepared by phenol treatment of whole yeast cells as described by Holley et al. (8). The procedure was followed until the purification step with DEAE cellulose.

Fractionation of yeast RNA by gel filtration. High-molecular-weight RNA, low-molecular-weight RNA, and tRNA were separated on Sephadex G-100 (Pharmacia, Uppsala, Sweden) according to the method described by Virmaux et al. (15). About 1,000 to 4,000 µg of RNA dissolved in 2 ml of 0.05 M ammonium acetate buffer (pH 5.0) was applied to a column (180 by 2 cm) of Sephadex G-100 made in the same buffer. Fractions (5 ml) were collected at a flow rate of 35 ml/hr; the first 200 ml of effluent was discarded. The optical density at 260 nm was read,

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and 1-ml samples were plated on planchets, dried, and counted in a Beckman Low Beta counter.

Separation of low-molecular-weight RNA components by chromatography on DEAE cellulose at 80 C. Separation of low-molecular-weight RNA species was also achieved by chromatography on DEAE cellulose at 80 C, as described by Comb and Zehavi-Willner (2). About 500 μg of low-molecular-weight RNA was applied on a column (10 by 1 cm) and eluted at 80 C, with 150 ml of a linear gradient of 0.25 M to 1.5 M NaCl in 0.02 M tris(hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (pH 7.6). Fractions (2.2 ml) were collected at a flow rate of approximately 1 ml/min. The optical density at 260 nm was read, and the radioactivity of the fractions was measured after the addition of 0.5 mg of bovine serum albumin as a carrier followed by precipitation and washing with cold trichloroacetic acid (4).

Incorporation of radioactive precursors into RNA. For the incorporation of adenine-8-14C into RNA, yeast cells were incubated at 30 C in a medium containing (per ml): 20 μmoles of potassium phosphate buffer (pH 6.2), 2 mg of Casamino Acids (Difco), 10 mg of glucose, 2 μmoles of MgCl2, and 20 μg of unlabeled adenine. Adenine-8-14C was added to a final concentration of 0.2 μc/ml. In the case of 14C-labeled methionine, the concentration of Casamino Acids was lowered to 0.5 mg/ml. 14C-methionine was added to a final concentration of 0.2 μc/ml. For the incorporation of 32P-labeled orthophosphate, the phosphate concentration of the medium was reduced to 0.5 μmoles/ml. For protoplasts, 13% mannitol was used as an osmotic stabilizer.

Estimation of the base composition of newly synthesized RNA. The base composition of newly synthesized RNA was estimated with 32P-labeled orthophosphate as described earlier (4).

Incorporation of 14C-labeled amino acids into tRNA. Experiments on the uptake of amino acids into tRNA were carried out as follows (3). The incubation medium contained, in a total volume of 0.5 ml: 1.0 μmole of adenosine triphosphate ATP; 5.0 μmoles of KCl; 5.0 μmoles of magnesium acetate; 10 μmoles of Tris-hydrochloride buffer (pH 7.6); 0.25 μc of leucine-1-14C (specific activity, 24.3 μc/mc/mole) and 100 μg of an activating enzyme preparation from yeast partially purified by centrifuging a yeast extract at 100,000 X g and chromatography on DEAE cellulose.

tRNA was added as indicated in Fig. 5. The incubation was carried out for 20 min at 25 C. RNA was precipitated and radioactivity was measured after the usual trichloroacetic acid precipitation and washing steps (4).

RESULTS

Gel filtration of yeast RNA synthesized in the presence of cycloheximide and FU. Figure 1 shows the results of experiments in which RNA prepared from yeast protoplasts was fractionated on Sephadex G-100. The yeast RNA used in these experiments was prepared with the cold phenol procedure which yields only RNA and low-molecular-weight RNA and leaves RNA with a deoxyribonucleic acid (DNA)-like base composition in the phenol phase (11a). The optical density pattern at 260 nm (Fig. 1) demonstrated that three main fractions can be obtained. Separate studies on the cellular location of the RNA fractions confirmed the results of Galibert et al. (7) and showed that the high-molecular-weight tRNA species emerge first from the column as a single peak, followed by low-molecular-weight tRNA and tRNA as two clearly resolved peaks. Quantitatively, the low-molecular-weight RNA species amount to less than 10% of the total RNA isolated by this procedure. Therefore, in many experiments on the
quantitative behavior of the inhibitors, extra-low-molecular-weight RNA was added as a carrier. Control experiments showed that treatment of protoplasts with cold phenol or phenol-sodium lauryl sulfate and direct phenol treatment of whole cells with phenol were equally effective in releasing the low-molecular-weight RNA species. Carried out in combination with sucrose density-gradient centrifugation to separate the 28S and 18S rRNA species, the data allow a quantitative estimation of the effect of both inhibitors on all stable RNA species. The results of a particular experiment are shown in Table 1. The data show clearly that cycloheximide has various effects on the formation of the different stable RNA species. Although the actual degree of inhibition varied for different experiments, it was always found that the formation of tRNA is least affected, followed, respectively, by the low-molecular-weight rRNA, the 28S rRNA component, and finally the 18S rRNA component which is synthesized very little. FU behaves differently, since the synthesis of both high-molecular-weight rRNA components is inhibited to an equal extent (11a). The formation of tRNA is least affected.

Fractionation of low-molecular-weight yeast RNA by chromatography on DEAE cellulose at 80 C. Results similar to those described above, employing gel filtration to separate the different RNA species, were obtained when the low-molecular-weight RNA components are separated on DEAE cellulose at 80 C. As shown in Fig. 2, low-molecular-weight RNA of yeast can be fractionated into two fractions. Control studies on the methylation, cellular localization, and the amino acceptor activity showed that the first-eluting component is tRNA, whereas the second component, which elutes at the higher salt concentration, is the low-molecular-weight rRNA component. These results are in agreement with those obtained for the low-molecular-weight RNA isolated from Escherichia coli and Blastocladia emersonii as described by Comb and Zehavi-Willner (2).

The results (Fig. 2) again show that cycloheximide inhibits the formation of low-molecular-weight RNA much more than it does the synthesis of tRNA. The results obtained when yeast cells were incubated with FU were quite unexpected. In this case, the newly synthesized low-molecular-weight RNA eluted from the column as one peak at salt concentration (±0.97 M) intermediary to the salt concentrations required for the elution of the two stable low-molecular-weight RNA components (±0.9 and 1.1 M). As Table 2 shows, the base composition of this abnormal RNA resembles closely the base composition of normal transfer RNA, except for a replacement of 60% of the uridylic acid residues by the analogue, a very low content of pseudouridylic acid, and a slightly lower cytidylic acid content. The base composition was found to differ considerably from the normal tRNA fraction eluting at the same salt concentration as the abnormal RNA, demonstrating that the abnormal elution pattern was not due to the increased synthesis of this particular tRNA fraction in the presence of the analogue.

Experiments on the incorporation of 14C-methyl-labeled methionine revealed that the RNA synthesized in the presence of the analogue is also methylated (Fig. 2d). A quantitative study revealed that the methylation and the synthesis were affected to an equal extent (60% in the particular experiment), indicating a tRNA-like methylation of the abnormally eluting RNA.

Effect of changing conditions of the chromatographic procedure on the separation of normal and analogue-containing tRNA. In attempts to characterize the analogue-containing tRNA further, and possibly to find a method for the purification of analogue-containing tRNA, the circumstances of the chromatographic procedure were varied. FU-containing tRNA was also found to elute slightly after normal tRNA at room temperature. Raising the temperature was found to improve the resolution considerably. The separation was not affected by changing the pH between 5.0 and 8.5. However, the separation of normal and analogue-containing tRNA can be improved considerably by adding 7 M urea to the gradient.

### Table 1. Inhibition of RNA synthesis in yeast protoplasts by cycloheximide and 5-fluorouracil

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fraction</th>
<th>28S</th>
<th>18S</th>
<th>LMW-rRNA</th>
<th>tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide...</td>
<td>93</td>
<td>98</td>
<td>81</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>5-Fluorouracil...</td>
<td>95</td>
<td>95</td>
<td>82</td>
<td></td>
<td>50</td>
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* Yeast protoplasts were incubated for 60 min with 14C-adenine as described in Fig. 1. RNA was isolated with the cold phenol procedure and fractionated by sucrose density-gradient centrifugation to separate 28S and 18S ribosomal RNA from the low-molecular-weight (LMW) RNA species and by gel filtration on Sephadex G-100 to separate low-molecular-weight rRNA and tRNA from the high-molecular-weight rRNA species. Fractions containing the different RNA species were pooled, and the specific radioactivity (counts per milligram of RNA) was estimated. Values are expressed as percentage inhibition with respect to the untreated control sample.
FIG. 2. Fractionation of low-molecular-weight RNA of yeast on DEAE cellulose at 80 C. Yeast cells were incubated for 90 min with 14C-adenine in 25 ml of the medium described in Materials and Methods (a) in the absence of both inhibitors, (b) in the presence of FU (100 µg/ml), (c) in the presence of cycloheximide (20 µg/ml), and (d), as (b), with 14C-adenine replaced by 14C-methionine. Low-molecular-weight RNA was isolated and fractionated on DEAE cellulose at 80 C.

<table>
<thead>
<tr>
<th>Table 2. Base composition of low-molecular-weight RNA components of yeast</th>
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<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Low-molecu-</td>
</tr>
<tr>
<td>lar-weight</td>
</tr>
<tr>
<td>rRNA</td>
</tr>
<tr>
<td>tRNA</td>
</tr>
<tr>
<td>tRNA'</td>
</tr>
<tr>
<td>FU-tRNA</td>
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</table>

Yeast cells were incubated for 90 min with 32P-labeled orthophosphate in a medium as described in Materials and Methods in the absence or presence of 5-fluorouracil. Low-molecular-weight RNA was isolated and fractionated on DEAE cellulose at 80 C and the base composition of the newly synthesized RNA was estimated as described before. Values represent moles per cent. Transfer RNA' = fraction of transfer RNA eluting at the same salt concentration as the peak fraction of FU-tRNA.

Abbreviations: CMP, cytidine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; UMP', pseudouridine monophosphate; FUMP, fluorouridine monophosphate.

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free of contaminating low-molecular-weight tRNA. Although the physical and biochemical properties of the analogue-containing tRNA will be the subject of a future publication, the preliminary estimation of the melting behavior showed that FU-tRNA melts at a lower temperature than does normal tRNA (54°C versus 52°C; Fig. 4) and that it is active in accepting at least leucine (Fig. 5).

**DISCUSSION**

The experiments described in this paper show that FU, as well as cycloheximide, inhibits the formation of low-molecular-weight rRNA less than the synthesis of the high-molecular-weight rRNA species and more than the formation of tRNA, thus suggesting that high- and low-molecular-weight rRNA species do not arise from the same high-molecular-weight precursor (11). A similar suggestion has been made by Knight and Darnell (9) as a result of their studies on the distribution of low-molecular-weight rRNA between the nucleus and the ribosomes of HeLa cells, and by Brown and Weber (1) on the basis of hybridization experiments of rRNA species with different DNA fractions of *Xenopus laevis*. An alternative explanation of the present data might be that, although the three rRNA species are transcribed from one operon, the low-molecular-weight rRNA is transcribed first, leaving the

**FIG. 3.** Effect of 7 M urea on the separation of FU-containing tRNA from normal tRNA. Yeast cells were incubated for 90 min in the presence of FU (100 μg/ml) and 14C-adenine. Low-molecular-weight RNA was isolated and fractionated on DEAE cellulose at 80°C in the presence of 7 M urea.

**FIG. 4.** Heat denaturation of tRNA formed in the presence of FU. tRNA containing FU was isolated by chromatography on Sephadex G-100 and DEAE cellulose at 80°C in the presence of urea. Samples containing 25 μg of tRNA per ml were heated in 0.2 M NaCl in 0.02 M Tris buffer (pH 7.2). Solid line, normal tRNA; dashed line, FU-containing tRNA.

**FIG. 5.** Amino acceptor activity of RNA synthesized in the presence of FU. Yeast cells were incubated for 180 min in a medium described in Materials and Methods, omitting 14C-labeled adenine and in the absence (a) or presence (b) of FU (100 μg/ml). Low-molecular-weight RNA was isolated and fractionated on DEAE cellulose at 80°C in the presence of urea. Acceptor activity for 14C-leucine was tested as described in Materials and Methods. An 0.05-ml amount of each fraction of the eluate was taken without prior removal of NaCl or urea.
other RNA species more liable to the action of the inhibitors. Finally, the difference in degree of inhibition might be the result of the appearance of degradation products of high-molecular-weight RNA species in the low-molecular-weight RNA fraction. The experiments with hot DEAE cellulose columns show that normal and FU-containing tRNA can be separated. Apparently yeast tRNA differs from E. coli tRNA, since Lowrie and Bergquist (10) were unable to separate the corresponding tRNA species from this organism on DEAE cellulose. The separation of normal and analogue-containing RNA on DEAE cellulose at high temperatures is most likely the result of differences in secondary structure between the two types of RNA under such conditions (2). This is confirmed by the slight difference in melting profiles. A contribution of FU itself having a lower $pK$ than uracil is also likely to be involved. The fact that 7 M urea lowers the salt concentration necessary for the elution and changes the resolution of the different RNA species shows again that the interaction between polynucleotides and DEAE cellulose is not purely ionic but involves other forces as well (14).

Finally, the availability of purified analogue-containing tRNA makes possible experiments which can contribute to the understanding of the function of tRNA in protein biosynthesis and the nature of the metabolic effects of FU, which are still poorly understood.

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LITERATURE CITED


ERRATUM

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Volume 97, no. 2, page 743, article heading, line 4, and running headings of pages 744, 746, and 748: Change "Hendricks" to "Hendrick."