ASSOCIATION OF SMALL NUCLEAR RNA WITH HnRNA ISOLATED FROM NUCLEAR RNP COMPLEXES CARRYING HnRNA

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Received 25 September 1978

1. Introduction

In the course of our studies dealing with the structure and function of the nuclear RNP complexes carrying HnRNA (informoferes according to [1], nuclear informosomes, according to [2]) we had detected the presence of small nuclear RNA (snRNA) tightly bound to these complexes [3–5]. The nature of the arrangement of the snRNA within the RNP complex was considered and evidence presented supporting the tight association of the small RNA with the specific particle proteins.

In the present communication we report experimental data demonstrating the snRNAs, in addition to their association with proteins, are also directly attached to HnRNA.

2. Materials and methods

2.1. *Isolation of HnRNA–protein complexes*

Rat liver nuclei were isolated by the method in [6] modified [7]. The HnRNA–protein complexes were isolated as in [4]. In short, the nuclei were suspended in 0.14 M NaCl solution containing 1 mM $MgCl_2$ and 10 mM Tris–HCl (pH 8.0) and submitted to ultrasonic treatment. The nuclear suspension was centrifuged at 10 000 rev./min for 10 min. The obtained supernatant was layered on a 15–30% sucrose gradient prepared in the 0.14 M NaCl solution mentioned above and submitted to centrifugation at 26 000 rev./min for 2.5 h in a SW 27 Beckman head. Fractions of the gradient, corresponding to particles with *S*-values of ~100–250 were pooled and submitted to RNA extraction. The RNP complexes were obtained in form of polymeric structures by performing the preparation starting from livers kept at –80°C, at least overnight. As shown [8], freezing of the liver, for yet unknown reasons, leads to a considerable decrease of the activity of nuclear nucleases, thus preserving the polymeric form of the RNPs.

2.2. *RNA-extraction*

After centrifugation of the informoferes on sucrose gradients, fractions sedimenting from 100–250 S were pooled, made 1% in SDS and proteinase K was added to final conc. 100 μg/ml. The proteinase K was pre-digested for 120 min at 37°C. After the incubation, the mixture was twice shaken with chloroform–isoamylalcohol and the RNA precipitated with 2.5 vol. ethanol overnight at –20°C. The RNA was pelleted and after washing twice with 70% ethanol, desalted on Sephadex G-50.

2.3. *Sucrose gradient centrifugation of RNA*

Both aqueous as well as formamide gradients were used. In the first case the gradient was 15–40% sucrose, 25 mM Tris–HCl, (pH 7.5) and 100 mM KCl (18 h at 80 000 × *g*). The formamide (70%) gradient was 5–20% sucrose, 3 mM Tris–HCl, (pH 7.5) and 3 mM EDTA (36 h at 170 000 × *g*).

2.4. *Electrophoresis of the RNA*

RNA was submitted to electrophoresis on 10% polyacrylamide gels in 98% formamide [9]. The gels were stained with toluidine blue and destained in

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water. Marker 23 S and 16 S *Escherichia coli* ribosomal RNA was also run.

2.5. *Labelling of RNA with [^{3}H]dimethylsulfate*

[^{3}H]Dimethylsulfate (NEN, spec. act. 4 Ci/mmol) 5 mCi, were taken into 250 μl 0.1 M phosphate buffer (pH 7.2) and incubated overnight at 5°C with 5 μg RNA isolated from fraction 5 of the formamide gradient shown in fig.1, containing traces if any, of degraded RNA. The non-bound radioactivity was separated from the RNA on Sephadex G-50 columns, and the RNA, eluting in the void volume, precipitated with ethanol or lyophilised. The specific activity of the RNA was 10 000 cpm/μg RNA, independent of the RNA amount used for the methylation.

2.6. *Hybridization of the RNA*

For the hybridization we used labelled snRNA and non-labelled HnRNA from fractions 15–25 of the formamide gradient depicted in fig.1. HnRNA, 10 μg, and 1 μg snRNA were mixed in hybridization buffer (1 × SSC, 40% formamide, pH 7.5), sealed in capillaries, boiled for 5 min and incubated at 37°C for 60 min. At the end of the incubation, the hybrids were separated using the cellulose system [10] as in [11].

T_1 ribonuclease treatment was carried out in 0.2 M NaCl, 0.1 M Tris–HCl (pH 6.85), 0.002 M EDTA at 37°C for 45 min. The concentration of T_1 ribonuclease was 0.5 μg/20 μg RNA and that of RNA was 50 μg/ml [11].

3. Results

HnRNA–protein complexes were isolated from rat liver nuclei under conditions yielding polymeric structures. The RNPs sedimenting with *S*-values of ~100–250 were pooled, the RNA extracted and then submitted to sucrose gradient centrifugation either in aqueous or in 70% formamide buffer (fig.1). The RNA of the RNPs, as has also been documented, shows an heterogeneous distribution, with values ranging from a few S up to 30–40 S. In acrylamide formamide gel electrophoresis, the presence of defined small molecular RNA with *S*-values of 4.5–6.5 could be seen (see [5]), as well as a smear, corresponding to HnRNA (see fig.2).

Analysis of the RNA fractions from the sucrose

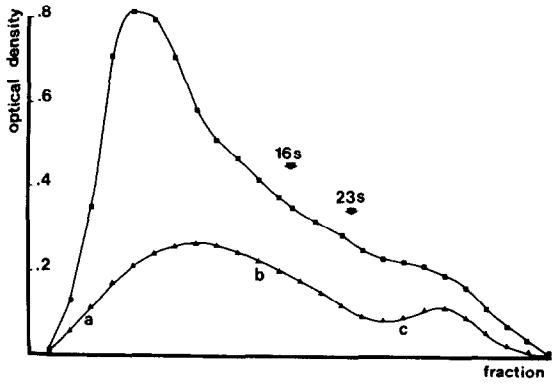

Fig.1. Aqueous and 70% formamide–sucrose gradient centrifugation of RNA isolated from nuclear HnRNA–protein complexes. Direction of centrifugation from left to right. The regions a, b and c, of the aqueous gradient (▲——▲) and every second fraction of the formamide gradient (■ — —■) were pooled and submitted to electrophoresis.

Fig.2. 10% acrylamide–98% formamide gel electrophoresis of total RNA isolated from nuclear RNP. Direction of migration from top to bottom.

gradients by acrylamide–formamide gel electrophoresis reveals that all fractions of the RNA separated on the aqueous gradient, independent of size class, contain both HnRNA and snRNA (fig.3). The distribution of snRNA in the 70% formamide gradients, however, is strikingly different (fig.4). Only the top fractions contain snRNA species, whereas the heavier fractions show mainly two, the larger in size, snRNA.

The effect of formamide on the association of snRNA to HnRNA suggested the involvement of base pair interaction between the two classes of RNA and tempted us to examine the homolarity between snRNA and HnRNA.

snRNA was isolated from gradient centrifugation of total RNA of the nuclear particles (see section 2)

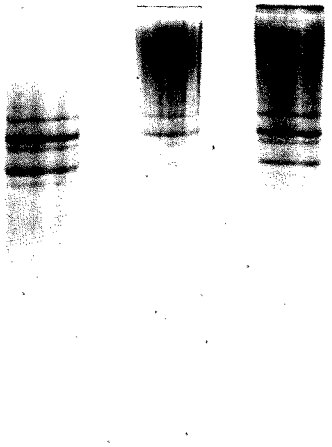

Fig.3. 10% acrylamide–98% formamide gel electrophoresis of RNA fractions a, b and c, pooled from the aqueous sucrose gradient of fig.1. Direction of migration from top to bottom.

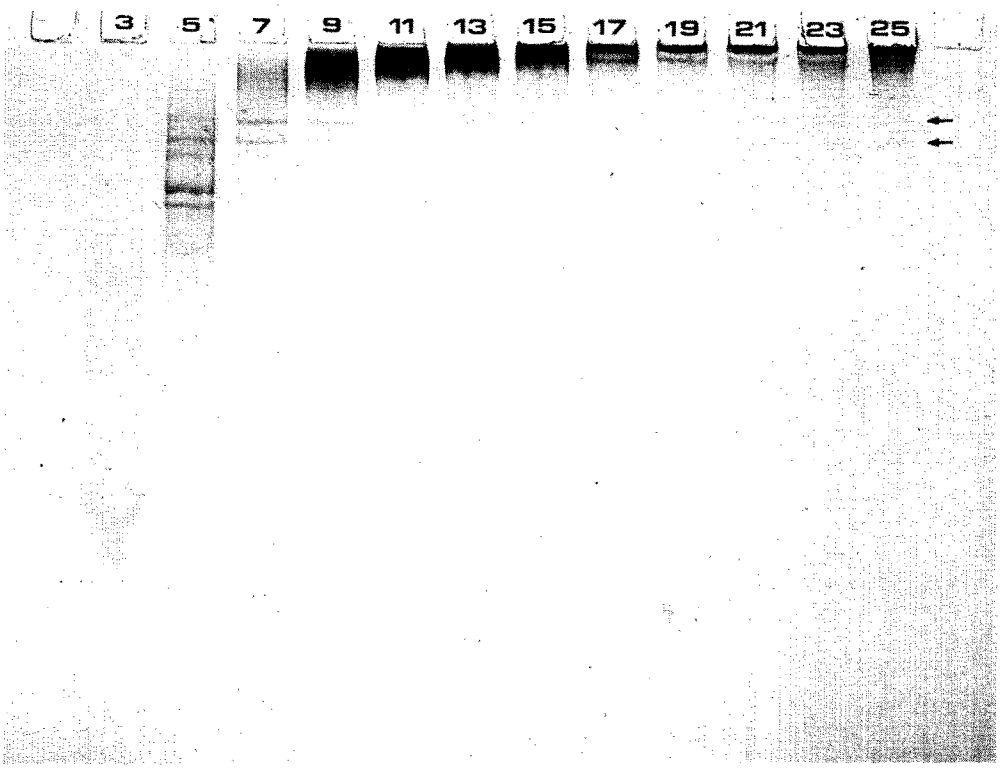

Fig.4. 10% acrylamide–98% formamide gel electrophoresis of RNA from every second fraction obtained from the formamide–sucrose gradient of fig.1. Direction of migration from top to bottom.

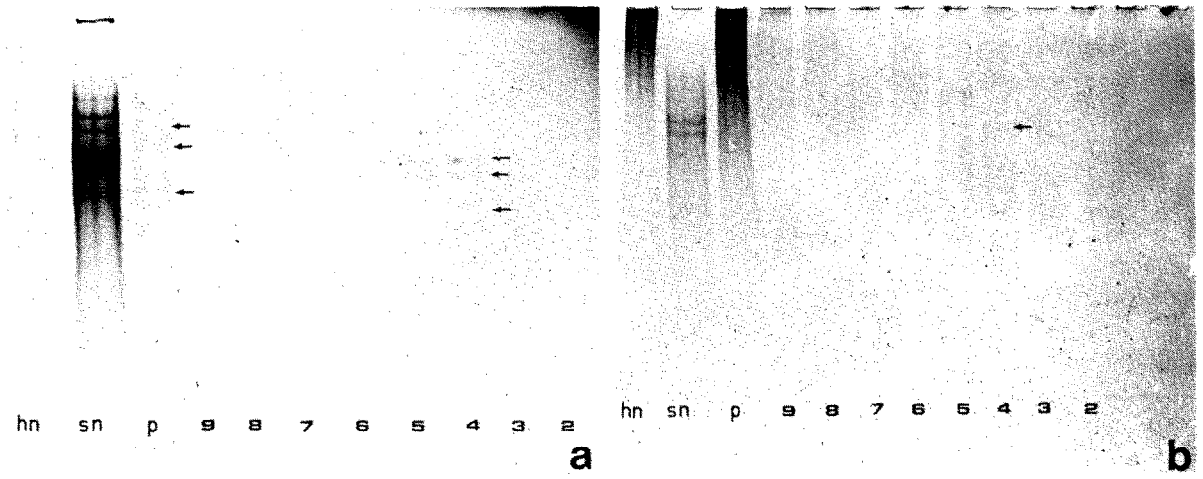

Fig.5. 10% acrylamide–98% formamide gel electrophoresis of the fractions and the pellet obtained after centrifugation on a 5–20% sucrose gradient in 40% formamide and 1 × SSC buffer of [^{3}H]snRNA annealed to HnRNA. P, pellet; sn, snRNA; Hn, HnRNA. (a) Fluorogram. (b) stained with toluidine blue.

and ^{3}H labelled by reacting with [^{3}H]dimethylsulfate.

In a first experiment 2 μg [^{3}H]snRNA was hybridized to 20 μg HnRNA (*S*-values, 20–40) as in section 2 and run on a 5–20% sucrose gradient in 40% formamide and 1 × SSC buffer (hybridization buffer). After 12 h centrifugation at 260 000 × *g* the large HnRNA was pelleted and separated from small nuclear RNA. Thereafter, the fractions of the gradient were desalted and submitted, with the pellet, to acrylamide–formamide gel electrophoresis. It is evident that labelled snRNA is found both in the top fractions of the gradient as well as in the pelleted HnRNA fraction, whereas the intermediate fractions are devoid both of HnRNA and of snRNA (see fig.5a,b). The snRNA is detected by fluorography of the gels (fig.5a) whereas HnRNA can be demonstrated by staining of the gel with toluidine blue (fig.5b).

In a further series of experiments [^{3}H]snRNA was hybridized to HnRNA and the amount of hybrid formed evaluated by the cellulose column method [10,11]. Two sets of experiments were performed. In one, the products of the hybridization reaction were immediately applied to the cellulose column, in the other they were treated with T_1 nuclease prior to the chromatography. The distinction between hybridized part and the non-hybridized tails of the molecule could thus be made. Furthermore, controls were also performed in which snRNA alone was subjected to reannealing conditions with or without prior treatment with T_1 nuclease.

The results of the experiments are shown in table 1. The first conclusion derived from the experiments involving reannealing of snRNA alone, is the existence of secondary structure on these small RNA molecules,

Table 1

	snRNA alone		snRNA + HnRNA	
	Hybridization	hybrid cpm	Hybridization	hybrid cpm
– T_1 nuclease	11.7%	620 (a)	22.2%	1178 (b)
+ T_1 nuclease	3.6%	194 (c)	4.7%	249 (d)

~10% of the base sequences of snRNA take part in hybrid formation with HnRNA. This has been calculated from the equation (d–c)/(b–a) × 100

as postulated [12] on the basis of the data on the primary structure of RNA U_1 and U_2. The T_1 digestion experiments also show that only a small amount of bases take part in hybrid formation (11.7% hybridization before, 3.6% after T_1 nuclease digestion). The addition of HnRNA to snRNA results in a significant increase in the quantity of hybrids formed (table 1) demonstrating a direct interaction between snRNA and HnRNA (increase of hybridization from 11.7–22.2%). The comparison of the radioactivity in the hybrid fraction before and after T_1 nuclease digestion demonstrates that the formed hybrids are very short, making up, on the average, ~10% of the length on the snRNA (length of snRNA ≃ 150–250 nucleotides, hybrid ≃ 15–25 nucleotide pairs).

4. Discussion

The existence in animal nuclei of small RNA species has been known for a long time [12–16], their functions, however, are still unresolved. The finding of snRNA in the HnRNA–protein complexes [3–5] suggested the involvement of the small nucleic acids in the organization of the RNP structure [3].

We have shown, that RNA components of the nuclear RNPs having a slower turnover than HnRNA, were less susceptible to RNase digestion when present in the RNP structure, than HnRNA [3]. We now know that the slower turning-over RNA corresponds to snRNA and have confirmed the partial resistance of snRNA to RNase digestion (A. Prüsse, unpublished). These findings led us to suggest that the RNase resistance of snRNA is due to its tight association with the particle proteins [3].

In the present study we demonstrate that HnRNA isolated from nuclear particles, even of very high molecular weight, is directly associated with the snRNA species of the particles. Three of the snRNAs (c,d,e, see [5]) are to a large extent dissociated in the presence of 70% formamide, whereas snRNA a and b, having a higher molecular size than c,d and e, are still attached to HnRNA under these conditions, being dissociated at higher concentrations (98%) of formamide. These results suggest a base pair interaction between snRNA and HnRNA, a suggestion strengthened by the finding that ^{3}H-labelled snRNA hybridizes to HnRNA.

The hybrids formed are quite small, ~15–25 nucleotides in length. This is a minimal estimation. The snRNA is found in all size classes of HnRNA. The smaller species of snRNA are less tightly attached to HnRNA than the two larger snRNA molecules.

The existence of snRNA in hybrid form with HnRNA raises a series of questions on the possible function of snRNA in the nucleus. The formation of hybrids could be one of the possible ways of attachment to HnRNA of the snRNA–protein complexes, carrying enzymes for the processing of HnRNA [17,18]. These hybrids could be sites of attack by nucleases preferentially attacking double-stranded RNA (RNase III) [19] which have been recently implicated in the processing of HnRNA. Double-stranded regions in HnRNA have been long recognized [20,21] having hairpin like structure and have been regarded as likely candidates for processing signals. These long double-helical regions are high in A+U sequences. Short G+C rich double-helical regions were also detected [21]. It is interesting that the distribution of these small sequences is independent of the size class of the HnRNA. It is tempting to speculate that these small helical regions correspond to the snRNA/HnRNA hybrids described here. Among the many questions still to be answered are the distribution of the hybrids within the HnRNA molecule, their fate during the processing of HnRNA and the possible appearance and role of snRNA–mRNA complexes in the cytoplasm.

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