DISTRIBUTION OF CODING SEQUENCES WITHIN THE NUCLEAR HnRNA–PROTEIN COMPLEXES

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1. Introduction

Most, if not all, of HnRNA is associated with a specific class of nuclear proteins [1–6]. The organization of the HnRNA within the RNP complexes is still unresolved. Depending on the nuclease activity of the respective tissues, the RNP complexes can be obtained in size classes > 250–30 S. The 30 S structures result from the degradation of that part of the HnRNA of the polymeric complexes which is easily accessible to the nuclear degradation enzymes [1,7]. The part of the HnRNA associated with the 30 S particles, very probably by virtue of its tight association with the protein moiety, is resistant to the action of the nucleases. As the HnRNA is composed both of coding sequences as well as of nucleotide stretches of unknown function, we were interested in obtaining data on whether there was a preferential enrichment of specific sequences in the 30 S RNP particles. To this end, we have compared the hybridization of cDNA synthesized from polysomal poly(A)$^+$ RNA to RNA isolated from 30 S and 80–150 S RNP particles. The results suggest that a higher proportion of coding RNA sequences is present within the 30 S RNP structures than in the regions of the HnRNA exposed to nuclear degrading enzymes.

2. Materials and methods

2.1. *Preparation of polymeric and monomeric RNP particles from rat liver*

Rat liver nuclei were isolated by the sucrose method in [8]. For the preparation of the 30 S particles fresh livers were used; for the preparation of polymers the livers were immersed in liquid nitrogen for 1 h and conserved at –80°C until use. Starting from purified nuclei, particles were isolated by the method in [9]. The RNA was extracted with the proteinase K method [10].

2.2. *Formamide–acrylamide electrophoresis*

The RNA was separated under completely denaturing conditions in either 10% acrylamide or 4–10% acrylamide gradient gels in 98% formamide as in [11].

2.3. *Isolation of polysomal poly(A)$^+$ RNA*

Polysomes from rat liver were isolated with Nonidet P-40 as in [12]. The RNA was extracted with phenol–chloroform [13], precipitated overnight with alcohol at –20°C, dried in a gentle stream of nitrogen and dissolved in 0.5 M KCl–10mM Tris, pH 7.2. The poly(A)-containing fraction was isolated after two passages over oligo (dT)-cellulose as in [14].

2.4. *Isolation of nuclear poly(A)$^+$ RNA*

Nuclei of rat liver were isolated with citric acid by the method in [15]. The isolated nuclei were free of cytoplasmic contamination as judged by electron microscopy. The RNA was extracted with phenol–chloroform as in [16]. The DNA was digested with DNase, pre-treated with iodoacetate [17]. The poly(A)-containing fraction was separated using the same procedure as above.

2.5. *Reverse transcription*

The poly(A)$^+$ RNA was reverse transcribed using AMV reverse transcriptase kindly supplied by Dr

J. Beard, Florida. The final mixture contains 35 μg/ml template RNA, 50 μg oligo (dT_{17})/ml, 800 μM deoxyribonucleotriphosphates (500 μCi; d[^{3}H]CTP/ml, spec. act. 41 Ci/mmol), 100 μg/ml actinomycin D, 50 mM Tris–HCl, pH 8.2, 50 mM KCl, 5 mM magnesium acetate, 10 mM dithiothreitol, and 70 units/ml reverse transcriptase. After 2 h incubation, the cDNA was separated from the triphosphates by chromatography on Sephadex G-50 and centrifuged on an alkaline sucrose gradient. Sedimentation coefficients were calculated using a computer programme and fractions greater than 300 nucleotides were pooled and the cDNA precipitated in the presence of *E. coli* DNA as carrier.

2.6. *Hybridization*

Appropriate quantities of RNA and cDNA were lyophylized, dissolved in hybridization buffer (0.24 M PB–0.1% SDS–1 mM EDTA), sealed in glass capillaries and incubated at 60°C for the desired time. At the end of the incubation the contents were flushed out with 250 μl buffer (0.14 M NaCl–0.07 M sodium acetate–2.8 mM $ZnSO_4$–14 μg/ml heat-denatured mouse DNA, pH 4.5) and added to 100 μl same buffer without DNA carrier containing 20 units nuclease S1 (Sigma). After 30 min at 37°C an aliquot was counted and the rest was precipitated with 5% TCA to calculate the non-degraded material.

2.7. *Melting curves of the RNA*

RNA from monomeric and polymeric informoferes were analysed for hyperchromicity in a Gilford spectrophotometer, equipped with a model 2527 Thermo programmer.

3. Results

HnRNA–protein complexes were isolated from rat liver as described. In the absence of RNase inhibitor, the applied procedure yields structures sedimenting at 30 S. To obtain polymeric structures [1,2,10] the addition of RNase inhibitor isolated from rat liver cytosol [18] to the extraction buffers was necessary. We have recently observed that if the livers are immediately frozen in liquid nitrogen and then kept at −80°C for 15–24 h, it is possible, for still unknown reasons, to obtain polymeric structures in the absence of the RNase inhibitor. In fig.1a,b, typical sedimentation profiles of monomeric and polymeric HnRNA protein complexes extracted from rat liver nuclei are depicted.

The fractions corresponding to the 30 S and the 80–150 S HnRNA–protein complexes (fractions 14–22 and 10–17 from fig.1a,b, respectively) were pooled, the RNA isolated and aliquots submitted to analytical acrylamide gel electrophoresis in the presence of formamide. As seen from fig.2a the RNA isolated

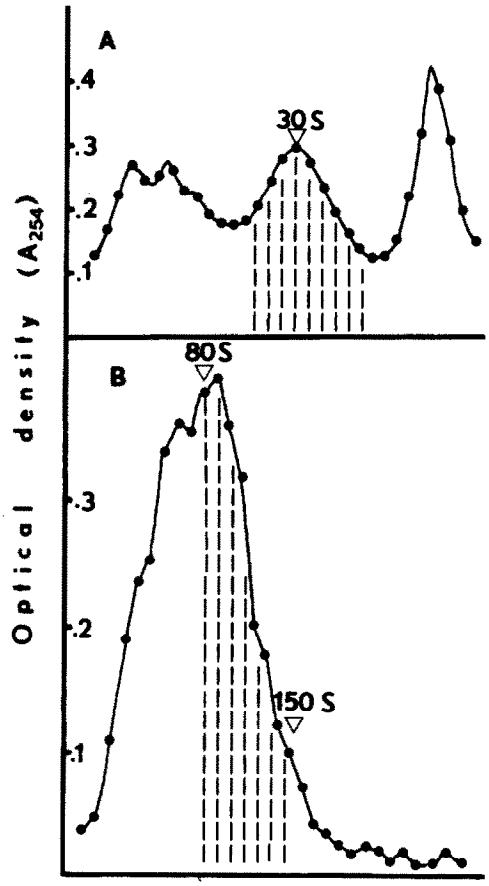

Fig.1a,b. Sucrose gradient centrifugation of HnRNA complexes from rat liver nuclei. (A) The complexes were isolated from nuclei prepared from freshly isolated livers. Centrifugation for 18 h at 26 000 rev./min. (B) The complexes were isolated from nuclei prepared from livers pre-immersed in liquid nitrogen then stored at −80°C. Centrifugation for 3 h at 26 000 rev./min. Sucrose gradient, 15–30% as in section 2. The fractions denoted in the figure were pooled for the isolation of RNA. Direction of sedimentation is from left to right.

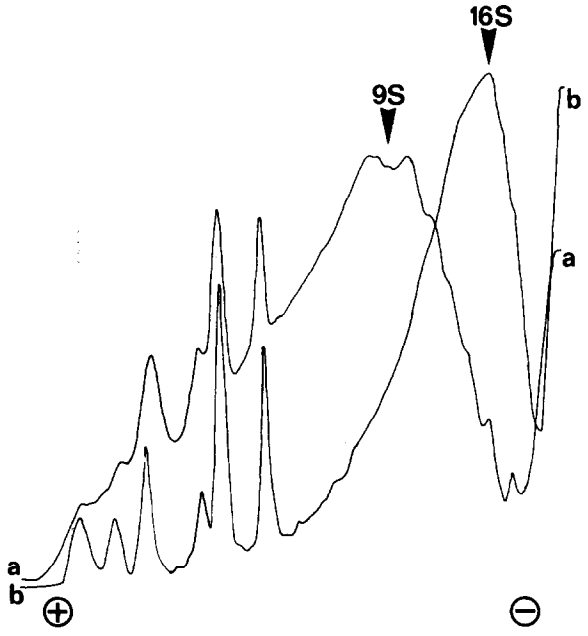

Fig.2. Gel-electrophoresis on a 10% acrylamide–98% formamide gel of RNA isolated from the RNA protein complexes. (A) RNA isolated from 30 S particles. (B) RNA isolated from 80–150 S particles. The ordinate gives the A_{600} nm value.

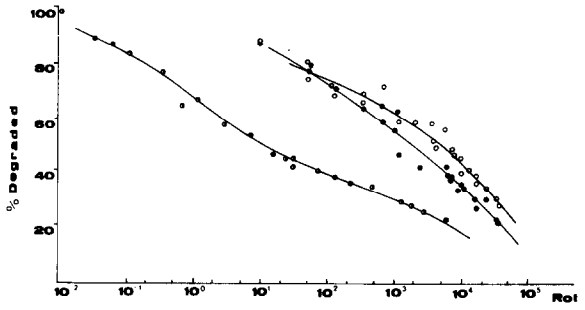

Fig.3. Hybridization of cDNA from polysomal poly(A)$^+$ RNA to HnRNA. Conditions of hybridization as in section 2. RNA was ≥ 25 000 times cDNA. The curves have been plotted on the basis of the results of 3 different experiments. The lines represent the best fit calculated with a polynomial regression computer program. (•) RNA from 30 S particles. (○) RNA from polymers. (◑) Polysomal poly(A)$^+$ RNA.

from the 30 S particles shows a size distribution between 4 S, 5 S and 12 S with a peak at 8–10 S whereas the RNA derived from the polymeric particles (fig.2b) is of an S-size between 4, 5 and 24. The small molecular weight RNA components of the RNP-particles show values from 4.5–6.5 S. To calculate the amount of small nuclear RNA (snRNA) present in the RNA derived from polymers and monomers we scanned the gels and integrated them. Both preparations contain similar amounts of snRNA from 26.5–29.1%.

We then proceeded to determine the content of sequences homologous to polysomal poly(A)$^+$ RNA in the two RNA fractions. We first isolated polysomal RNA and from this, the poly(A)-containing fraction. This RNA was used as template for a reverse transcription reaction. The prepared cDNA was hybridized to RNA, derived both from monomeric and polymeric informoferes, in a RNA-driven reaction. Figure 3 shows the computer analysed *Rot* curves of these reactions. In both reactions, the % hybridization reaches a similar value, near to completion, showing that the RNA from polymers and monomers are hybridizing to the same amount of polysomal cDNA. This indicates that both RNA preparations contain the same kind of sequences. However, the reaction between cDNA and the RNA derived from the 30 S particles is faster, specially in the low frequency population.

To check for parameters that could influence the interpretation of the curves, such as contamination with cytoplasmic RNA, we separated the RNA isolated from polymers and monomers electrophoretically on 4–10% acrylamide gradient–98% formamide gels (fig.4). No contamination with ribosomal RNA can be seen. However, this experiment does not exclude the possibility of non-ribosomal cytoplasmic contamination. To this end, we isolated crude rat liver nuclei, and mixed them with a cytoplasmic extract from rats which had obtained an 18 h pulse of [^{3}H]-orotic acid. The nuclei were then purified. No radioactivity could be detected in informoferes isolated from them.

It is known that HnRNA contains double-stranded sequences. These stretches, if present in different amounts in the RNA derived from the monomers and polymers, could influence their hybridization kinetics with the cDNA. We therefore analysed the hyperchromicity of both RNAs. The results are depicted in fig.5. The hyperchromicity of the two RNAs is similar,

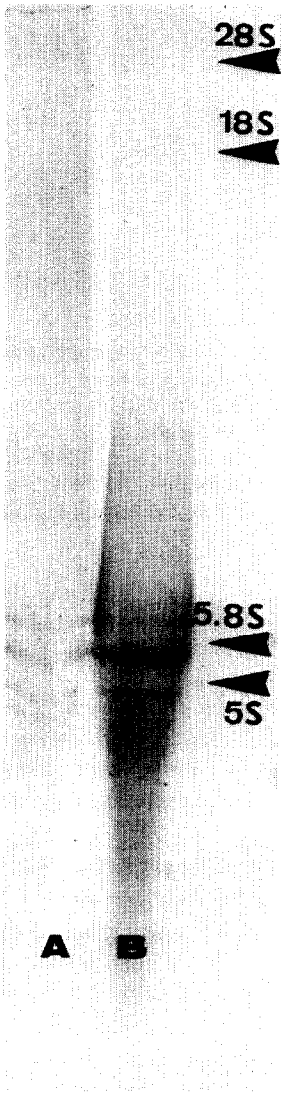

Fig.4. Gel electrophoresis on a 4–10% acrylamide gradient–98% formamide gel of RNA isolated from the RNA protein complexes. (A) RNA from polymers. (B) RNA from monomers. The arrows denote the migration distance of molecular weight markers.

19.5% for the RNA from monomers, and 16.5% for the RNA from polymers.

Finally we wished to investigate the concentration of nuclear poly(A)$^+$ sequences in the RNA from the monomers and the polymers. We prepared cDNA from nuclear poly(A)$^+$ RNA, using a 1 mM nucleotide

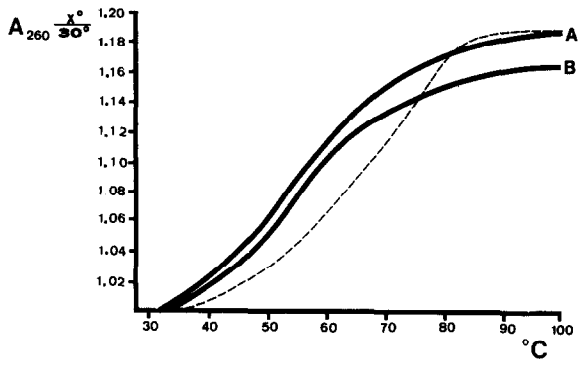

Fig.5. Melting curves of the RNA from informoferes, performed as in section 2. (A) RNA from monomers. (B) RNA from polymers. The dotted line shows the melting curve of tRNA.

in the final reverse transcription mixture. We have thus obtained cDNA with a mean sedimentation coefficient of 15 S, as analysed in an alkaline sucrose gradient. The cDNA was hybridized against RNA from both monomers and polymers. Figure 6 shows the results of the analysis. The reaction is clearly not going to completion, even at very high *Rot* values. Both RNAs reach a similar hybridization value, the reaction with the RNA derived from the 30 S particles being slightly slower. A possible explanation for this,

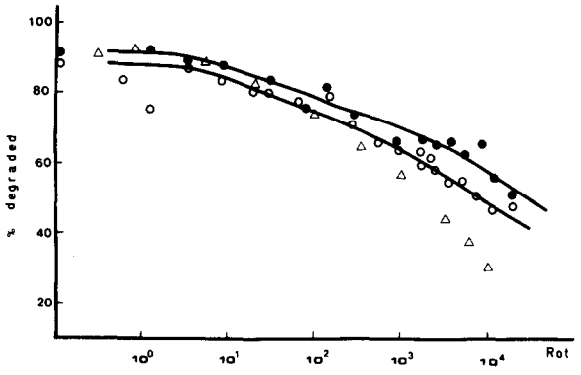

Fig.6. Hybridization of cDNA from nuclear poly(A)$^+$ RNA to HnRNA. Conditions of hybridization as in section 2. RNA was ⩾ 25 000 times cDNA. The curves have been plotted on the basis of the results of 3 different experiments. (●) RNA from 30 S particles. (○) RNA from 100 S particles. (△) Nuclear poly(A)$^+$ RNA.

is that in RNA from monomers, sequences have been eliminated, that are homologous to sequences present in nuclear cDNA but not in polysomal cDNA. This difference in the reaction rate can, however, be observed already in the high frequency sequences. When allowance is made for this the curves become coincident except at high *Rot* values.

4. Discussion

The association of specific non-histone nuclear proteins with HnRNA [1–7] and the recognition that HnRNA contains both precursor molecules for mRNA and non-coding sequences with still unknown function, raises the question of the organization of these functionally differing sequences of HnRNA. Samarina et al. first described the 30 S particles in rat liver nuclei, originating from larger structures by nucleolytic degradation [1]. Cleavage of these large structures either by endogenous or by added RNase, led to the formation of 30 S monomers, which could be further degraded only by excessive digestion. These experiments suggested the existence of a repeating RNP particle structure, composed of stretches of RNA tightly associated with proteins, thus imposing nuclease resistance and of exposed RNA stretches, easily susceptible to nucleolytic enzyme digestion. This would produce a preferential degradation of non-coding sequences in the monomeric structures, their RNA being therefore enriched in sequences homologous to polysomal poly(A)$^+$-cDNA.

If this assumption is true, the hybridization rate of RNA molecules present in the 30 S particles, being enriched in coding sequences, would be higher than that of the larger size RNA of the polymeric structures, which should contain a higher proportion of non-coding sequences. The hybridization rate of the polysomal poly(A)$^+$-cDNA with the RNA from the monomeric particles is indeed faster than that for the RNA from the polymers. This is more prominent in the low frequency population than in the high frequency one.

It could be argued that the slower hybridization rate of the RNA from the polymers is due to a higher degree of contamination with RNA other than HnRNA. Our results ruled out this possibility, since it was not possible to detect in any of the two RNA preparations contamination with rRNA or other cytoplasmic material. A possible effect of a differing content of snRNA in the two preparations could also be excluded, since we could quantitate the snRNAs from polymers and monomers and found them to be approximately equal.

HnRNA is known to contain double-stranded sequences which would not be available for hybridization. In addition, it was found [19] that the intact HnRNA can form intermolecular duplexes, thus making the amount of sequences free for hybridization smaller. It is also known, that some of the double-stranded sequences found in nuclear RNA can be found in the cytoplasmic RNA as single-stranded stretches. Therefore, different amounts of secondary structure in the RNAs from the polymers and the monomers could account for the different hybridization rate and thus cause misinterpretation of the curves. This was not the case as the hyperchromicity values of the RNA derived from polymers is somewhat lower than that for the monomeric RNA. Its hybridization rate, on the contrary was slower. These results taken together confirm our prediction, that 30 S particles are enriched in coding sequences.

It is unknown what proportion of poly(A)-containing sequences from HnRNA can be found in the RNA from nuclear RNPs. The RNA sequences in nRNP from *Dictyostelium* [20] hybridize in the same manner as total HnRNA in a total DNA-driven RNA hybridization. We have tried to analyse our 30 S and 80–150 S particles RNA for the concentration of poly(A)$^+$-*n*RNA sequences and the distribution of these sequences. The reaction could not go to completion, for technical reasons, as very high *Rot* values are necessary.

It is, however, possible to distinguish two different frequencies in the hybridization curve, similar to the frequencies which have been described for HnRNA.

The preferential association of the coding RNA sequences with nuclear proteins in a way imposing RNase resistance and the presence of the non-coding sequences in the exposed regions of the RNP structures could have implications for the processing of the HnRNA. It is therefore tempting to speculate that the specific nucleolytic and other enzymes involved in processing of HnRNA will preferentially interact with the exposed RNA stretches. Finally we want to point out that our model represents a first approach

to the organization of the coding sequences in the nucleus, and surely will be elaborated on in future work.

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