Antisuppressor mutations reduce the efficiency of nonsense suppressors. A mutation in the gene sin4 of Schizosaccharomyces pombe leads to loss of 5-(methyl-2-thiouridyl)-2-thiouridine (mcm5s2U) from the first anticodon position of tRNAs. This resembles the phenotype of sin3 (Heyer, W. D., Thuriaux, P., Kohli, J., Ebert, P., Kersten, H., Gehrke, C., Kuo, K. C., and Agris, P. F. (1984) J. Biol. Chem. 259, 2856-2862), but the mutations reside in different genes. In vivo 35S-labeled tRNA from the parental suppressor strain sup3, the antisuppressor strains sin3 and sin4, and the double mutant sin3 sin4 has been digested to nucleosides and analyzed with high performance liquid chromatography methods. The major sulfur-carrying nucleoside in wild-type S. pombe tRNA is mcm5s2U. It is reduced in the mutant strains. Two other thiolated nucleosides are also present: 2-thiouridine and a nucleoside of unknown structure. Neither was affected by the antisuppressor mutations. Thiocytidine has not been found. Independent from their effect on suppressors, the two mutations sin3 and sin4 reduce the growth rate of cells, and sin3 also increases cell length. In vivo decoding of the serine codon UCG by the UCA reading serine tRNA is not promoted by the two antisuppressor mutations.

Comparably little information exists on the function of modified nucleosides in tRNA (1). One approach for the study of modified nucleosides is screening of mutant strains with an altered translation apparatus (2). In the yeast Schizosaccharomyces pombe, informational suppression studies are well advanced (3). Many mutations reduce the efficiency of nonsense suppressor tRNAs (4). It was found that one of these antisuppressors (sin1) has lost tA next to the anticodon of several S. pombe tRNAs (5).

Recently, we have digested total tRNA from many mutants and subjected the nucleosides to HPLC analysis. The antisuppressor mutation sin3 leads to loss of mcm5s2U, a nucleoside found in the first anticodon position of eukaryotic tRNAs (6). This and related wobble base modifications restrict the decoding range of cytoplasmic tRNAs to triplets ending with A. The modification status of U in the wobble position is important for the definition of the decoding properties of a tRNA (1). With the present publication, we extend the earlier study of sin3 (6) and describe a mutation in the independent gene sin4 that leads also to loss of mcm5s2U from tRNAs. The occurrence of sulfur-carrying nucleosides in wild-type S. pombe tRNAs is also analyzed. The cumulative effects of the two mutations are studied in a double mutant strain sin3 sin4. Finally, the effect of the two mutations on growth rate and 137 cell length are quantitated. These mutant phenotypes are independent of the antisuppressor phenotype. No influence of the mutations on the decoding properties of nonsuppressor serine tRNAs could be detected with an in vivo assay. It allows detection of UCG reading by the serine isoacceptors normally assigned to the codons UCU, UCC, and UCA (7).

MATERIALS AND METHODS

RESULTS

The initial characterization of mcm5s2U loss in sin4, the construction of strains mentioned below, and further experiments are described in the Miniprint.

Sulfur-carrying Nucleosides in Suppressor and Antisuppressor Strains—For the identification and quantitation of 35S incorporation into modified nucleosides, in vivo-labeled tRNA from the strains sin+, sin3, sin4, and sin3 sin4 was digested to nucleosides, and these were fractionated by HPLC. The overall pattern of modification in the sin+ strain is displayed in Fig. 1 (top). There are significant differences between sin+ and the mutants for two modified nucleosides. One of them is mcm5s2U (see Fig. 1). The other reduced nucleoside elutes at 57 min following peak number 25 (Fig. 1, top). It is of unknown structure and absent from sin3, sin4, and the double mutant. It may well correspond to the unidentified compound at 18 min following peak number 18 (Fig. 1, top). Further studies on the function of sulfur-carrying nucleosides in eukaryotic tRNAs are presented in the Miniprint.

Portions of this paper (including "Materials and Methods," part of "Results," part of "Discussion," Table 2, and Figs. 2 and 3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-282, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Antisuppressors and tRNA Modification

TABLE 1
Sulfur-carrying nucleosides in S. pombe tRNA

<table>
<thead>
<tr>
<th>Identification and elution time (min)</th>
<th>sin+</th>
<th>sin3</th>
<th>sin4</th>
<th>sin3 sin4</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm %</td>
<td>cpm %</td>
<td>cpm %</td>
<td>cpm %</td>
<td>cpm %</td>
</tr>
<tr>
<td>Not nucleosides (3–5.5)</td>
<td>1,970</td>
<td>4</td>
<td>1,910</td>
<td>16</td>
</tr>
<tr>
<td>Unknown nucleoside (12.5–13.5)</td>
<td>4,190</td>
<td>8</td>
<td>1,400</td>
<td>12</td>
</tr>
<tr>
<td>s2U (24–25)</td>
<td>660</td>
<td>1</td>
<td>2,000</td>
<td>17</td>
</tr>
<tr>
<td>mcm5s2U</td>
<td>37,400</td>
<td>78</td>
<td>2,730</td>
<td>23</td>
</tr>
<tr>
<td>Total cpm recovered</td>
<td>44,220</td>
<td>89</td>
<td>8,040</td>
<td>67</td>
</tr>
<tr>
<td>Total cpm invested</td>
<td>49,440</td>
<td>100</td>
<td>11,970</td>
<td>100</td>
</tr>
</tbody>
</table>

that has been found to be missing in sin3 tRNA before (6). This nucleoside is devoid of sulfur, since no radioactivity eluted around 57 min.

Fractions of the column eluants were assessed for 35S radioactivity by liquid scintillation counting. Four peaks of radioactivity were observed for sin+ tRNA (Fig. 1 and Table 1). The first does not contain nucleosides as shown in a later experiment (see below). We have found before that 35S-labeled compounds bind noncovalently to tRNA (6), and that explains this peak. The major 35S-peak co-elutes with mcm5s2U and contains 78% of the radioactivity of the sin+ tRNA digest. The other two peaks are minor (8% and 1% radioactivity, respectively) and elute earlier than mcm5s2U. Nothing is known about the structure of the nucleoside corresponding to the 8% peak eluting after 13 min together with 5-methylcytidine. It is not mmn5s2U (elutes at a different position, see Fig. 1) nor thiocytidine which elutes between uridine and 1-methyladenine. The 35S-labeled nucleoside with the lowest abundance (1%) was suspected to be 2-thiouridine as judged from its elution position. In another experiment, synthetic s2U was added before chromatography. Then, the radioactivity peak coincided with the s2U peak that was identified as described under "Materials and Methods." This time the tRNA digest was also passed through a boronate column that discriminates between nucleosides and other compounds that lack cis-diol groups (8). The resulting chromatogram was devoid of the first radioactivity peak mentioned above. The remaining three radioactive peaks were altered in elution position and relative abundance. Thus, S. pombe tRNA carries mcm5s2U, s2U, and another nucleoside of unknown structure that all can be labeled with 35S. In comparison to the sin+ strain, the mutant strains contain strongly reduced amounts of mcm5s2U in their tRNAs. The double mutant sin3 sin4 is almost completely devoid of it. The two mutations have additive effects. The concentration of s2U in all three mutant

enlargements of the region where mcm5s2U elutes are given below. The positions of elution of the three radioactive nucleosides are indicated with arrows (see Table 1). Three peaks are visible in the region where mcm5s2U elutes. One of them persists in tRNA from mutant strains and corresponds to an unknown nucleoside (N). Upon loss of mcm5s2U, the small peak of A, becomes visible to the right of the elution position of mcm5s2U. The numbered peaks in the figures correspond to the following nucleosides: 1, pseudouridine; 2, cytidine; 3, uridine; 4, 1-methyladenosine; 5, 5-methylcytidine; 6, position of mmn5s2U; 7, 2′-O-methylcytidine; 8, 7-methylguanosine; 9, inosine; 10, 5-methyluridine; 11, guanosine; 12, position of s2U; 13, 2′-O-methyluridine; 14, 1-methylinosine; 15, 2′-O-methylguanosine; 16, 1-methylguanosine; 17, 4-acetylcytidine; 18, N2-methylguanosine; 19, adenosine; 20, N2,N2-dimethylguanosine; 21, mcm5s2U; 22, 2′-O-methyladenosine; 23, N-[9-(β-D-ribofuranosylpurin-6-yl) carbamoyl]-threonine (tPA); 24, N9-methyladenosine (m9A); 25, possibly mt9A; 26, Y nucleoside; 27, pA.

FIG. 1. HPLC of 35S-labeled tRNA. Unfractionated tRNA (250 μg) from the strains indicated was hydrolyzed and subjected to HPLC. Elution of nucleosides was monitored at two wavelengths (254 nm, 280 nm). The overall chromatogram for sin+ is shown at the top,
strains is slightly increased in comparison to sin+ (2–3-fold). Our earlier identification of s4U in S. pombe tRNAs (6) could not be confirmed by the present analysis and other unpublished experiments. The compound of unknown structure is reduced 3–7-fold in the mutant strains. No new 35S-labeled nucleoside was identified in the mutants.

DISCUSSION

In the sin+ strain of S. pombe, the major proportion (80% or more) of 35S label in tRNA is incorporated into mcm5s2U. A second nucleoside of unknown structure is 10-fold less abundant. The third compound occurs in the 1% range and most likely is s2U. Both mcm5s2U (tRNA8G18) and s2U (suppressor tRNA8ser, tRNA8eo) have been identified in the sequences of S. pombe tRNAs (9). This resembles the situation in Saccharomyces cerevisiae (10). In Candida tropicalis, 5-methyl-2-thiouridine has been identified besides mcm5s2U (11). The nucleoside s2C present in tRNA of higher eukaryotes is absent in these yeasts.

The mutations sin3 and sin4 reduce the abundance of mcm5s2U (6–13-fold) and of the unknown labeled nucleoside (3–4-fold). In the double mutant sin3 sin4, mcm5s2U is almost undetectable. In contrast, the mutant strains contain more s2U (3-fold) than the parental sin+ strain. No potential intermediates in the formation of mcm5s2U could be identified. It is unlikely that sin3+ and sin4+ gene products are involved in the formation of the mcm5 moiety of mcm5s2U, because mcm5 is present in similar amounts in tRNA from parental and mutant strains. In both mutants, a further unknown nucleoside is absent that cannot be labeled with 35S. Little new information on the pathway of mcm5s2U synthesis could be gained. It is not clear whether the sin3+ and sin4+ gene products are tRNA modification enzymes, or whether they affect tRNA modification indirectly.

It can be concluded from the electrophoresis pattern in Fig. 2 and from the RPC-5 chromatography data on aminoacylated tRNA8G18 (6) and tRNA8eo3, the two mutations lead to differential undermodification of the various tRNA species. To assess the effect of the sin3 and sin4 mutations on individual tRNAs, the modification state of purified tRNA species needs to be determined. Obviously, one target of the sin3+ and sin4+ gene products are the ochre and opal serine suppressor tRNAs of S. pombe (3). The mutations were identified as antisuppressors that reduce the efficiency of ochre suppressor tRNA8eo (4). The ochre suppressor tRNA8eo is only slightly affected (this work) and the opal suppressor tRNA8eo not at all (4).

Finally, since serine suppressor tRNA is inactive by the sin3 and sin4 mutations, we assumed that the corresponding wild-type tRNA8ser in nonsuppressor strains might also be undermodified and its decoding properties changed. Conceivably, the tRNA could then also read UCG beside UCA (7). The experiment carried out to answer this question indicates that UCG is not read by the UCA reading tRNA in cells with sin3 and sin4 backgrounds (see Miniprint). Other phenotypes of the antisuppressor strains were also investigated (growth rate and cell length). They may or may not be mediated through tRNA. These data and their discussion are given in the Miniprint Section.

Acknowledgments—We thank Marlies Fahrni for expert technical assistance, and Dat Phan and Carol Moyer for excellent assistance in chromatography.

REFERENCES


Continued on next page.
Antisuppressors and tRNA Modification

The PI5-labeled tRNA from the four strains were extracted and fractionated on a polyacrylamide gel, after staining with ethidium bromide and photographing. The sid gene was subjected to supershift immunoprecipitation (2). In comparison to SI5 both antisuppressors display loss of or reduction in PI5-labelling of several tRNAs, labelling of one of the two antisuppressor strains and the absence of immunodiffusion of the TNA from the double mutant (3) and SI5 strain when loss reduction.

Experimental procedures involving tRNA-modifying enzymes have been described previously (11). The isolation of the supernatant SUSP1 is based on an initial step involving the addition of chloroform to disrupt the tRNA:suppressor complex, followed by centrifugation of the two supernatants, and the latter stored at -20°C. To label the precursor tRNAs, the yeast strains were grown in the presence of [3H]UCA. This tRNA fraction contains antisuppressor activity and PCR. The function of the antisuppressor (tRNA modification (5)) in the genetic background. The experiments, performed with the analysis of the various SI5 and SI5 strains, indicate that the effectors are not associated with SI5. Further, the analysis of the antisuppressors in wild-type strains for the interaction with the two suppressor genes (4). The resulting undermodified tRNA, which is recognized by codon suppressor and tRNA modification (5).

The results show that the secondary mutation of the SI5 gene to a suppressor type (SU) or to the sup12 UGA leads to lethality of the host tRNA. Thus, in the case of the modifiable tRNA, the undermodified tRNA would not be decoded if the siR- or the sin- background, respectively, as a result of the interaction of the undermodified tRNA with the suppressor SI5. The resulting undermodified tRNA would then bind to the suppressor SI5 and be recognized by the codon suppressor.

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The growth data shown in Table 2 were obtained on glucose-supplemented medium where no suppression is required for growth. Under these conditions the presence of an ochre suppressor gene leads to a pronounced increase of doubling time (2.7 hours) as compared to the control (1.2 hours). This deleterious effect of the ochre suppressor is reduced when either of the antisuppressor mutations are crossed into the strain. Thus reduction of suppressor efficiency by the ochre suppressor mutant in the parental wild-type parallels improvement of growth rate on glucose-supplemented medium. However, this increased growth rate is not observed in the presence of either of the antisuppressor mutations. Thus, a correlation between the presence of antisuppressors and improved growth on glucose-supplemented medium was not observed. Neither the observed effect of antisuppressors on growth rate nor their effect on suppression was found to be dependent on the presence or absence of the ochre suppressor. The observed increase of cell size of the strains carrying the ochre mutation (Table 2) corresponds with a 25% increase of cell volume in wild-type cells upon 5-fluorodeoxyuridine treatment. This property of the ochre mutation is independent of the presence or absence of the ochre suppressor. Also the increase of cell size in ochre strains is independent of the genetic background. The ochre mutation carrying strains are normal concerning cell size and cell shape. Also ochre is notated over ISOL with respect to these phenotypes, indicating that the ochre mutation is not a suppressor of ISOL strains. It may be that tRNA modifications affected by ISOL are involved in the regulation of the cell cycle.