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# hnRNP PROTEINS INTERACT WITH NASCENT ADENOVIRUS RNA

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SUMMARY : By using Adenovirus 2 infected HeLa cells labeled during very brief pulses of ($^3$H)Uridine, we have shown that nascent chains of heterogenous nuclear RNA (hnRNA) were already associated with proteins to form ribonucleoprotein particles (hnRNP). It was also shown that the small Ad2 specific VA RNA was not associated with these hnRNP.

## INTRODUCTION

All or almost all hnRNA molecules seem to be associated with ribonucleoprotein particles within the nucleus of eucaryotic cells (1). This association seems to begin immediately on nascent RNA chains as they are transcribed from the chromatin template, i-e even before transcription is completed, according to electron micrographs of spread active chromatin (2-7). However, one cannot exclude the occurence of molecular rearrangements during the spreading process. We have therefore carried out a set of kinetic experiments intended to settle the question of how early do hnRNP proteins interact with nascent RNA.

Adenovirus 2 specific RNA has given us the opportunity for such a study : late during the viral infection cycle a large quantity of the total RNA synthesized in the nuclei of infected HeLa cells is virus specific (8-10). Furthermore this RNA is transcribed as a single species (11-13) which is subsequently matured into viral specific mRNA.

We have therefore studied the association of the Ad2 specific transcription products with the ribonucleoprotein particles during very brief pulses.

## MATERIALS AND METHODS

*Cell and virus growth* : procedures for growth of Ad2 infected Hela cells and virus purification have been described previously (14-15).
*Labeling and cell fractionation* : Hela cells, infected for 15 hours with 20 p.f.u. per cell, were concentrated to $10^7$ cells/ml in fresh medium and labeled for indicated times with ($^3$H)uridine (27Ci/mMole, 400µCi/ml). For longterm labeling with ($^{32}$P)$3.10^8$ cells were washed twice with phosphate-free medium and incubated for 5 hours with 20mCi($^{32}$P) at $3.10^6$ cells ml in the same medium. After labeling, cells were chilled collected by centrifugation and lysed in RSB (10mM Tris-HCl pH 7.4, 10mM NaCl, 1.5mM $MgCl_2$). Nuclei were washed with RSB con-

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taining 0.5% triton X100 and sonically disrupted as already described (16). The nuclear extract was freed of nucleoli and further fractionated by centrifugation on either 15-30% (w/v) sucrose gradient in RSB containing 0.1M NaCl (90 min. at 40K in the SW40 Spinco rotor) or 41% (w/v) metrizamide solution in RSB (60 hours at 30K in the SW60 Spinco rotor). The characteristic low density of hnRNP was checked by centrifugation to equilibrium in 4.5 ml 40% (w/v) CsCl RSB solution after fixation of the sample (0.5ml) with 6% glutaraldehyde (16).

*RNA extraction and hybridization* : RNA present in nuclear extracts or individual gradient fractions was extracted once with a mixture of phenol-chloroform (1:1) and twice with chloroform containing 1% isoamyl alcohol. Hybridization of RNA to filter-bound Ad2 DNA fragments was carried out in 0.5ml of 0.3M NaCl, 10mM EDTA, 0.2% SDS, 10mM TES buffer pH 7.4 for 36 hours at 65°C. RNAse resistant RNA was monitored as described (17) and DNA excess conditions were checked by rehybridization of the solution with fresh DNA filters.

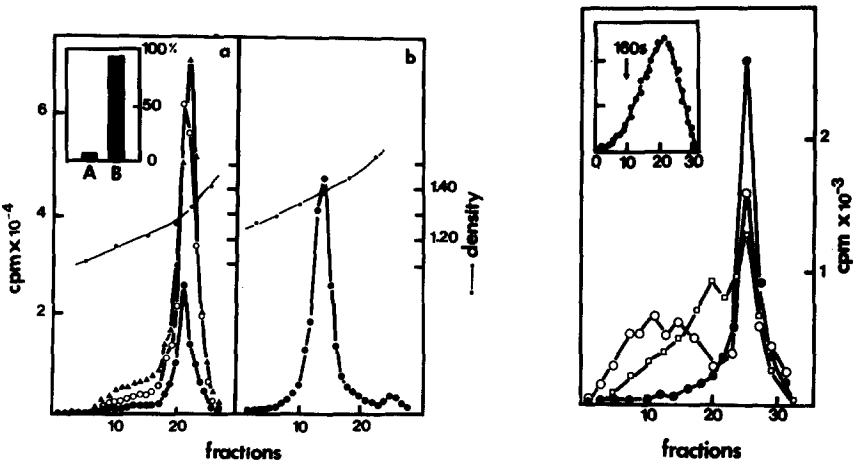
*Electrophoresis of RNA* : Small nuclear RNAs present in hnRNP were analysed by electrophoresis in 12.5% acrylamide- 0.4% bisacrylamide slab gels run in 90mM Tris-borate pH 8.3, 3mM EDTA, 8M urea and stained with ethidium bromide. Ad2 VA RNA was extracted from gels as described by Maxam and Gilbert (18) and labeled in vitro using ($^{32}$P) pCp and RNA ligase as described by Peattie (19). It was then hybridized to Ad2 DNA cut with Hind III or Sal I and transfered to DBM-paper after electrophoresis in 1% agarose slab gel according to Alwine et al(20)

## RESULTS and DISCUSSION

*Nascent chains of Ad2 specific RNA are present in hnRNP* : Ad2 infected Hela cells were pulse labeled for 45 sec, 2 min, 5 min and 20 min with ($^{3}$H)uridine and the nuclei prepared by hypotonic swelling as already described (16). Triton X100 cleaned nuclei were then sonically disrupted and fractionated into an RNP containing nuclear extract and a pellet of chromatin by centrifugation on 15-30% (w/v) sucrose gradients. The nuclear extract contains a heterogeneous population of hnRNP (insert to Fig. 1 right) which are highly enriched in Ad2 sequences as shown in Table 1.

To further separate hnRNP from free RNA or chromatin,nuclear extracts from pulse labeled Ad2 infected cells were analysed by centrifugation to equilibrium in metrizamide gradients. Most of the radioactivity was found to band at $\rho \sim$ 1.29-1.30 (Fig. 1a, left), a density characteristic of hnRNP (16) while free RNA or chromatin are readily separated with densities between 1.15 and 1.22 (21). For comparison the same ($^{3}$H)uridine labeled material has been centrifuged to equilibrium in a CsCl gradient after fixation with glutaraldehyde and showed the typical low density ($\rho \sim$ 1.40) of hnRNP (Fig. 1b, left).

Moreover when the RNA present in the different regions of the gradients was assayed by hybridization to Ad2 DNA immobilized on nitrocellulose filters 90-95% of the Ad2 specific RNA was shown to be associated with hnRNP (insert to Fig. 1a left). Because transcription of the late Ad2 transcription unit goes from 16.3 to 100 (22) (the Ad2 genome is arbitrarily divided into 100 units of approximately 350 base pairs each; see Fig. 2), Weber et al (12,13) have shown that RNA homologous to the different regions contained between those boundaries




For various times : ● -d ●, 45 sec
O - O : 5 min; ▲ - ▲, 2 min.

FIGURE 1 : (Left)

a) Metrizamide density gradient centrifugation of nuclear extracts from Ad2 infected HeLa cells pulse labeled with ($^3$H)uridine. insert : fractions 1 to 17 (A) and 18 to 28 (B) were pooled separately and their RNA extracted and hybridized to Ad2-DNA filters as described in materials and methods. The result is expressed as the percentage of total radioactivity in A or B which is Ad2 specific (only the result obtained with the sample labeled for 45 sec. is shown). ● - ● : 45 sec ; O - O : 5 min ; ▲ - ▲ : 20 min.

b) CsCl density gradient centrifugation of the nuclear extract labeled for 45 sec. after fixation with glutaraldehyde.

: (right)

Sucrose gradient fractionation of hnRNP pulse labeled with ($^3$H)uridine for 45 sec. 10µl aliquots were precipitated with trichloroacetic acid to determine the pattern of total radioactivity. The RNA present in every other fraction of the gradient was then hybridized to nitrocellulose filters bearing the following DNA restriction fragments : Eco C : O - O, Eco F : □ - □ , Sma F : ● - ●. Centrifugation was from right to left and the arrow shown in the insert corresponds to the position of poliovirion run in a parallel gradient as a marker.

are detected in nascent molecules whose size increases from promoter proximal regions, (Sma F 11.3-18.1) to those more promoter distal (Eco C: 89.7-100)(Fig.2). To detect such molecules, infected cells were labeled for 45 sec. with ($^3$H)uridine a procedure which results in the labeling of mainly the growing 3' termini and no completed chains (11,12).

RNA was phenol-chloroform extracted from briefly labeled hnRNP and hybridized to filters containing Sma F (11.3-18.1), Sma D (38.6-51.1), Eco F (70.7-75.9) and Eco E (83.4-89.7) fragments. The number of hybridized RNA molecules (expressed as the ratio of hybridized radioactivity to the size of the DNA fragment) is reasonably similar for the different selected fragments (Table 2). Moreover when pulse labeled particles were displayed on a sucrose gradient and

TABLE 1

VIRAL SPECIFIC FRACTION OF TOTAL PULSE LABELED RNA IN hnRNP

| Labeling time | % Ad2 specific RNA |
|---|---|
| 45 sec. | 44.8 |
| 2 min. | 57.9 |
| 5 min. | 63.9 |
| 20 min. | 55.5 |

RNA present in the nuclear extract was phenol-chloroform extracted, concentrated by ethanol precipitation and an aliquot corresponding to 1/100 of the preparation was hybridized to 20 µg of filter bound Ad2 DNA as described (14). The result is expressed as the percentage of the input radioactivity which remains RNAse resistant after hybridization.

labeled RNA monitored for hybridization to Sma F, Eco F and Eco C fragments, the pattern shown in Fig.1 right was obtained. Promoter distal sequences, scored with Eco C fragment were found in very large hnRNP contrary to the promoter proximal sequences, scored with the Sma F fragment, which were found in the smallest particles. An intermediate situation was obtained with the RNA hybridizing to Eco F fragment. This ordered increase in the size of pulse labeled RNA molecules within hnRNP shows that they actually represent nascent fibrils.

*Ad2 specific VA RNA is not present in hnRNP* : In this context, it is interesting to investigate the behaviour of this sequence with respect to its association with hnRNP proteins when it occurs as VA species (RNA-polymerase III transcript) or as part of a large premessenger molecule (RNA-polymerase II transcript).

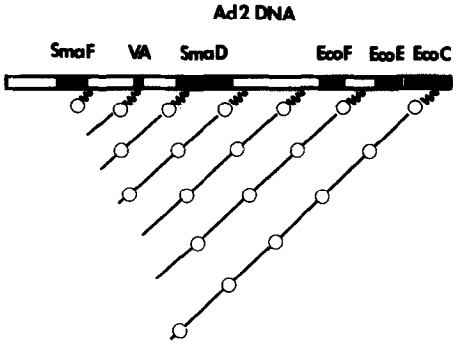



FIGURE 2 : Schematic representation of the late Ad2 right ward reading transcription unit showing the restriction fragments used in this work as well as the nascent RNP fibrils.

: Pulse labeled RNA; ● : RNA polymerase;—— : nascent RNA chains;
○ : hnRNP proteins.

TABLE 2

MOLAR RATIOS OF Ad2 RNA CONTAINED IN hnRNP

| DNA fragment | Map coordinates | cpm/map units |
|---|---|---|
| Sma F | 11.3 - 18.1 | 6070 ✱ |
| Eco F | 70.7 - 75.9 | 4000 |
| Sma D | 38.6 - 51.1 | 7000 |
| Eco F | 83.4 - 89.7 | 4500 |

Late Ad2 infected HeLa cells were pulse labeled for 45 sec. with ($^3$H) uridine and the RNA present in hnRNP was phenol-chloroform extracted as described in material and methods. The RNA was then alkali fragmented for 20 min. in 0.1 M NaOH at ice temperature and hybridized to filters containing 30 µg equivalent of DNA as described (14). The result is expressed as RNAse resistant cpm divided by the fragment length expressed in map units.

✱ In this case the length which was taken into account was 16.3 - 18.1 because transcription begins at 16.3.

Analysis by gel electrophoresis shows (Fig.3) that the same snRNA species are present in hnRNP from HeLa cells whether infected or not and that no stainable band could be detected at the position of VA (the identity of cytoplasmic VA was established by terminal labeling in vitro with $^{32}$pCp and hybridization to Ad2 DNA). The same analysis carried out on RNA pulse labeled in late phase (no cellular snRNA are labeled under these conditions) shows (results not presented) that only traces of VA are found in hnRNP as compared to the amounts present in nucleoplasm or cytoplasm (these traces were nevertheless identified as VA by fingerprint analysis). These results confirm those previously reported by others (23).

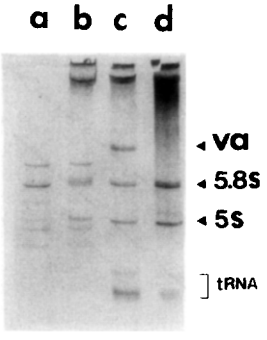



FIGURE 3 : Polyacrylamide-urea gel electrophoresis of small RNA (ethidium bromide staining). a) RNA from uninfected HeLa cells hnRNP; b) RNA from Ad2 infected HeLa cells hnRNP; c) cytoplasmic RNA from Ad2 infected HeLa cells; d) cytoplasmic RNA from uninfected HeLa cells.

Taken together with our findings that there is no significant difference in the protein complement of hnRNP from uninfected or late Ad2-infected HeLa cells (24,25), the above results suggest that viral sequences are packaged in the same kind of RNP structure as are cellular sequences.

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