The specific endoribonuclease activity of small nuclear and cytoplasmic α-RNPs

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Abstract For the first time small nuclear ribonucleoprotein particles (α-RNP) tightly bound to chromatin as well as cytoplasmic α-RNP are shown to possess strong and regulated endonuclease activity specific for mRNAs and hnRNAs. The enzymatic nature of this activity is confirmed, and the optimal conditions detected. This RNase activity is controlled by the action of a differentiating stimulus, dimethylsulfoxide, in human K562 cells. Small α-RNP involvement in the coordinated control of stability of pre-messenger RNA and messenger RNA molecules is suggested.

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Key words: Small α-ribonucleoprotein particle; Endoribonuclease activity; RNase; mRNA stability; Short interspersed repeat; Prosome; Proteasome

1. Introduction

One of the levels of the control of gene expression is the regulation of mRNA degradation rates. The range of mRNA stability in eukaryotic cells can vary over several orders of magnitude [1,2]. mRNA degradation mechanism specific to individual mRNAs or to classes of mRNAs include sequence-specific endonucleolytic cleavage and deadenylation-independent decapping [3]. The rate of RNA endonucleolysis depends on several factors: *cis*-regulatory elements in the mRNA molecules, protective proteins which bind at or near these elements, and the activity of ribonucleases which degrade mRNA molecules [3]. However, nucleases which degrade messenger RNAs in eukaryotic cells remain mainly undefined [1,2]. The mRNA decay rate can not only be regulated by the activity of protective proteins [4] but may also be directly controlled by changing the activity of ribonucleases [5,6]. Earlier we have characterized the specific class of small ribonucleoprotein particles (RNP) tightly bound to chromatin (nuclear α-RNP or n-αRNP) as well as their cytoplasmic analogs (c-αRNP) [7]. Here we present evidence that both n-αRNP and c-αRNP harbor regulated endoribonuclease activity specific for hnRNAs and mRNAs.

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Abbreviations: mRNA, messenger RNA; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethylsulfoxide; hmwRNA, high-molecular-weight RNA; lmwRNA, low-molecular-weight RNA; RNP, ribonucleoprotein particle; n-αRNP, nuclear α-RNP; c-αRNP, cytoplasmic α-RNP; hnRNA, heterogeneous nuclear RNA

2. Materials and methods

2.1. Animals and cell culture

Male Wistar rats (100–120 g) were used. All animals had been fasted for 24 h. Human proerythroleukemic K562 cells were grown in suspension in RPMI medium supplemented with 10% (v/v) fetal calf serum. The cells were incubated with 1.5% dimethylsulfoxide (DMSO) for 24 h. Under the action of DMSO an increase in benzidine staining of the cells was observed, indicating the induction of hemoglobin expression [8]. Lysis of the cells was performed in a buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS–Na, 100 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 40 min.

2.2. Isolation and analysis of RNA and RNP

Nuclei, chromatin, n-αRNPs and n-αRNAs were prepared as in [9,10]. Briefly, nuclei were isolated in 2.2 M sucrose with 0.5% sodium δ-glycerophosphate, 10 mM Tris–HCl and 2 mM $MgCl_2$, pH 7.0. The homogenate was put on a 5 ml cushion of this solution and centrifuged for 1 h at 135 000×*g*. The nuclei thus obtained were successively washed with: 0.35 M sucrose, 10 mM Tris–HCl, pH 7.0, 2 mM $MgCl_2$; 0.35 M NaCl, pH 7.0; 0.14 M NaCl; standard saline citrate, 1×SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and afterwards with 0.1×SSC and 0.01×SSC. All procedures were done at 0°C. The chromatin pellet was subjected to extraction with 2 M NaCl to isolate the histones and n-αRNP, and soluble components were fractionated by preparative gel electrophoresis according to [7]. Small n-αRNP was extracted from the electrophoretic band with 50 mM Tris–HCl, pH 7.5, 0.1 M NaCl, 5 mM EDTA, 0.75% SDS. C-αRNPs were obtained from the pellet of free cytoplasmic RNP isolated by differential centrifugation [7,11]. Electrophoretically pure α-RNPs were additionally purified by centrifugation in a cesium chloride gradient without formaldehyde fixation [7]. Nuclear, cytoplasmic and poly(A)$^+$ RNA were isolated and purified according to [9,10,12]. The RNA samples were labelled at the 3′ ends with 5′[^{32}P]pCp by T4 RNA ligase [13]. High molecular weight (hmw) RNA was separated in 3% PAG [14], whereas electrophoresis in 8% PAG with 7 M urea in TBE buffer (50 mM Tris–HCl, pH 8.3, 50 mM boric acid, 1 mM EDTA) [15] was used for separation of low molecular weight (lmw) RNA. CsCl and Cs_2SO_4 buoyant density gradient centrifugations of α-RNP particles were carried out as described [16]. SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was done according to Laemmli [17].

2.3. Molecular hybridization

Plasmid DNA isolated and purified according to [15] was treated with appropriate restriction endonucleases to split off the inserts and subjected to 1% agarose electrophoresis. DNA probes for examining specific binding were labelled using the random-priming technique [15]. DNA–RNA and RNA–RNA hybridizations were done according to [15]. The hybrids were finally washed under stringent conditions (0.1% SDS, 0.1×SSC, 65°C, 30 min).

2.4. In vitro RNA transcription

The c-*myc* 3′ UTR (untranslated region) RNA fragments labelled

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with [α-^{32}P]UTP were prepared by in vitro transcription of plasmid pGEM-3′-c-*myc* which carries an *Nso*I-*Aff*II insert from the 3′ end of the human c-*myc* gene at the *Hin*cII-*Pst*I sites of pGEM [18]. The plasmid was linearized at *Bam*HI and transcribed with SP6 RNA polymerase in the sense orientation according to the manufacturer's instruction (Maxiscript, Ambion), treated with DNase I, phenol–chloroform extracted and ethanol precipitated.

2.5. Cleavage of RNA by α-RNP

Poly(A)$^+$ RNA (1 μg) and total hmwRNA (1 μg) isolated from rat liver or K562 cells were incubated with α-RNP (0.7 μg) for 30 min (or other indicated intervals) at 25°C or at 37°C in buffer containing 20 mM HEPES, 50 mM KCl, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.5 mM PMSF, 2 mM dithiothreitol, pH 7.5. In the case of lmwRNA the same buffer without Mg^{2+} was used. After the end of incubation 0.2% SDS and 0.2 M NaCl were added, the reaction mixture was extracted with buffer-saturated phenol, phenol–chloroform and ethanol precipitated. The α-RNP RNase assay concerning c-*myc* RNA was carried out in 10 μl of reaction buffer containing 0.5 μg of α-RNP from DMSO-treated K562 cells, 5 μg of yeast tRNA and 50 ng of radiolabelled (5×10^4 cpm) c-*myc* mRNA 3′ UTR region, transcribed in vitro.

3. Results

Incubation of total cytoplasmic hmwRNA of rat liver cells with α-RNP from rat liver led to degradation of 28S and 18S ribosomal RNAs (Fig. 1A), although there was no visible RNA cleavage when the RNA was incubated without α-RNP under the same conditions (Fig. 1A, lane 2). At the same time the pattern of electrophoretic distribution shows that certain fractions disappeared and a well-defined pattern

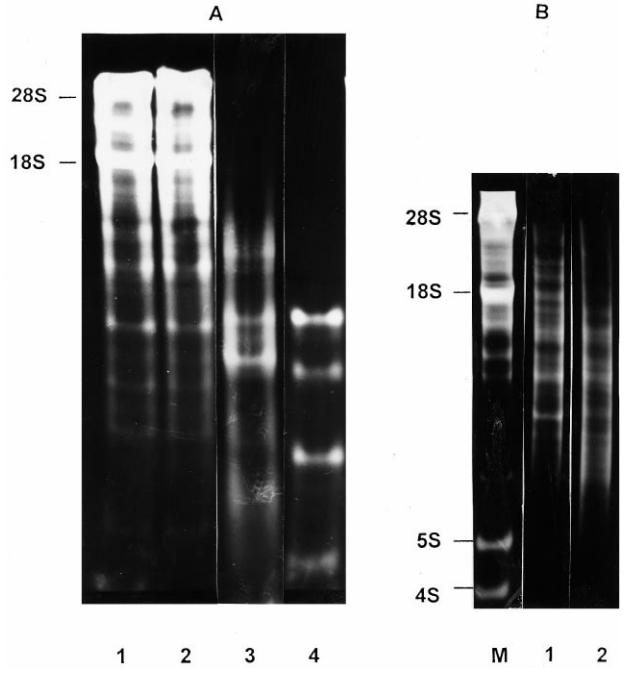


Fig. 1. Cleavage of total cytoplasmic hmwRNA (A) and cytoplasmic hmw poly(A)$^+$ RNA (B) of rat liver by n-αRNP from rat liver cells. A: Electrophoregram of total cytoplasmic hmwRNA (13.5 μg). unincubated RNA (lane 1); RNA incubated without RNP at 37°C for 30 min (lane 2); RNA treated with n-αRNP at 25°C for 20 min in buffer A, deproteinized with phenol and phenol–chloroform, ethanol precipitated and subjected to electrophoresis in 3% PAG [14] (lane 3); the same RNA treated with α-RNP at 37°C for 30 min (lane 4). B: Electrophoregram of cytoplasmic hmw poly(A)$^+$ RNA (15 μg). Lane 1, incubated without α-RNP; lane 2, incubated with α-RNP at 25°C for 30 min. M, marker: poly(A)$^-$ RNA.

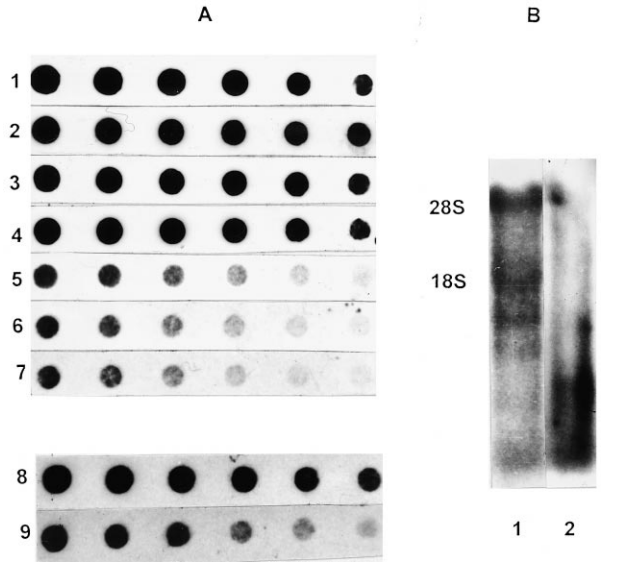


Fig. 2. Digestion of cytoplasmic hmw poly(A)$^+$ RNA and hnRNA of rat liver by α-RNP from rat liver analyzed by hybridization to the [^{32}P]ID probe. A: Dot hybridization. Poly(A)$^+$ RNA incubated at 25°C for 30 min without α-RNP (lane 1); with α-RNP heated at 100°C for 5 min (lane 2); poly(A)$^+$ RNA incubated with n-αRNP (lanes 3–5): at 0°C for 30 min (lane 3), at 25°C for 5 min (lane 4) and at 25°C for 30 min (lane 5); 100 units of RNasin were added to the incubation mixture incubated in the same conditions as in lane 5 (lane 6); poly(A)$^+$ RNA incubated with c-αRNP at 25°C for 30 min (lane 7). Lanes 8,9: ID-containing hnRNA digested by n-αRNP: lane 8, hybridization of undigested hnRNA; lane 9, hybridization of digested hnRNA (treatment with α-RNP carried out at 25°C for 30 min). Rat liver RNA was spotted onto Hybond N filter in amounts of 1.0, 0.5, 0.25, 0.13, 0.06, 0.03 μg. Hybridization with ^{32}P-labelled ID DNA insert [26,27] was performed. B: Analysis of degradation of rat liver cytoplasmic poly(A)$^+$ RNA by Northern hybridization to [^{32}P]ID DNA. Lane 1, undigested RNA; 2, RNA digested by α-RNP at 25°C for 30 min.

of discrete RNA fragments appeared as a result of treatment of cytoplasmic hmw poly(A)$^+$ RNA by n-αRNP (Fig. 1B). The formation of RNA fragments of definite sizes as well as the absence of a random set of RNA fragments confirms that the reaction under study is endonucleolysis.

To clarify the nature of the mRNA fractions which undergo nucleolysis with α-RNP RNase, dot and Northern hybridizations of the undigested and α-RNP-digested rat liver cytoplasmic hmw poly(A)$^+$ RNAs (mRNAs) were performed against the short interspersed repetitive (SINE) rat DNA sequence *ID*. The data presented in Fig. 2A (lanes 2–6) show that n-αRNPs from rat cells specifically digest *ID*-containing mRNA molecules. Not only nuclear but also cytoplasmic α-RNPs are capable of degrading specifically *ID*-containing mRNA (Fig. 2A, lane 7).

The properties of the α-RNP ribonuclease were characterized by the effects of various treatments on its activity in a standardized in vitro degradation assay. This assay measures the effectivity of degradation of mRNA hybridizing to *ID*-DNA. The optimal pH for activity of the nuclease was detected to be 7.5. No degradation of hmwRNA was observed after incubation of RNA with α-RNP treated at 100°C for 5 min, indicating that the RNase activity of α-RNP is thermolabile (Fig. 2A, lane 2). It is known that the activity of most ribonucleases of the RNase A family is suppressed by RNasin, a placental ribonuclease inhibitor. In contrast to

these RNases, recombinant RNasin (Sigma, USA) did not affect the nuclease activity of α-RNP (Fig. 2A, lane 6).

Nuclear α-RNPs specifically degrade not only mRNA but also *ID*-containing hnRNA (Fig. 2A, lanes 8 and 9). The data of Northern hybridization confirm the mRNA-degrading activity of α-RNP (Fig. 2B).

Human α-RNP prepared from proerythroleukemic cK562 ells possess endoribonuclease activity specific for mRNA containing human SINEs, *Alu* elements (Fig. 3). Importantly, this activity was induced by the action of DMSO, an agent stimulating cell differentiation, on K562 cells: α-RNP isolated from control (DMSO-untreated) K562 cells did not harbor this activity (Fig. 3A, lane 4) and α-RNP from DMSO-treated K562 cells specifically degrade *Alu*-containing mRNA (Fig. 3A, lanes 2,3).

The results of Northern hybridization of the products of limited digestion of cytoplasmic hmw poly(A)$^+$ RNA (20 min at 20°C) with α-RNP, isolated from DMSO-treated cells, revealed fragments hybridizing with *Alu* sequences (Fig. 3B); such fragments are likely to arise after realization of initial ruptures (not shown).

Further proof of the specificity of the RNase activity

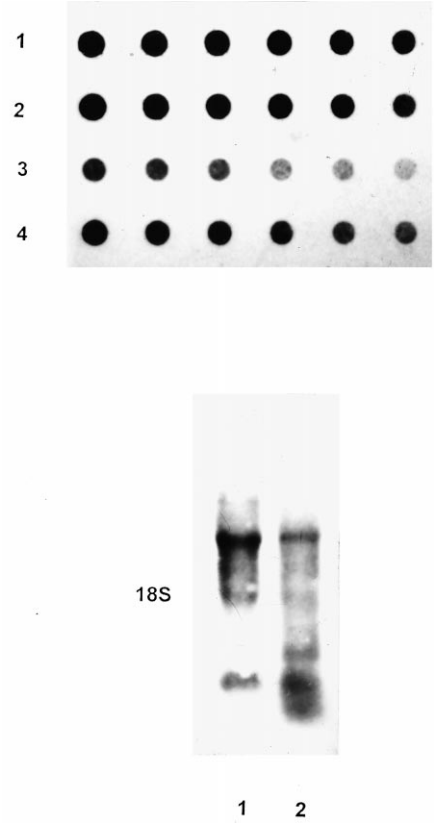


Fig. 3. Digestion of cytoplasmic hmw poly(A)$^+$ RNA from human K562 cells analyzed by hybridization to the ^{32}P-labelled *Alu* probe. A: Dot blot hybridization. Poly(A)$^+$ RNA isolated from K562 cells was incubated at 37°C for 30 min: without α-RNP (lane 1); with α-RNP from DMSO-treated K562 cells at 0°C (lane 2), with the same α-RNP at 37°C (lane 3), with α-RNP from DMSO-untreated K562 cells at 37°C (lane 4). RNAs were spotted onto Hybond N filter in amounts of 1.0, 0.5, 0.25, 0.13, 0.06, 0.03 μg and hybridized with ^{32}P-labelled *Alu* DNA insert (clone BLUR 8, [28]). B: Analysis of K562 poly(A)$^+$ RNA cleavage by Northern hybridization to the ^{32}P-labelled *Alu* probe. Lane 1, undigested RNA; 2, RNA digested with n-αRNP from DMSO-treated K562 cells at 20°C for 20 min.

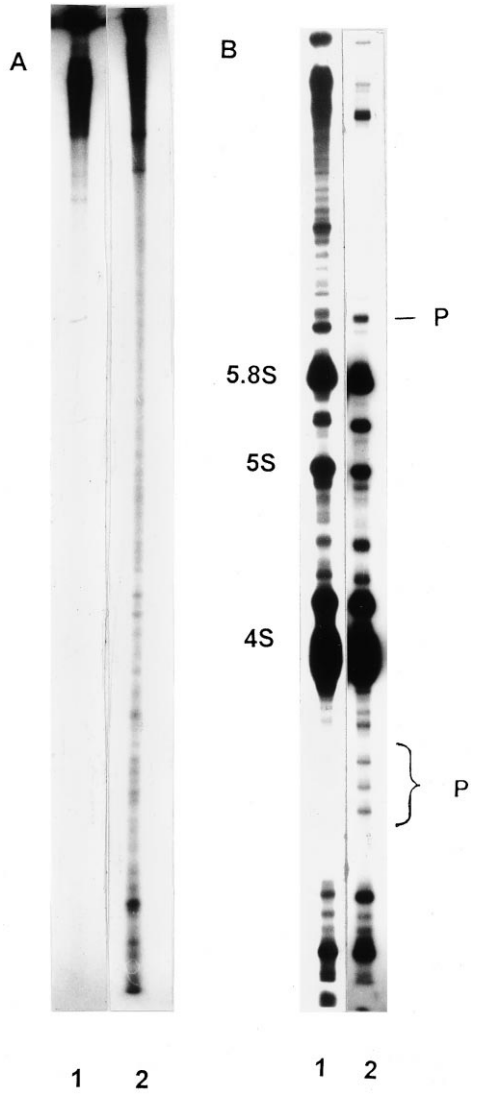


Fig. 4. Analysis of the specificity of the α-RNase activity. A: Cleavage of 3′ UTR c-*myc* RNA by α-RNP. Electrophoregram of ^{32}P-labeled 3′ UTR c-*myc* RNA untreated (lane 1) and treated with α-RNP (lane 2). B: Digestion of rat liver lmwRNA. Electrophoregram of RNA untreated (line 1) and treated by n-αRNP at 25°C for 30 min (lane 2). Electrophoresis in 8% PAG with 7 M urea, 1500 V for 5 h. P, reaction products with free 3′-OH ends (labelling was performed after cleavage).

studied was obtained in the analysis of the cleavage of the c-*myc* mRNA 3′ UTR region (transcribed in vitro). The 3′ UTR region of c-*myc* mRNA was used because this region was shown to be involved in the regulation of mRNA turnover [19]. The results show that α-RNase generates discrete c-*myc* cleavage fragments (Fig. 4A), suggesting that a specific sequence or structure of the RNA molecule is required for α-RNase activity.

One more piece of evidence for the specific endoribonuclease activity of α-RNP was obtained by examining the action of α-RNP on lmwRNA. In this experiment total cytoplasmic RNA preincubated with α-RNP was 3′ end-labeled and then separated by electrophoresis in 8% PAG in the presence of 7 M urea. Fig. 4B presents the results of preparative-scale RNase reaction with total rat liver cytoplasmic RNA as substrate. One can see that in contrast to hmwRNA, lmw 4S

and 5.8S RNAs were not cleaved by α-RNP, and 5S RNA was only partly digested. Besides, this figure demonstrates the appearance of reaction products with free labeled 3′-OH ends. When hmwRNA was cleaved by α-RNP, products with free 3′ end-labeled hydroxyl groups were formed also (not shown), thus α-RNP treatment generates free 3′-OH ends.

4. Discussion

In the present work the novel RNase activity of small α-RNPs is identified and initially characterized. Our data show for the first time that α-RNPs have mRNA-degrading activity and can be involved in the regulation of mRNA stability. We have earlier found that the action of DMSO on K562 cells causes a coordinated decrease of the content of *Alu*-containing RNA [20]. The correlation of this event with the DMSO-induced degrading activity of α-RNP towards mRNA and hnRNA, both containing *Alu* repeats, argues for the likely involvement of the particles in controlling the life time of inducible molecules or, in other words, for a new mechanism controlling RNA stability in the cell. We assume that the signals for directed degradation of mRNA and their precursors (hnRNA) may reside in the SINE sequences.

It has been shown earlier that the subunit composition of α-RNP is changed in response to DMSO treatment [20]. Whether or not these changes or modifications of subunits are responsible for the control of RNase activity remains to be seen. Our earlier results [7,11] demonstrated that α-RNA is *Alu*-like and can hybridize with specific mRNAs, and it is conceivable that complementary interactions of α-RNA with mRNA molecules contribute to the recognition of cleavage sites by α-RNP particles.

Small α-RNPs are similar to proteasomes in their size, protein composition, antigen determinants and peptidase activities [21]. And RNase activity has been described for prosomes (20S proteasomes), but with a different specificity (only for TMV viral RNA) [22,23]. Although we did not study the action of α-RNPs on this substrate, the existence of several ribonuclease centers in α-RNP seems to be quite possible (just as there are several proteolytic catalytic centers in proteasomes [24,25]). Endonucleolysis of hnRNA with α-RNP can be used by eukaryotic cells either for mRNA processing or for degradation of the hnRNA which does not go to the cytoplasm and degrades in the nucleus.

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