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HnRNP FROM HeLa CELLS CONTAIN A RIBONUCLEASE ACTIVE ON DOUBLE-STRANDED RNA

Jocelyne Rech, Claude Brunel and Philippe Jeanteur

Laboratoire de Biologie Moléculaire, Université des Sciences et Techniques du Languedoc, Place E. Bataillon, 34060 Montpellier Cédex and Laboratoire de Biochimie, C.R.L.C. Paul Lamarque, B.P. 5054, 34033 Montpellier Cédex, France

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SUMMARY

A ribonuclease D, i-e acting against double-stranded RNA structures like poly r(AU), was identified in ribonucleoprotein structures containing the heterogenous nuclear RNA (hnRNP) from HeLa cells. This activity could not however be detected in intact hnRNP but only after passage through a DEAE -cellulose column or digestion by a combination of ribonucleases $A+T_1$. This enzyme does not degrade poly r(A)-poly d(T) nor poly r(A), nor does it yield mononucleotides, excluding the possibility of a non-specific exonuclease type of activity like phosphodiesterase. It is inhibited by ethidium bromide and double-stranded RNA and resembles in all respects so far investigated the ribonuclease D previously isolated from Krebs cells by Rech et al (Nucl. Acids Res. 1976, 3, 2055-2065).

INTRODUCTION

Excision of extensive RNA sequences followed by religation through an accurate splicing mechanism is certainly the most drastic modification suffered by hnRNA when being processed into mRNA (1). We have been searching for the past few years for an enzyme which might act at the cleavage step of the splicing reaction. By analogy with *E. coli* RNAse III which exhibits in vitro specificity towards dsRNA (2) and has been unambiguously involved in the specific cleavage of primary transcripts of *E. coli* rRNA (3,4) as well as of T7 mRNA (3), we have chosen to look for a eucaryotic enzyme with a similar specificity. This working hypothesis was supported by the suggestion that dsRNA regions might be involved in the maturation of hnRNA into mRNA. Indeed such regions exist in hnRNA (5-16) and are not recovered from poly(A) containing cytoplasmic mRNA (9). Moreover, there is sequence complementarity between mRNA and hnRNA (17) whose ds regions can form duplexes with mRNA (18-20). This suggests that only one of the two strands of some ds regions is lost during processing and therefore that some important cleavage sites may occur within them.

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We have previously been able to detect and partially purify such an RNAse D activity from Krebs ascites cells (21). Should this enzyme play a role in the processing of hnRNA, it would be of interest to find it associated with hnRNP, i-e with the ribonucleoprotein structure in which hnRNA is engaged during all of its nuclear residence (22,23).

The present report demonstrates the occurence in HeLa hnRNP of an enzyme activity able to degrade exogenous poly r(AU) and which therefore resembles the previously described RNAse D from Krebs cells (21). However, in native hnRNP this activity appears to be masked as far as the hydrolysis of poly r(AU) is concerned and its detection requires prior elimination of endogenous nucleic acids.

MATERIALS AND METHODS

hnRNP either cold or pulse-labeled for 30 minutes with $|^{3}H|$uridine in the presence of 0.04 µg/ml actinomycin D were prepared as described previously (24) using a slight modification of the procedure of Kish and Pederson (25). For all enzymatic experiments, hnRNP were adjusted to 0.13 M NaCl in 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$ (buffer A). For DEAE-cellulose chromatography, they were adjusted to 50 mM NaCl and 6 mM 2-mercaptoethanol in 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$ (buffer B).

Radioactive poly r(AU) was prepared using $|^{3}H|$ATP (Amersham) as a precursor and poly d(AT) (P.L. Biochemicals) as template under the reaction conditions described by Berkower et al. (26). After incorporation reached a plateau, the poly d(AT) strand was destroyed by digestion with 10 µg/ml DNAse I (Worthington DPFF, RNAse free). After sodium dodecyl sulfate-phenol extraction and ethanol precipitation, the radioactive poly r(AU) was separated from unincorporated precursors by gel filtration on Sephadex G-50 fine in 20 mM Tris-HCl pH 7.5, 0.15 M KCl. $|^{14}C|$poly r(A) and $|^{14}C|$poly r(A)-poly d(T) were prepared as described previously (21).

The double-stranded RNA containing phage ϕ6 was grown on *Pseudomonas phaseolicola* according to Vidaver et al. (27). After sodium dodecyl sulfate-phenol-chloroform extraction, RNA was ehanol-precipitated, dissolved in buffer A and treated by 10 µg/ml DNAse I for 30 minutes at room temperature. After addition of 1/9th volume 10xSSC (0.15 M Na Citrate, 1.5 M NaCl) and further treatment with 10 µg/ml pancreatic RNAse A (Worthington RAF, boiled 5 minutes at 100°C) for 30 minutes at room temperature, it was reextracted with phenol and precipitated from ethanol by spooling around a glass rod.

RNAse D from Krebs ascites cells was prepared by a modification of the previously described procedure (21). RNAse T_1 was from Calbiochem.

Proteins were assayed either by absorbance at 280 nm or by Bradford's technique (28) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

When $|^{3}H|$poly r(AU) was incubated at 37°C in the presence of 30 micrograms of HeLa hnRNP, no significant hydrolysis was observed for incubation times up to 45 minutes (Figure 1). Varying the amount of hnRNP from 1.5 to 75 µg yielded the same results (not shown). We can therefore conclude that no RNAse D can be detected on exogenous substrate in hnRNP under these conditions. However, previous experience with Krebs RNAse D (21) taught us that the activity was

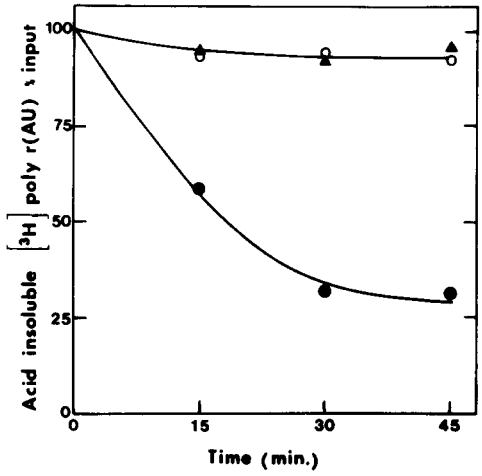

Fig. 1 :
Degradation of $|^3H|$poly r(AU) by hnRNP exposed to RNAses A+T_1. 20 microliter aliquots of hnRNP containing 30 micrograms of protein were incubated either alone (O) or in the presence of RNAses A (10 µg/ml) and T_1 (50 units/ml) (●) for one hour at 37°C. 80 microliters of standard RNAse D assay buffer (21) containing 4,000 cpm $|^3H|$poly r(AU) were added to each tube and incubation was continued for indicated times. At the end of the selected incubation period, 80 microliters of each tube were spotted on Whatman GF/A glass filters, washed extensively with cold 5% trichloracetic acid, dried and counted in a toluene based scintillation fluid using an Inter-technique SL30 scintillation counter. A control was made in which $|^3H|$poly r(AU) was incubated with RNAses A+T_1 in the absence of hnRNP (▲).

difficult to detect in crude fractions and especially nuclear ones and was drastically increased after chromatography on DEAE-cellulose, raising the possibility that an activity could be actually present in hnRNP although in a repressed form. Indeed, chromatography of hnRNP on a DE52 column revealed a peak of RNAse D against $|^3H|$poly r(AU) in the flow-through, no activity being found in the elution gradient (Figure 2). It is noteworthy that the Krebs enzyme also did not bind to DEAE-cellulose under the same conditions (21).

Should the effectiveness of DEAE-cellulose chromatography in revealing hnRNP RNAse D be due to the removal of endogenous RNA, the same result should be obtained by its enzymatic degradation. As expected, prior treatment of hnRNP by a combination of RNAse A (10 µg/ml) and T_1 (50 units/ml) led to the appearance of a degradative activity towards poly r(AU) while this substrate was fully resistant to these RNAses in the absence of hnRNP (Figure 1).

The possibility that this degradative activity could be due to a non-specific exonuclease (for example phosphodiesterase) is ruled out by the absence of mononucleotides in the digestion products of $|^3H|$poly r(AU) analysed by DEAE-Sephadex urea column chromatography (29) (results not shown). Furthermore, the

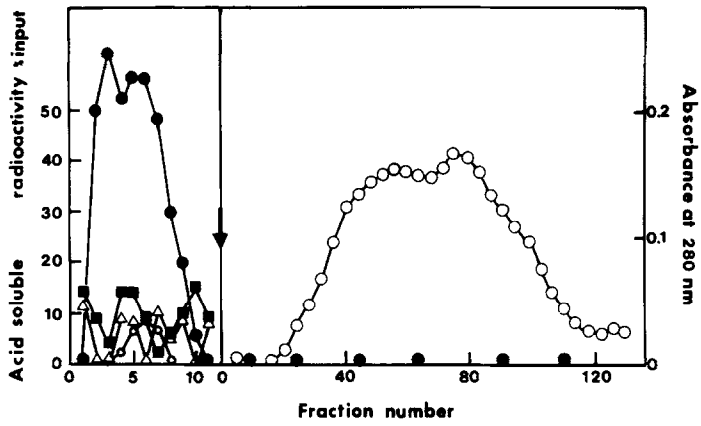

Fig. 2 :
Pattern of RNAse D activity after DE52 chromatography of hnRNP. 10 ml of hnRNP (15 mg) in buffer B were loaded onto a 5 ml (1 cm ϕ) Whatman DE52 column equilibrated with the same buffer. After washing until no more protein comes out, elution was then carried out at a flow rate of 10 ml/hr by a linear 150 ml gradient of NaCl (50-500 mM) in buffer B. Respectively 0.8 and 1.5 ml fractions were collected before and after starting the gradient and were assayed for absorbance at 280 nm (O) and hydrolytic activities against poly r(AU) (●), poly r(A)-poly d(T) (■) and poly r(A) (△) using 20 microliters aliquots.

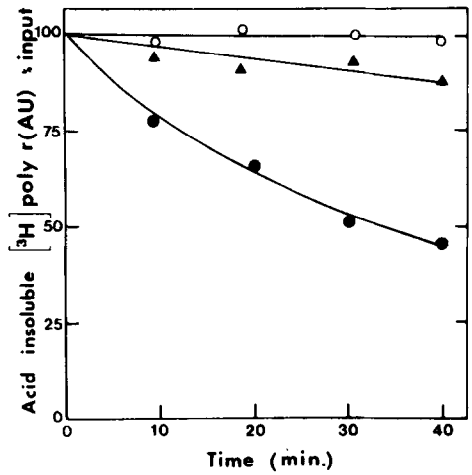

Fig. 3 :
Inhibition by ethidium bromide and phage ϕ6 RNA of RNAse D activity from hnRNP. To 20 microliters aliquots of the peak tube of activity from Figure 2 were added 80 microliters of standard RNAse D assay buffer (21) containing 4,000 cpm |^{3}H|poly r(AU) with no addition (●) or in the presence of 0.1 mM ethidium bromide (O) or 1.5 microgram ϕ6 RNA (▲) which represents about 100 fold excess over poly r(AU). Each tube was incubated and processed as in Figure 1.

DE52 flow-through fractions contained little, if significant, activity against either |^{14}C|poly r(A) or |^{14}C|poly r(A)-poly d(T) as compared to |^{3}H|poly r(AU)

(Figure 2). Inhibition by ds RNA from phage $\phi 6$ and by ethidium bromide (Figure3) which specifically intercalates into duplex structures (30) is also in keeping with the ds specific nature of the present enzyme.

It should be noted that close examination of Calvet and Pederson's data (31) on the digestion of hnRNP by various ribonucleases shows no indication of such an RNAse D activity against endogenous ds RNA even after digestion with RNAses $A+T_1$. This discrepancy may be due to the fact that we have not assayed for degradation of the endogenous substrate. This point is currently under investigation.

The relevance of the present enzyme to the endoribonuclease previously detected by Niessing and Sekeris (32) in rat liver hnRNP is not clear as no specificity similar to ours was reported by these authors. Their enzyme might well be responsible for the extensive degradation of bulk hnRNA that takes place during self-incubation of hnRNP (24).

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REFERENCES

1. TILGHMAN, S.M., CURTIS, P.J., TIEMEIER, D.C., LEDER, P. & WEISSMANN, C. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1309-1313
2. ROBERTSON, H.D., WEBSTER, R.E. & ZINDER, N.D. (1968) J. Biol. Chem. 243, 82-91
3. DUNN, J.J. & STUDIER, F.W. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3296-3300
4. NIKOLAEV, N., SILENGO, L. & SCHLESSINGER, D. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3361-3365
5. MONTAGNIER, L. (1968) C.R.H. Acad. Sci. Paris D267, 1417-1420
6. STERN, R. & FRIEDMAN, R.M. (1970) Nature (London), 226, 612-616
7. HAREL, J. & MONTAGNIER, L. (1971) Nature New Biology,229, 106-108
8. de MAEYER, E., de MAEYER-GUIGNARD, J. & MONTAGNIER, L. (1971) Nature New Biology, 229, 109-110
9. JELINEK, W. & DARNELL, J.E. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2537-2541
10. KRONENBERG, L.H. & HUMPHREYS, T. (1972) Biochemistry, 11, 2020-2026
11. RYSKOV, A.P., FARASHYAN, V.R. & GEORGIEV, G.P. (1972) Biochim. Biophys. Acta, 262, 568-572
12. PATNAIK, R. & TAYLOR, M.W. (1973) Biochemistry, 12, 1990-1995
13. JELINEK, W., MOLLOY, G., FERNANDEZ-MUNOZ, R., SALDITT, M. & DARNELL, J.E. (1974) J. Mol. Biol. 82, 361-370
14. MONCKTON, P.B. & NAORA, H. (1974) Biochim. Biophys. Acta 335, 139-154
15. TORELLI, U., TORELLI, G., CADOSSI, R. & FERRARI, S. (1974) Haematologica, 59, 393-400
16. ROBERTSON, H.D., DICKSON, E. & JELINEK, W. (1977) J. Mol. Biol. 115, 571-589
17. STAMPFER, M., ROSBASH, M., HUANG, A.S. & BALTIMORE, D. (1972) Biochem. Biophys. Res. Comm. 49, 217-224
18. NAORA, H. & WHITELAM, J.M. (1975) Nature (London), 256, 756-759
19. RYSKOV, A.P., KRAMEROV, D.A. & GEORGIEV, G.P. (1976a) Biochim. Biophys. Acta, 447, 214-229

20. RYSKOV, A.P., TOKARSKAYA, O.V., GEORGIEV, G.P., COUTELLE, C. & THIELE, B. (1976b) Nucl. Acids Res. 3, 1487-1498
21. RECH, J., CATHALA, G. & JEANTEUR, Ph. (1976) Nucl. Acids Res. 3, 2055-2065
22. DUCAMP, C. & JEANTEUR, Ph. (1973) Biochimie, 55, 1235-1243
23. PEDERSON, T. (1974) J. Mol. Biol. 83, 163-183
24. BLANCHARD, J.M., BRUNEL, C. & JEANTEUR, Ph. (1977) Eur. J. Biochem. 79, 117-131
25. KISH, V.M. & PEDERSON, T. (1975) J. Mol. Biol. 95, 227-238
26. BERKOWER, I., LEIS, J. & HURWITZ, J. (1973) J. Biol. Chem. 248, 5914-5921
27. VIDAVER, A.K., KOSKI, R.K. & VAN ETTEN, J.L. (1973) J. Virol. 11, 799-805
28. BRADFORD, M.M. (1976) Anal. Bioch. 72, 248-254
29. TENER, G.M. (1967) Methods in Enzymology, 12, 398-404 (Academic Press, New York and London)
30. LE PECQ, J.B. & PAOLETTI, C. (1967) J. Mol. Biol. 27,87-106
31. CALVET, JP. & PEDERSON, T. (1977) Proc. Natl. Acad. Sci. USA, 74, 3705-3709
32. NIESSING, J. & SEKERIS, C.E. (1970) Biochem. Biophys. Acta, 209, 484-492