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PRESENCE OF THE PRE-mRNA FOR THE 72K DNA BINDING PROTEIN IN hnRNP FROM EARLY ADENOVIRUS-2 INFECTED HeLa CELLS.

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Summary : nuclear ribonucleoprotein particles (hnRNP) extracted from early adenovirus-2 infected HeLa cells contain covalently intact pre-mRNA of adenovirus 72000 daltons DNA binding protein as well as mRNA which seems to be associated to typically nuclear structures.

INTRODUCTION

Several in vitro splicing systems have been worked out so far and with the exception of pre-tRNA, most of them involve incubation of more or less purified nuclei (1-4). This provided us some encouragement towards the goal of purifying the enzyme(s) that splices mRNA precursors. Our previous difficulty to observe in vitro splicing with purified RNA and fractionated cellular extracts has led us to address the problem of the relationships between messenger RNA processing and nuclear structure. It is now accepted that most nuclear RNA is associated with ribonucleoprotein particles within the cell nucleus. (For several recent reviews -see ref. 5-) moreover these RNA fibrils seem to be associated the nuclear skeleton (6-9), which could impose constraints on hnRNP molecules (10) and organize them to facilitate an accurate splicing while the RNP proteins could select the portions of the transcripts which are destined to be found in the mRNA, or just prevent undesirable RNA conformations like the formation of knots.

Whatever the role of these structures, their possible utilization in an in vitro splicing assay requires the RNA to be intact. We have therefore started a systematic analysis of adenovirus transcripts associated with these structures. The present work reports some preliminary results obtained with the RNA transcribed from the early regions E_2 which codes for the so called 72000 daltons DNA binding protein (72 K DBP) (11).

MATERIALS AND METHODS

Cell and virus growth : procedures for growth of Ad2 infected HeLa cells and virus purification have been described previously (12, 25).

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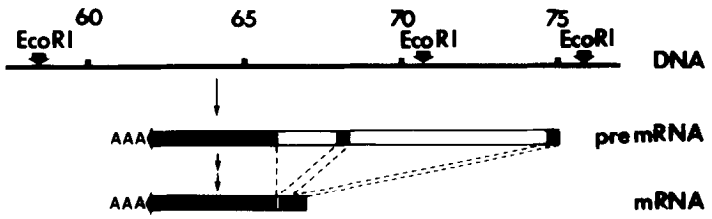

Figure 1 : The transcription unit for the mRNA of the Ad-2 72,000 daltons DNA binding protein (early region E2) showing the restriction fragments used in this study. The sequences contained in the spliced mRNA are shown in black. The numbers above the line indicate map distances from the left end of the genome.

Labeling and cell fractionation : Hela cells (3.10^8) were pulse labeled for 1 hour with 5mCi (^3H) uridine (27 Ci/mMole, 20 µCi/ml) 5 hours after infection with adenovirus 2 at a multiplicity of 50 p.f.u. per cell. After labeling the cells were collected by centrifugation and the nuclear extract prepared by sonication and fractionated by centrifugation for 90 min at 40 K (SW40) on a 15-30 % (w/v) sucrose gradient as described (24). The cytoplasm was freed of mitochondira by a low speed centrifugation, adjusted to 10 mM EDTA, 0.5 % Brij and fractionated by centrifugation for 5 hours at 40K (SW40) on a 7-47 % (w/v) sucrose gradient in 10 mM Tris-HCl pH 8.5, 0.15 M NaCl, 10 mM EDTA.
RNA extraction and hybridization : were as described (12, 25).
Electrophoresis of RNA : the RNA present in individual gradient fractions was subjected to electrophoresis on cylindrical 2.7 % polyacrylamide gels as described (25). The RNA was eluted from gel slices, hybridized to filter bound (58.5-70.7) Ad2 DNA and RNAse resistant hybrids were scored. Poly(A) + RNA from nuclei and cytoplasm as well as total RNA from large nuclear particles prepared according to Kish and Pederson (21) was run under denaturing conditions in 1.2 % agarose slab gels containing 10 mM methylmercuryhydroxyde and transfered on to diazobenzyl-oxymethyl paper as described (22). The RNA was detected by hybridization for 24 hours essentially as described by Stark et al (26) with (^{32}P) EcoRI B DNA labeled by nick-translation to a specific activity of 1.5×10^8 cmp per µg (27).

RESULTS AND DISCUSSION

Nuclear ribonucleoprotein particles (hnRNP) prepared from Ad-2 infected cells labeled with (^3H) uridine 5 hours post infection were fractionated sucrose gradient centrifugation. The radioactivity was found distributed in heterogenous structures sedimenting between 30 and 300S and having a characteristic low density upon centrifugation in CsCl gradient (not shown) (12-13). Gradient fractions were deproteinized and the labeled RNA was hybridized to Eco RI B (58.7-70.7) DNA fragment bound to nitrocellulose filter. As shown in figure 1, this fragment carries the 3' half of the gene coding for the 72K DBP and can be used to follow the primary transcript as well as the mRNA transcribed from this region. EcoRI B specific RNA was found in structures sedimenting between 70 and 200S according to a pattern different from that of the bulk hnRNA (figure 2-a). In contrast, hybridization of the same fractions with total Ad-2 DNA revealed a pattern that closely followed the total hnRNP profile (not shown) (14).

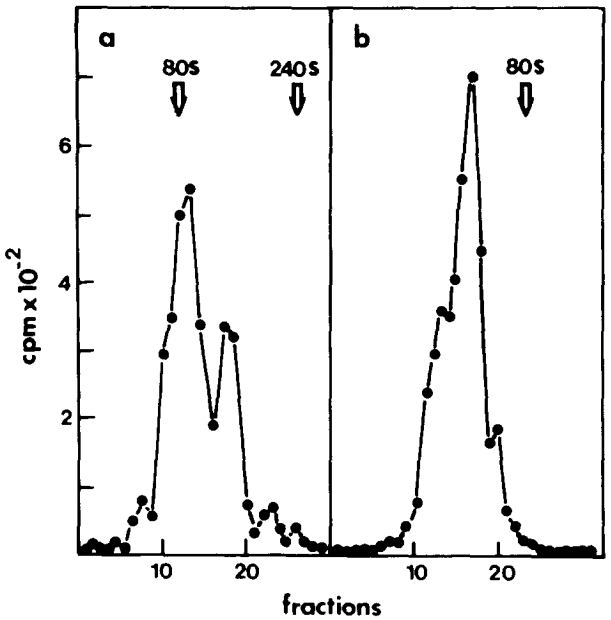

Figure 2 : Sucrose gradient centrifugation of nuclear (a) and cytoplasmic (b) RNP containing the early Ad2 region E2 transcripts. Centrifugation is from left to right and the markers : monosomes (80S), and SV40 virions (240S) were run on parallel gradients. The labeled RNA contained in each fraction was deproteinized by phenolchloroform extraction, concentrated by ethanol precipitation and 1/3 for the nuclear samples or 1/10 for the cytoplasmic samples hybridized to nitrocellulose filters bearing Eco RI B (58.5 - 70.1) restriction fragment as described (25).

The same experiment carried out on cytoplasmic RNP revealed the existence of a narrow distribution of the EcoRI B specific RNA centered around 50S (figure 2-b). The RNA contained in both nuclear and cytoplasmic RNP was analyzed by electrophoresis on polyacrylamide gels (figure 3). The same difference between the nuclear and the cytoplasmic pattern was obtained : cytoplasmic RNP contained a single 18S RNA species (figure 3-c) in contrast to nuclear RNP (figure 3-a, b) which showed a complex distribution of RNA molecules, with sizes ranging from 28S to 10S, the larger RNP containing the larger RNA species (figure 3-a). The heterodisperse sedimentation behavior of the E2 specific sequences indicates that they are present in a range of high molecular weight hnRNP as opposed to cytoplasmic RNP. However the distribution of EcoB specific sequences is different from that of the bulk nuclear particles, with these sequences being somewhat more concentrated in fractions 10-15, and showing very often a second peak around fractions 18-22 (precursor particles ?).

Previous studies have identified three size classes of nuclear RNA transcribed from the early region E2 : a 28S pre-mRNA molecule which is the first polyadenylated species likely to occur by cleavage of a 7Kb primary transcript and which contains two intervening sequences, a 23S intermediate and

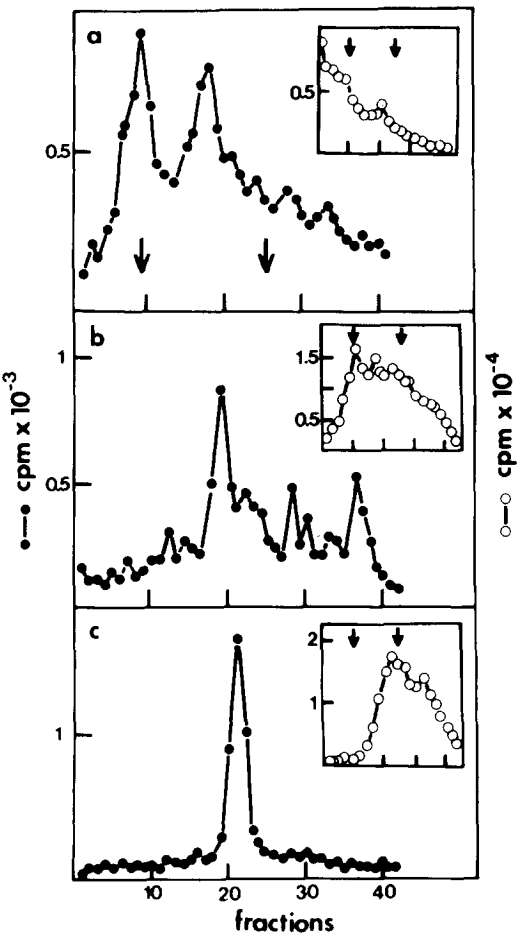

Figure 3 :Gel electrophoresis of pulse labelled early Ad2 E2 transcripts present in nuclear (a, b) and cytoplasmic (c) RNP.
RNA present in different fractions of the gradients shown figure 2 was denatured and subjected to electrophoresis through cylindrical polyacrylamide gels which were then sliced and processed as described (25). RNA was eluted, hybridized to filter bound (58.5-70.7) Ad2 DNA and RNAse resistant hybrids were scored.
a : RNA from large nuclear RNP (gradient fractions 13 to 25, figure 2a)
b : RNA from small nuclear RNP (gradient fractions 5 to 12, figure 2a)
c : RNA from cytoplasmic RNP (gradient fractions 10 to 20, figure 2b)
Total radioactivity present in each slice is shown in the inserts and the arrows indicate the position of ribosomal RNA run as markers. Migration is from left to right.

a 20S mature mRNA (15-20). We therefore conclude that, except for the species smaller than 18S which are degradation products, these data show that newly synthesized, intact transcripts of the gene coding for the adenovirus 72K DBP are present in large hnRNP. The integrity of these RNA sequences was further confirmed by analysis of the RNA from unlabeled hnRNP under denaturing conditions. Figure 4 shows the pattern of the RNA extracted from unlabeled large hnRNP prepared according to Kish and Pederson (21) and transfered on to diazobenzyloxymethyl

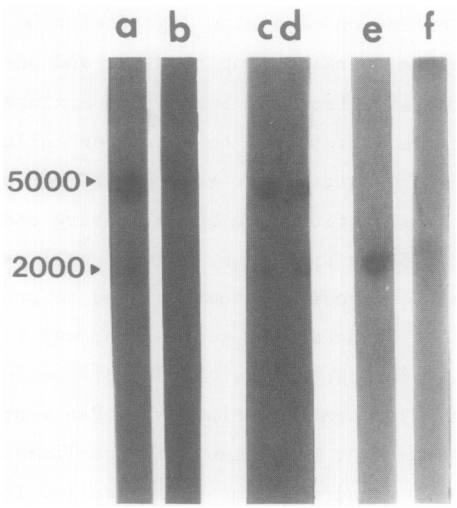

Figure 4 : Northern blot hybridization for early Ad2 E2 the transcripts in large nuclear particles. The approximate size for the different species (∿ 5000 and ∿ 2000 nucleotides) was obtained by comparison with the size of HeLa and E. Coli rRNA run in the same gel
a : total RNA from hnRNP ; b : 28S poly(A)$^+$ nuclear RNA ;
c and d : total RNA from hnRNP incubated respectively for 5 or 15 min at 37°C ; e : 18S poly(A)$^+$ cytoplasmic RNA ; f : total hnRNP RNA from uninfected HeLa cells.

paper after electrophoresis in agarose containing 10 mM methyl mercury hydroxyde (22). The RNA was revealed by hybridization to (^{32}P) Eco RI B restriction fragment labeled by nick-translation (27).

If we compare the RNA present in large hnRNP (lane a) to 28S nuclear poly(A) + RNA (lane b) as well as cytoplasmic poly(A)+ RNA (lane e), we can see that the EcoRI B probe hydridized to two components which correspond to the unspliced 28S pre-mRNA and to the mature 18S mRNA, this latter species being much less abundant than in total unfractionated RNP (not shown). This pattern, which corresponds to accumulated species is different from that of newly synthesized transcripts in that it does not show the 23S transitory species, but is in agreement with the data obtained by Goldenberg and Raskas on total poly(A) + accumulated nuclear RNA (28). While demonstrating the presence of intact unspliced 28S 72K DBP pre-mRNA this result suggests the existence of mature mRNA molecules in nuclear RNP which differ from cytoplasmic mRNP at least by their sedimentation coefficient.

Although it is difficult to rule out a possible ribosomal contamination of nuclei, the fact that the nuclear RNP centrifugation pattern was not affected by the presence of EDTA at variance with the cytoplasmic RNA pattern, where more than 70 % of EcoRI B specific RNA was found in structures larger than 80S in the absence of EDTA (result not shown), strongly supports this idea and corroborates a previous report by Pederson and Davis on β -globin-RNP (23).

hnRNP were then incubated for 5 or 15 minutes at 37°C in a buffer containing 0.14 M NH_4Cl and 10 mM $MgCl_2$ and the result is shown figure 4 -

lane c and d. With the exception of a slow degradation no processing occurred. This last result has several possible implications and one of them could be that some factors were missing from the incubation mixture, in which case hnRNP could be used in a complementation test to assay for cellular factors able to restore a correct processing pattern for the 28S 72K DBP pre-mRNA. This is suggested by the recent observation made by Goldenberg and Raskas that the pre-mRNA for the 72K DBP is spliced by a total cell extract (28). Alternatively, the isolation procedure that we used to prepare hnRNP particles and which involves nuclear breackage by sonication, was too severe and resulted in particles structurally improper for a correct processing. The latter explanation is supported by recent experiments by Van Venrooij and collaborators (29-30) who have shown that hnRNA can be cross-linked to the nuclear matrix via protein by U.V. irradiation of intact cells. Thus, the nuclear skeleton might provide us with a very useful structure for studies on the processing of pre-mRNA and could shed some light into this very intriguing question in eukaryotic mRNA biogenesis : how a restricted set of essentially nuclear proteins interacts with nascent RNA, selects specific sequences and leads to their export towards the cytoplasm after an exchange with an other set of proteins ?

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REFERENCES

1. J.M. Blanchard, J. Weber, W. Jelinek, J.E. Darnell (1978) Proc. Nat. Acad. Sci. USA 75, 5344-5348.
2. V.W. Yang, S. Flint (1979) J. Virol. 32, 394-403.
3. H. Hamada, T.I. Garashi, M. Muramatsu (1980) Nucl. Acid. Res. 8, 587-599.
4. A.J. Zaug, T.R. Cech (1980) Cell 19, 331-338.
5. The Cell Nucleus (1981) (Ed. H. Bush, Academic Press, New York) vol IX.
6. R. Berezney, D.S. Coffey (1977) J. Cell Biol. 73, 616-637.
7. T.E. Miller, C.Y. Huang, A.O. Pogo (1978) J. Cell Biol. 76, 675-691.
8. R. Herman, L. Weymouth, S. Penman (1978) J. Cell Biol. 78, 663-674.
9. B.H. Long, C.Y. Huang, A.O. Pogo (1979 Cell 18, 1979-1090.
10. J. Paoletti, J. Rech, C. Brunel, Ph. Jeanteur (1980) Biochemistry 19, 5223-5228
11. J. Flint (1977) Cell 10, 153-166.
12. J. M. Blanchard (1980) Biochem. Biophys. Res. Commun. 97, 524-529.
13. R. Gattoni, J. Stevenin, M. Jacob (1980) Eur. J. Biochem. 108, 203-211.
14. J.M. Blanchard, J. Weber (1981) Molec. Biol. Rep. 7, 107-133.
15. E.A. Craig, H.J. Raskas (1976) Cell 8, 205-213.
16. C.R. Kitchingman, S.P. Lai, H. Westphal (1977) Proc. Nat. Acad. Sci USA 74, 4392-4396.

17. A.J. Berk, P.A. Sharp (1978) Cell 14, 695-711.
18. C.J. Goldenberg, H.J. Raskas (1979) Cell 16, 131-138.
19. J. Weber, J.M. Blanchard, H. Ginsberg, J.E. Darnell (1980) J. Virol. 33, 286-291.
20. J.R. Nevins, J.M. Blanchard, J.E. Darnell (1980) J. Mol. Biol. 144, 377-386.
21. V. Kish, T. Pederson (1975) J. Mol. Biol. 95, 227-238.
22. J.C. Alwine, D.J. Kemp, G.R. Stark (1977) Proc. Nat. Acad. Sci USA 74, 5350-5334.
23. T. Pederson, N.G. Davis (1980) J. Cell Biol. 87, 47-54.
24. J.M. Blanchard, C. Brunel, Ph. Jeanteur (1977) Eur. J. Biochem. 79, 117-131.
25. J.R. Nevins (1980) in Meth. in Enzym. (L. Grossman, K. Moldave, Eds., Academi Press New York 1980) 65, 768-785.
26. G.M. Wahl, M. Stern, G.R. Stark (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 3683-3687
27. P.W.J. Rigby, M. Dieckmann, C. Rhodes, P. Berg (1977) J. Molec. Biol. 113, 237-251.
28. C.J. Goldenberg, H.R. Raskas, (1981) Proc. Nat. Acad. Sci. USA 78, 5430-5434.
29. C.A.G. Van Eekelen, W.J. Van Venrooij (1981) J. Cell Biol. 88, 554-563.
30. C.A.G. Van Eekelen, E.C.M. Mariman, R.J. Reinders, W.J. Van Venrooij (1981) Eur. J. Biochem. 119, 461-667.