Identification of the Ro and La antigens in the endoribonuclease VII–ribonucleoprotein complex

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45 S RNP (ribonucleoprotein) particles from calf thymus or L5178y mouse lymphoma cells contain the poly(A)-modulated and oligo(U)-binding endoribonuclease VII [Bachmann, Zahn & Müller (1983) *J. Biol. Chem.* **258**, 7033–7040]. From these particles a 4.5 S RNA was isolated that possesses an oligo(U) sequence. By using monospecific and non-cross-reacting antibodies directed against the La or Ro antigen, both proteins were identified in the endoribonuclease VII–RNP complex after phosphorylation *in vitro*. In a second approach, endoribonuclease VII activity was identified in immunoaffinity-purified Ro RNPs after preparative isoelectric focusing. Therefore we conclude that the 4.5 S RNA belongs to the Ro RNAs. The results indicate a possible function of endoribonuclease VII in activating stored mRNAs.

INTRODUCTION

In contrast with prokaryotic mRNA, the primary gene transcript of eukaryotes, termed heterogeneous nuclear RNA (hnRNA), must be modified by the following series of post-transcriptional processing steps before it can leave the nucleus as mRNA: addition of a 5'-terminal cap, addition of a 3'-terminal poly(A) tract, methylation of 5'-terminal and internal residues and removal of intervening sequences (splicing) [for reviews, see Darnell (1979), Müller *et al.* (1984) and Bachmann *et al.* (1984b)]. Such steps provide eukaryotes with the opportunity for regulation of mRNA synthesis at the level of post-transcription (Schröder *et al.*, 1985). Released into the cytoplasm, mRNA is found either to be translated in polyribosomes or to be in an inactive state as free mRNP complexes (Maundrell *et al.*, 1979). The factors that control the distribution of cytoplasmic mRNA into the translationally active or inactive form are not known. It is assumed that distinct cytoplasmic RNP particles, the so-called 'Ro RNPs', might play a role in this selection process (Kato *et al.*, 1982; Wolin & Steitz, 1983). Patients with autoimmune diseases such as systemic lupus erythematosus, mixed connective-tissue disease or Sjögren syndrome, often produce autoantibodies against nuclear or cytoplasmic antigens (for reviews, see Steitz *et al.*, 1983; Hardin & Mimori, 1985). In some cases the targets recognized by the antibodies associate with small nuclear or cytoplasmic RNAs (snRNAs or scRNAs), forming small nuclear or small cytoplasmic ribonucleoprotein complexes (snRNPs or scRNPs). These snRNPs or scRNPs can be immunoprecipitated by autoimmune patients' sera. The most familiar antigen–antibody systems are the Sm, RNP, La or Ro systems (Bachmann *et al.*, 1983a). Whereas the Sm antigen forms complexes with all UsnRNAs, the RNP antigen only associates with U1snRNA. In contrast with the UsnRNAs, which are transcribed by RNA polymerase II, the Ro and La RNAs are transcribed by RNA polymerase III (Hendrick

et al., 1981; Lerner & Steitz, 1981). The Ro RNPs are thought to be a subclass of the La RNPs (Hendrick *et al.*, 1981). Although immunofluorescent studies localize Ro RNPs in the cytoplasm, Ro RNAs are also found in the nucleus associated with hnRNAs (Kato *et al.*, 1982; Wolin & Steitz, 1983). In the cytoplasm, Ro RNPs are localized in what appear to be discrete organelles (Lerner & Steitz, 1981). Recently we succeeded in purifying the La and Ro antigens to homogeneity (Bachmann *et al.*, 1986). The purified antigens were used for the isolation of monospecific antibodies that did not cross-react (Bachmann *et al.*, 1986). Both antigens were successfully phosphorylated *in vitro* by the cytoplasmic protein kinase CII, although nuclear protein kinases were not able to phosphorylate these proteins (Bachmann *et al.*, 1986). The La and Ro antigens showed a high binding affinity to poly(U), which was strongly decreased by phosphorylation or by addition of ATP. About 3 years ago we described the purification of pyrimidine-specific endoribonuclease VII, which is modulated by poly(A) and binds to poly(U) (Bachmann *et al.*, 1983b). This enzyme was isolated from 45 S RNPs, which were assumed to be hnRNPs because they were found to be associated with 12 S UsnRNPs (Bachmann *et al.*, 1985). Here we show that the 45 S particles contain a 4.5 S RNA that carries an oligo(U) sequence. Besides the enzyme, the RNP complex contains the Ro and La antigens. Therefore we assume the 4.5 S RNA to belong to the Ro RNAs. In a further approach, endoribonuclease VII was isolated from immunoaffinity-purified Ro RNPs by preparative isoelectric focusing, indicating again that endoribonuclease VII is present in Ro RNPs.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: [³H]poly(U) with an average chain length of 135 UMP units (determined

Abbreviations used: RNP, ribonucleoprotein; snRNP, small nuclear ribonucleoprotein particle; scRNP, small cytoplasmic ribonucleoprotein particle; snRNA, small nuclear ribonucleic acid; scRNA, small cytoplasmic ribonucleic acid; hnRNA, heterogeneous nuclear ribonucleic acid; hnRNP, heterogeneous nuclear ribonucleoprotein particle; BSA, bovine serum albumin.

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by the method of Studier, 1965; specific radioactivity 528 mCi/mmol of phosphate) and poly(A)₉₅ were from Miles Laboratories, Elkhart, IN, U.S.A.; [γ - 32 P]ATP (3 Ci/mmol) and NaB 32 H₄ (21.8 Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.; RNAase A (from bovine pancreas; specific activity 50 Kunitz units/mg), snake-venom phosphodiesterase (specific activity 1.5 units/mg) and marker proteins from Boehringer Mannheim, Germany; Sephadex G-150, Sepharose 6B, poly(A)-Sepharose, CNBr-Sepharose 4B, carboxyhexyl (CH)-Sepharose 4B and protein A-Sepharose, from Deutsche Pharmacia, Freiburg, Germany; GF/C filters from Hormuth and Vetter, Heidelberg, Germany; Kodak X-Omat XR-5 X-ray film from Eastman-Kodak, Rochester, NY, U.S.A.; alkaline phosphatase (*Escherichia coli*; code P9761), insoluble RNAase A (from bovine pancreas, 101 Kunitz units/g; no. R-1626), 4-chloro-1-naphthol, protein A (no. C8890) and peroxidase-conjugated antibody [anti-(human γ -chain)-specific; no. A 8775] from Sigma, St. Louis, MO, U.S.A.; nitrocellulose sheets (BA 85; 0.45 μ m; no. 40 1180) from Schleicher and Schüll, Dassel, Germany. The purified monospecific anti-Ro or anti-La antibodies did not cross-react. Their preparation is described elsewhere (Bachmann *et al.*, 1986). As a control, antibody IgGs from a healthy human (Tr-IgGs) were prepared by (NH₄)₂SO₄ precipitation and DEAE-cellulose DE-52 chromatography (Bachmann *et al.*, 1984a).

Isolation of La, Ro, Ro RNPs, endoribonuclease VII and endoribonuclease VII-RNP complexes

About 5×10^9 L5178y mouse lymphoma cells were harvested from the ascites of 12 mice. The cells were collected by centrifugation (1000 *g* for 10 min at 4 °C). After being washed with 30 ml of buffer A [10 mM-Tris/HCl (pH 7.4)/350 mM NaCl], cells were resuspended in 60 ml of the same buffer, supplemented with 0.5% Nonidet P40) and frozen. After thawing the cells were disrupted in a Potter-Elvehjem-type homogenizer by ten strokes with a light (L)-pestle and kept on ice for 5 min, followed by ten strokes of a strong (S)-pestle. The homogenate was sonicated (using 15 ml portions) for 4×15 s in a Branson sonifier at setting 4. The supernatant obtained after centrifugation (10000 *g* for 30 min) was used as crude extract. This extract contains the Sm, U1snRNP (= RNP), La (= Ha and SSB) and Ro (= SSA) antigens (Bachmann *et al.*, 1986). After precipitation with 45–80% -satd. (NH₄)₂SO₄, this extract was used for the isolation of Ro, La, endoribonuclease VII, endoribonuclease VII-RNP complexes and Ro RNPs. For the isolation of endoribonuclease VII-RNP complexes and Ro RNPs, this extract was dialysed against 5 litres of buffer B [100 mM-Tris/HCl (pH 8.0)/2 mM-MgCl₂/1 M-NaCl/1 M-urea]. After dialysis the extract was passed through a column (4.5 cm $\times$ 26.5 cm) of Sephadex G-150. Endoribonuclease VII and endoribonuclease VII complexes (see below) were isolated from the void volume ($V_e/V_0 = 1.0$ –1.3) by DEAE-cellulose chromatography, phosphocellulose chromatography, preparative isoelectric focusing and sucrose-density-gradient centrifugation as described by Bachmann *et al.* (1983b). Ro RNPs were obtained from the same fraction by immunoaffinity chromatography using a monospecific anti-Ro column (see below). For the isolation of Ro and La antigens the extract was treated with insoluble RNAase A (10 units, 24 h, 4 °C) in the presence of

proteinase inhibitors [leupeptin, chymostatin and pepstatin (5 μ g/ml each)] and 0.5 mM-phenylmethanesulphonyl chloride and dialysed against buffer B. After gel filtration on Sephadex G-150 and dialysis against buffer C [10 mM-Tris/HCl (pH 7.4)/150 mM-NaCl] the antigens were isolated from the retarded fractions ($V_e/V_0 = 1.3$ –1.9) by immunoaffinity chromatography as described by Bachmann *et al.* (1986).

Immunoaffinity columns

Immunoaffinity columns (4 ml) were constructed by coupling 4 mg of non-specific Tr-IgGs (from healthy individuals with the initials Tr), or purified monospecific anti-La or anti-Ro IgGs to CH-Sepharose 4B as described previously (Bachmann *et al.*, 1983a). The columns were equilibrated with buffer D [10 mM-Tris/HCl (pH 7.4)/2.5 mM-MgCl₂/150 mM-NaCl]. After washing with buffer E (buffer C supplemented with 0.5 M-NaCl and 0.05% Tween 20), bound antigens were eluted by a pH change [buffer F: 0.1 M-glycine/HCl (pH 2.95)/1.5 M-NaCl]. Immediately after elution the pH was raised to pH 7.4 by addition of solid Tris. Then the eluate was dialysed against buffer C.

Binding affinity to poly(U)

Binding affinity of La, Ro and endoribonuclease VII to poly(U) was determined by using the binding assay of Stefano (1984) with the following modifications: mixtures containing the antigens (20 pmol of La or Ro) or 5 μ g of endoribonuclease VII complex or, for controls, 10 μ g of BSA, were incubated for 15 min at 20 °C with 250 pmol of [3 H]poly(U) in a final volume of 20 μ l of binding buffer (Stefano, 1984). After incubation the mixture was diluted to 100 μ l with binding buffer and added to 20 μ l of a Sepharose 4B suspension. The Sepharose 4B was coupled either with non-specific Tr-IgGs or with purified monospecific anti-La or anti-Ro IgGs (see above). After incubation (15 min on ice) the immunocomplexes were pelleted by centrifugation (5 min, 10000 *g*). Non-specific adsorbed radioactivity was removed by washing (three times with binding buffer). Bound material was then desorbed by addition of 1 ml of buffer H [0.1 M-glycine/HCl (pH 2.95)/1% SDS/0.5 M-NaCl].

Phosphorylation *in vitro* of Ro, La, Ro RNPs and endoribonuclease VII-RNP complexes

The Ro and La antigens were phosphorylated by cytoplasmic protein kinase CII (specific activity 1 μ mol/min per mg) as described by Bachmann *et al.* (1986). The enzyme was kindly provided by Professor K. Wagner (Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, 3000 Braunschweig, Germany). A 10 ng portion of Ro RNPs or of endoribonuclease VII complexes was incubated with the protein kinase by using the standard assay (Schröder *et al.*, 1983). Briefly, 10 μ l of antigens were diluted with 20 μ l of incubation buffer [100 mM-Mops (pH 7.0)/40 mM-MgCl₂] and added to 45 μ l of distilled water, 10 μ l of kinase (containing 0.5 unit) and 10 μ l of [32 P]ATP (specific radioactivity 6×10^3 d.p.m./pmol). Incubation was performed at 37 °C for 30 min. Then 80 μ l of monospecific antibodies coupled to Sepharose were added. The mixture was incubated for 15 min at 20 °C. After centrifugation (10000 *g*) for 5 min the pellets were resuspended several times in buffer D (each step with 1 ml) and re-centrifuged until the supernatants were free

of radioactivity. The supernatants were discarded. Bound antigens were eluted by the addition of 500 μ l of buffer H. After centrifugation (10000 *g* for 5 min) the supernatants were adjusted to a protein concentration of 50 μ g/ml by the addition of BSA. Then the phosphorylated proteins were precipitated with sodium deoxycholic acid and trichloroacetic acid (1 and 5% final concn. respectively). The pelleted proteins were washed twice with acetone and heat-denatured after resolution in gel-electrophoresis sample solution. Polyacrylamide-gel electrophoresis was then performed as described above. Subsequently the gels were dried and exposed for 8–48 h to Kodak X-Omat film. To check kinase activity in parallel, the phosphorylation procedure was performed by using 1 μ g of casein instead of the antigens. After phosphorylation, casein was precipitated with deoxycholate/trichloroacetic acid. The pellets were washed with buffer E until the supernatants were free of radioactivity. Then the pellets were counted for radioactivity.

Preparative isoelectric focusing

Preparative isoelectric focusing of the immunopurified Ro RNPs was performed as described for endoribonuclease VII (Bachmann *et al.*, 1983b) using 5–50%-(w/v)-sucrose density gradients containing 4 M-urea and 2 mM-dithiothreitol. Before isoelectric focusing the extracts were dialysed against 1 litre of 5% sucrose solution supplemented with 4 M-urea and 2 mM-dithiothreitol.

Nuclease assay and analysis of the nuclease degradation products

Nuclease activity was determined in the presence or absence of poly(A) by using the GF/C-filter test described for endoribonuclease VII (Bachmann *et al.*, 1983b). Analysis of the degradation products formed by the nucleases was performed by gel filtration as described elsewhere (Arendes *et al.*, 1977; Schröder *et al.*, 1980).

Analytical methods

Polyacrylamide-(10%, w/v)-gel electrophoresis in the presence of SDS was performed as described by Laemmli (1970). If necessary, before electrophoresis the samples were concentrated by precipitation with deoxycholate/trichloroacetic acid as described above. After electrophoresis the gels were silver-stained.

Polyacrylamide-gel electrophoresis of RNA in the presence of 8 M-urea was performed as described by Bachmann *et al.* (1984a). Samples (endoribonuclease VII-RNP particles) were phenol-extracted and ethanol-precipitated as described by Roop *et al.* (1978) and redissolved in 350 mM-NaCl. This procedure was repeated twice in order to remove residual amounts of EDTA and phenol. The RNA preparation was resuspended and stored at -20°C . Before electrophoresis, portions (0.2 ng–20 μ g of RNA) were taken, centrifuged, dried, redissolved in sample solution [containing 8 M-urea in electrophoresis buffer/1% xylene cyanol (Bachmann *et al.*, 1984a)] and denatured by heating to 95°C for 5 min.

Base analysis of RNA was performed by the procedure of Randerath *et al.* (1972, 1974). Briefly, the RNA sample was digested to nucleosides with ribonuclease A (10 μ g), phosphodiesterase I (10 μ g) and alkaline phosphatase (8 μ g). After oxidation with sodium meta-

Fig. 1. Analysis of the RNA present in the 45 S endoribonuclease VII particles

(a) A 2 ng portion of RNA isolated from 45 S endoribonuclease VII-RNP complexes was separated on 8 M-urea gels as described in the Materials and methods section. The asterisk (*) denotes 4.5 S RNA. The oligoribonucleotides (b, i and ii) isolated from the 45 S endoribonuclease VII-RNP complexes were degraded to nucleosides. The nucleosides were analysed by the Randerath *et al.* (1972, 1974) method and shown to consist predominantly of one kind of nucleoside (b, i). The radioactive spot was eluted and co-chromatographed with uridine labelled by the same method (b, ii). No additional radioactive spot was found, indicating the oligoribonucleotide to be an oligo(U) fragment.

periodate, the resulting dialdehydes were reduced with NaBH_4 ; 5 μ l of 0.1 M- NaBH_4 (8.8 Ci/mmol) were added to 70 μ l of RNA digest. The labelled digest was applied to cellulose sheets and chromatographed two-dimensionally by using the solvents acetonitrile/aq. 4 M- NH_3 (17:5, v/v) and 2-methylbutan-2-ol/butan-2-one/acetonitrile/ethyl acetate/formic acid (200:100:75:100:75:9, by vol.). The plates were exposed to X-ray film (Kodak X-Omat AR) at -80°C in a press.

RESULTS AND DISCUSSION

Characterization of the RNA present in the endoribonuclease VII-RNP complex

Endoribonuclease VII-RNP complexes were isolated by sucrose-gradient centrifugation as described above (see the Materials and methods section). A 100 μ g portion of RNPs was extracted with phenol/chloroform and RNA was precipitated with ethanol (Roop *et al.*, 1978). A 2 ng portion of RNA was separated on a 10%-polyacrylamide gel in the presence of 8 M-urea (Bachmann *et al.*, 1984a). As shown in Fig. 1(a) and in agreement with earlier estimations (Bachmann *et al.*, 1984a) the complexes contain a 4.5 S RNA. The RNP complexes were found to be very stable, even against 4 M-

Fig. 2. Chromatography of isolated 4.5 S RNAs on poly(A)-Sepharose

A 20 μ g portion of the 4.5 S RNA was separated on a 10% -polyacrylamide gel in the presence of 8 M-urea. The RNA band was eluted and, after freeze-drying, redissolved and applied to a poly(A)-Sepharose column. Fractions (0.5 ml each) were collected. The arrow indicates the buffer change.

urea and 1 M-NaCl (Bachmann *et al.*, 1983b). It was observed that Mg^{2+} stabilizes them, whereas they were destabilized at low ionic strength. Therefore 250 μ g of RNPs were dialysed against distilled water (500 ml). After dialysis, denatured material was removed by centrifugation (10000 g, 10 min). The supernatant, still containing endoribonuclease VII activity and RNA, was freeze-dried and phenol-extracted. In contrast with the RNA isolated from intact undialysed RNPs, no 4.5 S RNA was detected after polyacrylamide-gel electrophoresis (results not shown). Only small oligonucleotides with a chain length of 4–10 nucleotides by gel filtration were detected, indicating that the RNA was degraded by endoribonuclease VII during dialysis (results not shown). For further analysis these oligonucleotides were degraded to nucleosides and analysed by the Randerath *et al.* (1972, 1974) method as described in the Materials and methods section. After this procedure only one radioactive spot was found, as shown in Fig. 1(b, i). This spot was eluted and co-chromatographed with uridine labelled by the same procedure. No additional radioactive spot was found, indicating that the oligonucleotide is an oligo(U) fragment (Fig. 1b, ii). To investigate whether the isolated oligo(U) fragments are degradation products of the 4.5 S RNA, 20 μ g of RNA preparation was separated on a preparative 10% -polyacrylamide gel in the presence of 8 M-urea as described above. The 4.5 S RNA was eluted from the gel, freeze-dried and dissolved in 1 ml of 50 mM-Tris/HCl, pH 7.5, supplemented with 0.7 mM-NaCl, 10 mM-EDTA and 25% formamide (Ihle *et al.*, 1974). As shown in Fig. 2, more than 67% of the RNA was able to bind to a 1 ml of poly(A)-Sepharose column and was recovered after elution with 10 mM-potassium phosphate, pH 7.5, supplemented with 10 mM-EDTA and 90% (v/v) formamide. Fractions (0.5 ml each) were collected and the A_{260} was measured. From these results we conclude that the isolated 4.5 S RNAs (i) are not homogeneous and (ii) partially contain oligo(U) sequences. Because oligonucleotides of the mentioned molecular size should be lost during dialysis, we assume that an oligo(U)-binding protein has retained the oligonucleotides within the dialysis bag. Most RNA polymerase III transcripts, such as the 4.5 S

Table 1. Binding affinity of Ro, La and endoribonuclease (ERNase) VII to poly(U) determined by immunoprecipitation

Binding affinity was determined in assays containing 20 pmol of La or Ro or, instead of these proteins, 5 μ g of endoribonuclease VII complex or, for controls, 10 μ g of BSA, by incubation (15 min, 20 °C) with 250 pmol of [3 H]poly(U). After incubation, complexes formed were immunoprecipitated with monospecific anti-La or anti-Ro IgGs or with non-specific control Tr-IgGs coupled to Sepharose 4B. Bound radioactivity was eluted and counted.

Antigen	Immobilized Antibody	Bound radioactivity (d.p.m.)
La	Anti-La	84 570
Ro	Anti-Ro	62 160
ERNase VII-RNP complex	Anti-La	32 700
ERNase VII-RNP complex	Anti-Ro	34 500
BSA	Anti-La	17
BSA	Anti-Ro	11
La	Control IgG	20
Ro	Control IgG	38
La	Anti-Ro	24
Ro	Anti-La	52
ERNase VII-RNP complex	Control IgG	21

La and Ro RNAs, carry a 3' terminal oligo(U) sequence (Lerner & Steitz, 1981; Hendrick *et al.*, 1981). In the case of pre-tRNAs or 5 S RNA precursor these uridylyte residues have been shown to be removed in the nucleus during processing and maturation of the RNAs (Rinke & Steitz, 1982). Other RNA polymerase III transcripts, such as the Ro RNAs, still contain their oligo(U) sequence even after transport into the cytoplasm. However, they can also lose their oligo(U) tail in the cytoplasm (e.g. conversion of hY1 into hY2; Busch *et al.*, 1982). To prove that the 4.5 S RNA also belongs to the La or Ro RNAs, we tried to identify these antigens in the 4.5 S RNPs.

Immunoprecipitation of endoribonuclease VII-poly(U) complexes with monospecific La and Ro antibodies

It is known that the La and Ro proteins form immunoprecipitable complexes with poly(U) (Bachmann *et al.*, 1986). Therefore endoribonuclease VII complexes were incubated with [3 H]poly(U) in the standard binding assay. The complexes formed were incubated with monospecific anti-La or monospecific anti-Ro antibodies coupled to Sepharose 4B and precipitated. As shown in Table 1, the amounts of radioactivity found in the anti-La- or anti-Ro pellets were nearly the same, indicating that both antigens are present in the particles.

Endoribonuclease VII activity in the presence of monospecific Ro and La antibodies

To determine the influence of monospecific Ro or La antibodies on the activity of endoribonuclease VII, 5 units of purified enzyme were preincubated with the monospecific antibodies (2 μ g) for 10 min in 20 μ l of assay buffer as described in the Materials and methods section. Then 80 μ l of assay buffer containing 10 nmol of

Fig. 3. Identification of the Ro and La antigen in the 45 S endoribonuclease VII particles

(a) A 10 ng portion of immunoaffinity-purified Ro RNPs were separated by polyacrylamide-gel electrophoresis in the presence of SDS. (b) A 10 ng portion of these Ro RNPs or 10 ng of endoribonuclease VII-RNP complexes were phosphorylated by cytoplasmic protein kinase CII and immunoprecipitated with purified monospecific anti-Ro antibodies that did not cross-react with La. Lane (i), endoribonuclease VII particles; lane (ii), control; lane (iii), Ro RNPs. As a control the reaction mixture was immunoprecipitated with anti-Ro antibodies in the absence of Ro RNPs or endoribonuclease VII complexes. The arrows mark the positions of the following protein standards, which were run in parallel gels; from top to bottom: phosphorylase *a*, bovine serum albumin, ovalbumin and carbonic anhydrase B.

[³H]poly(U) was added and the enzymic activity was determined by using the GF/C-filter technique. No marked inhibition of nuclease activity was found in the presence of the antibodies; 96% of the enzymic activity was still present. In a second approach, endoribonuclease VII-RNP complexes, containing 8 units of enzyme

activity, were preincubated with the same amount of antibody, coupled to CH-Sepharose 4B, for 10 min in 100 μ l of assay buffer. After incubation the immuno-complexes formed were precipitated by centrifugation (5 min, 10000 *g*). Then 10 μ l of [³H]poly(U) (10 nmol) were added to 90 μ l of the supernatant and the remaining enzymic activity was analysed; 84% of the endoribonuclease VII activity was pelleted by the anti-Ro- and 78% by anti-La-Sepharose. These results indicate again that both antigens are present in the particles and that endoribonuclease VII is co-precipitated with them. In addition, the influence of the La antigen on nuclease activities was tested by using the standard assay of endoribonuclease VII. However, neither the poly(U) nuclease, nor the acidic endoribonuclease nor endoribonuclease VII was inhibited by La.

Identification of the La and Ro antigen in endoribonuclease VII-RNP complexes

To identify the Ro or La antigen in the 45 S endoribonuclease VII particles we used a highly sensitive and selective assay, which was recently described by us (Bachmann *et al.*, 1986). The assay is based on the phosphorylation *in vitro* of the Ro and La antigens with the cytoplasmic protein kinase CII, followed by immunoprecipitation of the labelled antigens, using purified, monospecific, antibodies coupled to CH-Sepharose 4B. As shown in Fig. 3(a), immunoaffinity-purified Ro RNPs contain predominantly the Ro and La proteins (as judged by staining with Coomassie Brilliant Blue). A 10 ng portion of these Ro RNPs or endoribonuclease VII complexes was incubated with 0.5 unit of cytoplasmic protein kinase CII. The phosphorylated antigens were immunoprecipitated by using the purified anti-Ro antibodies and separated on 10%-SDS/polyacrylamide gels. Previous estimations had shown that these antibodies precipitate only the Ro antigen, even from a mixture of homogeneous La and Ro proteins (Bachmann *et al.*, 1986). However, as shown in Fig. 3(b), lane (iii), both antigens were precipitated from Ro RNPs.

Table 2. Properties of endoribonuclease VII in comparison with the nuclease fractions isolated from Ro RNPs

A comparison of both endoribonucleases is summarized below. As shown in Fig. 3(a), Ro RNPs contain predominantly the Ro and La proteins. However, after silver staining (not shown) a 74 kDa protein is also detected. The enzymic activity of endoribonuclease VII towards poly(U) depends on the presence or absence of poly(A). Therefore poly(A) was added to the assays. The alterations of the activities at different molar ratios of poly(A) to poly(U) are shown in the Table. In the case of Ro RNPs, two enzymic activities were separated by isoelectric focusing. The extent of stimulation of the enzyme at pI 6.3 is given in parentheses. K_m values were determined as μ mol of poly(U) phosphate. Data concerning the endoribonuclease VII (molecules/cell) are taken from Bachmann *et al.* (1983b). The estimated concentration of Ro RNPs (molecules/cell) is taken from Hendrick *et al.* (1981).

Property	Endoribonuclease VII (45 S particles)	Ro RNP-associated nuclease activities
Source	Calf thymus	L5178y cells
M_r	74000	(74000 kDa protein is present)
$s_{20,w}$ value for particle (S)	45	12–20
Cofactors	None	None
pH optimum	7.2	7.4
pI	8.5	8.5 (6.3)
Substrate specificity	poly(U)/poly(C)	Poly(U)/poly(C)
Inhibitors	Double-stranded RNA, poly(A) (ratio 2:1)	Double-stranded RNA, poly(A)
Stimulation by poly(A)	140% (ratio 1:10)	180% (428%)
Product(s)	Oligo(U) ₁₀ ; 3'-OH; 5'-phosphate	Oligo(U) ₁₀ ; 3'-OH; 5'-phosphate
K_m (phosphate) (μ M)	5.11	4.8
Concentration (molecules/cell)	2.2×10^4	Around 10^5 Ro RNPs

Therefore the La protein must be co-precipitated with the Ro protein on account of its being present in the same particle. Moreover, both antigens were also identified in the 45 S endoribonuclease VII particles (Fig. 3*b*, lane i). The same results were obtained by using instead of anti-Ro antibodies the purified anti-La antibodies (results not shown).

Nuclease activities isolated from Ro RNPs

In a further approach we tried to isolate endoribonuclease VII activities from Ro RNPs. A 100 µg portion of immunoaffinity-purified Ro RNPs was subjected to preparative isoelectric focusing under the same conditions as described for the purification of endoribonuclease VII (Bachmann *et al.*, 1983*b*). Before isoelectric focusing the Ro RNPs were dialysed against 4 M-urea/5% sucrose/2 mM-dithiothreitol. After focusing, 0.5 ml fractions were collected and analysed for nuclease activity in the standard incubation assay for endoribonuclease VII. At a pH of 8.5 (the pI of endoribonuclease VII) and 6.3, nuclease activities were detected. To characterize these activities as endoribonuclease VII the degradation products were analysed. In addition the nuclease activity was tested in the presence of poly(A). Endoribonuclease VII is known to degrade single-stranded poly(U) to oligonucleotides with 3'-OH and 5'-phosphate groups and to be modulated by poly(A) in a concentration- and chain-length-dependent manner (Bachmann *et al.*, 1983*b*). No other nuclease activity is known to possess such properties. The degradation products of the pI-8.5 fraction were isolated by gel filtration and analysed by digestion with phosphodiesterase I or II as described previously (Arendes *et al.*, 1977; Schröder *et al.*, 1980; Bachmann *et al.*, 1983*b*). Only phosphodiesterase I was able to degrade the isolated oligonucleotides, indicating that the oligonucleotides carry a 3'-OH and a 5'-phosphate group. The same result was obtained from the degradation products of the pI-6.3 fraction (Table 2). Both enzymic fractions were found to be stimulated by poly(A) (pI-8.5 fraction, stimulation to 187%; pI-6.3 fraction, to 428%). A comparison of endoribonuclease VII and the nuclease activities isolated from Ro RNPs is given in Table 2. From these results we conclude that the isolated nuclease is endoribonuclease VII.

Final implications

We have shown that the 45 S endoribonuclease VII-RNP particles contain an oligo(U)-carrying 4.5 S RNA and the Ro and La antigens. Additionally we succeeded in isolation of endoribonuclease VII from Ro RNPs. Therefore we assume that the 4.5 S RNA belongs to the Ro RNAs. The properties of the enzyme and its existence in Ro RNPs point to a function in mRNA regulation. Presently we favour the idea that the interaction of hnRNA molecules with 4.5 S Ro RNAs is a signal for storage of processed mRNA in cytoplasmic 'Ro organelles' (Lerner & Steitz, 1981) until use. This implies that the isolated Ro RNP particles are related to the 'Ro organelles'. They may also be related to the 'prosomes' described by Schmid *et al.* (1984). The prosomes are also RNP particles and are thought to function in the storage and repression of distinct mRNAs. Like the 45 S RNPs, prosomes are stable in the presence of high salt. The 45 S endoribonuclease VII particles also associate with the Sm antigen that is

removed during the final preparative isoelectric-focusing step (Bachmann *et al.*, 1984*a*, 1985). Future experiments will have to establish whether endoribonuclease VII RNPs are substructures of the Ro organelles.

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