THE INCORPORATION OF URIDINE LABEL INTO THE RNA OF MOUSE EMBRYO CELLS DOES NOT ALWAYS REFLECT THE LABELLING OF THE MAJOR CELLULAR UTP POOL

Peter W. PIPER
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, England

Received 6 June 1981

1. Introduction

Using the restriction enzyme digestion fragments of a specific DNA as hybridisation probes, techniques now exist whereby the RNAs within a cell having complementary sequences can be mapped relative to the restriction enzyme cleavage map of the DNA [1–3]. Quantitation of the different RNA–DNA hybrids formed gives a measure of the steady state proportions of the various complementary RNAs in total cellular RNA. To place such RNAs within a processing pathway describing how the transcripts of a specific gene are synthesised, matured or broken down, it is necessary to analyse the kinetics of labelling of these RNAs in either a continuous label or a pulse-chase type of experiment. The purpose of this paper is to highlight certain considerations that must be made if the labelling of a specific RNA in such experiments is to be used as a measure of its turnover.

Greenberg [4] has derived a relationship that can be used in calculating half-lives of individual RNA species. The specific activity of a cellular RNA, A, is assumed to follow the relationship:

$$A/A^{\infty} = 1 - \exp\left[-1n2(^{1}/TD - {}^{1}/t_{1/2})t\right]$$

Here $t_{1/2}$ is the half-life of the RNA, TD the cell doubling time, and A^{∞} the final specific activity reached with increasing time, t. This relationship requires both that the cells are in exponential growth and that the RNA is being labelled from a precursor pool of constant specific activity. It is often difficult to assess when the latter condition is being met and whether correction of RNA labelling for fluctuations in the specific activity of the precursor pool needs to be made before the Greenberg relationship can be used to calculate RNA stabilities. Nucleoside triphosphate pools within cells are comparatively large and their labelling frequently does not stabilise for several minutes after addition of labelled nucleoside to the culture medium. If glucosamine is added to the cells and uridine is used for labelling, stabilisation is considerably more rapid. This is because there is a lowering of the intracellular UTP pool, through UDP-*N*-acetylglucosamine formation, this pool thereby responding much more rapidly to changes in the specific activity of uridine in the medium ([5–8]; see also fig.1).

Certain estimates of RNA stabilities [8–10] have used the procedure in [11] to correct the raw data for label incorporation into RNA for both the specific activity of the triphosphate pools and the contribution of radioactivity in the RNA due to nucleosides other than that used for labelling (i.e., [^{3}H] CMP residues when [^{3}H]uridine is the label, or [^{3}H]GMP residues when [^{3}H]adenosine is the label). Using [^{3}H]-uridine for labelling the specific activity curve of total cellular UTP (cpm in UTP/A_{260} of total pool at each time point) is integrated at each time point and divided by the time to give the average UTP pool-specific activity. The base composition of the labelled RNA corresponding to each time point is determined to subtract for label in CMP residues, and the radioactivity present in the RNA as UMP residues at each time point is then divided by the integrated UTP pool-specific activity [11]. In studying the turnover of RNAs in mouse embryo cells by [^{3}H]uridine labelling I observed fluctuations in the labelling of the total cellular UTP pool which were not reflected in RNA labelling. As this pool does not therefore directly supply the precursors for RNA synthesis, its labelling should not be used to derive quantitative measurements of RNA turnover rates. I describe here the labelling of both the total UTP pool and stable RNA

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

in mouse embryo cells, and also the effect of glucosamine treatment and extracellular uridine concentration upon such labellings. Labelling cells in medium of low uridine concentration, [^{3}H]uridine incorporation into the stable RNA of the cells became linear long before labelling of the major UTP pool had stabilised. This linearity probably shows that there is a minor UTP pool that provides the immediate precursors for RNA synthesis and which rapidly responds to changes in the specific activity of uridine in the medium while the major UTP pool of the cells responds more slowly. However reasonable this assumption, it must be remembered that there is at present no way of testing it directly by experimental measurements of the specific activity of the RNA precursor UTP pool.

2. Materials and methods

Secondary mouse embryo fibroblasts were grown at 37°C in 90 mm Petri dishes containing 10 ml Dulbecco's modified Eagle's medium (DMEM) containing 3% foetal calf serum. At the start of a labelling experiment dishes were taken from the incubator and the medium removed by aspiration. 2 ml DMEM containing 3% foetal calf serum and 150 μCi/ml [5,6-^{3}H]uridine (Radiochemical Centre, Amersham, 45 Ci/mmol; diluted to 4.5 μM or 300 μM final concentration with non-radioactive uridine) was then added to all the dishes simultaneously. To avoid temperature fluctuations these manipulations were performed in a 37°C room.

At various times following the addition of label the cells from two 90 mm dishes were rapidly chilled for preparation of total RNA [12], and a third dish was rapidly washed twice in ice-cold DMEM and left containing 0.5 ml 0.5 N perchloric acid for 15 min at 20°C. These acid extracts were transferred to microfuge tubes containing 25 μl 10 N KOH, and the tubes vortexed before being allowed to stand on ice for 15 min. The resultant precipitates were removed by centrifugation. The absorbance of each supernatant at 260 nm was measured. Aliquots of the supernatants were spotted onto a plastic-backed polyethylinimine-impregnated cellulose sheet (Macherey-Nagel), together with non-radioactive UTP, UDP and CTP markers, and separated by thin-layer chromatography using 0.85 M NaH_2PO_4 (pH 3.4) as solvent. The markers were identified under an ultraviolet lamp and the UDP, UTP and CTP regions of the chromatogram corresponding to each time point were cut out of the plate and counted.

The specific activity (cpm/A_{260}) was measured for the samples of total cellular RNA corresponding to each time point. Portions of these RNAs were loaded into alternate sample slots of a 10% polyacrylamide slab gel [13]. After electrophoresis, the tRNA regions of each gel track were located by staining for 20 min in a solution of 0.1% methylene blue in 10% acetic acid and destaining in 10% acetic acid. They were then excised and counted [14]. To analyse the relative labellings of uridine and cytidine residues within RNA samples aliquots of these RNAs were hydrolysed for 18 h in 0.3 N KOH at 37°C, neutralised with HCl, and the labelled Up and Cp within hydrolysates separated by high-voltage paper electrophoresis at pH 3.5. The dried papers were sliced and counted.

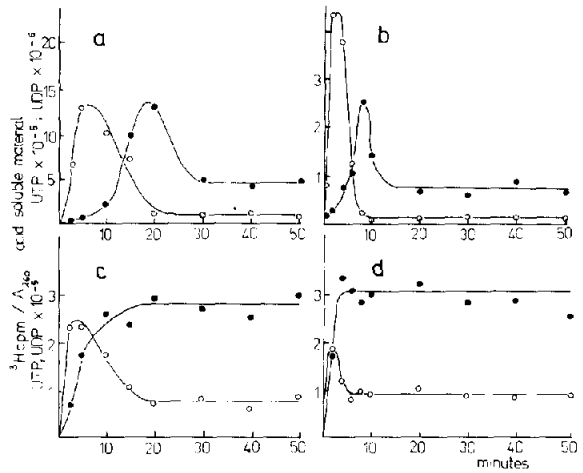

Fig.1. The labelling kinetics of the uridine nucleotide pools of mouse embryo cells immediately after the addition of [^{3}H]uridine to the medium: (a) The labelling, with [^{3}H]-uridine present in the medium at 4.5 μM, of the total UDP (○) and UTP (●) pools of cells incubated in the absence of glucosamine, expressed as cpm/A_{260} of total nucleotide pool for each time point. UMP labelling was <5% of the total uridine nucleotide label throughout the period shown and did not change appreciably (not shown); (b) as for (a), except that the cells had been both preincubated for 1 h and then labelled in the presence of 20 mM glucosamine; (c) the labelling of the total UDP and UTP pools of cells which had been both preincubated for 1 h and then labelled in the presence of 300 μM uridine; (d) as for (c) except that the cells were preincubated for 1 h in the presence of 300 μM uridine and 20 mM glucosamine and then labelled in the presence of both compounds.

3. Results

When [^{3}H]uridine is added to mouse embryo cells it is initially phosphorylated to UDP and this in turn is converted to UTP in a fairly slow reaction (fig.1). With 4.5 μM uridine in the medium the labelling of the total cellular UDP and UTP pools shows a feedback inhibition of the transport mechanism whereby uridine is taken up by the cells and does not stabilise until 25–30 min after addition of isotope (fig.1(a)). The kinetics of this inhibition are consistent with it being brought about by cellular UTP. When the UTP pool is depleted by pretreatment of cells with 20 mM glucosamine [5] the labelling of the uridine nucleotide pools stabilises more rapidly (10–15 min in fig.1(b)). However, these pools have not been reduced to a level where the feedback inhibition of uridine uptake is no longer operative.

By subjecting the cells to a higher uridine concentration the uridine transport system of the plasma membrane becomes saturated (fig.1(c,d)). Up to about 10 μM uridine in the medium incorporation of externally added uridine increases in direct proportion to the uridine concentration, while above 100 μM uridine the uridine-transport system is saturated [15]. At still higher concentrations most uridine uptake occurs by diffusion, and with 300 μM uridine in the culture medium no feedback inhibition of the labelling of the uridine nucleotide pools was observed either in the absence of glucosamine (fig.1(c)) or its presence (fig.1(d)). Under such conditions the specific activity of the total cellular UTP stabilises more rapidly than with a low concentration of uridine in the medium and becomes constant after 10–15 min in the absence of glucosamine or <5 min in its presence (fig.1(c,d)).

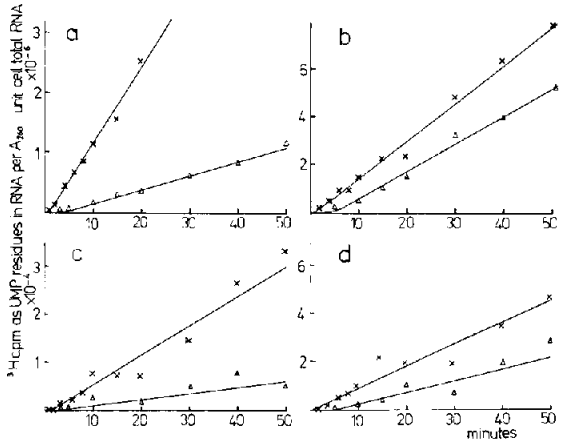

Fig.2. The kinetics of labelling of the total cellular RNA and transfer RNA of mouse embryo cells after addition of [^{3}H]-uridine to the medium: (a) The labelling of total cellular RNA (cpm/A_{260}) at low (4.5 μM) medium uridine concentration, determined both in the absence of glucosamine (△) and for cells incubated in 20 mM glucosamine for 1 h before and during labelling (x); (b) as for (a) except a high concentration (300 μM) of uridine was present throughout labelling; (c) the labelling of total tRNA in the RNA samples of (a); (d) the labelling of total tRNA in the RNA samples of (b).

The incorporation of uridine label into the total RNA of the cells and into transfer RNA was also measured in these experiments (fig.2). Transfer RNA was chosen as a model for stable RNA since the transcripts of tRNA genes are very rapidly processed to mature tRNAs and exported from the nucleus to the cytoplasm following their synthesis [7,8], whereas the nuclear maturation of ribosomal RNAs and messenger RNAs can take several minutes. Also it is comparatively easy to measure the labelling of the entire 4 S region of several RNA samples separated on polyacrylamide gels (see section 2). [^{3}H]Uridine started to be incorporated into total RNA (fig.2(a,b)) and 4 S RNA (fig.2(c,d)) at a constant rate within about 5 min of the addition of isotope irrespective of whether the uridine concentration of the medium was low (4.5 μM, fig.2(a,c)) or high (300 μM, fig.2(b,d)). In particular it should be noted that with the extracellular concentration of uridine low, the specific activity of the total cellular UTP stabilised considerably later (fig.1(a,b)) than the time that label incorporation into total RNA and 4 S RNA became linear (fig.2(a,c)). However with a high concentration of uridine in the medium the UTP pool stabilised (fig.1(c,d)) at about the same time that incorporation of isotope into total RNA and 4 S RNA became linear (fig.2(b,d)). The lag period between the time of addition of isotope to the cells and the time when incorporation of label into RNA became linear was possibly not affected by extracellular uridine concentration and was appreciably diminished by glucosamine treatment of the cells (fig.2).

4. Discussion

Many kinetic studies in the future will be directed towards understanding the maturation and processing of specific gene transcripts within the cell nucleus. It will be necessary to label cells with high specific

activity nucleosides which it would be prohibitively expensive to have at high concentrations in the culture medium. By maximising incorporation of label into the transcript of interest both the detection and labelling kinetics of its various processing intermediates can be more readily analysed by RNA–DNA hybrid formation [1–3]. Some pioneer studies of this nature have already extended our understanding of how the transcripts made of certain viral genes are processed to messenger RNA [10,17]. Because such investigations often involve labelling cells with a low concentration of labelled uridine due regard must be paid to how the actual labelling of UMP residues within a specific RNA has to be corrected for UTP pool labelling in calculations of the half-life of the RNA. This is especially apparent when the total cellular UTP is showing large labelling fluctuations that are not reflected in RNA labelling (fig.1(a,b)2(a,c)) and this pool and the pool supplying RNA synthesis are not labelled identically. The specific activity of the pool that supplies the precursors for RNA synthesis presumably stabilises at the time that incorporation of uridine label into stable RNA becomes linear. There is as yet no way by which this assumption can be tested directly. However using the Greenberg equation [4] to calculate the half-lives of polyoma virus transcripts in mouse embryo cells (in preparation) we have observed that the data points fit straight line plots considerably better using this assumption than if the data correction procedure in [11] is employed.

The results of fig.1(a,b) and fig.2(a,c) probably indicate that uridine is incorporated into at least two different UTP pools inside the cell, only one of which serves as a primary source of nucleotides for RNA synthesis. A similar situation has been observed in Novikoff rat hepatoma cells [15,16] and even in simpler organisms like *Escherichia coli* [18] and *Saccharomyces cerevisiae* [19] exogenous nucleosides appear to be incorporated into RNA without passing through the main nucleotide pools of the cell. During active synthesis of RNA the pool supplying RNA synthesis must be very small and respond relatively rapidly to changes in the nucleoside composition of the medium (fig.2). Preincubation of hepatoma cells with 500 μM unlabelled uridine had little or no effect upon subsequent [^{3}H]uridine incorporation [15]. That the major pool of UTP (presumably the cytoplasmic pool) only becomes available for RNA synthesis at a slow rate is also suggested by the absence of a reflection of the feedback inhibition of UTP labelling (fig.1(a,b)) in the onset of RNA labelling (fig.2(a,c)). I have not investigated the uptake and incorporation of nucleosides other than uridine in mouse embryo cells, but in other cell types adenosine also appears to be incorporated as two independent nucleotide pools whereas thymidine does not [15,16, 19]. Thymidine inhibits uridine uptake by cells possibly because it enters by the same transport mechanism [20].

References

[1] Berk, A. J. and Sharp, P. A. (1977) Cell 12, 721–732.
[2] Sharp, P. A., Berk, A. J. and Berget, S. M. (1980) Methods Enzymol. 65, 750–768.
[3] Favaloro, J., Treisman, R. and Kamen, R. (1980) Methods Enzymol. 65, 718–749.
[4] Greenberg, J. R. (1972) Nature 240, 102–104.
[5] Scholtissek, C. (1971) Eur. J. Biochem. 28, 70–75.
[6] Wertz, G. W. (1975) J. Virol. 14, 1340–1344.
[7] Levis, R. and Penman, S. (1977) Cell 11, 105–113.
[8] Herman, R. C. and Penman, S. (1977) Biochemistry 16, 3460–3465.
[9] Brandhorst, B. P. and McConkey, E. H. (1974) J. Mol. Biol. 85, 451–463.
[10] Nevins, J. R. and Darnell, J. E. (1978) Cell 15, 1477–1493.
[11] Brandhorst, B. P. and Humphries, T. (1971) Biochemistry 10, 877–881.
[12] Piper, P. W. (1979) J. Mol. Biol. 131, 399–407.
[13] Piper, P. W. and Wasserstein, M. (1977) Eur. J. Biochem. 80, 103–109.
[14] Ward, S., Wilson, D. L. and Gilliam, J. J. (1970) Anal. Biochem. 38, 90–97.
[15] Plagemann, P. G. W. (1971) J. Cell Physiol. 77, 213–240.
[16] Plagemann, P. G. W. (1971) J. Cell Physiol. 77, 241–258.
[17] Nevins, J. R. (1979) J. Mol. Biol. 130, 493–506.
[18] Rake, A. V. and Graham, A. F. (1962) J. Cell. Comp. Physiol. 60, 139–147.
[19] Hynes, N. E. and Phillips, S. L. (1976) J. Bacteriol. 125, 595–600.
[20] Steck, T. L., Nakata, Y. and Bader, J. P. (1969) Biochim. Biophys. Acta 190, 237–249.