Free Pyrimidine Nucleotide Pool of Ehrlich Ascites-Tumour Cells

COMPARTMENTATION WITH RESPECT TO THE SYNTHESIS OF HETEROGENEOUS NUCLEAR RNA AND PRECURSORS TO RIBOSOMAL RNA

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The incorporation of [^{14}C]orotate and [^{14}C]uridine into UMP residues of hnRNA (heterogeneous nuclear RNA) and pre-rRNA (precursors to rRNA) of Ehrlich ascites-tumour cells was compared: orotate was incorporated at a markedly higher rate into hnRNA. On the other hand, the rate of incorporation of uridine into pre-rRNA was even somewhat higher than into hnRNA. The ratio of specific radioactivities of CMP to UMP residues in pre-rRNA and hnRNA was studied. At all times of labelling this ratio was similar for both RNA species independently of the precursor used. On addition of excess unlabelled uridine, the CMP/UMP labelling ratio in both pre-rRNA and hnRNA rose. However, this increase was much more pronounced with hnRNA. It is concluded that the nuclear pyrimidine nucleotide pool for RNA synthesis is compartmentalized. The synthesis of hnRNA and pre-rRNA is supplied preferentially by the large and the small compartment, respectively. A detailed model for the cellular compartmentation of uridine nucleotide precursors to RNA is proposed.

Two groups of rapidly turning-over high-molecular-weight RNA molecules are synthesized in the cell nucleus: precursors to rRNA and hnRNA, at least part of the latter being precursors to mRNA (see Perry, 1976; Hadjiolov & Nikolaev, 1976; Revel & Groner, 1978). Comparisons of the synthesis rates of pre-rRNA and hnRNA are dependent on the assumption that there is a single nuclear pool of precursor ribonucleoside 5'-triphosphates. The possibility for compartmentation of the nuclear pyrimidine nucleotide precursor pool was studied in HeLa cells. Wu & Soeiro (1971) analysed the time course of labelling of CMP and UMP residues in the two nuclear RNA species and concluded that they are synthesized from a common precursor pool. These results were confirmed (Puckett & Darnell, 1977), and the recommended approach was used to study compartmentation of pyrimidine nucleotide precursor pools (Soeiro & Ehrenfeld, 1973; Dämmgen & Scholtissek, 1975). However, Wieggers *et al.* (1976), using continuous

labelling of cytoplasmic mRNA and rRNA to isotopic equilibrium with exogenous [^3H]uridine, concluded that the nuclear pyrimidine nucleotide precursor pool in HeLa cells is compartmentalized.

In the present work, making use of a new approach, we compared the incorporation of uridine and orotate into nuclear RNA of Ehrlich ascites-tumour cells. The results demonstrate that hnRNA and pre-rRNA are synthesized from partly independent pyrimidine nucleotide precursor pools.

Methods and Materials

Cell labelling

Ehrlich ascites-tumour cells were obtained and labelled *in vitro* with radioactive uridine or orotate as described in the accompanying paper (Genchev, 1980). The experimental details are given in the legends to the corresponding Figures.

Isolation and fractionation of RNA

At specified labelling times samples containing cells at a known concentration were withdrawn from the incubation suspension. They were mixed immediately with several volumes of ice-cold 0.14M-NaCl and a known quantity of unlabelled cells as a source of marker RNA. The cells were collected by low-speed centrifugation for 3 min at 850g in the

Abbreviations used: hnRNA, heterogeneous nuclear RNA; pre-rRNA, precursor to rRNA; 4°C RNA, 50°C RNA and 85°C RNA, RNA extracted with phenol at the indicated temperatures; 2'(3')-CMP, mixture of the two isomers cytidine 2'-phosphate and cytidine 3'-phosphate; 2'(3')-UMP, mixture of the two isomers uridine 2'-phosphate and uridine 3'-phosphate.

cold, washed once with 0.14 M-NaCl and subjected to phenol extraction of RNA at different temperatures (see Georgiev, 1967). Slight modifications of the procedures previously described in detail (Markov & Arion, 1973; Hadjiolov *et al.*, 1974) were used. The cell pellet was suspended in 15–20 vol. of ice-cold 0.1 M-Tris/acetate buffer (pH 5.4), containing 0.01 M-EDTA. An equal volume of phenol containing 0.1% 8-hydroxyquinoline and saturated with 0.14 M-NaCl was added. The mixture was shaken for 15 min at 4°C and centrifuged in the cold. The interphase layer, which contains the cell nuclei, was washed once at 4°C with a Tris/acetate buffer/phenol mixture (1:1, v/v) and then extracted consecutively at 50°C and 85°C with the same mixture. Sodium dodecyl sulphate (0.5%) was present during the extraction at 85°C. To the water phases (containing RNA) sodium dodecyl sulphate was added to a final concentration of 1%. The mixture was deproteinized two or three times with phenol, once with phenol/chloroform (1:1, v/v) and once with chloroform, and finally the RNA was precipitated by the addition of 2.5 vol. of ethanol containing 0.1 M-sodium acetate. It is established that the RNA fractions thus obtained (subsequently designated 4°C RNA, 50°C RNA and 85°C RNA) correspond roughly to cytoplasmic RNA, nuclear rRNA and hnRNA respectively (Georgiev, 1967; Markov & Arion, 1973; Hadjiolov *et al.*, 1974; see also the Results section).

All RNA were additionally purified by several-fold precipitation with ethanol/acetate (see above) and freed from low-molecular-weight RNA and DNA contaminants by dissolution in a minimum volume of water and precipitation with an equal volume of 4 M-LiCl at –5°C overnight.

Analyses of RNA mononucleotides

The base composition of RNA was determined by the method of Katz & Comb (1963). In most of the experiments, when only the pyrimidine mononucleotide residues of RNA were analysed, an acid hydrolysis of RNA (60 min at 100°C in 1 M-HCl) was done. The derived UMP and CMP were purified by successive cation- and anion-exchange chromatography (Genchev, 1980). Their specific radioactivities were corrected for dilution by unlabelled cells. In some of these experiments, when the amount of RNA was too small for a direct spectrophotometric measurement of its mononucleotides, marker 2'(3')-UMP and 2'(3')-CMP were added before the acid hydrolysis. Their total radioactivity was determined and the ratio of their specific radioactivities [subsequently designated, according to Wu & Soeiro (1971), as specific C:U ratio] was then calculated, using the data for the nucleotide composition of the respective RNA fractions (Table 1).

Other methods

The cell number and viability, the content of the nucleic acids and the radioactivity of the aqueous samples were determined as described in the preceding paper (Genchev, 1980).

Agar-gel electrophoresis of RNA was done as described by Tsanev & Staynov (1964). For radioactivity analysis the electrophoretograms were cut into 2 mm slices, each slice was digested with a Protosol/toluene/water mixture and counted for radioactivity as described by Hadjiolov *et al.* (1974).

Materials

Analytical-grade reagents were used throughout. The phenol was freshly distilled and protected from oxidation by the addition of 8-hydroxyquinoline. The [5-³H]uridine was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.), and the specifications and sources of ¹⁴C-labelled uridine and orotic acid, as well as other materials used, are given in the accompanying paper (Genchev, 1980).

Results

General characteristics of the RNA fractions and their labelling

The incorporation of labelled uridine and orotate into total RNA of Ehrlich ascites-tumour cells, as well as some general characteristics of the cell system under study, are described in detail by Genchev (1980). The RNA fractionation applied in the present study discloses that for the times investigated most of the incorporated radioactivity from both precursors is in unstable nuclear RNA species. The electrophoretic patterns of 50°C RNA and 85°C RNA are shown in Fig. 1. Most of the label of the 50°C RNA fraction is in 45S and 32S pre-rRNA components, whereas a distribution typical for hnRNA is observed with the 85°C RNA fraction (see Markov & Arion, 1973; Hadjiolov *et al.*, 1974). Similar electrophoretic patterns are obtained with either [¹⁴C]uridine or [¹⁴C]orotate as precursors (results not shown). The correspondence of the 50°C RNA to nuclear rRNA and of 85°C RNA to hnRNA is confirmed by their nucleotide compositions (Table 1).

Comparison of the labelling kinetics of nuclear rRNA and hnRNA by [¹⁴C]uridine and [¹⁴C]orotate

It is known that both uridine and orotate are converted ultimately into UTP (see Hauschka, 1973). Therefore, if the synthesis of rRNA and hnRNA is fed by a common nuclear compartment, the relative labelling of these two RNA species should be independent of the radioactive precursor used and should reflect only their relative synthesis rates. However, several experiments in which the

Fig. 1. Agar-gel electrophoresis of 50°C RNA (a) and 85°C RNA (b) of cells labelled with [³H]uridine Ehrlich ascites-tumour cells were labelled *in vitro* with [³H]uridine (25 μCi/ml) for 2 h. After removal of the cytoplasmic RNA by cold phenol extraction, the ‘interphase nuclei’ were extracted at 50°C and 85°C and the RNA fractions obtained were freed from low-molecular-weight components and subjected to agar-gel electrophoresis as described in the Methods and Materials section. The arrows indicate the position of marker 28S, 18S and 4S RNA.

Table 1. Mononucleotide composition of RNA fractions isolated from Ehrlich ascites-tumour cells by thermal phenol extraction

The RNA fractions were obtained by thermal phenol extraction at the temperatures indicated and their base compositions determined by direct spectrophotometric analysis as described in the Methods and Materials section.

RNA fraction	Molar ratio of the bases (% of the total)				Ratio $\frac{G+C}{A+U}$
	C	A	G	U	
4°C RNA	28.6	18.6	33.7	19.1	1.65
50°C RNA	29.2	18.7	30.5	21.6	1.48
85°C RNA	21.9	28.9	22.7	26.5	0.84

labelling kinetics of 50°C RNA and 85°C RNA by uridine and orotate were compared (results not shown) do not confirm this assumption. To eliminate the possibility of artifacts owing to incomplete RNA purification and to make the results with both precursors more directly comparable, the specific radioactivities of 2'(3')-UMP isolated from 50°C RNA and 85°C RNA were determined. The results of a typical experiment (Fig. 2) show that the labelling patterns with [¹⁴C]uridine and [¹⁴C]orotate are quite different. When [¹⁴C]orotate is used it is incorporated more intensively into 85°C RNA than into 50°C RNA, indicating an apparent rate of hnRNA synthesis severalfold higher than that of the pre-rRNA. However, incorporation of [¹⁴C]uridine displays the opposite relation. The distinct relative

rates of labelling for nuclear rRNA and hnRNA observed with [¹⁴C]orotate and [¹⁴C]uridine, both entering RNA through UTP, demonstrate unequivocally the compartmentation of the precursor pool for these two RNA species. Previous data (Genchev, 1980) show that [¹⁴C]orotate is preferentially incorporated into a larger compartment of the free pyrimidine nucleotide pool, whereas [¹⁴C]uridine enters mainly into a smaller compartment. Accordingly, the synthesis of hnRNA and rRNA is supplied mainly by the large and the small uridine nucleotide compartment, respectively. Using an entirely different approach, a similar conclusion for the compartmentation of mRNA and rRNA precursor pools in HeLa cells was reached by Wieggers *et al.* (1976).

Fig. 2. *Specific radioactivity of UMP residues of nuclear RNA labelled with $[^{14}\text{C}]$ uridine (a) or $[^{14}\text{C}]$ orotate (b)* The cells were suspended at about 15×10^6 cells/ml in a standard medium (phosphate-buffered saline supplemented with 18 mM-glucose and 2 mM-L-glutamine) and labelled at 37°C with $[^{14}\text{C}]$ uridine (0.07 $\mu\text{Ci}/\text{ml}$) or $[^{14}\text{C}]$ orotate (6.85 $\mu\text{Ci}/\text{ml}$). At the time indicated with an arrow a portion of the cell suspension was transferred into a flask containing unlabelled uridine to give a final concentration of 0.4 mM ('chase'). At the specified times cells were collected from the medium and subjected to RNA extraction as described in the Methods and Materials section. The specific radioactivity of 2'(3')-UMP isolated from 50°C RNA (Δ , $\blacktriangle$) and 85°C RNA (O, $\bullet$) was determined after successive cation- and anion-exchange chromatography. Open symbols, standard incubation; closed symbols, uridine chase.

The experiment illustrated on Fig. 2 further shows that the addition of unlabelled uridine to cells pre-labelled with either uridine or orotate displays a similar 'chase' effect on both 50°C RNA and 85°C

RNA. This observation indicates that: (1) both the larger and the smaller compartments of the free uridine nucleotide pool expand in the presence of high extracellular uridine concentration, and (2) pre-rRNA and hnRNA are rapidly turning over, possessing similar (very short) half-lives (see Genchev, 1980).

Changes of the specific C:U ratio during RNA labelling under standard conditions and after uridine chase

The specific C:U ratio (see Wu & Soeiro, 1971) for isolated hnRNA and rRNA was determined under standard labelling conditions. As shown in Fig. 3, the specific C:U ratio increases slowly with

Fig. 3. *Ratio between the specific radioactivities of CMP and UMP residues in 50°C RNA and 85°C RNA during labelling with $[^{14}\text{C}]$ uridine (a) and $[^{14}\text{C}]$ orotate (b)* The cells were labelled with $[^{14}\text{C}]$ uridine or $[^{14}\text{C}]$ orotate and chased with unlabelled uridine (0.4 mM) at the times indicated with an arrow (for details see the legend to Fig. 2). The 2'(3')-CMP and 2'(3')-UMP were isolated from 50°C RNA (Δ , $\blacktriangle$) and 85°C RNA (O, $\bullet$) as described in the legend of Fig. 2 and the ratio of their specific radioactivities was determined. Open symbols, standard incubation; closed symbols, uridine chase.

time and no significant differences are observed between the curves for the two nuclear RNA species, labelled with either [^{14}C]orotate or [^{14}C]uridine. Further, the results shown in Fig. 3 demonstrate that on chase with unlabelled uridine the specific C:U ratio increases rapidly. This increase is observed with both RNA species, but is more pronounced with hnRNA than with nuclear rRNA. The rise of the specific C:U ratio on uridine chase is due to the rapid equilibration of free and RNA-bound uridine nucleotides, whereas their conversion into cytidine nucleotides is relatively slow (Genchev, 1980). The established divergence of the specific C:U ratio for hnRNA and rRNA supplies additional evidence for the compartmentation of the nuclear precursor pools of pyrimidine nucleotides.

Discussion

This work exploits a simple approach towards the problem of compartmentation of the nuclear pools of mononucleotides. This approach is based on the parallel use of two pyrimidine precursors, uridine and orotate, for the labelling of nuclear RNA species. Our analyses demonstrate the compartmentation of the nuclear pools for hnRNA and rRNA. The same conclusion was inferred from studies by labelling to isotopic equilibrium (Wiegiers *et al.*, 1976). However, the latter approach is suitable only for rapidly dividing cells. It requires very long (4–5 days) incubations, during which the radioactive precursor in the medium should be maintained at constant concentration and specific radioactivity.

As shown by our results, investigation of the changes in the specific C:U ratio (used in several earlier studies; see the introduction) is of limited applicability. It supplies a decisive conclusion only in cases where different kinetics for separate RNA species are found.

According to the original proposal of Plagemann (1971), two partly independent free nucleotide compartments exist in animal cells: a large (cytoplasmic) and a small (nuclear), the latter supporting RNA synthesis (see review by Hauschka, 1973). The results obtained in the present and the preceding (Genchev, 1980) papers allow the proposal of a more detailed model, at least in respect to the free uridine nucleotides. The larger compartment, labelled predominantly by exogenous orotate, serves mainly the synthesis of hnRNA. Thus it is plausible that the mononucleotides in the cytoplasm and nucleoplasm are in rapid equilibrium and may be considered as a single compartment. On the other hand, uridine is incorporated preferentially into a small intranuclear (nucleolar ?) compartment, used in the synthesis of pre-rRNA. However, when

uridine in the extracellular medium is present in high concentrations, it enters both compartments, causing their expansion and possibly rapid equilibration. The exact mechanism by which uridine is channelled directly into the nucleolar compartment remains to be elucidated.

The proposed model explains our results, as well as some conflicting data in the literature. For example, it has been suggested by other authors too that uridine enters selectively a small compartment feeding RNA synthesis (Bölsföldi *et al.*, 1971; Plagemann, 1971). However, it is considered that uridine causes only the expansion of the large (cytoplasmic) compartment (Plagemann, 1971, 1972). A distinct penetration of uridine, at low and high concentrations, in the small and the large cellular compartments explains this apparent discrepancy. More recent results by Khym *et al.* (1978) suggest also that at high concentrations of exogenous uridine the compartments feeding RNA synthesis expand and equilibrate very rapidly. Another study on the labelling of pyrimidine nucleotides and total RNA in cultured rat hepatoma cells reached the conclusion that RNA synthesis is fed by the larger rather than the smaller pyrimidine precursor pool (Losman & Harley, 1978). It is possible that in this case hnRNA, fed by the large precursor pool, was predominantly labelled.

In any case, the fact that the synthesis of hnRNA and pre-rRNA is supplied by separate pyrimidine nucleotide precursor pools should be taken into consideration in studies on transcription and turnover of these RNA species.

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