SYNTHESIS OF UDP-GLUCURONIC ACID IN AN ESTABLISHED RAT HEPATOMA CELL LINE

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1. Introduction

Cultured cells of hepatic parenchymal origin have many advantages over perfused rat liver or liver slices for the study of liver specific functions [1]. Primary cultures of rat liver parenchymal cells retain differentiated functions effectively but are difficult to maintain for more than a few days without complex manipulations, as, for example, growing the cells on collagen membranes [2], which increases the duration of viability to about three weeks. An ideal system would be an established multiplying cell line which still retained the differentiated functions of interest. Transformed cells, and cell lines derived from hepatomas, multiply well but tend to lose differentiated functions [1].

We report here the use of a simple, sensitive approach which demonstrates the synthesis of UDP glucuronic acid (UDPGA) in an established line of rat hepatoma cells, DAB-1 [3]. This cell line is known to retain a number of liver specific functions, such as glycogen formation [3] and active orotate uptake (in preparation).

2. Materials and methods

2.1. *Cells*

The DAB-1 hepatoma cell line was obtained from Dr C. Albrecht, and SV40 Hamster Tumour (SVH), and CV-1 cells from Flow Laboratories. Cell lines were propagated as monolayer cultures in 25 cm^2 or 75 cm^2 Falcon Flasks using Eagles minimum essential medium supplemented with 5% calf serum and monitored for mycoplasma contamination as described [4].

2.2. *Preparation and analysis of acid soluble derivatives*

Cell monolayers were incubated with [2-^{14}C]-uridine (2 μCi/ml) or [U-^{14}C]galactose (2.5 μCi/ml) in 6 ml medium for 1 h. The monolayer was washed quickly in warm saline and frozen in situ after addition of 0.5 M perchloric acid (PCA). Cells were removed by shaking, were centrifuged, and the supernatant, representing the PCA-soluble fraction, was neutralized with KOH.

White Wistar rats were injected intra-peritoneally with [5-^{3}H]orotic acid (100 μCi); they were killed by decapitation and the liver was immediately removed and frozen in liquid nitrogen. It was then homogenized in 0.5 M PCA, centrifuged at 15 000 × g and the supernatant neutralized with KOH. Analysis by Biogel P2 and paper chromatography was as described for pyrimidine derivatives [5]. Dowex 1 X-8 chromatography was based on the method [6] using a single gradient system with increasing concentration of sodium formate from 0–1 M.

2.3. *UDPG dehydrogenase assay*

Cells, 5–6 × 10^7, were swollen and homogenized as described [4]. Rat liver was homogenized in the same buffer and centrifuged at 10 000 × g and then at 100 000 × g for 1 h to pellet the microsomal fraction. Final supernatants were tested for UDP glucose (UDPG) dehydrogenase using an adaptation of the technique [7]. The reaction mixture consisted of 0.7 ml 0.1 M glycine buffer, pH 9, 0.8 μmol NAD

in 0.1 ml, and 200 μl post-microsomal supernatant. Reaction was started by addition of UDPG, 0.24 μmol in 0.1 ml, and $A_{340\,nm}$ at 21°C. The microsomal fraction was assayed for glucuronidation of para-nitrophenol as described [8].

3. Results

Post-microsomal supernatants of DAB-1 cells were assayed for UDPG dehydrogenase activity, with similar preparations from rat liver, SVH cells, and

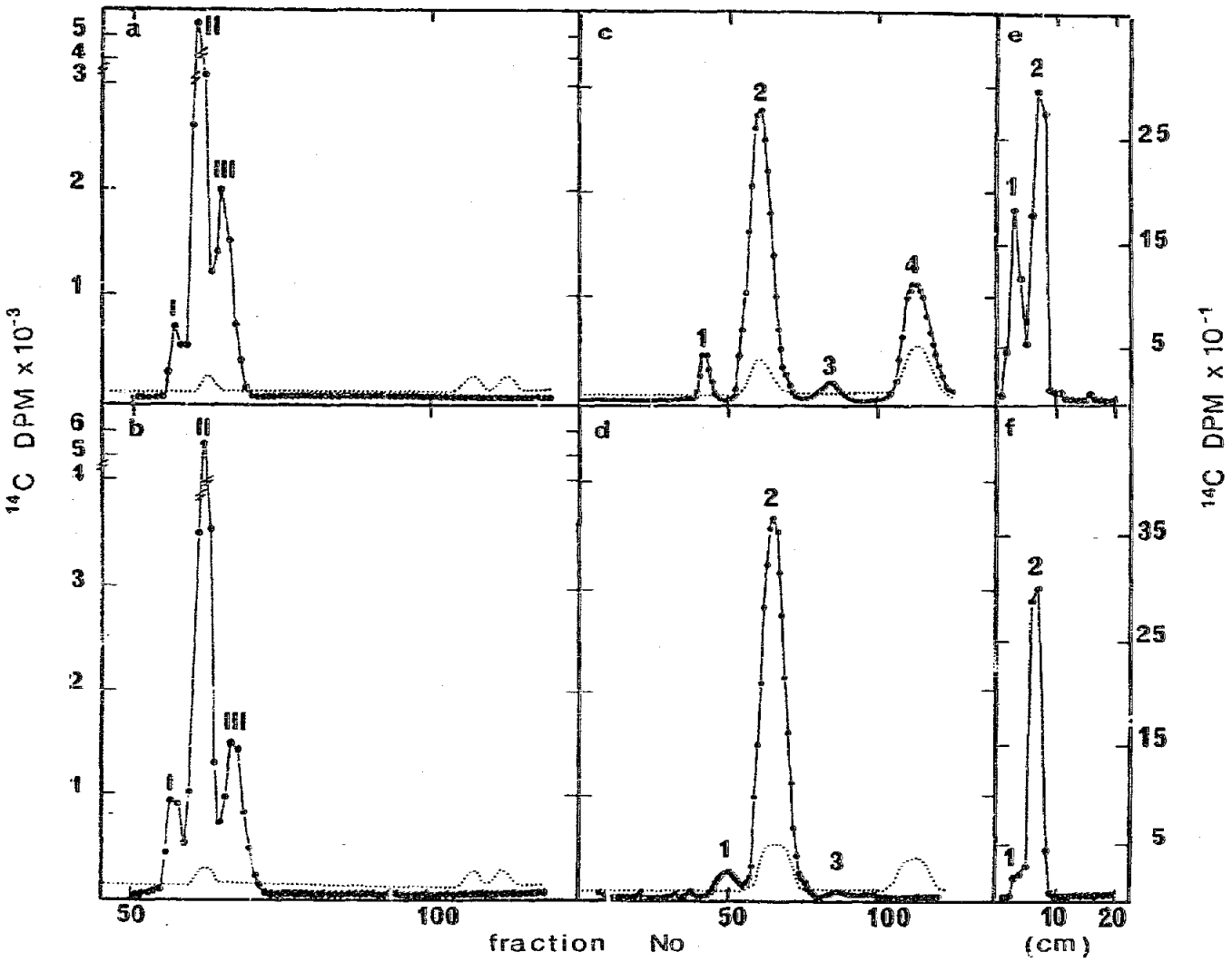

Fig.1. Analysis of PCA-soluble extracts of 15–18 X 10^6 DAB-1 cells (a,c,e) and SVH cells (b,d,f) labelled for 1 h with 2.5 μCi/ml of [^{14}C]uridine. (a,b) Biogel P2 elution profile; the A_{254} profile in arbitrary units depicts elution positions of markers of, from left to right, UTP, uridine and uracil, 10 μl 10 mM solution in each case. (c,d) Analysis of Biogel P2 peak III (Fr 64-69 and Fr 66-71 for DAB-1 and SVH cells respectively) on Dowex 1 X-8; 50 μl 10 mM solutions of UDPG and UDPGA were added as $A_{254\,nm}$ markers. (e,f) Paper chromatography of Biogel P2 peak III; 1 and 2 correspond to the migration positions of markers of UDPGA and UDPG/UDP, respectively, identified by short wave ultraviolet illumination (. . . . .) A_{254} (arbitrary units); (●————●) ^{14}C dpm.

CV-1 cells as controls. UDPG dehydrogenase activity was found to be 4.9 μM/min/mg protein rat liver preparation, whereas no activity could be demonstrated in any of the three cultured cell lines. It could be estimated that this represented less than 0.2 μM/min/mg protein UDPG dehydrogenase activity detectable in any of the three cell lines. Similarly, no demonstrable glucuronidation of para-nitrophenol was found in any of these cell lines. Cells were then labelled with [2-^{14}C]uridine and the acid-soluble nucleotide pool was analysed by chromatographic methods designed to optimise the separation of pyrimidine derivatives, including UDP sugars. A preliminary analysis of DAB-1 nucleotides on Biogel P2 is shown (fig.1a,b) using SVH cells labelled in the same way for comparison. The labelled profiles show in each case three major peaks: peak 1 elutes in the position of an unlabelled marker of CTP, peak II corresponds to the position of UTP together with UDP-*N*-acetyl glucosamine (UDPAG), and peak III corresponds to the elution position of markers of UDP, UDPG, UDPGA and UMP [5]. Subsequent analysis of peak fractions of peak I and II by paper chromatography confirmed that labelled CTP, UTP and UDPAG were present in similar proportions in both cell lines. On the other hand, paper chromatographic analysis of fractions from peak III showed that whereas a species migrating as UDPGA was present as a major constituent in DAB-1 cells, it was absent from the control cells (fig.1e,f). Analysis of the same peak III fractions from the Biogel P2 eluate on a third, ion-exchange, chromatographic system, (fig.1c,d) also showed that a labelled species (peak 4) comigrating with a UDPGA marker was a major constituent. UMP (peak 1), UDPG (peak 2), and UDP (peak 3) were present in similar proportions in the two cell lines. It would therefore seem that despite the absence of detectable UDPG dehydrogenase activity by conventional assay, there is readily demonstrable synthesis of UDPGA in the hepatoma cell line DAB-1 when cells are labelled with [5-^{3}H]-uridine. To confirm this, DAB-1 and SVH cells were

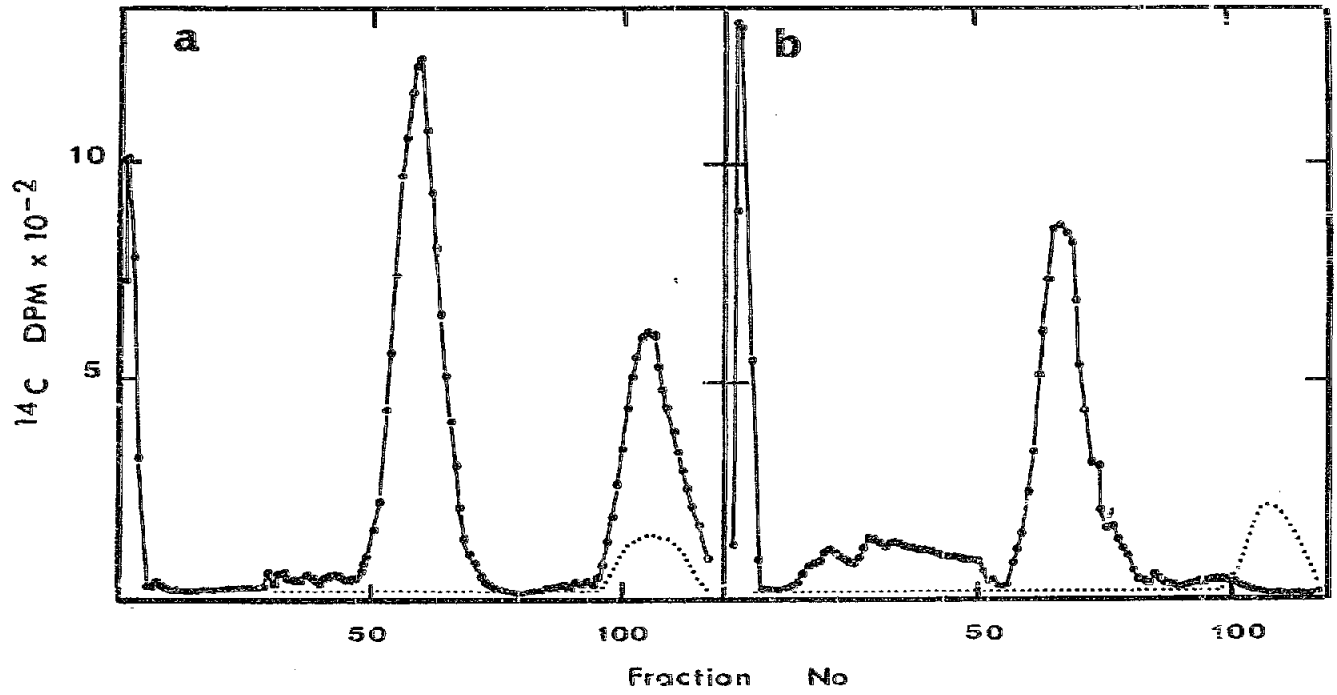

Fig.2. Dowex 1 X-8 chromatograms of Biogel P2 fractions corresponding to elution positions of UDPGA markers from PCA extracts of cells labelled for 1 h with 2.5 μCi/ml [U-^{14}C]galactose. A_{254} profile in arbitrary units depicts the elution position of a UDPGA marker. (a) DAB-1 cells. (b) SVH cells (. . . . . .) A_{254} (arbitrary units); (●——●) ^{14}C dpm.

labelled with [U-^{14}C]galactose which is readily metabolized to UDPGA in liver via UDPG [9]. After preliminary separations of the neutralized PCA extracts on Biogel P2, the peak fractions eluting with markers of UDPGA were analysed by ion-exchange chromatography and the profiles are shown in fig.2. The presence of a major labelled species, coeluting with a marker of UDPGA confirms the identity of this species as UDPGA in DAB-1 cells. Again there was negligible labelled UDPGA demonstrable in the control cells. In order to assess whether the DAB-1 cell line could utilise the UDPGA produced for glucuronidation reactions, cells were labelled for 1 h with [^{14}C]galactose in the presence or absence of 0.01 mM bilirubin. PCA extracts were analysed by Biogel P2 followed by ion-exchance chromatography of the UDPG/UDPGA peak, and the relative quantities of these labelled species was measured. In the absence of bilirubin UDPGA represented 45% of the total label in these two species, whereas in the presence of bilirubin this fell to 14% representing a decrease of 80% in the total quantity of labelled UDPGA detectable under these conditions.

4. Discussion

The evidence for the active UDPGA synthesis in the DAB-1 cell line is based on the detection of a species which can be labelled with both uridine and galactose, and which has similar characteristics to unlabelled UDPGA on 3 separate chromatographic systems. It is difficult therefore to avoid the conclusion that this species is indeed UDPGA despite the lack of detectable UDPG dehydrogenase activity in the DAB-1 cell line. However, the lack of any detectable labelled UDPGA in the other cell lines examined indicates that this does represent at least a partial expression of this liver specific pathway in the DAB-1 cells.

The isotopic approach used here to demonstrate UDPGA synthesis is much more sensitive than the standard enzyme assay for UDPG dehydrogenase activity, and it is therefore most likely that the UDPGA labelled in the DAB-1 cells is being synthesized by the standard pathway via UDPG but with UDPG dehydrogenase operating at less than 5% of its activity in a rat liver homogenate.

The marked decrease in the accumulation of labelled UDPGA in the presence of bilirubin is an indirect indication of a glucuronidating capability in the DAB-1 cells. It would seem that in tissue culture there are few substrates for glucuronidating enzymes, allowing UDPGA to accumulate. It is of interest in this regard that practically no labelled UDPGA can be demonstrated in rat liver after labeling for 1 h with [5-^{3}H]orotate injected intraperitoneally, despite good labelling of UDPG (unpublished observations). Presumably UDPGA in functioning rat liver is very rapidly consumed.

The DAB-1 cell line is therefore characterised by its ability to synthesize, and in the absence of appropriate substrates to accumulate, UDPGA. There is also evidence that it retains glucuronyl transferase activity with respect to bilirubin. This cell line has been maintained continuously by serial passage twice a week for over two years in our laboratory, and therefore has value as a stable tissue culture model system for a number of liver specific differentiated functions.

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