AN EVALUATION OF SMALL NUCLEAR RNA IN hnRNP

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1. Introduction

Small nuclear RNA (snRNA) were shown to be hydrogen bonded to hnRNA [1,2]. They were also shown to be present in hnRNP [3–5] which are assumed to be the site of premessenger RNA processing. Therefore, it might be postulated that snRNA play a role in such processing. However, snRNA are not solely localized in hnRNP but were described in other nuclear structures [6–9]. Moreover, there are several small nuclear RNA which do not necessarily perform the same function.

Before starting a study of the possible role of snRNA in processing or splicing, we felt that some additional informations should be available and we tried to answer several questions: (1) is the presence of snRNA (or of a fraction of them) in hnRNP not due to aspecific adsorption during preparation? (2) are snRNA quantitatively important in hnRNP as compared to other nuclear structures or to hnRNA? (3) is there a specific distribution of the various snRNA in hnRNP or in their constitutive units?

The results indicate that aspecific adsorption of snRNA to hnRNP is unsignificant under currently used experimental conditions. At least 25% of the nuclear small RNA are present in hnRNP and there is 1 molecule of snRNA per 2500 nucleotides of hnRNA, on the average. In addition, a specific distribution of the various snRNA was observed in different nuclear fractions suggesting the possibility of specific roles.

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2. Materials and methods

2.1. *Preparation and fractionation of a nuclear extract*

A brain nuclear extract containing hnRNP and nucleosol was prepared as previously described [10]. It was centrifuged on 10–40% linear sucrose gradients for 16 h at 24 000 rev./min in a SW25–2 rotor (70 000 × *g*). The sucrose solutions contained 10 mM triethanolamine–HCl (pH 7.4), 25 mM KCl and 1 mM $MgCl_2$. In certain experiments, the KCl concentration was raised to 100 mM and/or the $MgCl_2$ was replaced by EDTA (see text). Fractions of 2 ml were pooled as indicated in fig.2 and precipitated overnight at −20°C in the presence of 0.1 M NaCl and 2 volumes of ethanol.

2.2. *Extraction of RNA*

RNA from the nuclear extract or from the pellets of pooled fractions was extracted at pH 8.3 in the presence of 0.5% sodium-dodecylsulfate at 37°C for 10 min [11,12]. After deproteinization, the RNA was precipitated with ethanol in the presence of 0.24 M ammonium acetate [13].

2.3. *Slab-gel electrophoresis of RNA*

Linear gradients of acrylamide (2.2–15%) were used. Buffers were those of Loening [14]. Just before electrophoresis, the samples were denatured by heating at 65°C for 10 min [15]. When the quantitative estimation of hnRNA was required, the samples were treated with 10–20 μg/ml of ribonuclease-free deoxyribonuclease [16]. Migration was for 3 h–3 h 15 min at 10 V/cm. The RNA were stained with

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methylene Blue [17] and destained in a methanol–water mixture [3].

The gels were recorded with a Vernon densitometer (Paris, France). snRNA and hnRNA were determined by planimetry as indicated in fig.1. Nomenclature for snRNA was that of Ro-Choi and Busch [18]. Yeast 4 S and 5 S cytoplasmic RNA, *E. Coli* 16 S and 23 S ribosomal RNA were used as markers.

2.4. *Preparation of* ^{125}I *snRNA*

snRNA were prepared from a nuclear extract by phenol extraction at pH 7.6 and 4°C. The RNA were treated with 20 μg/ml of ribonuclease-free deoxyribonuclease (15 min, 37°C) [16]. Deoxyribonuclease was eliminated with 50 μg/ml of proteinase K (20 min, 20°C). After deproteinization, the 4–7 S RNA were purified by 2 successive sucrose gradients [15]. The RNA were labelled with ^{125}I according to Prensky et al. [19] and repurified on sucrose gradients. The specific activity was 6×10^5 counts/min/μg. After electrophoresis, the radioactivity and absorbance profiles were superimposable. Detection of iodinated snRNA on gels was achieved by fluorography (film RP X-OMAT-Kodak) according to Laskey et al [20].

3. Results

Our standard method of preparation of hnRNP consists in an ultrasonic lysis of purified nuclei followed by a high speed centrifugation allowing the obtainment of a nuclear extract containing hnRNP and small molecular weight constituents such as soluble proteins [21]. Chromatin, nucleoli, nuclear membranes are eliminated in the pellet of centrifugation [22]. snRNA were determined in the nuclear extract and 'chromatin pellet' (fig.1). On an average, 50% of the snRNA were found in each nuclear fractions. As a relatively important fraction of hnRNP remained bound to chromatin under our experimental conditions [23], it is likely that 50% is an underestimation. The electrophoretic profiles of the 2 fractions were close, with the exception of the presence of U3 RNA and of 1 small band between U1 RNA and the major 5 S RNA preferentially localized in the pellet. These RNA were shown to be of nucleolar origin [7].

For determination of the proportion and distribution of snRNA in hnRNP, a nuclear extract was fractionated on a sucrose gradient (fig.2). The fractions were pooled as follows: (A) pellet, hnRNP above 100 S, (B) 50–100 S hnRNP, (C) 30–50 S hnRNP, (D) 10–25 S material, (E) material smaller than 10 S. hnRNA was found as low mobility heterogeneous material in fractions A, B, C and its

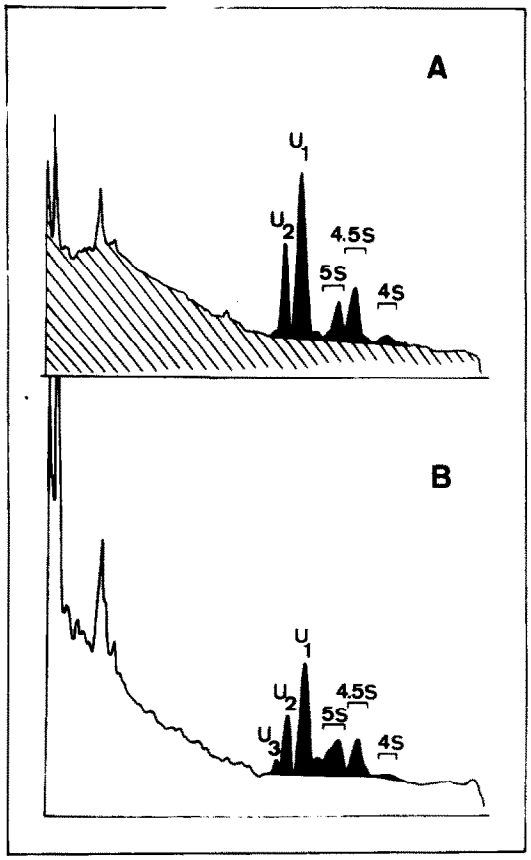

Fig.1. snRNA in nuclear extract and nuclear pellet. Purified brain nuclei were lyzed by ultrasonication [10]. After centrifugation, the RNA were extracted from the supernatant (nuclear extract A) and the pellet ('chromatin pellet', B). Phenol extraction was carried out at pH 8.3, 30°C in the presence of 0.5% sodium dodecylsulfate in A. In order to avoid the obtainment of large amount of DNA from B, extraction was at pH 7.6, 4°C in the absence of sodium dodecylsulfate. Recovery of snRNA was 90–95% in A and B, that of hnRNA was of the same order of magnitude in A but lower in B. RNA were electrophoresed and the gels recorded after staining. Areas were determined by planimetry as indicated. Black areas: snRNA, shaded area: hnRNA. Background was determined on a blank gel.

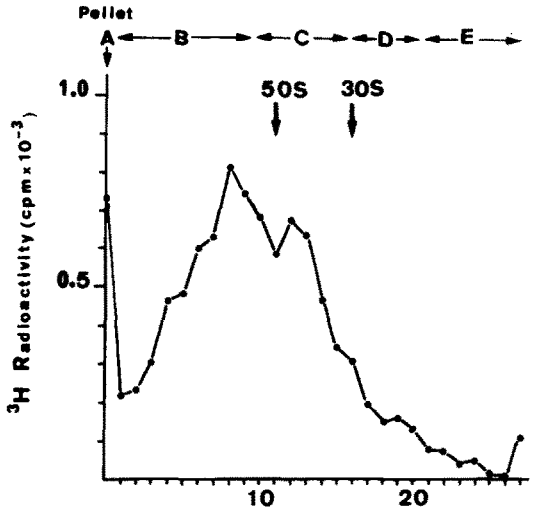

Fig.2. Standard fractionation of a nuclear extract on sucrose gradient. Rats were injected intracisternally with [^{3}H]uridine and sacrificed 16 h after injection [10]. A nuclear extract was prepared and fractionated on a 10–40% sucrose gradient as indicated in section 2. Ribosomal subunits served as centrifugation markers. The bulk of phenol extracted snRNA sedimented in region E. Acid insoluble radioactivity was determined on aliquots and fractions were pooled as indicated.

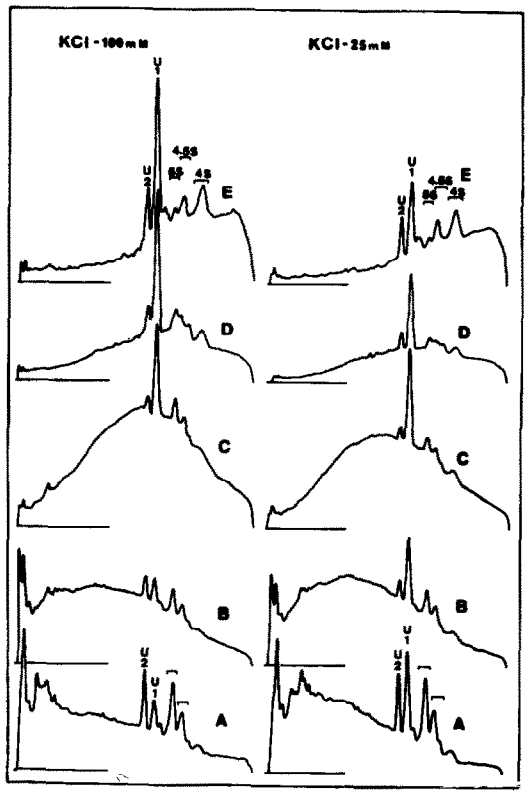

Fig.3. RNA analysis of sucrose gradient fractions. RNA was extracted from pooled fractions as indicated in fig.2 and electrophoresed on slab gels. Right panel: standard gradient buffer (see section 2). Left panel: the KCl concentration was raised to 100 mM. In order to achieve proportionality for determination of RNA, aliquots of each fraction were analyzed: (A) 50%, (B) 33%, (C) 37%, (D) 43%, (E) 100%. Similar proportions of the fractions were analyzed in each gradient so that direct comparison between the 2 panels is possible.

size decreased with sedimentation coefficient of hnRNP, as expected (fig.3). Protein analysis confirmed that the 28 000–38 000 M_r proteins, typical of hnRNP [24] were present only in fractions A to C. Fractions A and B contained polymeric structures, fraction C primarily monomers [10,25].

The various snRNA were not equally distributed in the fractions (fig.3). hnRNP of 30–50 S (C) were highly enriched in U1 RNA as compared to hnRNP of higher sedimentation coefficient (A and B). snRNA were also present in fractions D and E devoid of hnRNP. The snRNA sedimenting at 10–25 S (D) are probably associated to proteins as snRNP, whereas fraction E is expected to contain free snRNA. The electrophoretic profiles, in particular in the 4–5 S region of fractions D and E, suggested the presence of snRNA species absent from or scarce in hnRNP.

Centrifugation was carried out in buffers containing 25 mM or 100 mM KCl. The distributions of snRNA along the sucrose gradient were similar but not identical in the 2 buffers (fig.3). The most noticeable difference was that a large fraction of U1 RNA was released from the largest hnRNP (A, B) and found in the upper parts of the gradients (D, E) when the KCl concentration was raised from 25 to 100 mM (fig.3 and table 1). The extent of release was smaller or unsignificant for the other snRNA. It is worth mentioning that almost 50% of U1 RNA was found in the snRNP fraction (D) at 100 mM KCl.

The possibility of aspecific adsorption of a fraction of snRNA in particular U1 on hnRNP at low salt concentration was suggested by these experiments. However, this could not be verified at KCl concentrations higher than 100 mM as dissociation

Table 1
Distribution of U1 RNA along a sucrose gradient after centrifugation of a nuclear extract

KCl (mM)	EDTA (mM)	Fractions (%)			
		A + B	C	D	E
25[a]	0	52	27	16	5
100[a]	0	16	23	50	11
25	0	45	28	19	8
100	0	15	26	41	18
25	0.2	16	24	49	11
100	0	15	22	41	22
100	0.2	8	23	52	17
100	1,0	11	19	53	17

[a] Average of 2 experiments

Fractions were defined in fig.2

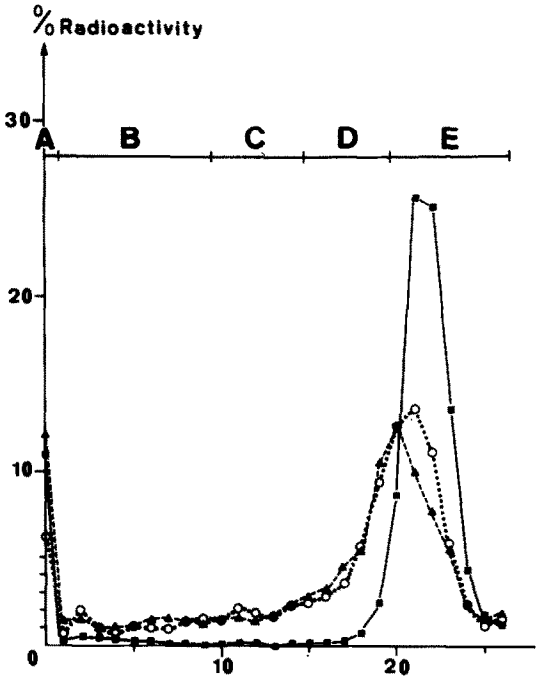

Fig.4. Centrifugation of [^{125}I]snRNA in the presence or absence of nuclear extract. Conditions of centrifugation as in fig.2. [^{125}I]snRNA were centrifuged without any addition (■—■) or were added before nuclear lysis and centrifuged in the presence of standard buffer (25 mM KCl, ▲— – —▲) or of buffer containing 100 mM KCl (o···o). Acid-insoluble radioactivity was determined. Results are expressed in per cent of total acid insoluble radioactivity.

of the major monoparticle proteins was already important at 250 mM KCl [26] and started at 150–200 mM. Therefore, another way to detect spurious adsorption was looked for. Previous experiments had shown that EDTA provoked a slight decrease of sedimentation coefficient of hnRNP without modifying their protein to RNA ratio [27]. A release of snRNA (which was not analyzed in these early experiments) possibly with associated proteins might have provoked the sedimentation shift. Sucrose density gradient fractionation of hnRNP was therefore performed in the presence of 0.2 mM EDTA in a buffer containing 25 mM KCl. Quantitatively and qualitatively, the results were similar to those obtained by increasing the KCl concentration from 25 to 100 mM (table 1). Increasing the EDTA concentration from 0.2 to 1.0 mM or adding EDTA to a 100 mM KCl buffer did not provoke any further release of snRNA (table 1).

The experiments show that EDTA releases the same snRNA in particular U1 from hnRNP as does the increase of KCl concentration from 25 to 100 mM. Though the mechanism of action of EDTA and KCl might be different, the similarity of final results is in favor of an easy accessibility of certain snRNA. It is not possible to decide whether the released snRNA correspond to an aspecifically adsorbed fraction or whether they are loosely bound to hnRNP. In practice, we shall consider that only the snRNA which sediment with the hnRNP at 100 mM KCl or at 25 mM KCl, 0.2 mM EDTA belong to these structures. Under such conditions the snRNA from the nuclear extract were distributed between the fractions as follows: 28% in A plus B, 21% in C, 35% in D and 17% in E.

The possibility of aspecific adsorption was also controlled with exogeneous RNA. ^{125}I-snRNA was added to a nuclear suspension before lysis. The labelled snRNA amounted to approximately 3% of the snRNA present in the soluble fraction (E). About 50% of the labelled RNA remained in that fraction against 80% when snRNA were centrifuged in the absence of nuclear extract (fig.4).

A relatively large proportion of the radioactivity (19–27%) was found in fraction D corresponding to snRNP, against 4–5% when nuclear extract was omitted. Fluorography showed that the radioactivity

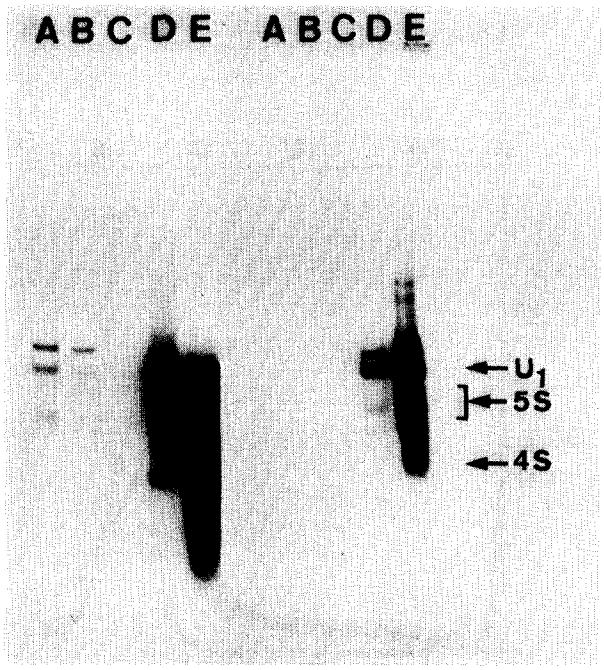

Fig.5. Fluorography of [^{125}I]snRNA after slab gel electrophoresis. RNA were extracted from pooled fractions (A to E) of the sucrose gradients shown in fig.4 and electrophoresed. Labelled RNA were visualized after fluorography. Left panel: [^{125}I]snRNA plus nuclear extract. Right panel: [^{125}I]snRNA without nuclear extract. Results were similar at 25 mM and 100 mM KCl. Films were overexposed for D and E in order to detect small amounts of radioactivity in A, B, C.

was indeed due to the presence of snRNA (fig.5). This indicated that a relatively high proportion of the snRNP might arise from associations of snRNA with proteins in vitro.

25–35% of the radioactivity was found in the hnRNP region (fraction A, B, C). However, fluorography showed that only 5–12% of this radioactivity was due to snRNA in contrast to the results obtained for fraction D (fig.5). When snRNA were centrifuged alone, 12–15% of the radioactivity was recovered in regions A, B, C but did not correspond to snRNA. This suggested that iodinated impurities such as radiolysis products present in the preparation of ^{125}I-snRNA cosedimented with and/or adsorbed on hnRNP. Assuming that 10% of the free snRNA may adsorb on hnRNP and that free snRNA represent 20% of the total snRNA from a nuclear extract, it was estimated that the proportion of snRNA from a nuclear extract which may aspecifically bind to hnRNP during preparation at 100 mM KCl was 2%. As hnRNP contain 50% of the snRNA from the total extract, at most 4% of the snRNA present in hnRNP may not belong to these structures.

The results were essentially the same at 25 mM and 100 mM KCl or when the quantity of free snRNA was doubled by addition of unlabelled snRNA. A systematic study of effect of salt and snRNA concentrations was not attempted.

Together, our data show that approximately half of the snRNA of the nuclear extract were present in hnRNP, i.e., 25% of the total nuclear pool, this being a minimal estimation. We now determined the proportion of snRNA versus hnRNA. It was found to be 5.9% ± 1.1 in total hnRNP (average of 7 experiments). It was not significantly different in large hnRNP (6.1% ± 1.0) and in 30–50 S monoparticles (5.8% ± 1.8). If we consider that snRNA are 150 nucleotides long, this would indicate that there is 1 molecule of snRNA per 2500 nucleotides of hnRNA, on an average.

As already observed in fig.3, this proportion is different for the various snRNA according to their localization. Thus, it was estimated that there was about 1 molecule of U1 RNA or U2 RNA (171 and 196 nucleotides) [28,29] per 15 000 nucleotides of hnRNA in large hnRNP. In monoparticles, 1 molecule of U1 RNA was found per 6000 nucleotides of hnRNA, but 1 molecule of U2 RNA per 35 000 nucleotides.

4. Discussion

Under our conditions of preparation of hnRNP, snRNA were distributed in various nuclear fractions: chromatin-nucleoli, hnRNP, free snRNP, free snRNA. This is consistent with previous work showing their presence in chromatin [6], nucleoli [7], hnRNP [3–5] and snRNP [30]. However, snRNA were also shown to be all bound to the nuclear skeleton together with hnRNA [8,9]. The discrepancy between these 2 sets of data must probably be related to the methods of preparation, relatively drastic in the first case, milder in the second. It is likely that nuclear lysis (in our case, ultrasonication), which is necessary for the

isolation of hnRNP, provoked a disruption of the skeleton with a concomitant release of the bound hnRNP and snRNA. Our experimental conditions do not permit to decide whether free snRNA or snRNP are present as such in the nucleus. They may have been associated to the skeleton in vivo and released after lysis. In addition, snRNP might be formed artefactually at least partially as suggested by the experiments illustrated in figs.4 and 5.

As snRNA were seemingly distributed in several nuclear fractions after lysis, an important point was to show that the presence of snRNA in hnRNP reflected an in vivo situation. It must be first recalled that hnRNP prepared by our standard method correspond to perichromatin fibrils described in situ [22,31]. We showed here that free snRNA did not significantly associate with hnRNP under similar conditions. The proportion of snRNA versus hnRNA was fairly constant (6%) as well as the relative distribution of the various snRNA in hnRNP fractions and hnRNP-free fractions. Only the proportion of U1 RNA was significantly modified upon the small changes of ionic environment compatible with persistence of hnRNP. Together, these experiments strongly suggest that snRNA are regular constituents of hnRNP. The nature of the binding to hnRNA, to protein or to both is currently under investigation.

We find that at least 25% of the small nuclear RNA are present in hnRNP and that there is 1 molecule of snRNA per 2500 nucleotides of hnRNA which is far from negligible. If we consider 2500 nucleotides as an average size for hnRNA, this would indicate 1 molecule of snRNA per average hnRNP. However, we ignore whether all individual hnRNP contain snRNA or whether there is a non random distribution, related to certain steps of processing for instance.

The relative distribution of the various snRNA is strikingly different in the large hnRNP and in soluble fractions. This suggests that snRNA associate with hnRNP in vivo according to specific rules. If we assume that snRNA are hydrogen bonded to hnRNA [1,2], a recognition of specific sequences is implied. Therefore, the frequency of certain sequences in pre-messenger RNA or intermediary products of processing, may govern the snRNA distribution in hnRNP. However, all molecules of a given snRNA may not be hybridized to hnRNA in hnRNP, nor all snRNA species and other factors may be involved in their distribution.

It is also worth mentioning that the distribution of snRNA is different in 30–50 S monoparticles and in the large hnRNP which contain the constituents designated as 'heterogeneous complexes' in addition to monoparticles [10]. In particular, U1 RNA represents 50% of the snRNA from monoparticles and only 20% of that of large hnRNP. U2 of 5S RNA are comparatively enriched in the large hnRNP. A new structural difference between the 2 classes of hnRNP constituents is thus shown here and may help towards an understanding of the function of these ribonucleoproteins.

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