Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 457-462

ISOLATION OF A PURE U_1 snRNP FROM HELA CELLS

Joannes SRI-WIDADA, Chantal ASSENS, Jean-Pierre LIAUTARD, Philippe JEANTEUR and Claude BRUNEL.

Laboratoire de Biochimie, Centre Paul Lamarque, 2 avenue Bertin Sans, BP 5054 34033 MONTPELLIER CEDEX and Laboratoire de Biologie Moléculaire, Université des Sciences et Techniques du Languedoc, Place Eugène Bataillon, 34060 MONTPELLIER CEDEX. FRANCE .

Received November 17, 1981

We had previously reported the extensive purification of snRNPs (ribonucleoprotein complexes containing small nuclear RNAs) from HeLa hnRNP (ribonucleoprotein complexes containing the heterogenous nuclear RNA) by taking advantage of their ability to withstand centrifugation in cesium chloride containing 0.5% sarkosyl (C. BRUNEL et al 1981, Nucleic Acids Research, 9, 815-830). Such purified snRNPs contained the five nucleoplasmic snRNAs (U_1, U_2, U_4, U_5 and U_6) and a very simple protein complement of 4-5 polypeptides between 10 and 14,000 daltons. The possible involvement of U_1 in messenger RNA splicing makes it crucial to obtain in pure form the individual U_1 snRNP, if an in vitro splicing system is to be reconstructed. The present work reports the purification of U_1 snRNP and shows that it contains exactly the same polypeptides as the mixture of all five snRNPs suggesting that the specific function,if any, depends on the RNA moiety.

INTRODUCTION

It is well established that the small nuclear RNAs (snRNA) species U_2, U_1, U_4, U_5 and U_6 exist as RNA-protein complexes (snRNP)(1)which have now been characterized by their reactivity towards antibodies from patients with autoimmune rheumatic diseases (2,3) . We have also recently succeeded in purifying them from hnRNP by taking advantage of their ability to withstand a combination of high ionic strength as prevails in cesium chloride density gradients and of a moderately strong anionic detergent (sarkosyl) (4,5). In view of the current hypothesis that U_1 snRNA (6,7) in its RNP form (3), could be involved in splicing by base pairing of its 5' end with the intron borders of intron-exon junctions of hnRNA, it was of interest to obtain a pure U_1 snRNP which should provide a useful handle to approach splicing mechanism in vitro. This was accomplished by taking advantage of previous observations(8,9) which showed that U_1 snRNA species is selectively released from hnRNP under isotonic conditions. Under these conditions, U_1 RNA still in RNP form (this work) can be purified utilizing a combination of centrifugations

0006-291X/82/020457-06$01.00/0

in cesium chloride and sucrose in the presence of sarkosyl similar to that used previously (4) to obtain the mixture of purified snRNP. This U_1 snRNP turned out to have a protein complement identical to that of the mixture,suggesting that all five individual snRNPs have the same protein composition and therefore that the specificity of their function should depend only on their snRNA component.

MATERIALS AND METHODS

Conditions for cell growth and labeling, preparation of hnRNP and acrylamide gel electrophoresis of RNA were as previously described (4,5). Proteins were analysed on acrylamide gels after labeling *in vitro* by the ^{125}I Bolton and Hunter reagent according to Amersham recommendations except that snRNPs were in 30mM Tris HCl pH 8.0 containing 0.1M ammonium chloride, 5mM $MgCl_2$ and 1mM β-mercaptoethanol in place of the borate buffer. All other specific techniques are described in the text or in the legends to figures.

RESULTS AND DISCUSSION

^{32}P labeled hnRNP from HeLa cells were purified in RSB buffer through a discontinuous sucrose gradient, adjusted to 0.1 M NaCl by dialysis, and centrifuged through a sucrose gradient as shown in Fig. 1 A. The fractions were pooled as indicated and subjected to the electrophoretic analysis shown in Figure 1 B (see also Figure 2 track b). As expected, it was observed that fraction I essentially contained U_1 in addition to some hnRNA and U_1^* which is generated during the purification process by removal of the cap and seven adjacent nucleotides from the 5' end of U_1 (2,4).

All snRNAs species (including U_1) were present in the other fractions except U_2 which was almost exclusively concentrated in fraction III. These two last fractions were not further analysed .

In order to see whether U_1 and U_1^* snRNAs are present in fraction I in the form of snRNP, we have submitted it to the purification protocol previously used to purify the mixture of snRNPs (4). As seen in Figure 1C, centrifugation of fraction I in cesium chloride yielded a peak banding at a density of 1.43 g/cm^3 as previously observed for the mixture of snRNPs (4). This peak was further subjected to a second cycle of centrifugation in cesium chloride containing 0.5% sarkosyl and a final step of purification by velocity sedimentation in sucrose gradient containing 0.5% sarkosyl and 0.5 M KCl instead of 0.5 M cesium chloride as previously used (4). The sharp peak around 12 S in the final sucrose gradient was concentrated by high speed centrifugation.

Results of the electrophoretic analysis of the RNA species present at each stage of purification are shown in Figure 2.Several observations can

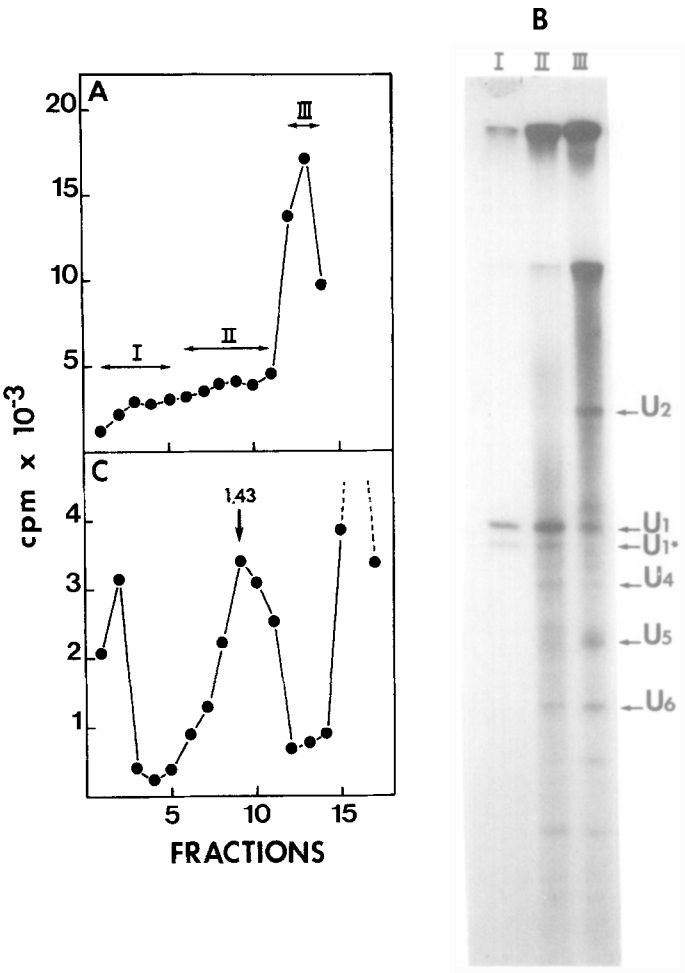

FIGURE 1 : Extraction of U_1 snRNP from hnRNP of HeLa cells.

A - Centrifugation of hnRNP in sucrose gradient. hnRNP from HeLa cells labeled for 36 hours with $H_3{}^{32}PO_4$ (10 µCi/ml) were prepared as described previously (10), dialysed against 0.1M NaCl in RSB buffer and incubated for 30 min. at 25°C. They were then layered on top of a 12 ml 15-30% (w/w) sucrose gradient in RSB buffer containing 0.1M NaCl and centrifuged at 35,000 rev/min in a SW 40 Beckman rotor for 16 hours. Aliquots of each fraction were analysed for RNA ^{32}P counts which are the difference between cold and hot trichloracetic acid-precipitable counts.

B - Electrophoretic analysis of RNA from fractions I, II and III : Pooled fractions I, II, III were analysed for RNA content as described previously(4) on a 12.5% polyacrylamide gel in Tris-Borate buffer containing 8 M urea which was then autoradiographed.

C - Centrifugation of fraction I in cesium chloride gradient. Sample was adjusted to 45% (w/w) CsCl and introduced at the bottom of a 10 ml column of 40% (w/w) CsCl. Centrifugation was for 65 hours at 33,000 rev/min. and 20°C in the SW 40 Beckman rotor.

be made. First, only U_1 and U_1^* and no other snRNAs are present in fraction I from the initial sucrose gradient. The several minor bands of smaller size are degradation products of U_1 as suggested by their increasing proportion concomitantly with the decrease of U_1. This was confirmed by fingerprint analysis

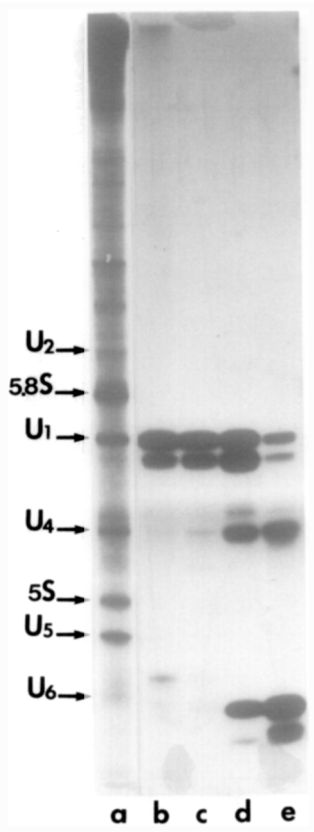

FIGURE 2 : Electrophoretic pattern of RNA at each stage of purification of U_1 snRNP.

Fraction I from the sucrose gradient described in Figure 1 A was subjected to essentially the same purification protocol previously used for the mixture of snRNP (4) and the RNA content was monitored as described in Figure 1 B.

a) Mixture of total nuclear snRNAs.
b) Fraction I from Figure 1 A.
c) Buoyant peak from the cesium chloride gradient in Figure 1 C.
d) Buoyant peak from the cesium chloride gradient in the presence of 0.5% sarkosyl (not shown).
e) 12 S peak of the sucrose gradient in 0.5 M KCl + 0.5% sarkosyl (not shown).

which revealed that all these small bands are derived from U_1 by removal of various lengths of 5' terminal sequences (results not shown). Second, the proportion of these small fragments increases drastically during the second cycle of cesium chloride centrifugation and the final sedimentation in sucrose (tracks d & e) coïnciding with the presence of sarkosyl at both steps. The proportion of U_1^* also rise sharply (track d) before being also degraded (track e).

It therefore appears that there exist several discrete sites in U_1 RNA where preferential and reproducible nuclease cleavage occurs during the course of purification. The level of this nuclease activity is very low

considering the fact that the purification protocol spans about ten days, most of this time being spent at 20°C. It may be important to note that degradation becomes significant only after sarkosyl has been introduced, raising the possibility that a nuclease inhibitor has been removed at this stage. Whether such a nuclease is a contaminant or an integral part of the snRNP remains to be established. A clue to answering this question would be to compare the location of these cleavage sites with those which can be introduced by exposure to an exogenous RNAse. In this context, Epstein et al (11), recently showed that treatment of a nuclear extract by T1 RNAse followed by immunoprecipitation of snRNP by anti Sm antibody resulted in the presence of discrete cleavages in U_1. At first glance, these sites do not closely correspond to those which we (unpublished results) observed. A detailed investigation of the structural organization of U_1 snRNA within snRNP is currently under way in our laboratory. In any case, these cleavages do not seem to interfere much with the antigenicity of the snRNP either under the conditions of Esptein et al (10) or in the present case (see below).

Proteins of the purified U_1 snRNP were analysed on 17.5% SDS acrylamide gels after labelling *in vitro* by the ^{125}I Bolton and Hunter reagent (Fig. 3 B Lane 1) and compared to proteins corresponding to a mixture of snRNP (lane 2). In both cases, the autoradiograph revealed five identical bands corresponding to molecular weight betwenn 10,000 and 14,000 *, demonstrating that U_1 snRNP has a protein complement identical to that of the mixture and suggesting that all individual snRNPs have the same protein composition.This finding agree with the previous suggestion of Lerner and Steitz (2) who identified the same protein complement of seven polypeptides (with three in the 20,000-35,000 range) using both an anti-RNP antibody which reacts only with U_1 snRNP and an anti-Sm antibody with immuno-precipitates RNPs containing all snRNA species.

At this stage, it has become crucial to know whether these most extensively purified U_1 snRNP as well as the entire mixture of all individual snRNPs have still retained their antigenic determinants after this drastic purification protocol. Preliminary results of our laboratory seem to indicate that a large fraction of purified U_1 snRNP can still be precipitated by anti-Sm while only a small fraction is precipitable by anti-RNP. This discrepancy suggests that Sm and RNP determinants are different in keeping with the recent study by Lerner et al (12) using monoclonal antibodies. The protein complement of purified U_1 snRNP being undistinguishable from that of the other snRNP, the determinants recognized by anti-RNP antibodies must therefore depend on those few additional proteins of larger size which are found in immunoprecipitated snRNP (2) but are absent from the purified material used here. A detailed account of the antigenicity of U_1 snRNP will be forthcoming soon.

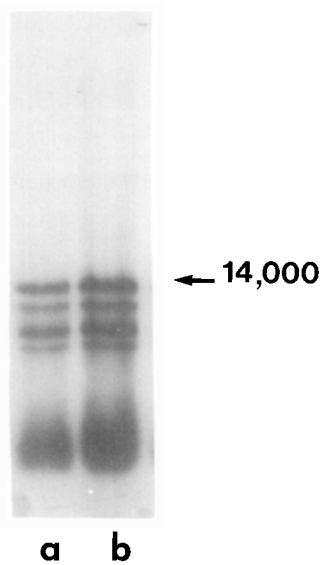

FIGURE 3 : Comparaison of protein components of purified U_1 snRNP and of that of the purified snRNP mixture.

Proteins from U_1snRNP and a mixture of snRNP were labeled *in vitro* with 200 µCi of ^{125}I Bolton and Hunter reagent. Electrophoresis and autoradiography were carried out as previously described (4). (a) proteins from U_1 snRNP, (b) proteins from the mixture of snRNP (4).

ACKNOWLEDGMENTS

We thank R. DIETZ for invaluable technical assistance and R. FILLOT for typing the manuscript. This work was supported by grants from I.N.S.E.R.M. (CRL N°80-1013), C.N.R.S., D.G.R.S.T., Ligue Contre le Cancer and Fondation pour la Recherche Médicale.

REFERENCES

1 - RAJ, N.B., RO-CHOI, T.S. and BUSCH, H. (1975) Biochemistry, 14, 4380-4385
2 - LERNER, M.R. and STEITZ, J.A. (1979) Proc. Natl. Acad. Sci. USA, 76, 5495-5499
3 - LERNER, M.R., BOYLE, J., MOUNTZ, S., WOLIN, S. and STEITZ, J. (1980) Nature, 283, 220-224.
4 - BRUNEL, C., SRI-WIDADA, J., LELAY, M.N., JEANTEUR, Ph. and LIAUTARD, J.P. (1981) (1981) Nucl. Acids. Res., 9, 815-830.
5 - SRI-WIDADA, J., LIAUTARD, J.P., ASSENS, Ch. and BRUNEL, C. (1981) Molecular Biology Reports (in press).
6 - MURRAY, V. and HOLLIDAY, R. (1979) FEBS Lett., 106, 5-7.
7 - ROGERS, J. and WALL, R. (1980) Proc. Natl. Acad. Sci. USA, 77, 1877-1879.
8 - GALLINARO, H. and JACOB, M. (1979) FEBS Lett., 104, 176-182
9 - ZIEVE, G. and PENMAN, S. (1981) J. Mol. Biol., 145, 501-523.
10 - BLANCHARD, J.M., BRUNEL, C. and JEANTEUR, Ph. (1977) Eur. J. Biochem., 99, 273-283
11 - EPSTEIN, P. REDDY, R. and BUSCH, H. (1981) Proc. Natl. Acad. Sci. USA, 78, 1562-1566.
12 - LERNER, E.A., LERNER, M.R., JANEWAY, E.A. and STEITZ, J.A. (1981) Proc. Natl. Acad. Sci. USA, 78, 2737-2741.