Transfer RNA and ribosomal RNA are synthesized from the same pyrimidine nucleotide pool

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We have tested the hypothesis [Wiegers, Kramer, Klapproth & Hilz (1976) *Eur. J. Biochem.* **64**, 535–540] that the synthesis of rRNA in the nucleolus may have a partially independent supply of nucleoside triphosphates that is not completely suppressed when cells are supplied with exogenous nucleosides. For the slime mould, *Physarum polycephalum*, and Chinese hamster ovary cells in culture, the specific activities ($^3\text{H}/^{32}\text{P}$) of UMP and CMP in tRNA, which is synthesised in the nucleoplasm, and rRNA were compared after continuous labelling with [^3H]uridine and [^{32}P]phosphate. No differences were found, suggesting that transcription throughout the nucleus draws on a common supply of pyrimidine triphosphates that is uniformly labelled from exogenous [^3H]uridine. As a control of the radioactive labelling schedule, a similar experiment was carried out with the prokaryote, *Escherichia coli*, and identical results were obtained.

The incorporation of radioactivity from a labelled pyrimidine or purine nucleoside into the RNA of a growing cell is easily accomplished. Nucleosides as such are not intermediates in the biosynthesis of nucleotides, but a simple kinase reaction is all that is required to effect conversion to the corresponding 5'-mononucleotide. However, quantitative evaluation of the rate of incorporation of radioactivity into RNA is difficult, because of the complexities of RNA metabolism and the spatial separation of different reactions, particularly in eukaryotic cells. Even in bacteria, early studies of RNA synthesis by radioactive tracer techniques were beset with problems caused by recycling of nucleotides from the degradation of mRNA (McCarthy & Britten, 1962; Buchwald & Britten, 1963).

In experiments with eukaryotes, a major question has been whether or not there are separate cytoplasmic and nuclear pools of ribonucleoside triphosphates. Two main themes can be identified in the literature of the past decade. One argues that there are distinct pools of nucleoside triphosphates and, moreover, that the nuclear pool is labelled preferentially from exogenous, radioactive nucleosides (Plagemann, 1971, 1972; Falkenthal & Lengyel, 1981), whilst the other states that separate pools cannot be distinguished (Wu & Soiero, 1971; Soiero & Ehrenfeld, 1973; Puckett & Darnell, 1977). A variety of experimental strategies have been employed, each with its individual assumptions, and it is not easy to decide which 'model' to accept as a

general description for the incorporation of exogenous nucleosides into the RNA of eukaryotic cells. This difficulty is highlighted in the work of Khym *et al.* (1978) who, after comparing the incorporation of [^3H]uridine into the nucleotide pool and RNA of several lines of mammalian cells, concluded that 'there is compartmentalisation of nuclear and cytoplasmic ribonucleotide pools in all cells, but in most kinds of cells, there is rapid equilibrium between these pools'.

If the object of a labelling experiment is simply to compare the relative rates of synthesis of RNA species all of which have their origin in the nucleus, it could be argued that differential labelling of nuclear, as opposed to cytoplasmic, ribonucleoside triphosphates should not interfere with interpretation of the data. However, there is the implicit assumption that the synthesis of different classes of RNA (hnRNA, rRNA, tRNA and 5S rRNA) all draw upon ribonucleoside triphosphates with identical labelling kinetics from the exogenous, radioactive nucleoside. Although this is usually taken for granted, there are studies suggesting the existence of discrete nucleotide pools for nucleolar, as opposed to nucleoplasmic, RNA synthesis. Thus, Wiegers *et al.* (1976), from experiments with Hela cells, postulated that the synthesis of rRNA in the nucleolus has an endogenous supply of pyrimidine nucleoside triphosphates that cannot be totally suppressed, even when the cells are provided with excess uridine.

In an examination of the regulation of transcription in the slime mould, *Physarum poly-*

cephalum, it became important for us to know whether or not there is a common supply of nucleoside triphosphates for both nucleolar and nucleoplasmic RNA synthesis. As an alternative to the procedure of Wiegiers *et al.* (1976), who compared the labelling of rRNA with that of mRNA, we decided to use tRNA as an index of nucleoplasmic transcription, thereby avoiding the problem of obtaining sufficient mRNA for unambiguous measurements. In *Physarum*, as in other organisms, the genes for tRNA are not linked to the nucleolar DNA, but are dispersed amongst the remainder of the genome (Hall & Braun, 1977), and transcription from them may be considered as representative of RNA synthesis in the nucleoplasm. We find that, in steady-state labelling with [6-³H]uridine, UMP and CMP residues in both tRNA and rRNA attain the same specific radioactivities, and we conclude that transcription throughout the nucleus draws upon a supply of ribonucleoside triphosphates that is uniformly accessible to labelling from exogenous uridine.

As a control of the procedure, we show that labelling of tRNA and rRNA in the bacterium *Escherichia coli* gives no distinction between the two classes of RNA. Furthermore, we also obtained the same result with Chinese hamster ovary cells (CHO-K1) in culture. Possible reasons for the different results obtained by Wiegiers *et al.* (1976) and ourselves are discussed.

Experimental

Radioactive labelling

For all three organisms used in this study, the objective was to achieve continuous labelling of RNA with [6-³H]uridine and [³²P]phosphate, so that the specific radioactivities of pyrimidine nucleotides, isolated after alkaline hydrolysis of RNA, could be expressed as ³H/³²P ratios. Both isotopes were purchased from Amersham International.

(i) *Escherichia coli*. Strain B/r was grown with aeration in a Tris-buffered salts medium (Meynell & Meynell, 1965), supplemented with vitamin-free casamino acids (1% w/v) and with glucose (0.4% w/v) as carbon source. Carrier-free [³²P]phosphate was added to give a specific radioactivity of 3 μ Ci $\cdot$ μ mol⁻¹ and [6-³H]uridine to a specific radioactivity of 8 μ Ci $\cdot$ μ mol⁻¹. With carrier, the concentration of uridine was 0.12 mM. It has been shown (Bremer & Yuan, 1968) that endogenous synthesis of pyrimidine nucleotides is effectively suppressed by concentrations of uridine above 1 μ M. Bacteria were harvested from a 50 ml culture at a density of (3–4) $\times$ 10⁸ cells $\cdot$ ml⁻¹ after at least three generations of exponential growth in the radioactive medium, at which time slightly more than half the [³H]uridine

remained in the medium. An extract was prepared by treating the cells with lysozyme and Triton X-100 (Turnock, 1970) and stored frozen at -80°C.

(ii) *Physarum polycephalum*. Strain M₂CVIII was grown in a standard medium (Plaut & Turnock, 1975) containing 1% Bacto-Soytone and with the concentration of KH₂PO₄ reduced to one-tenth of the normal value. This change enabled an adequate [³²P]phosphate (100 μ Ci in 20 ml of medium) specific radioactivity to be attained without affecting growth. Uridine was added to give a concentration of 0.2 mM together with [6-³H]uridine (100 μ Ci in 20 ml of medium); the actual specific radioactivity in the medium cannot be stated precisely because of the contribution of nucleosides by the Bacto-Soytone. The rate of utilization of uridine by surface plasmodia of *Physarum* is a linear function of its concentration (Birch & Turnock, 1976). Therefore, to ensure a uniform supply of the nucleoside throughout an experiment, plasmodia were transferred to fresh, radioactive medium every 12 h.

A plasmodium was initiated from 30 μ l of a concentrated suspension of microplasmodia and grown successively on two portions of 1.4 ml and finally on 15 ml of radioactive medium, the total period of growth being 36 h (2.5–3 mass doublings). At the end of this time, the plasmodium was scraped off the filter paper support and frozen in dry ice.

(iii) *Chinese hamster ovary (CHO-K1) cells*. To 60 ml of Ham's F12 medium (Faik & Morgan, 1977) containing 10 mM-glucose and 10% (v/v) foetal bovine serum, treated with Sephadex G-100 (Faik & Morgan, 1977), were added 120 μ Ci of [³²P]phosphate and 300 μ Ci of [6-³H]uridine (25 μ Ci $\cdot$ μ mol⁻¹). The medium was then sterilized by filtration. The line of CHO-K1 cells used was stained for the presence of mycoplasmas with Hoechst 33258 (Chen, 1977), with negative results.

Two 15 cm dishes were each seeded with approx. 2 $\times$ 10⁶ cells and supplied with 15 ml of radioactive medium. This was replaced with fresh, radioactive medium after 24 h and the cells harvested after a further 18 h. The cells underwent 2–3 doublings in the labelled medium and the schedule ensured that the concentration of uridine in the medium did not decrease by more than 10–15%.

After harvesting, the cells were dispersed in 20 mM-Tris chloride (pH 7.5)/100 mM-NaCl/1 mM-EDTA/1% (w/v) naphthalene 1,5-disulphonic acid, and lysis was completed by adding 0.1 vol. of 10% (v/v) Nonidet P40. The extract was kept at 0°C for 5 min and then centrifuged to remove unbroken cells and nuclei. RNA was extracted from the supernatant.

Extraction and chromatography of RNA

RNA was extracted from all three types of cell with standard methods based on phenol (Parish &

Kirby, 1966). After precipitation with ethanol, the RNA was dissolved in 0.02 M-Tris chloride (pH 7.5)/0.1 M-NaCl and fractionated by ion exchange chromatography on methylated serum albumin-coated kieselguhr (Mandell & Hershey, 1960), using the simplified procedure described by Osawa & Sibatani (1967). To facilitate recovery of RNA from the column fractions by precipitation with ethanol, 20 mM-Tris chloride, pH 7.5, was substituted for the phosphate buffer of the original recipe.

The column provides effective separation of tRNA (and 5S rRNA) from rRNA. The peak fractions of both classes of RNA were pooled and precipitated with 2 vol. of ethanol at -80°C. The 5S rRNA will constitute approx. 10% of the total RNA in the tRNA peak. However, the validity of the results should not be affected, since this small rRNA is also synthesised in the nucleoplasm; indeed the synthesis of both tRNAs and 5S rRNA is directed by the same specific RNA polymerase III (Sklar & Roeder, 1977).

Alkaline hydrolysis of RNA and separation of nucleotides

RNA was dissolved in 0.3 M-NaOH and incubated at 37°C overnight to effect complete hydrolysis. After neutralization of the solution with HCl, portions (usually 20 µl) were fractionated to give the constituent nucleotides of the RNA either by paper electrophoresis (Smith, 1967) or by chromatography on paper impregnated with (NH₄)₂SO₄ (Lane, 1963). Where possible, hydrolysates were subjected to duplicate analyses by both methods of separation. Spots were located under u.v. light and UMP and CMP were cut out for assay of ³H and ³²P.

Two methods for separating nucleotides were used to insure against inadvertent interference by non-standard nucleotides, particularly in the case of tRNA. [6-³H]Uridine (rather than 5- or 5,6-³H) was

chosen as the vehicle for labelling with the same problem in mind. For example, ribothymidylic acid, pseudouridylic acid and dihydrouridylic acid should all retain the label and yet, in the electrophoretic method at least (Smith, 1967), they are not resolved from 2',3'-UMP.

Assay of radioactivity

Nucleotides were eluted from the paper into 1.5 ml of 0.01 M-HCl and duplicate 0.5 ml samples were assayed for ³H and ³²P in a standard scintillant designed for aqueous samples in a Packard 3385 scintillation spectrometer. The spillover (3%) of ³²P in the ³H channel was corrected for, although the ratio of ³H to ³²P was always at least 2:1. Samples were counted to 5000 counts in the ³²P channel.

Results and discussion

We decided that the simplest way to monitor the incorporation of uridine into the pyrimidine nucleotides of RNA would be to label cells with both [6-³H]uridine and [³²P]phosphate and to measure the specific radioactivities of individual nucleotides from tRNA and rRNA as ³H/³²P ratios. As a control of the procedure, we first of all carried out an experiment with *E. coli*, assuming that in a prokaryote all classes of RNA synthesis must draw on the same supply of UTP (and CTP). Expt. 1 (Table 1) shows that this expectation was fulfilled; UMP from both tRNA and rRNA has, within the limits of experimental error, the same specific radioactivity after separation of nucleotides either by electrophoresis or chromatography. The same is true for CMP from the two types of RNA and, furthermore, the specific activities of UMP and CMP appear to be identical, consistent with the synthesis of CTP by amination of UTP.

Having established the validity of the radioactive labelling schedule, we used it to compare the

Table 1. Specific radioactivities of UMP and CMP after continuous labelling of RNA with [6-³H]uridine and [³²P]phosphate

Specific radioactivities were determined after alkaline hydrolysis of RNA and separation of nucleotides either by electrophoresis (E) or by chromatography (C) and are expressed as ³H/³²P ratios. The average coefficient of variation for the determination of specific activity was 3.02% for UMP and 3.12% for CMP. For comparison, in the last two columns of the Table, the measurements of specific radioactivities are expressed as tRNA/rRNA ratios for UMP and CMP respectively.

Experiment and organism	Method	Specific radioactivity				tRNA/rRNA specific radioactivity ratios	
		UMP		CMP		UMP	CMP
		tRNA	rRNA	tRNA	rRNA		
1. <i>E. coli</i>	E	1.97	2.12	1.93	2.07	0.929 ± 0.064	0.932 ± 0.090
	C	2.13	2.15	1.99	2.07	0.992 ± 0.026	0.964 ± 0.015
2. <i>Physarum</i>	C	3.10	3.43	3.08	2.95	0.903 ± 0.039	1.044 ± 0.053
3. <i>Physarum</i>	E	4.23	4.17	4.21	4.19	1.016 ± 0.059	1.004 ± 0.067
	C	4.82	4.93	4.22	4.41	0.978 ± 0.039	0.957 ± 0.043
4. CHO cells	C	4.78	4.93	4.59	4.65	0.970 ± 0.027	0.987 ± 0.019

labelling of tRNA and rRNA in the plasmodial phase of *Physarum*. The results (Table 1, expts. 2 and 3) indicate the same simple model assumed to describe the supply of substrates for RNA synthesis in *E. coli*, namely that the synthesis of tRNA (in the nucleoplasm) and rRNA synthesis (in the nucleolus) draw on pools of UTP and CTP that attain identical specific radioactivities in continuous labelling with exogenous [^3H]uridine.

Finally, we carried out an experiment (Table 1, expt. 4) with CHO-K1 cells in culture, as an example of a mammalian cell line, and obtained results indistinguishable from those given by *E. coli* and *Physarum*.

Table 1 also summarizes the results for the three organisms in the form of tRNA-nucleotide/rRNA-nucleotide ratios. It shows a slight tendency for this parameter to lie just below 1.0. However, as the bias is evident in RNA from *E. coli*, as well as in RNA from the two eukaryotic systems, it is unlikely to reflect compartmentalization of nucleotide pools for tRNA and rRNA synthesis.

If it is accepted that tRNA can be used as an index of RNA synthesis in the nucleoplasm, our results show that in long-term labelling of *Physarum* and CHO-K1 cells transcription throughout the nucleus draws upon pyrimidine nucleoside triphosphates that receive radioactivity to the same extent from exogenous [^3H]uridine. This gives a different view of the supply of substrates for RNA synthesis to that postulated by Wiegiers *et al.* (1976). From their work with Hela cells, they suggested that the nucleolus possesses a multienzyme complex for synthesis *de novo* of pyrimidine nucleotides, including the synthesis of CTP from UTP, and that it is not subject to feedback inhibition by UTP made available by supplying the cells with excess uridine. It is possible, although it does not seem very likely, that the organization of pyrimidine biosynthesis is dramatically different in human cells by comparison with other eukaryotes. If this is not the case, there has to be a technical explanation for their finding that UMP and CMP from rRNA have only about half the specific radioactivity of UMP and CMP from mRNA after long-term labelling of Hela cells with 200 μM -[^3H]uridine. The principal comment that can be made is that they did not have enough material to allow direct determination of nucleotide specific radioactivities; this parameter was calculated from measurement of radioactivity in individual nucleotides and the u.v. absorbance of the RNA, using standard values for the base composition of the mRNA and rRNA fractions. Any indirect determination such as this could be biased by inadvertent interference with one (or more) of the assays, and it is for this reason that we decided to use the labelling schedule described in this paper.

A further point is that ribosomal RNA, because it constitutes the bulk of the RNA of growing cells,

provides the major drain on the supply of pyrimidine nucleotides. If the nucleolus did indeed possess its own collection of enzymes for pyrimidine synthesis, the nucleus would be expected to be a major source of such enzymes. This, however, is not the case (Shoaf & Jones, 1973). More seriously, a central enzyme (dihydro-orotate dehydrogenase) of the pathway is specifically located in the mitochondria (Chen & Jones, 1976). The spatial separation between nucleolus and mitochondria also calls into question the hypothesis that the former could have a local supply of pyrimidine nucleotides.

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