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SMALL NUCLEAR RNA AND VA RNA IN NUCLEAR RIBONUCLEOPROTEIN FIBRILS FROM ADENOVIRUS-2 INFECTED HeLa CELLS

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SUMMARY : The small RNA of hnRNP[1] were studied in HeLa cells infected with adenovirus-2. At 15 h post-infection, when 50-60 % of the hnRNA was of viral origin, all the small nuclear RNA of hnRNP from non-infected cells were present in hnRNP from infected cells. The small, virus-encoded VA RNA could not be detected by staining like the snRNA but only after labeling. It represented less than 1 % of the small nuclear RNA in hnRNP. The low level of VA RNA in hnRNP as compared to that of the small nuclear RNA does not favor the hypothesis of a similar function for these 2 classes of small RNA.

Small nuclear RNA (snRNA) were shown to be present in hnRNP from rat liver, rat brain and HeLa cells (1-4). A fraction of them seems to be hydrogen bonded to hnRNA (5, 6). Sequence complementarities between U1 RNA and series of intron sequences close to splicing sites were described (7). On the other hand, sequences of the VA RNA encoded by the adenovirus genome were shown to be complementary to intron and messenger sequences at the leader-hexon junction (8). Therefore, it was proposed that small nuclear RNA and VA RNA may serve as splicer RNA for the processing of premessenger RNA (7, 8), but this hypothesis was not experimentally verified.

At first fiew, it seems difficult to assimilate VA RNA and small nuclear RNA. SnRNA are preferentially localized in the nucleus and only relatively small amounts were found in the cytoplasm (9). In contrast, 90 % of VA RNA were shown to be present in the cytoplasm (10). SnRNA are synthesized by RNA polymerase II (11) and VA RNA by RNA polymerase III (12). VA RNA is more actively synthesized (10) than snRNA (13). In addition, the sequence complementarity of VA RNA to the leader-hexon junction as proposed by Murray and Holliday (8) requires a longer sequence than that of mature VA RNA, probably that of V200 assumed to be a precursor to VA RNA and detected up to now only in *in vitro* RNA-synthesizing systems (12).

Nevertheless, before rejecting the possibility of a similar function of VA RNA and snRNA, we searched for VA RNA in hnRNP from adenovirus infected

[1] Abbreviations used : hnRNP : ribonucleoprotein containing heterogeneous nuclear RNA, snRNA : small nuclear RNA ; VA RNA : virus associated RNA.

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HeLa cells. At the late period of productive infection, a large fraction of the nuclear RNA is encoded by the virus genome (14-15). We showed that adenovirus transcripts representing 60 % of the nuclear RNA at 14-16 h post-infection were part of hnRNP having the same characteristics as those of non infected HeLa cells (16). At that period of infection, VA RNA is still actively synthesized and is relatively abundant in the cytoplasm of infected cells (10). If VA RNA plays the same role versus the adenovirus transcripts as snRNA versus the cellular premessenger RNA, it would replace snRNA in the hnRNP containing the viral transcripts and, therefore, represent about 60 % of the population of small RNA in the hnRNP from infected cells. This proportion would be lower but at least of the same order of magnitude as that of the other small RNA, if one assumes that different classes of small RNA are specific to different classes of junctions.

Our experiments showing that VA RNA is not detectable in hnRNP under conditions where the small nuclear RNA are routinely demonstrated do not favor a function of VA RNA similar to that of snRNA.

METHODS

HeLa cells were grown in suspension and infected with adenovirus-2 at 100 plaques forming units per cell. Infection was stopped at 15 h post-infection and the cells were harvested as previously described (16, 17). For labeling experiments, 10 µCi/ml of (^{3}H) uridine was added at 13 h post-infection and incorporation was stopped after 2 h.

After washing, the cells were suspended in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$ (RSB) and broken with a tight-fitting Dounce pestle. The nuclei were sedimented at 600 x g for 3 min. The supernatant served as source of cytoplasmic RNA. The pellet was washed in RSB containing Triton X-100. The purified nuclei were broken by ultrasonication in RSB. In order to avoid release of adenovirus replication complexes, 75 mM NaCl was added to the lysate before centrifugation at 100,000 x g for 5 min (16). The supernatant contained hnRNP and nucleosol and was designated as "nuclear extract". The pellet whose major component was chromatin was designated as "nuclear residue".

The nuclear extract was fractionated on a 10-40 % linear sucrose gradient made in 10 mM Triethanolamine-HCl, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$. Centrifugation was for 16 h at 24,000 rmp in a SW 25-2 rotor.

Fractions were pooled as follows : (A) pellet, large size hnRNP ; (B) 60-100 S hnRNP ; (C) 30-50 S hnRNP (or monoparticles) ; (D) 10-25 S ; (E) 0-10 S. Pools (D) and (E) contained snRNP and free snRNA, respectively (4). The material of the pools was precipitated overnight in the presence of 2 volumes of ethanol and the RNA was phenol-extracted from the pellets at pH 7.6, 0-4°C for 30 min.

RNA was electrophoresed in polyacrylamide slab gel as previously described (4). The conditions are indicated in the legend of figures. Fluorography (film Fuji RX) was according to Laskey *et al* (18). Nomenclature was that of Roi-Choi *et al* (19) for nuclear RNA and of Mathews and Pettersson for cytoplasmic RNA (10).

RESULTS

Nuclear extracts were prepared from infected and non infected HeLa cells and fractionated on sucrose gradients. The small-size RNA of the various fractions (A to E) as well as those from cytoplasm (F) and from nuclear residue

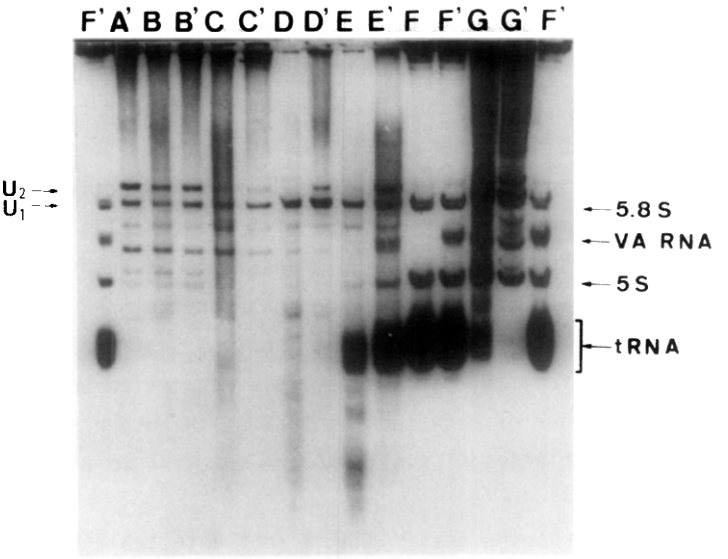

Fig. 1 : Slab gel electrophoresis of small RNA after staining. Gels (8 M urea, 10 % polyacrylamide) were 11 cm long. The samples were heated at 65°C for 10 min in the presence of 6 M urea and 1 % sodium dodecylsulfate just before electrophoresis. Samples A to G were from non infected HeLa cells. Samples A' to G' corresponded to the same fractions after infection with adenovirus. (A') : > 100 S, hnRNP ; (B-B') : 60-100 S, hnRNP ; (C-C') : 30-50 S, hnRNP ; (D-D') : 10-25 S, snRNP ; (E-E') : 0-10 S, soluble material ; (F-F') : cytoplasmic RNA ; (G-G') : nuclear residue. VA RNA is detected only in the cytoplasm of infected cells (F').

(G) were analyzed in denaturing polyacrylamide slab-gels (Fig. 1) and the gels were stained with Methylene Blue.

As previously described in brain hnRNP (4), there were several snRNA species in hnRNP of HeLa cells (B, C). The same snRNA were detected after infection (A', B', C') and there was no additional band. The VA RNA appeared as a prominent band in the cytoplasm of adenovirus-infected cells (compare F' to F) and no band migrating at this level was detectable in hnRNP (A', B', C').

VA RNA was also undetectable in the snRNP (D, D') and free snRNA (E, E') fractions. In the nuclear residue, a faint band with the same migration as VA RNA was present in infected cells and absent from non infected cells (G, G')

As 50-60 % of the hnRNP RNA were adenovirus transcripts, the results indicate that VA RNA does not replace snRNA in the hnRNP containing such transcripts. A snRNA species representing 1 % of the snRNA population can be detected under our experimental conditions provided that it might be separated electrophoretically which is the case for VA RNA. We may conclude that VA RNA is present at a very low level in or absent from hnRNP.

In contrast to snRNA (13), VA RNA is rapidly labeled (10). Therefore, labeling experiments were performed to allow detection in hnRNP (Fig. 2).

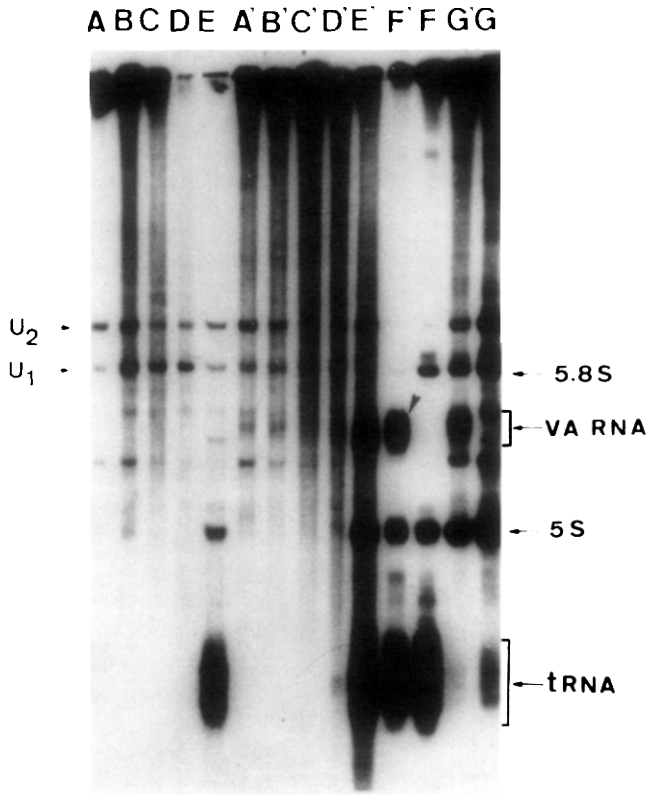

Fig. 2 : Slab gel electrophoresis of small RNA after fluorography. Gels (6 M urea - 12 % polyacrylamide) were 28 cm long. Denaturation and symbols as in figure 1. The arrowhead indicates the cytoplasmic VA RNA which served as migration marker.

Longer and more concentrated polyacrylamide gels were used to improve resolution VA RNA was highly labeled in the cytoplasm of infected cells (F'), more than 5 S RNA for exemple. A band migrated at the level of VA RNA in hnRNP from infected cells (A', B', C') and was absent in those from non infected cells (A, B, C). Higher amount of the same species were found in the other nuclear fractions including nuclear residue (D', E', G'). VA RNA II which is a minor cytoplasmic RNA also encoded by the adenovirus genome (10) and which migrates slightly faster than the major VA RNA I that we are studying, did not seem to be present in hnRNP.

In order to verify whether the additional RNA band in hnRNP was VA RNA, we made use of the considerable change of migration of VA RNA versus the other small RNA when analyzed in non-denaturing gels (10). Cytoplasmic VA RNA migrated slower than U1 RNA in non-denaturing gels and faster in denaturing gels (F' in Fig. 2 and 3). The additional band in hnRNP (A', B', C') and in the other fractions (D', E', G') had this same behavior and it is reasonable to deduce

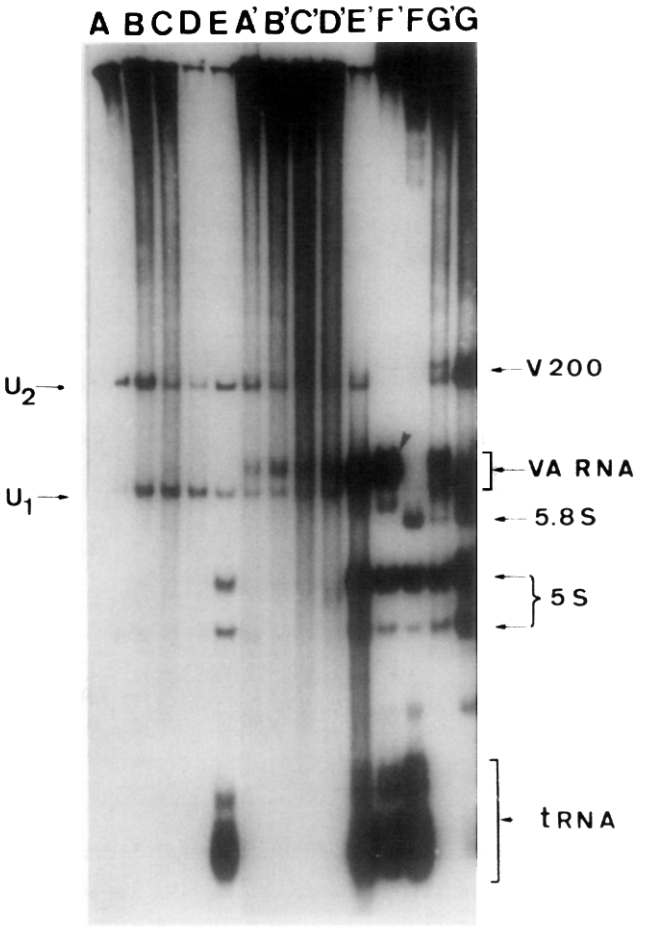

Fig. 3 : Slab gel electrophoresis of small RNA after fluorography. The conditions were the same as in figure 2, except that urea was omitted from denaturing medium and from gel buffer. The additional band migrating slower than U2 RNA was designated as V200 though its identification as authentic V200 was not yet confirmed.

that it represents VA RNA. Another additional band which was not detected in denaturing gels was found in hnRNP and nucleosol from infected cells (Fig. 3, A' to E'). It migrated slightly slower than U2 RNA (196 nucleotides) and may represent V200 RNA (200 nucleotides) (12). It is likely that it comigrated with U2 RNA in denaturing gels and therefore, escaped detection (Fig. 2). V200 RNA seemed less abundant in hnRNP (or less labeled) than the major VA RNA.

Labeled VA RNA was quite abundant in the nucleosol (E', Fig. 2 and 3) and aspecific adsorption on hnRNP would be possible. However, previous work showed that adsorption was limited under our experimental conditions (4). Moreover, 5 S and 4 S RNA which are as abundant as VA RNA in the nucleosol did not trail in hnRNP. Therefore, it seems likely that VA RNA is associated with hnRNP.

DISCUSSION

Our experiments show that the population of small RNA from hnRNP containing adenovirus transcripts is primarily that of the host cell. In a previous work, we also demonstrated that the cellular hnRNP proteins were associated with the adenovirus transcripts in spite of the considerable change of RNA sequences (16). It might be concluded that the cellular machinery is used for the processing of premessenger RNA whatever their origin. In this respect, it is worth mentioning that the 5' terminal sequence of U1 RNA shown to be complementary to a large number of intron sequences at cellular or viral junctions (7) is also complementary to the intron sequences at the leader 3-hexon messenger and at the leader 4-fiber messenger junctions (unpublished observations). The use of the cellular machinery does not exclude that viral factors are implied in regulation of the processing as might be the case for the small amount of viral proteins (16) or the very small amount of VA RNA found associated to hnRNP (present work).

Due to its low amount, the proportion of VA RNA in hnRNP relative to snRNA is difficult to determine accurately. It certainly does not exceed 1 % and is probably less, against 3-30 % for the individual snRNA species. If one assumes that there is 1 molecule of snRNA per 2,500 nucleotides of hnRNA (4) i.e. 1 molecule per hnRNP fibril, VA RNA would be present in no more than 1 fibril out of 100 and even less for V200 RNA. As already stressed (7), a participation to RNA processing implies a relatively high abundance and it seems difficult to conciliate such low level of VA RNA with the splicing of the premessenger RNA of an actively synthesized viral protein such as hexon (8). However, as the function of the snRNA in splicing has not been demonstrated yet, the main conclusion of our work is only that the amount of VA RNA or V200 RNA relative to snRNA in hnRNP from adenovirus infected cells is too low to account for a same function in agreement with other arguments already enumerated in the introduction. More experimental evidence is certainly needed to determine what is exactly the function of the 2 classes of small RNA.

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