The mRNAs for cyclin A, c-myc and ribosomal proteins L4 and S6 are associated with cytoskeletal-bound polysomes in HepG2 cells

Randi HOVLAND,* Gill CAMPBELL,† Ian PRYME* and John HESKETH†‡

*Department of Biochemistry and Molecular Biology, University of Bergen, Arstadveien 19, N-5009 Bergen, Norway and †Intracellular Targeting Group, Rowett Research Institute, Greenburn Rd., Bucksburn, Aberdeen AB2 9SB, U.K.

Cytosolic, cytoskeleton and membrane fractions were extracted from HepG2 cells by a sequential detergent/salt extraction procedure. The cytosolic fraction contained 93% of the lactic dehydrogenase activity while the cytoskeleton fraction was enriched in actin and vimentin. The distribution of mRNAs for c-myc, glucose transporter 1, ribosomal proteins L4 and S6 and cyclin A were investigated by Northern hybridization of total RNA extracted from polysomes isolated from cytosolic,

cytoskeleton and membrane fractions. The membrane-bound polysomes were enriched in the glucose transporter 1 mRNA and the cytoskeleton-bound polysomes were enriched in the mRNAs for the two ribosomal proteins, c-myc and cyclin A. The results suggest that the mRNAs for nuclear proteins are one class of mRNAs which are translated on polysomes associated with the cytoskeleton; this may be related to the requirement to transport the newly synthesized protein to the nucleus.

INTRODUCTION

A major proportion (approximately 70–80%) of cell mRNAs and polyribosomes (polysomes) are retained in the cell matrix when cells are treated with non-ionic detergents [1–3] and, on the basis of such observations, it has been suggested that many polysomes are associated with the cytoskeleton. Treatment of the cell matrix with salt concentrations above 100 mM leads to disruption of the microfilaments [1–4] and release of both actin and polysomes [5]. Salt extraction thus provides a method to separate polysomes associated with the cytoskeleton from those on the endoplasmic reticulum, which are also present in the cell matrix. Using an initial treatment with non-ionic detergent at 25 mM salt concentration, followed by extraction with 130 mM salt and then deoxycholate, it has proved possible to isolate from ascites cells, fibroblasts and MPC-11 cells three polysome populations which, on the basis of markers such as lactic dehydrogenase, actin, vimentin, phospholipid content and β -2-microglobulin mRNA, have been characterized as free (FP), cytoskeleton-bound (CBP) and membrane-bound (MBP) polysomes [6–9].

Hybridization data have shown that certain specific mRNAs, such as those coding for actin and histones, are associated with the cell matrix and released by cytochalasins which depolymerize microfilaments [10–13]. Results from *in vitro* translation experiments suggested that the CBPs released by 130 mM KCl contained mRNAs coding for a different pattern of proteins from those made on FPs or MBPs [7]. Furthermore a cytoskeletal fraction from *Xenopus* oocytes has been shown to be enriched in Vg1 mRNA [14] and CBPs isolated by 130 mM KCl extraction of the cell matrix are enriched in c-myc mRNA [8]. It thus appears that CBPs are a distinct population of polysomes enriched in specific mRNAs [6,15]. Furthermore, the presence of the c-myc mRNA in CBPs depends upon the 3' untranslated region (3'UTR) of the mRNA, indicating that there are targeting signals within the c-myc 3'UTR which direct it to CBPs [16].

However, the physiological significance of cytoskeleton-polysome interactions and the CBP population remain to be

fully investigated; do CBPs represent a population of polysomes which are spatially segregated in the cell or are they polysomes which are associated with the cytoskeleton for transport? Further insight into the functional significance of CBPs will be gained by a description of the mRNA species which are translated on such polysomes. The aim of the present work was to investigate, using HepG2 hepatoma cells, the association of mRNAs for a series of nuclear proteins with the cytoskeleton.

MATERIALS AND METHODS

Cell fractionation

HepG2 cells were grown in 90-mm-diam. Petri dishes (Nunc) in Williams E medium supplemented with 10% fetal-calf serum and in an atmosphere of 5% CO₂. Cells were grown to confluence and treated with emetine (2×10^{-7} M in the medium) for 30 min prior to extraction of polysomes; concentrations of this order have been reported previously to be effective in maintaining polysomes and mRNA-ribosome interactions [17]. Polysome fractions were isolated from 1×10^8 cells, essentially as described previously [7,8]. After rinsing cells three times in 10 mM sodium phosphate buffer, pH 7.4, containing 0.9% NaCl (PBS), cells from 10 dishes were harvested into 2 ml of lysis buffer (10 mM Tris, pH 7.6, 0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂, 0.5 mM CaCl₂) containing 0.5% Nonidet P-40 (NP-40), and after 5 min at 4 °C the suspension was centrifuged at 1000 g for 5 min. The supernatant (free polysome fraction; FP) was removed and stored on ice. The pellet was washed once in lysis buffer and then resuspended in 2 ml of the same buffer containing 130 mM KCl. After incubation for 10 min at 4 °C, the suspension was centrifuged at 2000 g for 5 min and the supernatant (CBP fraction) removed and stored on ice. MBPs were solubilized by incubation of the pellet in 2 ml of the same 130 mM KCl buffer containing 0.5% NP-40 and 0.5% deoxycholate for 15 min and collected by centrifugation at 3000 g for 5 min. Polysomes were separated from monosomes and lighter material by centrifugation at 16000 rev./min (30000 g) for 16 h through a 15 ml cushion

Abbreviations used: 1 × SSC, 0.15 M NaCl + 0.015 M sodium citrate; FP, free polysome; CBP, cytoskeleton-bound polysome; MBP, membrane-bound polysome; 3'UTR, 3' untranslated region; NP-40, Nonidet P-40.

‡ To whom correspondence should be addressed.

of 40% sucrose [18,19] and the separation was verified by recentrifugation of both the material retained above the cushion and the resuspended pellet from below the cushion on 15–45% sucrose gradients as described below; these showed that the material at the top of the gradient consisted of 80 S monosomes and lighter material but no polysomes, while the pellet corresponded to some 90% of the original polysomes. All buffers and cushion solutions were autoclaved where appropriate; extraction buffers contained the RNase inhibitor RNasin (133 units/ml; Promega, U.K.).

Polysome sedimentation profiles were obtained for the three fractions. Fractions (10 A_{260} units of FP fraction; same volume of CBP and MBP fractions) were layered on to 15–40% sucrose gradients and centrifuged at 40000 rev./min (280000 *g*) for 1 h in an SW41 rotor and the profiles were monitored by measuring the absorbance at 260 nm using a flow cell.

Lactic dehydrogenase was measured spectrophotometrically [20]. Actin and vimentin were assayed by enzyme-linked immunosorbent assay [5,7] using anti-actin from Amersham International Ltd. (Amersham, U.K.) and anti-vimentin from Sigma Chemical Co. (Poole, Dorset, U.K.).

RNA extraction and hybridization

Total RNA was extracted by the acid/guanidinium/phenol/chloroform method of Chomczynski and Sacchi [21], and the preparations were assessed by the A_{260}/A_{280} absorbance ratio. RNA species were then separated by electrophoresis through a denaturing 2.2 M formaldehyde, 1.2% agarose gel [22] and transferred to nylon membrane (Genescreen from NEN Dupont Ltd.) by capillary blotting. RNA was fixed to the membrane by exposure to UV light and the membranes stored dry until required. Membranes were prehybridized overnight at 42 °C with 0.1 mg/ml denatured salmon sperm DNA in 50% deionized formamide, containing 10% dextran sulphate, 0.2% BSA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS and 50 mM Tris/HCl, pH 7.5.

The *c-myc* probe, a gift from Dr. M. Cole (Princeton University, NJ, U.S.A.), was the cDNA of the three exons of the mouse *c-myc* gene. The 2.3 kbp cDNA was cloned into pT7 and a 1.8 bp *HindIII* fragment used in hybridization studies after separation on 0.6% low-melting-point agarose gels. The cyclin A probe, a gift from Dr. Berthold Henglein (INSERM U-75, Paris), was a 1.8 kb *XbaI* fragment of the cDNA of the human cyclin A gene [23]; the glucose transporter 1 probe was a 2.0 kb *BglII* fragment from the rat cDNA (a gift from Dr. G. Gould, Glasgow); the ribosomal protein S6 probe was a 750 bp fragment of the cDNA [24] to the human protein (a gift from Dr. G. Mackie, Western Ontario) and the ribosomal protein L4 probe, a gift from Dr. F. Amaldi (Rome, Italy), was an *EcoRI*

fragment corresponding to almost the full-length cDNA for the human protein. 50–100 ng of the DNA probes were labelled with [³²P]dCTP by random priming (Multiprime kit from Amersham International, U.K.) and the labelled DNA was then separated from free nucleotides by gel filtration on Sephadex G-50 (elution buffer: 1 × SSC, 0.1% SDS); probe specific radioactivities were approximately 10⁹ c.p.m./μg of DNA. The labelled probes were added to the prehybridization mix and hybridized at 42 °C for 24 h. The membranes were washed to remove non-specific hybridizations twice in 2 × SSC at room temperature for 5 min, followed by 0.5 × SSC, 1% SDS at 65 °C for 2 h with one change of wash solution. Specific hybridization was then detected either by autoradiography using Hyperfilm-MP (Amersham International, U.K.) at –70 °C or with a Packard Instantimager which allowed quantification of hybridization by direct measurement of specifically bound probe. After autoradiography membranes were washed in 0.1% SDS for 5–7 min at 95 °C before rehybridization to other probes. Quantification of the RNA loaded in each lane was assessed either from scans of the ethidium bromide-stained agarose gel or by hybridization with a 1.4 kb probe for 18 S rRNA [25].

RESULTS AND DISCUSSION

It was found necessary to modify the initial non-ionic detergent extraction conditions in the sequential detergent/salt procedure [7] for separation of FPs, CBPs and MBPs from HepG2 cells. Treatment of HepG2 cells with 0.05–0.1% NP-40, which was found to be sufficient to release cytosolic components from 3T3 fibroblasts or MPC-11 cells [8,9], failed to release all of the lactic dehydrogenase activity from HepG2 cells. However, treatment of HepG2 cells with low-salt extraction buffer containing 0.5% NP-40 released more than 93% of the activity; specific activities (arbitrary units/mg of protein) were 11.2, 3.5 and 0.8 respectively in the NP-40/25 mM KCl, 130 mM KCl and NP-40/deoxycholate extracts. This indicates that such treatment released the bulk of the cytosolic material and on this basis the low-salt/0.5% NP-40 extract was considered as a cytosolic fraction. The difference in detergent susceptibility presumably reflects differences in cell membrane lipid composition between the cell lines; interestingly, in the case of Krebs II ascites cells 1% NP-40 was required in order to release the majority of cytosolic lactic dehydrogenase activity and FPs [7]. Treatment of the non-ionic-detergent-insoluble cell matrix with 130 mM KCl caused a release of actin and vimentin to such an extent that the concentration was some 5-fold higher than in the cytosolic fraction (Table 1). As shown in Figure 1(a), sucrose gradient analysis of the released material showed the presence of polysomes both in the cytosolic fraction and in the material released by 130 mM KCl. Thus it appears that, as in other cell lines [6–8], 130 mM salt treatment

Table 1 Characterization of cytoskeletal and membrane fractions

Values shown are means ± S.E.M. from three separate preparations.

Extract	Marker proteins ($A_{492}/\mu\text{g}$ of protein)		Marker mRNAs (arbitrary units per unit of 18 S rRNA)	
	Actin	Vimentin	<i>c-myc</i> mRNA	Glucose transporter-1 mRNA
NP-40/25 mM KCl	0.10 ± 0.03	0.10 ± 0.01	562 ± 53	811 ± 69
130 mM KCl	0.54 ± 0.08	0.34 ± 0.10	1115 ± 279	600 ± 30
Deoxycholate/NP-40	0.13 ± 0.03	0.10 ± 0.02	606 ± 178	1817 ± 225

Figure 1 Sedimentation profiles of cytosolic, cytoskeleton and membrane fractions from HepG2 cells

(a) Polysome sedimentation profiles of cytosolic, cytoskeleton and membrane fractions. Fractions were isolated from HepG2 cells, layered on to 15–40% sucrose gradients and centrifuged at 40 000 rev./min for 1 h. Polysome profiles were monitored by measuring the absorbance at 260 nm using a flow cell: (A) cytosolic fraction, FPs; (B) cytoskeleton fraction, CBPs; and (C) membrane fraction, MBPs. (b) Sedimentation profile of material retained on top of the 40% sucrose cushion. 2 ml of cytosolic fraction was layered on to a 40% sucrose cushion and centrifuged at 16 000 rev./min for 16 h as described in the Materials and methods section. The material remaining above the cushion was then layered on top of a 15–40% linear sucrose gradient and centrifuged at 40 000 rev./min for 1 h, and the A_{260} profile monitored. The profile shows the presence of 80 S monosomes and lighter material but no polysomes; the arrow indicates the position of migration of 80 S monosomes in the presence of polysomes.

of the cell matrix releases both cytoskeletal material and polysomes; the enrichment of the fraction in actin and vimentin indicates that these polysomes represent the CBPs. Solubilization of the endoplasmic reticulum with deoxycholate released further polysomes (Figure 1a) and some remaining actin and vimentin (Table 1).

In subsequent experiments mRNAs present in total RNA extracted from the three polysome fractions were analysed by Northern hybridization. Polysomes were isolated from each fraction by centrifugation through a sucrose cushion. This centrifugation procedure, similar to those used previously to prepare polysomes and to distinguish between polysomes and other ribonucleoprotein complexes [18,19], separated polysomes (pellet) from monosomes and smaller ribonucleoprotein particles which were retained at the top of the sucrose cushion. As shown in Figure 1(b) recentrifugation of the material retained above the cushion showed that this consisted of 80 S monosomes and lighter material but no polysomes. Sucrose gradient analysis of the pellet involves resuspension and inevitable breakdown of polysomes, but such recentrifugation showed the presence of polysomes in the pellet, corresponding to some 90% of those in the original fraction. Crucially, when the material retained at the top of the cushion was layered on to a sucrose cushion and recentrifuged, it remained on the top of the cushion and no A_{260} -absorbing material was recovered from the cushion and there was no pellet (results not shown), showing that monosomes or lighter material do not enter the cushion. Clearly therefore, the pellet produced by centrifugation of fractions through a sucrose cushion contains material which is heavier than monosomes, i.e. polysomes, uncontaminated by non-polysomal, untranslated ribonucleoprotein particles.

Polysome fractions were characterized by assessing the distribution of two marker mRNAs: *c-myc*, which has been found previously on CBP [8], and glucose transporter 1, which because its protein is an integral membrane protein [26] is a marker for those polysomes which are derived from the rough endoplasmic

Figure 2 Northern hybridization analysis of the mRNAs for *c-myc*, ribosomal proteins L4 and S6 and cyclin A in free, cytoskeletal-bound and membrane-bound polysomes

Results shown are for 20 μ g of total RNA isolated from free (FP), cytoskeletal-bound (CBP) and membrane-bound (MBP) polysomes. Each hybridization was carried out on duplicate samples and on the same nylon membrane.

reticulum (i.e. MBP). The results showed that, as in fibroblasts and ascites cells, the *c-myc* mRNA was enriched in those polysomes released by 130 mM salt (Table 1; Figure 2), supporting the data on cytoskeletal proteins (Table 1) in suggesting that the polysomes released by 130 mM KCl were originally associated with the cytoskeleton (i.e. CBPs). The mRNA for glucose transporter 1 was recovered largely in the polysomes released by deoxycholate (Table 1), showing that, as found previously, this fraction consists largely of MBPs. The results show that with minor adjustments the sequential detergent/salt extraction procedure used in ascites cells [7], MPC-11 cells [9] and fibroblasts [8,16] can also be used in a liver-derived cell line. This should allow the investigation of the function of CBPs in the synthesis of specific liver proteins.

In a further series of experiments the distribution of three additional specific mRNAs was studied by Northern hybridiz-

Table 2 Quantification of mRNA distribution between FP, CBP and MBP

The mRNA levels in each polysome fraction are presented per unit of 18 S rRNA; each mRNA hybridization was quantified by direct measurement of the radioactivity (c.p.m.) in the specific band detected by the Packard Instantimager and corrected for any loading differences by expressing the results per unit of 18 S rRNA. Results are presented as arbitrary units because of differences in data acquisition/hybridization efficiency between experiments. Results shown are means of duplicate determinations from two separate fractionation experiments. S6, ribosomal protein S6; L4, ribosomal protein L4.

mRNA species		mRNA per unit 18 S rRNA (arbitrary units)		
		FP	CBP	MBP
<i>c-myc</i>	Expt. 1	437	754	229
	Expt. 2	95	260	102
S6	Expt. 1	432	711	235
	Expt. 2	115	222	79
L4	Expt. 1	222	617	275
	Expt. 2	112	275	67
Cyclin A	Expt. 1	238	376	87
	Expt. 2	64	208	52

ation: cyclin A, ribosomal protein L4 and ribosomal protein S6. In each case visual inspection of the autoradiographs showed that the mRNA was found at highest abundance in the CBP fraction (Figure 2). Quantification of the data, and correction for any potential differences in loading by expressing the data per unit of 18 S rRNA, confirmed that the CBP fraction was enriched in cyclin A, ribosomal protein L4 and ribosomal protein S6 mRNAs, as well as the *c-myc* mRNA (Table 2). The results extend the previous observation that *c-myc* mRNA is associated with CBPs by showing that the mRNAs for cyclin A and two ribosomal proteins are also present in CBPs. These mRNAs code for proteins which, like *c-myc*, are transported to the nucleus. Indeed, in transfected cells *c-myc* mRNA is found to be both perinuclear in location and associated with CBP [16], while retargeting of the mRNA to the FPs by exchanging the normal 3'UTR for that of the β -globin mRNA leads to a smaller proportion of the protein in the nucleus [27]. It is our hypothesis therefore that the mRNAs for nuclear proteins represent one class of mRNAs which are translated on the cytoskeleton [8] and this view is supported not only by the present data but also by the

observation that *c-fos* mRNA is released from the cell matrix by actin depolymerization induced by cytochalasin [10]. The targeting of these mRNAs to the cytoskeleton would allow their retention in the perinuclear cytoplasm and promote efficient transport of the newly synthesized proteins to the nucleus.

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