c-myc mRNA in cytoskeletal-bound polysomes in fibroblasts

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3T3 fibroblasts were treated sequentially with 25 mM-KCl/0.05 % Nonidet P40, 130 mM-KCl/0.05 % Nonidet P40 and finally with 1 % Nonidet P40/1 % deoxycholate in order to release free, cytoskeletal-bound and membrane-bound polysomes respectively. The membrane-bound fraction was enriched in the mRNA for the membrane protein β_2 -microglobulin, whereas the cytoskeletal-bound polysomes were enriched in c-myc mRNA. Actin mRNA was present in both free and cytoskeletal-bound polysomes. The results suggest that cytoskeletal-bound polysomes are involved in the translation of specific mRNA species.

INTRODUCTION

Using subcellular fractionation techniques, cellular polyribosomes (polysomes) can be separated into two populations, the membrane-bound polysomes attached to the endoplasmic reticulum and the free polysomes in the cytosol (Shires & Pitot, 1973). Evidence from electron microscopy and the fact that polysomes can be extracted from the non-ionic detergent-insoluble cell matrix has suggested that there is a third population of polysomes, bound to the cytoskeleton (Wolosewick & Porter, 1976; Lenk *et al.*, 1977; Nielsen *et al.*, 1983). In most biochemical studies the presence of polysomes in the non-ionic-detergent-insoluble cell matrix has been taken as sufficient evidence that such polysomes are cytoskeletal-bound (e.g. Lenk *et al.*, 1977; van Venrooij *et al.*, 1981). Although such non-ionic-detergent-insoluble material does contain cytoskeletal filaments, it is a most heterogeneous fraction which also contains unsolubilized remnants of the rough endoplasmic reticulum (Dang *et al.*, 1983; Ramaekers *et al.*, 1983).

It has been observed that 130 mM-KCl caused a concomitant release of actin and polysomes from the non-ionic detergent-insoluble cell matrix (Hesketh & Pryme, 1988), and this observation was used to develop a sequential extraction procedure to separate free, cytoskeletal-bound and membrane-bound polysomes (Vedeler *et al.*, 1991); on the basis of actin content and sensitivity to cytochalasin and phalloidin, which both affect microfilament stability, the polysomes released by salt were defined as a cytoskeletal-bound fraction. These results, together with observations from electron microscopy and immunohistochemical data showing co-distribution of ribosomes or initiation factors with filaments (Toh *et al.*, 1980; Heuveljans *et al.*, 1989), now present a considerable body of evidence showing that a proportion of cellular polysomes is bound to the cytoskeleton (see Hesketh & Pryme, 1991). The physiological significance of these polysomes, however, is still unclear. One possibility is that they are involved, as are the membrane-bound population, in the synthesis of specific proteins. The aim of the present work was to address this question by using Northern hybridization techniques to investigate whether certain mRNAs are present specifically on cytoskeletal-bound polysomes isolated using a sequential detergent/salt extraction technique.

MATERIALS AND METHODS

Cell fractionation

3T3 fibroblasts were grown in 90 mm-diam. Petri dishes in Dulbecco's minimal Eagle's medium supplemented with 12 %

(v/v) foetal calf serum and in an atmosphere of 10 % CO₂. Cells were transferred to medium containing 4 % serum for 48 h (Hesketh *et al.*, 1986) and insulin (1 munit/ml final concentration) or saline was added for 1 h before harvesting. Polysome-containing fractions were isolated by sequential treatment of the cells with lysis buffer (10 mM-Tris, pH 7.6, containing 0.25 M-sucrose, 25 mM-KCl, 5 mM-MgCl₂, 0.5 mM-CaCl₂ and 0.05 % Nonidet P40), the same buffer containing 130 mM-KCl, and finally lysis buffer containing 1 % deoxycholate and 1 % Nonidet P40, as described previously (Vedeler *et al.*, 1991). In the present experiments all buffers and gradient solutions were autoclaved and contained 135 units of placental RNAase inhibitor/ml (Boehringer Mannheim Ltd.). After rinsing cells three times in 10 mM-sodium phosphate buffer, pH 7.4, containing 0.9 % NaCl, cells were harvested from 24–30 dishes using a rubber policeman and resuspended in 1.5 ml of lysis buffer. After 5 min at 4 °C the suspension was centrifuged at 1000 g for 5 min and the supernatant (free polysomes) was removed and stored on ice. The pellet was washed once in the same buffer and then resuspended in 1.5 ml of buffer containing 130 mM-KCl. After a 10 min incubation at 4 °C, the suspension was centrifuged at 2000 g for 5 min and the supernatant (cytoskeletal-bound polysomes) was removed and stored on ice. Membrane-bound polysomes were solubilized by incubation in lysis buffer containing 1 % Nonidet P40 and 1 % deoxycholate for 15 min and collected by centrifugation at 3000 g for 5 min. RNA was extracted directly from the fractions or from polysomes prepared by centrifugation at 32000 g for 16 h through a 5 ml sucrose cushion.

RNA extraction and analysis

Total RNA was extracted by the acid/guanidinium/phenol/chloroform method of Chomczynski & Sacchi (1987), and the preparations were assessed by the A_{260}/A_{280} absorbance ratio. RNA was then separated on denaturing 2.2 M-formaldehyde/1.2 % agarose gels and transferred to nylon membranes (Genescreen) by capillary blotting (Sambrook *et al.*, 1989). The RNA was fixed to the membrane by exposure to u.v. light and then prehybridized (6–16 h, 42 °C) with 0.1 mg of salmon sperm DNA/ml in 50 % formamide, 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. c-myc and β_2 -microglobulin probes were labelled with [³²P]dCTP by random priming. The β -actin probe was a 27-mer oligonucleotide obtained from Clontech Ltd., and it was end-labelled with [³²P]ATP following the manufacturer's instructions. The c-myc probe, a cDNA of exons 1, 2 and 3 of the mouse myc gene, was generously provided by Dr. M. Cole (Princeton University,

Princeton, NJ, U.S.A.). The β_2 -microglobulin probe (a gift from Dr. K. Lipson, Temple University, Philadelphia, PA, U.S.A.) was a 900 bp *HindIII*-*EcoRI* fragment of the mouse β_2 -microglobulin gene. All hybridizations were carried out for 18 h at 42 °C in cylindrical bottles which were slowly rotated. After hybridization, filters were washed either with 0.5 × SSC/1 % SDS at 65 °C for 1 h (twice) in the case of the *c-myc* and β_2 -microglobulin probes or 6 × SSC/0.1 % SDS at 66 °C for the actin probe (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate). Specific hybridization was detected by autoradiography at -70 °C and the bands were then quantified by image analysis using a QUIPS image-processing workstation (Torch Computers, Cambridge, U.K.) operating with VCS image processing software (Vision Dynamics, Hemel Hempstead, Herts., U.K.). After autoradiography, filters were washed in 0.1 % SDS at 95 °C before rehybridization. All filters were hybridized successively with the three probes.

RESULTS AND DISCUSSION

Sequential extraction (Vedeler *et al.*, 1991) of 3T3 cells produced a soluble fraction containing free polysomes (F), a salt extract containing cytoskeletal-bound polysomes (C) and a deoxycholate-solubilized fraction containing membrane-bound polysomes (M). Northern hybridization of total RNA from the three fractions showed that the cytoskeletal fraction was considerably enriched in *c-myc* mRNA compared with the soluble and membrane fractions (Fig. 1). Further experiments were carried out in which the polysomes in each fraction were pelleted by centrifugation through a sucrose cushion. Northern hybridization again showed the *c-myc* mRNA to be present largely in the cytoskeletal-bound polysomes released by 130 mM-KCl (Fig. 2); per unit of total RNA, there was approximately twice as much present in this fraction as in the free polysomes. This association of the *c-myc* mRNA with the cytoskeletal-bound polysomes was equally striking when account was taken of the greater amount of RNA recovered in the cytoskeletal-bound polysome fraction (Table 1); thus 55–70 % of the *c-myc* mRNA was found in the cytoskeletal fraction. This proportion recovered in the cytoskeletal fraction is much larger than the 35 % reported in the case of the Vg1 mRNA which was enriched in a cytoskeletal fraction from *Xenopus* oocytes (Pondel & King, 1988). The small amounts recovered in the other fractions are most probably due to breakdown of the cytoskeleton during cell lysis and incomplete removal of cytoskeletal-bound polysomes before treatment with deoxycholate.

Actin mRNA was found in all three fractions (Fig. 2), but only at a low concentration in the membrane-bound polysome fraction, and this probably reflects contamination of the fraction with polysomes which have been trapped in the pellet after salt extraction. Both visual inspection and quantification of the autoradiograms (Table 1) showed that, per unit of total RNA, there were similar amounts of the actin mRNA in both free and cytoskeletal-bound polysomes. However, when account was taken of the different amounts of RNA recovered in the three polysome fractions, it was apparent (Table 1) that approx. 60 % of the actin mRNA associated with polysomes was recovered in the cytoskeletal-bound polysomes and 18 % in the free polysomes. This distribution of actin mRNA between both cytoskeletal-bound and free polysomes is consistent with earlier studies of HeLa cells and myoblasts in which the majority of actin mRNA was found in cytoskeletal-bound polysomes and 10–30 % was associated with free polysomes (Bird & Sells, 1986; Bag & Pramanik, 1987). Together with such previous work and results from *in situ* hybridization studies (Singer *et al.*, 1989), the

present data suggest that actin mRNA is primarily translated on cytoskeletal-bound polysomes. It is not clear why the mRNA also appears to be associated with polysomes in the soluble fraction; it may be that actin mRNA is translated on a population of polysomes which are more easily removed from the cytoskeleton.

The mRNA for β_2 -microglobulin was found to be recovered in the deoxycholate-solubilized polysomes (Fig. 2, Table 1). Only very small amounts of this mRNA were found in the other two

Fig. 1. *c-myc* mRNA levels in soluble (F), cytoskeletal (C) and membrane (M) fractions from 3T3 fibroblasts

Each lane was loaded with 20 µg of total RNA from each fraction. Fractions and RNA were prepared as described in the Materials and methods section.

Fig. 2. Northern hybridization analysis of *c-myc*, actin and β_2 -microglobulin mRNAs in polysomes prepared from soluble, cytoskeletal and membrane fractions

Results shown are for 20 µg of total RNA from free (F), cytoskeletal-bound (C) and membrane-bound (M) polysomes isolated as described in the Materials and methods section. Each hybridization was carried out using the same nylon membrane. (a) β_2 -Microglobulin, (b) actin, (c) *c-myc*.

Table 1. Quantitative analysis of mRNAs present in free, cytoskeletal-bound and membrane-bound polysomes

The mRNA levels for each fraction are presented both per μg of RNA, to illustrate relative enrichment of the specific mRNAs, and per fraction, to show the proportion recovered in each fraction. Values are means from two separate preparations. $\beta_2\text{MG}$, β_2 -microglobulin.

Polysomes	Total RNA recovered in polysomes (μg)	mRNAs present in polysomes (arbitrary units)					
		(per 20 μg of RNA)			(per fraction)		
		c-myc	Actin	$\beta_2\text{MG}$	c-myc	Actin	$\beta_2\text{MG}$
Free	56	0.11	0.19	0.06	0.31	0.52	0.17
Cytoskeletal-bound	149	0.20	0.24	0.10	1.40	1.64	0.71
Membrane-bound	197	0.08	0.09	0.39	0.65	0.75	3.81

fractions. Since β_2 -microglobulin is a membrane protein whose mRNA would therefore be expected to be translated on membrane-bound polysomes (Shires & Pitot, 1973), this observation confirms that the deoxycholate-solubilized polysomes represent membrane-bound polysomes initially associated with the rough endoplasmic reticulum. The presence of only small amounts of this mRNA in the other fractions shows that there is little contamination of the other fractions with membrane-bound polysomes.

Treatment of 3T3 cells with insulin for 1 h had little or no effect on the distribution of the three mRNAs studied (Fig. 2). Quantification of the autoradiograms showed, however, that insulin did increase the amount of c-myc mRNA in the cytoskeletal-bound polysomes; thus the amount (expressed as arbitrary units) of c-myc mRNA/20 μg of RNA increased from 0.2 in control cells to 0.33 in the insulin-treated cells, and the total amount of c-myc mRNA recovered in this fraction increased by 144% from 1.4 to 3.4. This suggests that insulin, although not mitogenic in these cells, can increase the amount of c-myc mRNA being translated. The results are consistent with recent data from other cell lines in which insulin has been shown to increase c-myc mRNA levels (Banskota *et al.*, 1989).

In conclusion, both c-myc and actin mRNAs are largely associated with cytoskeletal-bound polysomes. Furthermore, the c-myc mRNA is enriched in such polysomes compared with those recovered from soluble and membrane-derived fractions, and this suggests that the cytoskeletal-bound polysomes are involved in the synthesis of a specific set of proteins. The physiological significance of c-myc synthesis on such polysomes is unclear, but one possibility is that it is related to the need to transport the synthesized protein back to the nucleus.

REFERENCES

- Bag, J. & Pramanik, S. (1987) *Biochem. Cell Biol.* **65**, 565–575
- Banskota, N. K., Tamb, R., Zellner, K., Olsen, P. & King, G. L. (1989) *Diabetes* **38**, 123–129
- Bird, R. & Sells, B. (1986) *Biochim. Biophys. Acta* **868**, 215–225
- Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Dang, C. V., Yang, D. C. H. & Pollard, T. D. (1983) *J. Cell Biol.* **96**, 1138–1147
- Hesketh, J. E. & Pryme, I. F. (1988) *FEBS Lett.* **231**, 62–66
- Hesketh, J. E. & Pryme, I. F. (1991) *Biochem. J.*, in the press
- Hesketh, J. E., Campbell, G. P. & Reeds, P. J. (1986) *Biosci. Rep.* **6**, 797–804
- Heuveljans, J. H., Pieper, F. R., Ramaekers, F. C. S., Timmermans, L. J. M., Kuipers, H., Bloemendal, H. & van Venrooij, W. J. (1989) *Exp. Cell Res.* **181**, 317–330
- Lenk, R., Ransom, L., Kaufmann, Y. & Penman, S. (1977) *Cell* **10**, 67–78
- Nielsen, P., Goelz, S. & Trachsel, H. (1983) *Cell Biol. Int. Rep.* **7**, 245–254
- Pondel, M. D. & King, M. L. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7612–7616
- Ramaekers, F., Benedetti, E., Dunia, I., Vorschenbosch, P. & Bloemendal, H. (1983) *Biochim. Biophys. Acta* **740**, 441–448
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Shires, T. K. & Pitot, H. C. (1973) *Adv. Enzyme Regul.* **11**, 255–272
- Singer, R. H., Langevin, G. L. & Lawrence, J. B. (1989) *J. Cell Biol.* **108**, 2343–2353
- Toh, B., Lolait, S., Mathy, J. & Baum, R. (1980) *Cell Tissue Res.* **211**, 163–169
- van Venrooij, W. J., Sillekens, P. T. G., van Eekelen, C. A. G. & Reinders, R. T. (1981) *Exp. Cell Res.* **135**, 79–91
- Vedeler, A., Pryme, I. F. & Hesketh, J. E. (1991) *Mol. Cell Biochem.*, in the press
- Wolosewick, J. J. & Porter, K. R. (1976) *Am. J. Anat.* **147**, 303–324

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