Volume 247, number 1, 51–54 FEB 06986 April 1989

In vitro reconstitution of hnRNP particles

Johannes Schenkel and Peter M. Kloetzel*

University of Heidelberg Medical School, Im Neuenheimer Feld 324, Raum 150 and **Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), INF 282, D-6900 Heidelberg, FRG*

Received 16 January 1989

The assembly of hnRNP-like particles was studied by in vitro reconstitution, UV-crosslinking and CsCl-equilibrium centrifugation. Using total nuclear protein and RNA extracts from HeLa cells for RNP reconstitution, RNP particles sedimenting with the same buoyant density of $\rho = 1.4$ g/cm^3 as 'native' 40 S core hnRNPs were obtained. Under the stringent reconstitution conditions used, hnRNP complexes containing only the C1-core hnRNP protein could be identified.

hnRNP; Ribonucleoprotein; Crosslinking; Density gradient centrifugation

1. INTRODUCTION

The assembly of nuclear RNA/protein complexes is essential for the post-transcriptional modification of pre-mRNA. Not only are the individual binding behaviour of the proteins and the nature of RNA prerequisites to the formation of the complex of hnRNP but also the sterical structure of these complexes is responsible for the activation of mRNA. hnRNP are usually isolated in a monomeric form sedimenting at ≈40 S. They are composed of nascent RNA and of proteins of which the 'core proteins' predominate [1]. The nature of the association between core proteins and RNA has been the subject of previous reports [1–9]. In those studies, however, the binding properties of either a mixture of all core proteins to in vitro synthesized nucleic acids or the binding of the individual core proteins to exogenous RNA was investigated. Here, we have studied the assembly of hnRNP using nuclear proteins and native nuclear RNA which participate in vivo in RNP complex formation. To monitor the complex-forming proteins, covalent links between proteins and RNA were induced by UV crosslinking. The RNP particles formed were purified by CsCl density gradient centrifugation and the RNA-binding proteins examined by SDS-PAGE.

Correspondence address: J.S. Schenkel, University of Heidelberg Medical School, Im Neuenheimer Feld 324, Raum 150, D-6900 Heidelberg, FRG

Abbreviations: hnRNA, heterogenous nuclear ribonucleic acid; hnRNP, heterogenous nuclear ribonucleoprotein particles; pre-mRNA, pre-messenger ribonucleic acid; snRNA, small nuclear ribonucleic acid; UV, ultraviolet

2. MATERIALS AND METHODS

2.1. *Cell culture and RNA labelling*

HeLa-S-3 cells were grown as described [3]. For RNA labelling 10^9 cells were resuspended in serum-free MEM medium and 1 mCi [5,6′-^{3}H]uridine added for 1 h to label rapidly synthesized RNA.

2.2. *Isolation of proteins and RNA*

Nuclei and hnRNP were isolated as in [3,12]. Purified nuclei were homogenised in buffer of pH 8, applied to centrifugation on 4–6 M CsCl gradients in Tris/EDTA buffer [10 mM Tris (pH 8.0), 1 mM EDTA] (Beckman SW 60 rotor, $\omega^2 t = 2 \times 10^{12}$ rad^2/s^{-1}). Proteins were collected from the top and RNA from the bottom of the gradient. Proteins and RNA were dialysed vs Tris/EDTA and dissolved in Tris/EDTA with 8 M urea and 2 M NaCl [13,14].

2.3. *Reconstitution of RNP-complexes*

Dissolved proteins and RNA were combined and dialysed vs 100 ml of 8 M urea, 2 M NaCl in Tris/EDTA buffer. For reconstitution, the ionic strength was slowly reduced to 0.14 M NaCl without urea by dialysis.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2.4. *UV crosslinking and density gradient centrifugation; SDS-PAGE*

Reconstituted RNP complexes were UV-irradiated for 6 min as described in [4] and ethanol precipitated. The RNP pellets were dissolved in buffer containing 8 M urea, 0.1% β-mercaptoethanol and Tris/EDTA, incubated for 2 min at 80°C and diluted to 4 M urea. Non-irradiated RNP material was dissolved in Tris/EDTA buffer with 2% NP-40 and 0.1% β-mercaptoethanol. RNP probes were applied to CsCl or Cs_2SO_4 gradient centrifugation (as described above).

The gradients were fractionated and the radioactivity determined by precipitation with trichloroacetic acid.

Before analysis of the proteins by SDS-PAGE, free RNA was digested with 500 U micrococcal nuclease and 25 μg RNase A for 1 h at 37°C. After electrophoresis, the gels were stained, treated with ^{3}H-enhancer (NEN), dried and exposed for 5–10 days [15].

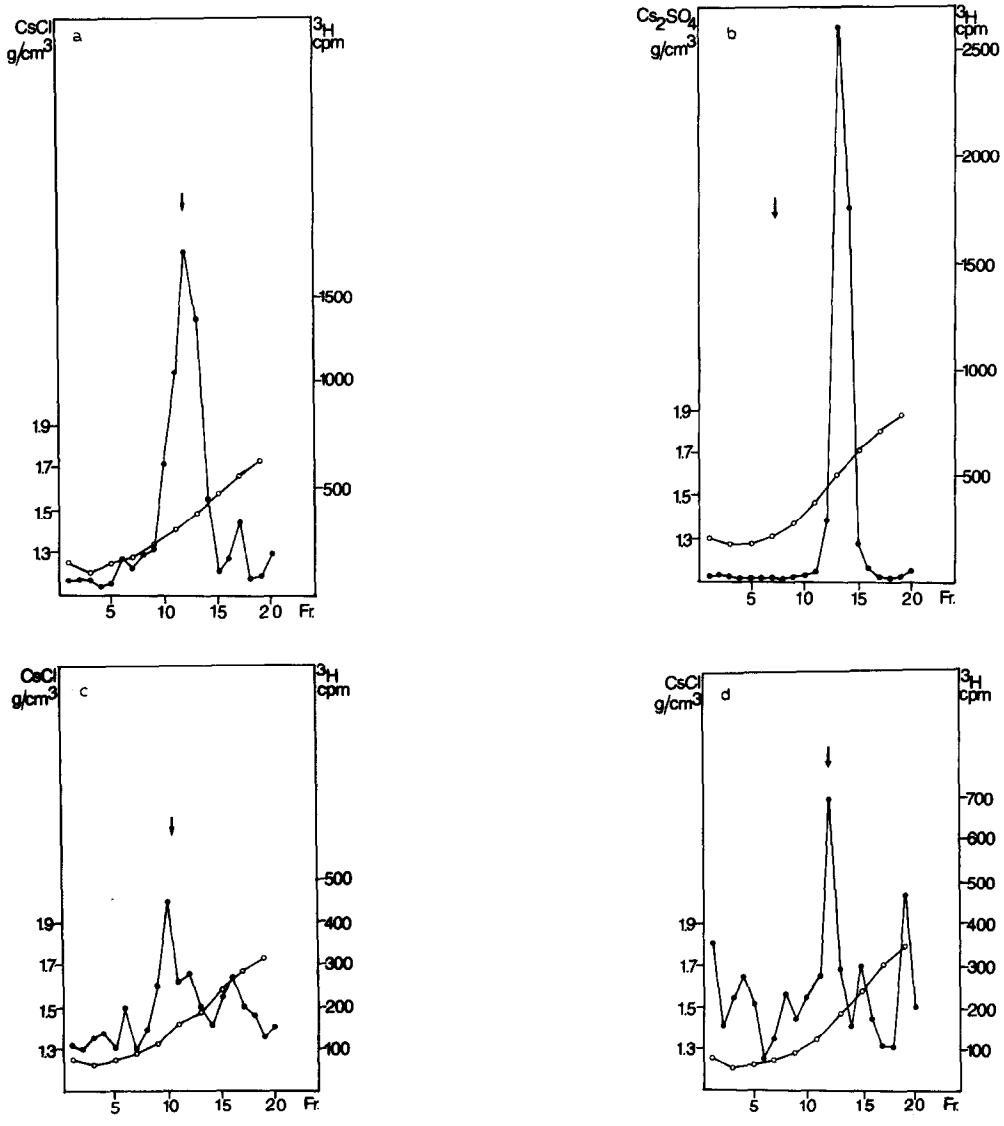

Fig.1. Isopycnic equilibrium centrifugation of hnRNP. (•) Radioactivity of acid-precipitable material; (○) salt density. The arrow indicates the buoyant density expected for hnRNP. (a) CsCl equilibrium centrifugation of UV-treated native 40 S core hnRNP. (b) Cs_2SO_4 equilibrium centrifugation of dissociated 40 S hnRNP. (c) CsCl equilibrium centrifugation of hnRNP reconstituted from 40 S hnRNP RNA and 40 S hnRNP protein after UV crosslinking. (d) CsCl equilibrium centrifugation of UV-treated hnRNP reconstituted from the total nuclear protein and RNA extract.

3. RESULTS

[5,6′-^{3}H]Uridine-labelled 40 S core hnRNP was isolated and UV crosslinked. After CsCl gradient centrifugation ^{3}H-labelled hnRNP bands were observed at a buoyant density of ϱ = 1.4 g/cm^3, reflecting an RNA/protein ratio of 1:4 (fig.1a) [16,17]. For initial reconstitution experiments and control ^{3}H-labelled 40 S core hnRNP complexes were dissociated into protein and RNA. To test for complete dissociation, an aliquot was subjected to Cs_2SO_4 density gradient centrifugation (fig.1b). hnRNP complexes were reconstituted from dissociated RNP material by dialysis, subjected to UV crosslinking and applied to CsCl gradients. As shown in fig.1c, the reconstituted labelled hnRNP gave bands at a buoyant density of ϱ = 1.4 g/cm^3, which is identical to that observed for native hnRNP core particles. An identical result was obtained when hnRNP was reconstituted from total nuclear protein and RNA extracts, i.e. at molar protein/RNA ratios close to those in isolated nuclei (fig.1d). Without UV irradiation of reconstituted hnRNP, no significant amounts of [^{3}H]uridine activity were detected at a buoyant density of ϱ = 1.40 g/cm^3 (not shown). To identify proteins bound to the rapidly labelled RNA in reconstituted hnRNP, proteins were marked by residual ^{3}H-labelled nucleotides still covalently bound to the RNP-proteins after nuclease and RNase treatment. Under the stringent conditions used, core protein C_1 was found to be the only protein bound to labelled RNA in hnRNP complexes reconstituted from either total nuclear protein and RNA extracts (fig.2, lane C), hnRNP reconstituted from separated protein and RNA components of 40 S core hnRNP particles (lane B) and in 40 S core hnRNP complexes (lane D).

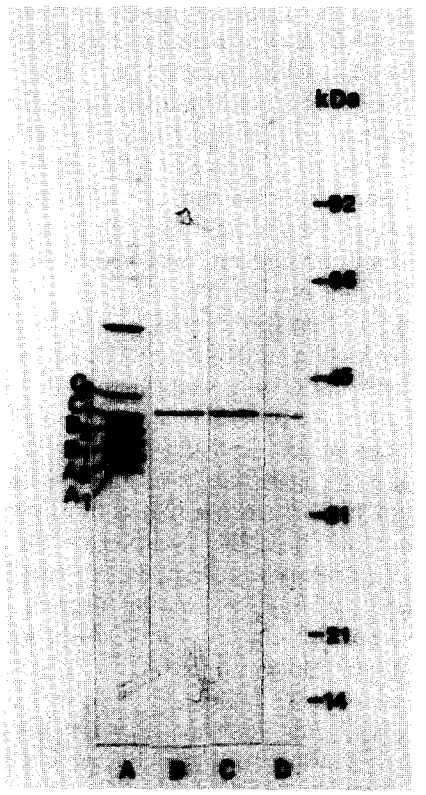

Fig.2. SDS-PAGE of hnRNP proteins: (lane A) Coomassie blue-stained hnRNP core proteins. Radioactivity fluorescence of RNA-binding proteins after UV crosslinking: hnRNP reconstituted from 40 S hnRNA and 40 S hnRNP proteins (B), hnRNP reconstituted from nuclear extracts (C) and from 'native' 40 S core hnRNP (D).

4. DISCUSSION

To avoid artificial RNA-protein interaction due to unphysiological RNA/protein ratios, hnRNP complexes were reconstituted from total nuclear protein and RNA extracts under conditions which closely resemble the molar RNA/protein ratios in vivo. This is in contrast to other reports [6,9,10] employing isolated protein and in vitro synthesized RNA. In order to detect only those proteins which interact strongly with nascent RNA, hnRNP complexes were reconstituted and analysed under stringent experimental conditions. The participating proteins were then detected after induction of covalent links between the protein moiety and the RNA by UV crosslinking [3,4]. Depending on the amount of energy applied during UV irradiation, different members of RNP proteins can be crosslinked to RNA. Thus, under the low UV energy conditions employed in the present experiments, only those RNA-binding proteins are detected which bind most readily and tightly to nascent RNA. Our data show that under low-energy conditions and independently of the nuclear RNA-protein extracts, the C_1 core protein is the only hnRNP protein found to be tightly associated with nascent RNA. Interestingly, the reconstituted hnRNP complexes possess a buoyant density of ϱ = 1.4 g/cm^3, i.e. an RNA/protein ratio of 1:4,

which is identical to that of native hnRNP. Thus, a single hnRNP core protein can form RNP complexes which, with respect to their overall RNA/protein ratio, are indistinguishable from RNP complexes containing the whole set of core proteins. This is in accord with previous data showing that a 60 kDa nuclear RNP protein of *Xenopus laevis* which may function as a core protein in the amphibian, can form RNP complexes of normal buoyant density [20].

The finding that the C_1 core protein on its own can form stabile hnRNP complexes with an RNA/protein ratio of 1:4 appears to be most interesting, since the protein is known to play an important role in the splicing process [2]. Since ribosomal RNA does not form hnRNA-like complexes with these proteins [20] (not shown), the formation of hnRNP complexes at molar RNA/protein ratios of 1:4 appears to be an intrinsic property of hnRNA-binding proteins and a result of specific, thus far unknown, structural features of the nascent hnRNA.

Acknowledgements: We thank Professor E.K.F. Bautz for excellent working conditions and helpful discussions and Professor F.A. Bautz for providing us with HeLa cells. This work was supported by the Stiftung Volkswagenwerk.

REFERENCES

[1] Beyer, A.L., Christensen, M.E., Walker, B.W. and Le Stourgeon, W.M. (1977) Cell 11, 127–138.
[2] Choi, Y.D., Grabowsky, P.J., Sharp, P.A. and Dreyfuss, G. (1986) Science 231, 1534–1539.
[3] Schenkel, J., Sekeris, C.E., Alonso, A. and Bautz, E.K.F. (1988) Eur. J. Biochem. 171, 565–569.
[4] Van Eekelen, C.A.G. and Van Venrooij, W.J. (1981) J. Cell Biol. 88, 554–563.
[5] Economides, I.V. and Pederson, T. (1983) Proc. Natl. Acad. Sci. USA 80, 1599–1602.
[6] Pullman, J.M. and Martin, T.E. (1983) J. Cell Biol. 97, 99–111.
[7] Sekeris, C.E. and Niessing, J. (1975) Biochem. Biophys. Res. Commun. 62, 642–650.
[8] Le Stourgeon, W.M., Lothstein, L., Walker, B.W. and Beyer, A.L. (1981) The Cell Nucleus (Bush, H. ed.) vol.9, pp.49–87, Academic Press, New York.
[9] Wilk, H.E., Angeli, G. and Schäfer, K.P. (1983) Biochemistry 22, 4592–4600.
[10] Conway, G., Wooley, J., Bibring, T. and Le Stourgeon, W.M. (1988) Mol. Cell Biol. 8, 2884–2895.
[11] Hügle, B., Guldner, H., Bautz, F.A. and Alonso, A. (1982) Exp. Cell Res. 142, 119–126.
[12] Louis, C. and Sekeris, C.E. (1976) Exp. Cell Res. 102, 317–328.
[13] Flytzanis, C., Alonso, A., Louis, C., Krieg, L. and Sekeris, C.E. (1978) FEBS Lett. 96, 201–206.
[14] Northeman, W., Seifert, H. and Heinrich, P.C. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 877–888.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Niessing, J. and Sekeris, C.E. (1971) Biochim. Biophys. Acta 247, 391–403.
[17] Pederson, T. (1974) J. Mol. Biol. 83, 163–183.
[18] Pederson, T. and Davis, N.G. (1980) J. Cell Biol. 87, 47–54.
[19] Economides, I.V. and Pederson, T. (1983) Proc. Natl. Acad. Sci. USA 80, 4296–4300.
[20] Kloetzel, P.M., Johnson, R.M. and Sommerville, J. (1982) Eur. J. Biochem. 127, 301–308.