Subcellular localization of low-molecular-weight RNA components in rat liver

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The subcellular localization of the four major low-molecular-weight RNA components, D, C, A and L, was studied in rat liver cells. The cells were fractionated by a non-aqueous technique into a nuclear and a cytoplasmic fraction. The cytoplasm contained 43% of component D, 57% of component C and more than 80% of component L.

Vertebrate cells contain metabolically stable low-molecular-weight RNA components (Naora, 1977; Hellung-Larsen, 1977; Hellung-Larsen & Frederiksen, 1977; Frederiksen & Hellung-Larsen, 1979). The four major low-molecular-weight RNA components are called D, C, A and L, and consist of from 171 to about 300 nucleotides. The subcellular localization of the four low-molecular-weight RNA components has been studied by several investigators, and the components were originally found in the cell nucleus and claimed to be absent from the cytoplasm (Dingman & Peacock, 1968; Larsen *et al.*, 1968; Weinberg & Penman, 1968; Ro-Choi & Busch, 1974; Raj *et al.*, 1975). Other reports describe the presence of component L in the cytoplasm (Walker *et al.*, 1974; Zieve & Penman, 1976; Gunning *et al.*, 1979) and the presence of host-cell component L in the budding-type viruses (Bishop *et al.*, 1970; Erikson *et al.*, 1973; Emanoil-Ravicovitch *et al.*, 1973). In CHO cells 50% of components D and C are present in ribonucleoprotein particles in the cytoplasm (Enger & Walters, 1970), in chick forebrain 20% of component C and 8% of component D are in the cytoplasm (Gunning *et al.*, 1979) and in HeLa cells 25–40% of component C is in the cytoplasm (Zieve & Penman, 1976). In BHK cells components D, C and A are about equally distributed between the nuclear and cytoplasmic compartments (Frederiksen & Hellung-Larsen, 1975), and in the rat liver cells low-molecular-weight RNA components are shown to be part of the nuclear skeleton and to exist in the cytoplasm associated with membranes of the endoplasmic reticulum (Miller *et al.*, 1978). By a non-aqueous glycerol technique, components C and D were found in the nuclear fraction (Eliceiri & Gurney, 1978). These apparently contradictory

findings suggested a reinvestigation of the localization of low-molecular-weight RNA components in mammalian cells. In order to understand the function of this RNA, knowledge about the localization is important. The present paper describes the results obtained by a non-aqueous fractionation of rat liver cells.

Materials and methods

Wistar rats weighing 200 g were fed *ad libitum*, but starved for 24 h before excision of the liver. The animals were injected with 2.5 mCi of [5-³H]orotic acid (sp. radioactivity 0.25 Ci/mmol) intraperitoneally and labelled for 20 h.

Non-aqueous fractionation

The rats were anaesthetized with diethyl ether and the livers removed and frozen within a few seconds to –196°C in a freeze-clamp which had been precooled in liquid nitrogen. The 1–2 mm-thick liver pieces were freeze-dried in a desiccator over P₂O₅ under vacuum (oil pump) for 16–18 h at room temperature. The dried material was then broken to pieces by treatment with porcelain balls (diameter 2 cm) in a slowly rotating desiccator. The desiccator contained a steel net (mesh diameter 0.2 mm) which allowed small particles to filter to the bottom of the desiccator, thereby avoiding unnecessary mechanical treatment of the tissue. The powder was further dried under vacuum (oil pump) over P₂O₅ for 1 day at 4°C. The dried cellular material was homogenized in light petroleum (boiling range 60–80°C) and separated into a nuclear and several cytoplasmic fractions by centrifugation in the following mixtures of cyclohexane and tetrachloromethane as described by Siebert (Siebert, 1964,

1967; Shakoory *et al.*, 1972). Three centrifugations (900 g, 20 min) in 49.0% (v/v) tetrachloromethane gave cytoplasm A. The pellet was then suspended in 62.2% (v/v) tetrachloromethane and centrifuged twice (2000 g, 60 min) to give cytoplasm B. The nuclear pellet was purified further by centrifugation twice in 64.1% (v/v) tetrachloromethane (cytoplasm C), and finally the nuclei were floated in 69.0% (v/v) tetrachloromethane (2000 g, 60 min). The pure nuclei were obtained from the latter mixture by adding cyclohexane to lower the density and pelleting the nuclei by centrifugation (36 000 g, 5 min).

Aqueous fractionation

Rat livers were excised under ether anaesthesia and homogenized in 2 vol. of ice-cold 0.25 M-sucrose in TKM buffer (25 mM-KCl, 5 mM-MgCl₂, 0.05 M-Tris/HCl, pH 7.5) by a Potter-Elvehjem homogenizer. The homogenate was centrifuged in a step gradient of 1.67 M- and 2.3 M-sucrose in TKM buffer as described by Blobel & Potter (1967).

RNA extraction and purification

Aqueous solutions were routinely treated with diethyl pyrocarbonate.

Cell fractions obtained in non-aqueous media were treated as follows. About 200 mg of dry powder was stirred for 5 min in 1 ml of chloroform with 25 μ l of diethyl pyrocarbonate, and 0.5 ml of 10% (w/v) sodium dodecyl sulphate in 0.05 M-sodium acetate (pH 5.1) was then added to the suspension. The mixture was stirred for 30 s, and 10 ml of 0.05 M-sodium acetate (pH 5.1) and 10 ml of phenol saturated with water and containing 0.1% 8-hydroxyquinoline were then added. The material was extracted at 0°C for 20 min by vigorous shaking, or for 5 min at 55°C. After centrifugation for 5 min at 1000 g, the RNA was precipitated from the aqueous phase with 2.5 vol of ethanol containing 0.2% potassium acetate. RNA was then dissolved in 5 ml of a mixture of 1% (w/v) tri-isopropyl-naphthalenesulphonate, 6% (w/v) sodium 4-aminosalicylate, 1% (w/v) NaCl and 6% (w/v) butan-2-ol, and extracted with 5 ml of a mixture of 100 g of phenol, 14 ml of *m*-cresol and 11 ml of water containing 0.1% 8-hydroxyquinoline (Parish & Kirby, 1967). After extraction at 22°C for 10 min, the mixture was centrifuged for 10 min at 19 000 rev./min (44 000 g). RNA was precipitated from the aqueous phase by addition of 2 vol. of ethanol and the nuclear RNA purified by reprecipitation (Hellung-Larsen & Frederiksen, 1972). Cytoplasmic RNA was centrifuged on a 5–20% (w/v) sucrose gradient (in 0.1 M-NaCl/1 mM-EDTA/5 mM-Tris/HCl; pH 7.2) to obtain the 4–9 S fraction of RNA containing the low-molecular-weight RNA components (Truman & Frederiksen, 1969).

Cell fractions obtained in aqueous media were extracted at 0°C with reticulocyte standard buffer (10 mM-NaCl, 1.5 mM-MgCl₂, 10 mM-Tris/HCl, pH 7.4) and phenol saturated with water and containing 0.1% 8-hydroxyquinoline, or with 0.5% (w/v) sodium dodecyl sulphate in 0.05 M-sodium acetate (pH 5.1) and phenol containing 0.1% 8-hydroxyquinoline at 55°C (Frederiksen *et al.*, 1974).

Analysis of RNA

Electrophoresis on 3% and 10% polyacrylamide gels (Hellung-Larsen & Frederiksen, 1972; Frederiksen *et al.*, 1974), scanning of u.v. absorption and counting of radioactivity in gel slices (Hellung-Larsen, 1971a,b) have been described previously.

Results

The aim of the present investigation was to examine whether low-molecular-weight RNA components, which were originally isolated from the cell nuclei of different mammalian cells, are present in the cytoplasm in amounts exceeding those released from the nuclei during mitosis (Rein, 1971). In order to prevent leakage of nuclear material during isolation of the cell fractions, we have used a non-aqueous technique developed by Siebert (Siebert, 1964, 1967; Shakoory *et al.*, 1972) for the isolation of nuclear and cytoplasmic fractions from liver cells. The livers are frozen to –196°C within a few seconds, freeze-dried and homogenized in light petroleum, and a nuclear fraction was obtained by means of several density-gradient centrifugations in different mixtures of cyclohexane and tetrachloromethane.

The nuclei were suspended in 0.1 M-citric acid, stained with Crystal Violet and examined in the optical microscope. The nuclei had the morphology of intact nuclei obtained by the aqueous-sucrose method described below, and only a little cytoplasmic material adhered to the nuclei.

The purest cytoplasmic fraction was analysed in the present experiments (cytoplasm A). Since leaking out of nuclear material cannot occur, the only possible contamination of the cytoplasm would be from nuclear fragments with a density too low to be removed by the centrifugation. The purity of the cytoplasmic fraction was therefore checked by measuring the content of DNA. No DNA could be detected by the Burton (1956) method, which shows that no nuclear material was present in this fraction.

It was very difficult to extract pure undegraded RNA from cellular fractions obtained by this non-aqueous technique. Different phenol extraction procedures (Hellung-Larsen & Frederiksen, 1972; Frederiksen *et al.*, 1974), the Kirby extraction (Parish & Kirby, 1967), and attempts to suspend the

dry powder in buffers with 5% (w/v) sodium dodecyl sulphate and diethyl pyrocarbonate at 0°C followed by phenol or phenol/chloroform extractions, led to varying extents of degradation of RNA. Degradation was determined from the gel-electrophoretic analysis of rRNA on 3%-polyacrylamide gels. These findings are in line with previous results, which demonstrates that ribonucleases can be activated up to 8-fold (Giannitis *et al.*, 1967) when liver cells are treated with the organic solvents used for the isolation of nuclei and cytoplasm from liver cells in the present paper.

In order to prevent degradation of RNA, the dry powders containing nuclear and cytoplasmic material were suspended in chloroform containing diethyl pyrocarbonate. After stirring at 0°C for 5 min, the conventional phenol extraction procedures could be applied and RNA obtained undegraded (Fig. 1). Diethyl pyrocarbonate in the concentrations used here will not ethoxycarbonylate

Fig. 1. Radioactivity profile of rRNA extracted from different fractions of rat liver cells obtained by a non-aqueous technique

Rat liver cells were labelled *in vivo* by injection of 2.5 mCi of [^3H]orotic acid per rat. The livers were excised, quickly frozen by a freeze-clamp technique and freeze-dried. The dry powder was homogenized in light petroleum and the homogenate separated into a nuclear fraction and three cytoplasmic fractions by centrifugations in mixtures of cyclohexane and tetrachloromethane as described in the Materials and methods section. The dry cellular material (homogenate or cytoplasm A) was suspended in chloroform and treated with diethyl pyrocarbonate. RNA was extracted with sodium dodecyl sulphate and phenol and precipitated from the aqueous phase with ethanol. RNA was re-extracted by the method described by Parish & Kirby (1967) and electrophoresed on 3%-polyacrylamide gels, and the radioactivity in 1 mm slices determined (Hellung-Larsen, 1971a,b).

RNA (Öberg, 1971) to any extent that will change the gel-electrophoretic mobility of the RNA (Hellung-Larsen & Frederiksen, 1975). One more purification step has to be applied before the RNA can be analysed well on 10% polyacrylamide gels. After phenol extraction, the RNA preparations still contain contaminating material, which interferes with the electrophoresis of RNA. This material is removed by the Kirby extraction (Parish & Kirby, 1967).

The A_{254} patterns of low-molecular-weight RNA from rat liver nuclei and the cytoplasmic fraction A obtained by the non-aqueous technique are shown in Fig. 2. Little or no component L is present in the nuclei, whereas the pattern of components A, C and D is the usual one, taking into account that this RNA is extracted by the hot-phenol procedure, which will extract 5.5S RNA and partly convert 5S into the denatured 5S conformer (5'S) as described by Aubert *et al.* (1968) and Weinberg & Penman (1968). In the cytoplasmic fraction components L, A, C and D are all present.

This non-aqueous fractionation procedure gives rise to the three cytoplasmic fractions A, B and C. Fraction A is the purest fraction and contains no nuclear material, as judged from the absence of DNA, whereas fraction C contains debris of broken nuclei. This fact makes it impossible directly to determine the distribution of low-molecular-weight RNA between the nuclei and cytoplasm unless relatively large correction for cross-contamination is made. We have therefore determined the relative amount of low-molecular-weight RNA in the cytoplasmic fraction after labelling of rat liver RNA *in vivo* with [^3H]orotic acid. The radioactivity profile of a purified RNA sample is shown in Fig. 3. The radioactivity found in components L, C and D relative to the amounts of radioactivity in 5S RNA in the cytoplasm was compared with these ratios in RNA preparations from whole liver homogenate. In two experiments with homogenates and four experiments with cytoplasm, it was found that 80% of component L, 57% of component C and 43% of component D are in the cytoplasm. The radioactivity measurements may not measure the exact amounts of RNA, owing to the labelling conditions *in vivo*, but since the distribution in the fractions is measured relative to an internal marker of 5S RNA, which is predominantly a cytoplasmic component, this method should give a good estimate of the distribution of low-molecular-weight RNA components. The amount of radioactivity incorporated into component A is too small to be measured reliably.

The low-molecular-weight-RNA patterns in Figs. 2 and 3 show that liver cells contain 2–3 times more L and H components compared with components, A, C and D than is found in other cell types

Fig. 2. Absorption profile of low-molecular-weight RNA from rat liver nuclei and cytoplasm obtained by a non-aqueous technique

RNA was obtained as described in the legend to Fig. 1 and analysed on 10% polyacrylamide gels. The gels were scanned in a Joyce-Loebl scanner, and the absorption profiles show the low-molecular-weight components L, A, C, D and H, as well as tRNA, 5S RNA, 5'S RNA and 5.5S RNA. The 5.5S RNA is partly released from rRNA during the extraction with phenol at 55°C, and this temperature also partly convert 5S RNA into the denatured conformer 5'S RNA (Aubert *et al.*, 1968; Weinberg & Penman, 1968). The components were characterized by co-electrophoresis with low-molecular-weight RNA components from Ehrlich ascites-tumour cells (Hellung-Larsen & Frederiksen, 1977).

Fig. 3. Radioactivity profile of low-molecular-weight RNA from rat liver cytoplasm obtained by a non-aqueous technique

RNA was obtained from liver cells labelled *in vivo* as described in the legend to Fig. 1, except that the temperature of phenol extraction was 0°C. The cytoplasmic RNA was centrifuged on a 5–20% (w/v) sucrose gradient and the 4–9S RNA fraction precipitated with ethanol. This RNA was electrophoresed on a 10%-polyacrylamide gel and the radioactivity in 1 mm slices determined.

Fig. 4. Radioactivity profile of low-molecular-weight RNA from a homogenate from rat liver cells and from nuclei isolated by an aqueous technique

Rat liver cells were labelled *in vivo* by injection of 2.5 mCi of [^3H]orotic acid per rat. The livers were excised and homogenized in ice-cold sucrose by a Potter-Elvehjem homogenizer and the nuclei obtained by centrifugation in a step gradient of sucrose as described by Blobel & Potter (1967). RNA was extracted with phenol at 55°C, purified and electrophoresed on 10%-polyacrylamide gels and the radioactivity in 1 mm slices determined.

(Hellung-Larsen & Frederiksen, 1977; Frederiksen & Hellung-Larsen, 1979). In order to confirm these findings, we labelled rat liver cells with [^3H]orotic acid and isolated the nuclei by the sucrose-gradient

technique of Blobel & Potter (1967). The homogenate contains 2–3 times more component L than components A, C, D and 5S RNA (Fig. 4), and the nuclei show a large component-H peak and almost complete absence of component L.

Discussion

In rat liver cells some of the low-molecular-weight RNA species are found in ribonucleoprotein particles associated with the nuclear skeleton. The components are also found associated with the endoplasmic reticulum to a varying degree (Miller *et al.*, 1978). That paper also stresses the importance of proteolytic activity for the release of RNA from the nuclei isolated in aqueous media. When nuclei are isolated in aqueous media, it is impossible to control completely the leakage of low-molecular-weight RNA during the isolation. We have therefore applied a non-aqueous isolation technique (Siebert, 1964, 1967; Shakoori *et al.*, 1972), which prevents any leakage of nuclear material into the cytoplasm. Rat livers were quickly frozen in liquid nitrogen, freeze-dried and mechanically disrupted, and the nuclei separated from the cytoplasm on the basis of difference in density. By this non-aqueous method we demonstrated the presence of components L, A, C and D in the cytoplasm of rat liver cells. The cytoplasm contain 57% of component C, 43% of component D and 80% of component L. This latter finding is in agreement with a previous demonstration of component L in the cytoplasm of different cells (Walker *et al.*, 1974; Zieve & Penman, 1976; Gunning *et al.*, 1979; Frederiksen & Hellung-Larsen, 1975). The low-molecular-weight RNA pattern obtained by the non-aqueous technique showed relatively more of components L and H than has been demonstrated in other cell types (Hellung-Larsen & Frederiksen, 1977; Frederiksen & Hellung-Larsen, 1979). When nuclei were isolated from liver cells by the sucrose-gradient technique of Blobel & Potter (1967), we confirmed that these cells contained 2–3 times more components L and H than other cells and also confirmed the almost complete absence of component L from the nuclei.

It can be firmly concluded from this and other investigations that component L is not a nuclear low-molecular-weight RNA, but is predominantly localized in the cytoplasm. This should obviously lead to a search for a function of component L in the cytoplasm. One possibility is that component L is involved in protein synthesis, since the component is partly associated with the polyribosomal fraction (Walker *et al.*, 1974; Gunning *et al.*, 1979). In rat liver tissue only about 2% of the cells are in mitosis, but the cells are actively engaged in protein synthesis and they are rich in endoplasmic reticulum. This could explain why liver cells contain relatively more

of component L than do other cells. The present demonstration that significant amounts of components C and D are present in the cytoplasm does not necessarily suggest that these components exert their function in the cytoplasm. However, it adds a question mark to the hitherto very strong emphasis put on the nuclear appearance of low-molecular-weight RNA.

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References

- Aubert, M., Scott, J. F., Reynier, M. & Monier, R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 292–299
- Bishop, J. M., Levinson, W., Sullivan, D., Fanshier, L., Quintrell, N. & Jackson, J. (1970) *Virology* **42**, 927–937
- Blobel, G. & Potter, V. R. (1967) *Science* **154**, 1662–1665
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Dingman, C. W. & Peacock, A. C. (1968) *Biochemistry* **7**, 659–668
- Eliceiri, G. L. & Gurney, T., Jr. (1978) *Biochem. Biophys. Res. Commun.* **81**, 915–919
- Emanoil-Ravicovitch, R., Larsen, C. J., Bazilier, M., Robin, J., Peries, J. & Boiron, M. (1973) *J. Virol.* **12**, 1625–1627
- Enger, M. D. & Walters, R. A. (1970) *Biochemistry* **9**, 3551–3562
- Erikson, E., Erikson, R. L., Henry, B. & Pace, N. R. (1973) *Virology* **53**, 40–46
- Frederiksen, S. & Hellung-Larsen, P. (1975) *FEBS Lett.* **58**, 374–378
- Frederiksen, S. & Hellung-Larsen, P. (1979) *Specific Eukaryotic Genes: Alfred Benzon Symp.* **13**, 457–471
- Frederiksen, S., Pedersen, I. R., Hellung-Larsen, P. & Engberg, J. (1974) *Biochim. Biophys. Acta* **340**, 64–76
- Giannitis, V. D., Kessebring, K., Schwarzhaupt, W. & Siebert, G. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 1133–1140
- Gunning, P. W., Austin, L. & Jeffrey, P. L. (1979) *J. Neurochem.* **32**, 1725–1736
- Hellung-Larsen, P. (1971a) *Acta Chem. Scand.* **25**, 1359–1369
- Hellung-Larsen, P. (1971b) *Anal. Biochem.* **39**, 454–461
- Hellung-Larsen, P. (1977) Ph.D. Thesis, University of Copenhagen
- Hellung-Larsen, P. & Frederiksen, S. (1972) *Biochim. Biophys. Acta* **262**, 290–307
- Hellung-Larsen, P. & Frederiksen, S. (1975) *Int. J. Biochem.* **6**, 361–370
- Hellung-Larsen, P. & Frederiksen, S. (1977) *Comp. Biochem. Physiol.* **58B**, 273–281
- Larsen, C. J., Galibert, F., Hampe, A. & Boiron, M. (1968) *C. R. Hebd. Seances Acad. Sci., Ser. D* **267**, 110–113
- Miller, T. E., Chen-Ya, H. & Pogo, A. O. (1978) *J. Cell Biol.* **76**, 692–704

- Naora, H. (1977) in *The Ribonucleic Acids* (Stewart, P. R. & Letham, D. S., eds.), pp. 61–71, Springer Verlag, New York, Heidelberg and Berlin
- Öberg, B. (1971) *Eur. J. Biochem.* **19**, 496–501
- Parish, J. H. & Kirby, K. S. (1967) *Biochim. Biophys. Acta* **142**, 273–275
- Raj, N. B. K., Ro-Choi, T. S. & Busch, H. (1975) *Biochemistry* **14**, 4380–4385
- Rein, A. (1971) *Biochim. Biophys. Acta* **232**, 306–313
- Ro-Choi, T. S. & Busch, H. (1974) in *Cell Nucleus* (Busch, H., ed.), vol. 3, pp. 151–208, Academic Press, New York
- Shakoori, A. R., Romen, W., Oelschläger, W., Schlatterer, B. & Siebert, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 1735–1748
- Siebert, G. (1964) in *Biochemische Taschenbuch* (Raun, H. M., ed.), 2nd edn., vol. 2, pp. 541–546, Springer Verlag, New York, Heidelberg and Berlin
- Siebert, G. (1967) *Methods Cancer Res.* **2**, 287–301
- Truman, J. T. & Frederiksen, S. (1969) *Biochim. Biophys. Acta* **182**, 35–45
- Walker, T. A., Pace, N. R., Erikson, R. L., Erikson, E. & Behr, F. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3390–3394
- Weinberg, R. A. & Penman, S. (1968) *J. Mol. Biol.* **38**, 289–304
- Zieve, G. & Penman, S. (1976) *Cell* **8**, 19–31