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Studies on Microbial Ribonucleic Acid

VI. Appearance of Methyl-deficient Transfer Ribonucleic Acid During Logarithmic Growth of *Saccharomyces cerevisiae*

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Transfer ribonucleic acid (tRNA) that is deficient in methyl groups may be detected in logarithmically growing *Saccharomyces cerevisiae*. The amount of methyl-deficient tRNA is not constant throughout the logarithmic phase, but is maximal about one generation before the onset of the late growth phase. During this latter phase, the tRNA is fully methylated. The methyl-deficient tRNA is present during a period of high metabolic activity of the cell, characterized by increased RNA and protein content.

The final stage in the cellular synthesis of transfer ribonucleic acid (tRNA) is the enzymatic modification of the product of deoxyribonucleic acid (DNA) transcription (20). Most intensively studied among the enzymes involved in the modification reactions are those catalyzing tRNA methylation, using *S*-adenosylmethionine as the methyl donor (8, 20, 21). Usually, tRNA isolated from cells contains a full complement of methyl groups as shown in homologous systems with tRNA and methylating enzymes from the same species (19). Methyl-deficient tRNA is found only under special circumstances, such as methionine deprivation of methionine-requiring *Escherichia coli* with relaxed control over RNA synthesis (20), or methionine deprivation of certain methionine auxotrophs of *Saccharomyces cerevisiae* (14; Kjellin-Stråby and Phillips, *in preparation*). This study shows that methyl-deficient tRNA can be isolated from cells of *S. cerevisiae* at a certain stage in the logarithmic phase of growth.

Modifications of tRNA in different growth phases of *Bacillus subtilis* have been investigated by elution of charged tRNA from methylated albumin-kieselguhr columns. Experiments have been reported (7, 12, 15) which suggest that there may be differential synthesis of tRNA that accepts a particular amino acid at different times in the process of sporulation, or in different growth media. Other modifications of tRNA are known to occur on phage infection of bacteria. For example, T-even phage infection of *E. coli* leads to alteration of leucyl-acceptor tRNA (13, 24), tRNA methylating enzymes (22), and tRNA labeling by sulfur in the thiol transferase reaction (10). Induction of phage λ leads to inhibition of tRNA methylases (23). These observations suggest a role for tRNA in cellular control mechanisms. This work demonstrates a variation in the degree of tRNA methylation, at a period of intense metabolic activity, during logarithmic growth of the eucaryotic organism *S. cerevisiae*.

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MATERIALS AND METHODS

Materials. [^{14}C - *methyl*] - *S* - adenosyl - L - methionine (50.2 mc/mmole) was obtained from New England Nuclear Corp., Boston, Mass. [*8*-^{14}C]-adenine (11.3 mc/mmole), [^{14}C-*methyl*]-L-methionine (15.6 mc/mmole), and [*5,6*-^{3}H]-uracil (4.8 c/mmole) were purchased from the Radiochemical Centre, Amersham, England. [*2*-^{14}C]-DL-methionine (0.57 mc/mmole) was obtained from Calbiochem, Los Angeles, Calif. Ribonuclease, deoxyribonuclease (electrophoretically purified), and *E. coli* alkaline phosphatase were obtained from the Sigma Chemical Co., St. Louis, Mo. Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Membrane filters MF50 were obtained from Göttingen, Germany. Methylated purine nucleosides were a gift from R. K. Robins of Salt Lake City, Utah.

Strains and growth conditions. Strains D38, D84, MG331, and MG334 of *S. cerevisiae* were used. All were isolated and characterized by M. Grenson. Their genealogy has been described previously (17). All strains are haploid and require methionine; D38 also requires adenine, and D84 requires histidine and uracil. All cells were grown at 30 C, on a rotatory shaker, in minimal media as described previously (14). Growth was followed by a Klett-Summerson photometer (filter no. 66, 13-mm path length). Supplementation of media with [^{14}C-*methyl*]-L-methionine was as described by Isaksson and Phillips (11).

Incorporation of metabolites in vivo. A culture of

actively growing yeast cells in the early logarithmic phase was divided into two parts. The radioactive precursor was added to one portion; the other was used to observe growth. The culture was incubated at 30 C overnight (a period encompassing six or seven generations). Incorporation of the metabolites was then observed for the last three or four generations of logarithmic growth by simultaneous withdrawal of duplicate 150-μliter samples into 3 ml of ice-cold 0.5% sodium chloride and 5% trichloroacetic acid. The samples were mixed and filtered through membrane filters. The filters were washed with several volumes of saline or trichloroacetic acid and then dried; radioactivity was determined in a Mark I liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Appropriate blanks were prepared with heat-inactivated cells (14). A measure of the RNA or protein per cell was obtained by dividing the radioactivity found in the RNA or protein of 150 μliters of the culture (trichloroacetic acid precipitate) by the Klett reading of the culture at the time of sampling.

Methylating enzymes, tRNA, and assay conditions. The preparation of tRNA and of the crude RNA methylating enzyme extracts, the assay of methyl group incorporation using [^{14}C-*methyl*]-*S*-adenosyl-L-methionine as the methyl donor, and the analysis of the methylated components of tRNA have been described (11, 14, 17). Incubation mixtures (1 ml) for the determination of methyl group acceptance of tRNA contained 100 μmoles of tris(hydroxymethyl)-aminomethane (Tris)-chloride (*p*H 8.0), 10 μmoles of $MgSO_4$, 20 μmoles of NH_4Cl, 0.1 μmole of ethylenediaminetetraacetate (EDTA), 2 μmoles of glutathione, and tRNA, methylating enzymes, and [^{14}C-*methyl*]-*S*-adenosyl-L-methionine as specified. All values given for levels of incorporation of methyl groups into tRNA in vitro are derived from time curves corrected for enzyme blanks and continued to plateau levels of incorporation, using two to five different concentrations of tRNA. Hot trichloroacetic acid-precipitable material is isolated in 10% trichloroacetic acid after incubation for 15 min at 100 C. Separation of ribosomal RNA (rRNA) and tRNA by gel filtration has been described by Boman and Hjertén (1). An optical density (OD) unit is defined as the amount of material which, in a 1-ml volume, gives A(1 cm) = 1.00 at 260 or 280 nm, as specified.

RESULTS

Distribution of methyl groups in tRNA in vivo. The yeast strains used were all haploid methionine auxotrophs. Growth in a minimal medium, supplemented with methionine as described in Materials and Methods, proceeds as shown in Fig. 1. Such a growth curve, with small variations in generation time, is typical for all strains referred to in this work. At a certain cell density, the growth rate slows down markedly; adhering to the terminology of Croes (4), we refer to the two growth phases as logarithmic and late growth, respectively. The major source of energy during logarithmic growth is glucose, which acts as a substrate for glycolysis. The ethyl alcohol produced is then metabolized aerobically during the late growth phase (4, 25). The total cell count and the viable count continue to increase during the late growth phase to reach a plateau before the onset of the true stationary phase, which may be delayed longer than illustrated in Fig. 1.

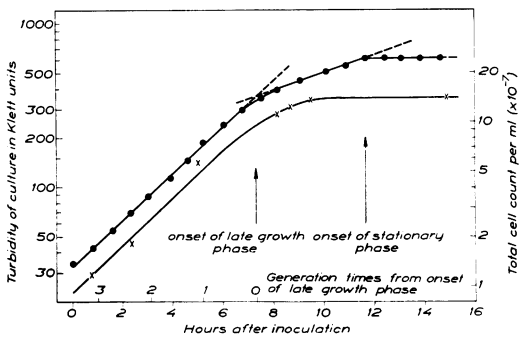

FIG. 1. *Growth (●) of yeast strain MG331 in minimal medium containing methionine. At the time shown, 20 ml of medium was inoculated with cells from an overnight culture near the end of the logarithmic growth phase. At intervals, 1-ml samples were removed and added to 20 μliters of formaldehyde. After an appropriate dilution in 0.5% saline, cells were counted in a blood cell-counting chamber (×).*

Supplementation of the growth medium with [^{14}C-*methyl*]-L-methionine leads to incorporation of [^{14}C]-methyl groups into tRNA. Isolated tRNA may be subjected to hydrolysis and the methylated components can then be separated by two-dimensional chromatography (11). Detailed analysis of tRNA from cells of strains D38, MG331, and MG334, harvested both one generation before and 1 hr after the onset of the late growth phase (17), has shown that the methylated base composition of tRNA is the same at each time, within the limits of accuracy of the method. Since the method for tRNA isolation presumably gives all species of tRNA, we may conclude that there is no major change in the composition of the tRNA in these two growth phases, although the assay method is not sufficiently sensitive to detect a change in the proportion of a single tRNA species.

Incorporation of methyl groups into tRNA in vitro. Cells harvested during the logarithmic growth phase were used for the preparation of crude methylating enzymes; these preparations contained fair amounts of rRNA and tRNA. Methylating enzymes catalyze the incorporation of methyl groups from [^{14}C-*methyl*]-*S*-adenosyl-L-methionine into homologous tRNA only if the tRNA is methyl-deficient. Usually, there is no incorporation into tRNA that is isolated from exponentially growing cells. We observed,

however, that crude deoxyribonuclease-treated enzyme preparations from *S. cerevisiae* strain D38 incorporated large amounts of radioactivity from [^{14}*C-methyl*]-*S*-adenosyl-L-methionine. The incorporation of methyl groups was linearly dependent upon the amount of crude enzyme added, and about 50% of the incorporation was ribonuclease-sensitive (Fig. 2). The same proportion was also rendered soluble by hot trichloroacetic acid. These results indicate that the enzyme preparation contained methyl-deficient RNA.

Because both rRNA and tRNA might function as methyl acceptors, RNA was isolated (11) from an incubation mixture of crude enzymes and labeled methyl donor, and was subjected to gel filtration on Sephadex G-200 (1). Figure 3 shows that the methyl acceptor is tRNA; no methylated product is found in the rRNA region of the chromatogram (fractions 14–22). The results presented in Fig. 4 show that the incorporation of methyl groups into the crude enzyme preparation was stimulated by the addition of tRNA that had been isolated from the same batch of cells used for the enzyme preparation. By contrast, the addition of tRNA isolated from cells harvested in the late growth phase (Fig. 5), or from cells harvested after methionine starvation, leads essentially to no stimulation of incorporation. This indicates that

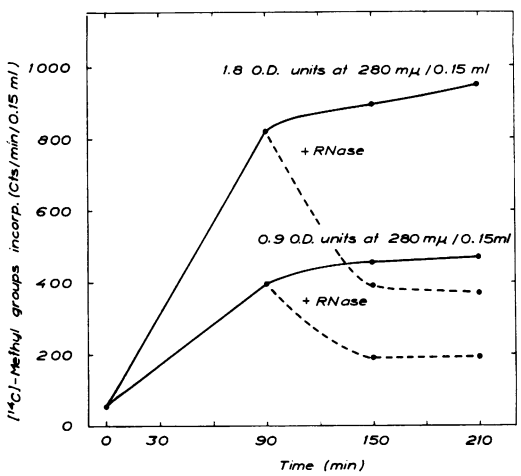

FIG. 2. *Time curves for incorporation of methyl groups in vitro into two different amounts of the crude preparation of methylating enzymes* (A_{280}/A_{260} = 0.67) *made from cells of strain D38, harvested half a generation time before onset of late growth phase. The methyl donor was* [^{14}*C-methyl*]-*S-adenosyl-L-methionine (3 nmoles/ml). At the time shown, pancreatic ribonuclease was added to a final concentration of 33 μg/ml. Samples of 150 μliters were removed for assay of radioactivity incorporation. The incubation temperature was 30 C. No extra tRNA was added to the incubation mixture.*

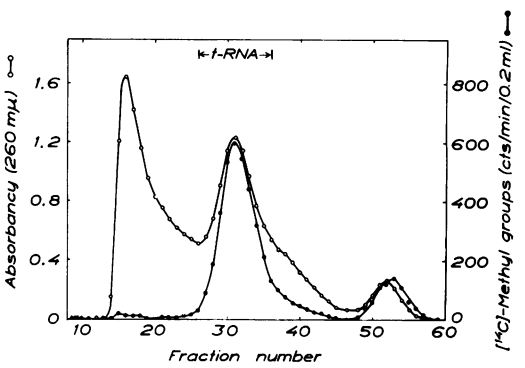

FIG. 3. *Gel filtration of RNA labeled in vitro with* [^{14}*C*]-*methyl groups. Incubation mixture (5.0 ml, pH 8.0) contained 60.2* OD_{280} *units of crude methylating enzyme preparation from cells of strain D38 harvested half a generation time before the onset of the late growth phase, and* [^{14}*C-methyl*]-*S-adenosyl-L-methionine (15 nmoles/ml) as the methyl donor. Incubation was terminated after 210 min at 30 C. RNA contaminating the enzyme preparation was isolated by phenol treatment and ethyl alcohol precipitation, and analyzed by gel filtration on a column of Sephadex G-200 (1.8 × 55 cm) equilibrated with 0.05 M triethylamine-acetic acid buffer at pH 5.4, fraction-volume 1.8 ml. Samples of 200 μliters were removed for estimation of radioactivity in a liquid scintillation counter.*

tRNA is partly methyl-deficient during the logarithmic growth phase, even though the cells contained methylating enzymes at that time. The tRNA from late growth phase cells, or even from cells starved for methionine, appeared to be almost fully methylated. These results were in contrast to those obtained with strain D84, another methionine auxotroph of *S. cerevisiae*, where methyl-deficient tRNA appeared only after removal of methionine from the growth medium (14).

The extent of incorporation of methyl groups into the enzyme preparation of strain D38 was found to vary with the time of harvesting of the cells used for the preparation. Therefore, cells from D38 were harvested at different stages of growth, and tRNA was isolated and assayed for methyl group incorporation in vitro. The enzyme preparation used was from cells harvested at about one generation before the onset of the late growth phase. The tRNA and enzyme preparation made a homologous system in that both were isolated from D38 cells grown in methionine-supplemented minimal medium. By comparison, the same experiment was performed with strain D84, in which tRNA previously was found to be not methyl-deficient in the logarithmic phase (14).

Figure 5 shows that the incorporation of methyl groups in vitro into D38 tRNA reached a marked

peak when tRNA was isolated from cells harvested about one generation before the onset of the late growth phase. A similar curve for the methyl-acceptor ability of these tRNA preparations was established when the enzymes were prepared from D38 cells harvested in the late growth phase. Although the incorporation as measured in vitro was very small, it appears significant when compared to the D84 system, where the incorporation of methyl groups into tRNA was low and almost constant throughout the logarithmic growth phase. That the D84 enzyme preparation was in fact competent was shown by control experiments using methyl-deficient tRNA from D84 (14). The differences found between the D38 and the D84 systems appear to have arisen from their tRNA; that from D38 was, to a small extent, methyl-deficient during a period of the logarithmic growth, while that from D84 contained almost a full complement of methyl groups throughout the logarithmic phase.

Although D38 and D84 are both methionine auxotrophs, they contain blocks in different positions in the methionine pathway. D38 is blocked

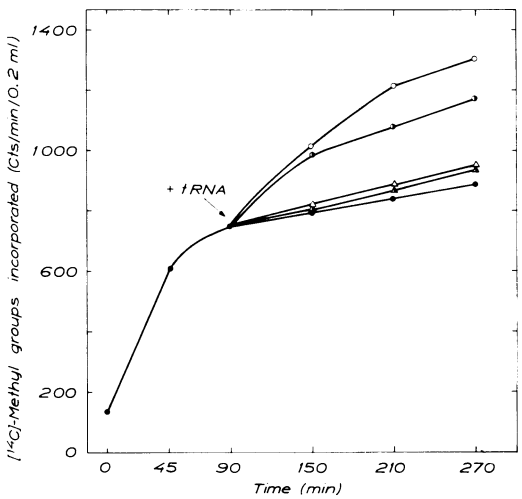

FIG. 4. *Time curves for incorporation of methyl groups in vitro into tRNA from yeast strain D38. Log phase tRNA and crude methylating enzymes were isolated from D38 cells harvested half a generation time before onset of late growth phase. Initial incorporation (●) is partly into tRNA contaminating the enzyme preparation (6.0 OD_{260} units/ml) using [14C-methyl]-S-adenosyl-L-methionine as the methyl donor (10 nmoles/ml). At the time shown, additions were made of (i) tRNA from log phase D38 cells (4.6 OD_{260} units/ml, ○ and 2.8 OD_{260} units/ml, ◑), (ii) tRNA from D38 cells starved for methionine for* 9 hr) *(3.4 OD_{260} units/ml, △ and 1.7 OD_{260} units/ml, ▲). Samples of 200 μliters were removed for assay of radioactivity incorporation. The incubation temperature was 30 C.*

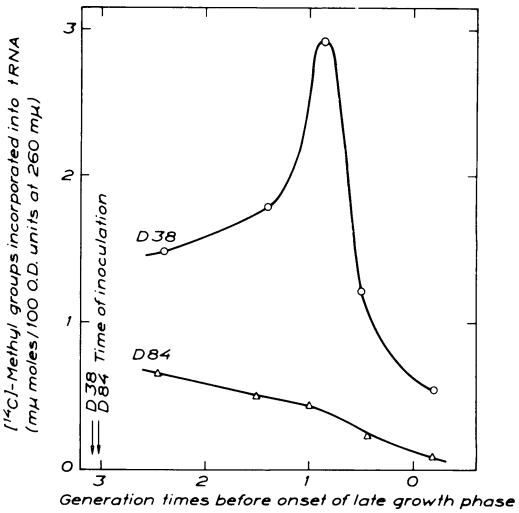

FIG. 5. *Incorporation of methyl groups into tRNA from yeast strains D38 (○) and D84 (△) during logarithmic growth. Cultures (15 liters) of the yeast strains were grown at 30 C with forced aeration and constant stirring. Generation time for D38 was 120 min and for D84, 160 min. The strains reached late growth phase at Klett readings of 320 for D38 and 200 for D84. Samples were withdrawn for isolation of tRNA at the times shown. Methylating enzymes were prepared from cells harvested about one generation before the onset of the late growth phase. Incubation mixtures (pH 8.0) for incorporation of methyl groups in vitro contained [14C-methyl]-S-adenosyl-L-methionine (10 nmoles/ml) as the methyl donor, methylating enzymes from D38 cells (A_{280}/A_{260} = 0.63, 10 OD_{280} units/ml) or from D84 cells (A_{280}/A_{260} = 0.59, 9.5 OD_{280} units/ml), and varying amounts of the different tRNA preparations. Incorporation of methyl groups to plateau levels and tRNA dependence were as described in Materials and Methods.*

in the synthesis of cysteine; D84 is derived from a strain (D6) which has been shown to lack homoserine-*O*-transacetylase (5). In order to investigate whether the different behavior of the two strains was due to the position of the methionine block, two isogenic sister strains were studied. These were MG331 and MG334, blocked at the same position as D84 and D38, respectively, as shown by complementation tests (Grenson, *personal communication*). Results are presented in Fig. 6 for incorporation of methyl groups into tRNA derived from these mutants at different stages of logarithmic growth, using homologous enzyme preparations from cells harvested one generation before the onset of the late growth phase. It can be seen that there was incorporation of methyl groups into logarithmic phase tRNA from each strain. The incorporation into tRNA from MG331 was maximal one generation before

the onset of the late growth phase; for strain MG334, the maximum was achieved somewhat earlier. Judging from the incorporation of methyl groups into the tRNA of the crude enzyme preparations, 7 of the 10 methionine auxotrophs of *S. cerevisiae* that have been investigated contain methyl-deficient tRNA during logarithmic growth.

The methylated bases formed in vitro in the D38 system were analyzed by two-dimensional chromatography after acid hydrolysis of the tRNA (11). Table 1 shows an analysis of tRNA from logarithmic D38 methylated in vitro. For comparison, analyses are presented of D38 tRNA labeled with [^{14}C]-methyl groups in vivo and isolated from cells harvested both one generation before and 1 hr after the onset of the late growth phase. However, analyses of tRNA methylated in vitro that was derived from different growth

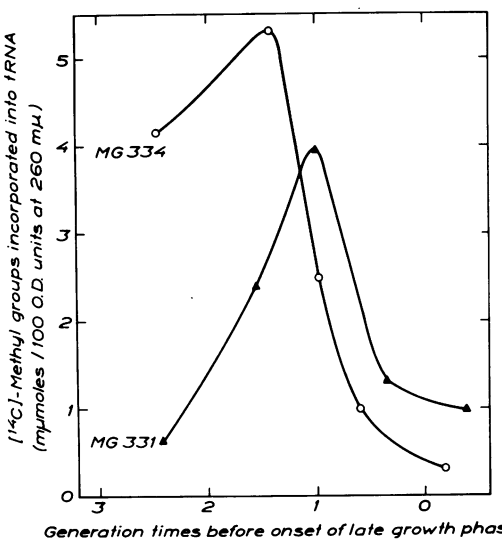

FIG. 6. *Incorporation of methyl groups into tRNA from yeast strains MG331 (▲) and MG334 (○) during logarithmic growth. Growth conditions were as described in Fig. 5. Generation time for MG331 was 155 min and for MG334, 140 min. The strains reached late growth phase at Klett readings of 280 for MG331 and 350 for MG334. Samples for isolation of tRNA were withdrawn at the times indicated. Methylating enzymes were prepared from cells of strains MG331 and MG334 harvested one generation before the onset of the late growth phase. Incubation mixtures (pH 8.0) for incorporation of methyl groups in vitro contained [^{14}C-methyl]-S-adenosyl-L-methionine (10 nmoles/ml) as the methyl donor, methylating enzymes from MG331 cells (A_{280}/A_{260} = 0.67, 8.2 OD_{280} units/ml) or from MG334 cells (A_{280}/A_{260} = 0.64, 7.8 OD_{280} units/ml), and varying amounts of the different tRNA preparations. Incorporation conditions were as described in Fig. 5.*

TABLE 1. *Distribution of methyl groups in tRNA from S. cerevisiae strain D38*[a]

Methylated component	Labeled in vivo		Labeled in vitro (log growth)
	Log growth	Late growth	
1-Methyladenine	9.9[b]	8.8	1.1
2-Methyladenine	0	0	0
N^6-Methyladenine	0.1	0	0
N^6-Dimethyladenine	1.3	0	0
1-Methylguanine	14	16	31
N^2-Methylguanine	11	9.3	7.0
N^2-Dimethylguanine	0.4	0.9	0.9
7-Methylguanine	5.5	5.9	11
1-Methylhypoxanthine	0.8	1.0	1.4
Methylcytosine	21	23	13
Methyluracil	23	23	19
2′-*O*-Methylribose	13	12	16

[a] The tRNA labeled in vivo was analyzed from cells grown in medium supplemented with [^{14}C-*methyl*]-L-methionine and harvested either one generation before (log) or 1 hr after the onset of the late growth phase (14). The third column is an analysis of tRNA isolated from cells harvested one generation before the late growth phase and methylated in vitro in the presence of enzyme from another batch of cells harvested at a similar time, and with [^{14}C-*methyl*]-*S*-adenosyl-L-methionine as the methyl donor.

[b] All figures are percentages of total counts/min recovered in methylated compounds. All values above 1% have been corrected to two significant figures. The experimental error involved, however, is approximately ±10% of each value. Counts/min recovered from chromatograms: log, 9 × 10^3; late growth, 5 × 10^3; in vitro 3 × 10^3.

experiments did not show absolute quantitative agreement. The figures show that at least five methylated bases are formed in vitro. The absence of 1-methyladenine may be due to the lability of the enzyme catalyzing its formation.

Metabolic activity in logarithmic phase cells. The period between one and two generations before the onset of the late growth phase is one of intense metabolic activity in which the synthesis of oxidative enzymes is induced, and other fundamental changes occur (3, 4). It has been shown that yeast ribosomes have maximal activity in this period (6). Therefore, the protein and RNA contents of logarithmically growing yeast were measured using radioactive precursors. The turbidity of the culture (Klett value) was taken as a measure of the number of cells present, proportionally, during this period (Fig. 1). There tended to be an increase in both cellular RNA and protein content at this time (Fig. 7), in agreement with results from chemical determinations (16). There was a

marked decrease in RNA, as well as in protein content, as the growth rate decreased.

Concomitant measurements were made of the amount of radioactive material free in the cell pools. To obtain a value for this, portions of equal size were taken simultaneously and placed in 0.5% saline and 5% trichloroacetic acid solutions. Each sample was filtered, and subtraction of the acid-precipitable radioactivity from the total radioactivity of the saline-washed cells was taken as an indication of the pool size. The value obtained includes radioactivity in all derivatives of methionine which were present (e.g., *S*-adenosylmethionine and other metabolites, small peptides, etc.). As expected, a substantial proportion of the radioactive material present in cells labeled with methionine was present in the acid-soluble pool.

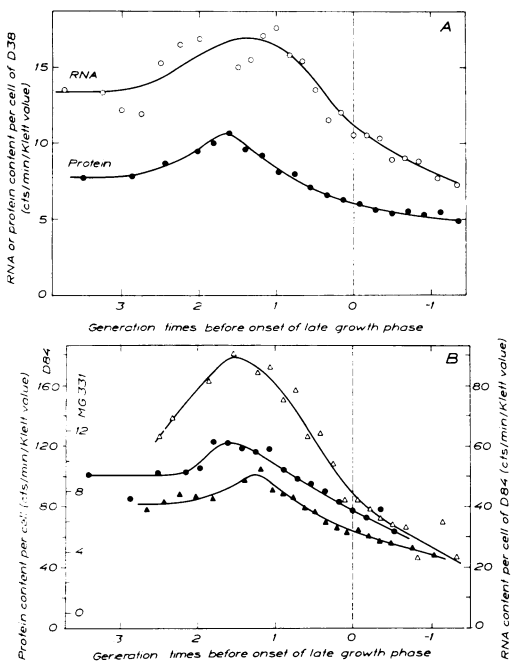

FIG. 7. *(A) RNA* (○) *and protein* (●) *content per cell of yeast strain D38 during logarithmic growth. Growth media were supplemented with* [8-^{14}C]*-adenine (0.13 μc/ml) or* [2-^{14}C]*-DL-methionine (0.16 μc/ml); incorporation of radioactivity was assayed as described in Materials and Methods. Values are expressed in counts/min incorporated in a 150-μliter sample of the culture, divided by the Klett value of the culture. (B) RNA* (△) *and protein* (▲) *content per cell of yeast strain D84, and protein content* (●) *per cell of MG331 during logarithmic growth. Growth media were supplemented with* [5,6-^{3}H]*-uracil (33 μc/ml) or* [^{14}C-methyl]*-L-methionine (0.31 μc/ml) in the case of D84, and* [2-^{14}C]*-DL-methionine (0.16 μc/ml) in the case of MG331.*

If a nonrequired amino acid, such as phenylalanine, was supplied, a comparatively small amount was in the pool (9). Figure 8 shows that, towards the end of the logarithmic phase, the pool of methionine derivatives decreased rapidly and became a small fraction of its normal size in the late growth phase. This occurred in spite of excess methionine in the growth medium. Uptake of methionine by the cells slows greatly between one and two generations before the growth rate decreases.

DISCUSSION

The experiments that have been described show that methyl-deficient tRNA accumulates in yeast cells in the period of high metabolic activity that precedes the end of the logarithmic growth phase. This effect, shown by all but one of the yeast strains examined in detail, appears to be independent of the mating type and of the known metabolic blocks present in these mutants.

It is not possible at present to assess the significance of this observation. When grown in media containing excess methionine, yeast cells accumulate large amounts of *S*-adenosylmethionine (18), and this component accounts for a high proportion of the radioactivity in the pool (Fig. 8). It seems unlikely, therefore, that a lack of this component, which is the methyl donor, could account for an accumulation of methyl-deficient precursor tRNA which then becomes methylated by the time the cells enter the late growth phase. It is conceivable, however, that only a portion of the *S*-adenosylmethionine may be accessible to the enzymes if the pool is physically compartmentalized (2) or if the delay between the synthesis of precursor tRNA and its methylation is increased during periods of intense RNA synthesis. The tRNA of strain D84, however, does

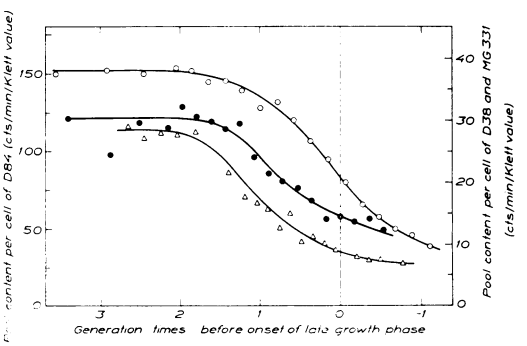

FIG. 8. *Pool size per cell of metabolites derived from methionine during log phase growth of yeast strains D38* (○), *D84* (△), *and MG331* (●). *Data are taken from the experiments illustrated in Fig. 7, as described in Materials and Methods.*

not show this effect even though an increase of RNA and protein synthesis is found during logarithmic growth as in strains where the methyl deficiency appears.

The fact that methyl-deficient tRNA from D38 in the logarithmic growth phase contains sites for the formation in vitro of at least six of the seven major methylated constituents of D38 tRNA (Table 1) may indicate that the methyl-deficient tRNA is uniformly submethylated, in spite of the fact that the proportions of the bases found did not correspond exactly to those found in vivo. Assuming that the incorporation in vitro on the polynucleotide level gives a measure of the extent of the deficiency in vivo, only a very small proportion of the tRNA in the cell is devoid of methyl groups. An incorporation of 3 nmoles per 100 OD units of tRNA corresponds to one site of methylation on about 2% of the tRNA molecules. This very small effect may indicate that only one or a few species of tRNA are involved. This suggests the possibility that changes in elution profiles of amino acyl tRNA species occurring during sporogenesis or phage infection may be correlated with variations in tRNA methylation, even though there is no lack of methionine in the growth medium (24).

ACKNOWLEDGMENTS

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